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EXPRESSION OF HISTONE DEACETYLASE ENZYMES IN MURINE AND CHICK OPTIC NERVE

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LIST OF ABBREVIATIONS

Accrediatation of Laboratory Animal care	ALAC
B cell lymphoma	Bcl2
Bicinchoninic acid assay	BCA
Blood brain barrier	BBB
Bone morphogenic proteins	BMP
Central nervous system	CNS
Cerebrospinal fluid	CSF
Ciliary neurotrophic factor	CNTF
Coastal2	Cos2
Co-repressor element transcription factor	Co-REST
Dimethyl sulphoxide	DMSO
Dulbecco's Modified Eagle Medium	DMEM
Embryonic day 16	E16
Embryonic day 5	E5
Embryonic day 8	E8

Epidermal growth factor	EGF
Fibroblast growth factor	FGF
Fused	FU
Glial fibrillary acidic protein	GFAP
Glial growth factor	GGF
Glial restricted precursors	GRPs
Glioma associated factors	GLI
Heat shock protein	HSP
Heterochromatin protein 1	HP1
Histone acetyl transferase	HAT
Histone deacetylases	HDACs
Histone methyl transferases	HMT
Hours	Hrs
Interferon β	IFNβ
Janus kinase	JAK
Leukemia inhibitory factor	LIF
Lysine specific demethylases	LSD
Methyl CpG binding protein 2	MeCP2
Mouse mammary tumor virus	MMTV
Neural/GlialAntigen2	NG2

Neuroepithelial precursor	NEP
Neurotransmitters	NTs
Oligodendrocyte type2 astrocyte precursor	O2A
Optimal cutting temperatures	OCT
Paired homeo box2	*pax*2
Paired homeobox2 transcription factor	PAX2
Patched	PTC
Phenylmethane sulfonylfluoride	PMSF
Platelet derived growth factor α	PDGFRα
Polyvinylidene difluoride	PVDF
Post natal day 5	P5
Post natal day 30	P30
Protein kinase A	PKA
Radio-Immunoprecipitation Assay	RIPA
Rat neural antigen 2	Ran2
Repressor element transcription factor	REST
Retinal pigment epithelium	RPE
Real time quantitative polymerase chain reaction	RT-qPCR
Ribonucleic acid	RNA
Ribo nucleic acid polymerase ll	RNA Pol ll
Sonic hedgehog	SHH
Signal transducer and activator of transcription	STAT3

Smoothened	SMO
SRY-related HMG box	SOX
SWItch/Sucrose non fermentable factors	SWI/SNF
Transforming growth factorβ	TGFβ
TATA binding protein	TBP
Tail less	TLX
Trichostatin A	TSA

ABSTRACT

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Epigenetic alterations have been shown to control cell type specification and differentiation leading to the changes in chromatin structure and organization of many genes. HDACs have been well documented to play an important role in both neurogenesis and gliogenesis in ganglionic eminence and cortex-derived cultures. However, the role of HDACs in glial cell type specification and differentiation in the optic nerve has not been well described. As a first step towards understanding their role in glial cell type specification, we have examined histone acetylation and methylation levels as well as the expression levels and patterns of the classical HDACs in both murine and chick optic nerve. Analysis of mRNA and protein levels in the developing optic nerve indicated that all 11 members of the classical HDAC family were expressed, with a majority declining in expression as development proceeded. Based on the localization pattern in both chick and murine optic nerve glial cells, we were able to group the classical HDACs: predominantly nuclear, nuclear and cytoplasmic, predominantly cytoplasmic. Nuclear expression of

HDACs during different stages of development studied in this project in bothmurine and chick optic nerve glial cells suggests that HDACs play a role in stage-dependent changes in gene expression that accompany differentiation of astrocytes and oligodendrocytes. Examination of localization pattern of the HDACs is the first step towards identifying the specific HDACs involved directly in specification and differentiation of glia in optic nerve.

CHAPTER 1. INTRODUCTION

1.1 Background

Epigenetics is the study of various alterations that regulate the transcriptional activity of genes without changing the nucleotide sequence on DNA (Holliday 2006). Histones proteins can be modified in different ways; acetylation/ deacetylation, methylation, phosphorylation, ubiquituination and somoylation. Study of these epigenetic modifications has been an area of interest because of their implication in both development and disease processes, such as cancers, neurological disorders and diabetes (Egger, Liang et al. 2004; Peterson and Laniel 2004; Esteller 2008). Environmental factors such as stress, nutrition and heavy metal exposures have also been documented to induce histone modifications that can subsequently alter gene expression (Wade and Archer 2006).

Modification of histones by histone deacetylase enzymes (HDACs) has been described to play an important role in glial cell development in central nervous system in general (Hsieh and Gage 2004). The role of these modifications in ocular disorders has been an area of interest over the past decade (Cvekl and Mitton 2010). However, there is very little is known of their role in differentiation

of astrocytes and oligodendrocytes within the optic nerve. The focus of this study was to begin to determine the epigenetic state of optic nerve glia by studying histone modifications and histone modifying enzymes during the development of the optic nerve. Understanding the normal epigenetic state of the optic nerve in development will lay the groundwork for understanding potential role in disease processes such as diabetes and glaucoma.

The portion of this text describing the expression patterns of the classical HDACs in both murine and chick optic nerve is prefaced by an introduction to glial cells in the central nervous system and optic nerve. Understanding the concept of epigenetic modifications and their role in transcription of genes will further help to correlate our findings with the differentiation process of the glial cells within the optic nerve.

1.2 Cells in the central nervous system

The cells in the mature central nervous system have been broadly grouped as the neurons and the glial cells (Fig.1) (Bergmann, Lane et al. 2006). The progenitor cells of the nervous system give rise to various populations of cells at specific but overlapping time points, starting with the generation of neurons, then astrocytes, and finally oligodendrocytes. During the embryonic development of the cortex, the neuroepithelial cells lining the ventricular and the sub-ventricular zone give rise to the radial glial cells. Through the process of neurogenesis these radial glial cells give rise to two different type of cells; neurons and glial precursor

cells (Gage 2000; Temple 2001). Based on the extrinsic and intrinsic signals, glial precursors further differentiate into the various types of the glial cells. Glial cells are further categorized as macroglia and microglia. Macroglia are further subdivided into astrocytes, oligodendrocytes and ependymal cells each of which have their own functions to be covered in the next sections (Ridet, Malhotra et al. 1997; Qian, Shen et al. 2000; Okano 2002; Zawadzka, Rivers et al. 2010).

1.3 Glial cells of the nervous system

1.3.1 Microglia cells

Commonly known as the scavenger cells of the central nervous system, microglia help to protect against the microbial organisms. They play an important role in removing the dead tissue fragments and provide support to the neurons. They have been classified into different types based on their reactivity to inflammatory signals (Cameron and Rakic 1991; Hurley, Walter et al. 1999; Grossmann, Stence et al. 2002; Czeh, Gressens et al. 2011).

1.3.2 Macroglial cells

1.3.2.1 Ependymal cells

Ependymal cells line the ventricles and the central canal of the spinal cord (Morest and Silver 2003). Their main function is secretion, movement, and recycling of cerebrospinal fluid (CSF) into the ventricles of the brain and creation of the brain-CSF barrier. CSF is secreted from a structure called the choroid plexus; a network of blood vessels lined with ependymal cells that is located in all the four ventricles of the brain. The tight junctions of the ependymal cells lining the vessels thereby help in the formation of CSF through the filtration of the blood from the capillaries (Serot, Zmudka et al. 2012). In addition to secretion and movement of CSF, the cells also act as a filter for some chemicals such as neurotransmitters (NT's). Improper functioning of the ependymal cells leads to the development of hydrocephaly due to abnormality in the clearing of the cerebrospinal fluid (Rothfus and Latchaw 1985; Taulman, Haycraft et al. 2001; Jacquet, Salinas-Mondragon et al. 2009; Del Bigio 2010). Ependymal cells have also been documented to act as a stem cell under certain situations (Carlen, Meletis et al. 2009).

1.3.2.2 Oligodendrocytes

Oligodendrocytes are the other type of macroglial besides ependymal cells and astrocytes, widely studied in the central nervous system. They are the myelinating cells of the central nervous system. Insulation of axons by myelin

sheath provides an increase in the speed of the neuronal conduction, nutritional support to the axons, and maintains neuronal growth and survival (Weatherby, Davis et al. 2000). Defective myelination of the central nervous system has been known to be associated with various demyelinating diseases such as multiple sclerosis where focal loss of myelination leads to severe functional deficits (Bunge 1968; Waxman 1992; Furusho, Dupree et al. 2012; Soldan and Pirko 2012).

1.3.2.3 Astrocytes

Astrocytes are well known as one of the major macroglial cell types of the central nervous system. These glial cells originally thought to be inert but have now been shown to be dynamic and interactive (Butt, Pugh et al. 2004). (Sofroniew, Vinters et al. 2010). Their functions include: 1) providing extracellular matrix proteins and adhesion molecules, which help to develop and maintain structural integrity of the central nervous system, 2) providing trophic support to neurons and neurite promoting factors in the form of soluble and substrate bound factors, 3) serving as an energy reserve by storing glycogen, and supplying energy to glucose deprived neurons, 4) aiding in the formation of tight junctions between endothelial cells that form the blood brain barrier (BBB), and providing structural support and help in transport of materials across the BBB, 5) maintaining homeostasis within the nervous system by taking up molecules such as ATP, glutamate, glycine and regulating extracellular ion concentration and maintaining pH and osmolarity,

6) metabolizing neurotransmitters and heavy metals and thus prevent these molecules from reaching toxic levels in the environment and 7) assistance in development of neuronal synapses during nervous system development (Montgomery 1994; Freeman 2010).

1.4 Classification of the astrocytes

Astrocytes like oligodendrocytes are widely studied glial cell types. Unlike other glial cells their classification has been also studied in great detail. Astrocytes are broadly classified into fibrous and protoplasmic astrocytes based on the differences in their morphology and their distribution (Levison, Hudgins et al. 1998; Sofroniew and Vinters 2010). Fibrous astrocytes are the star-shaped cells with relatively fewer intracellular organelles found mainly in the white matter of the CNS. The long unbranched processes are symmetrically distributed on their cell surface and have abundant intermediate filaments. Protoplasmic astrocytes, on the other hand, have a more complex morphology. Their processes are branched with fewer intermediate filaments as compared to fibrous types. These cells are localized to the grey matter have spherical to oval nuclei, and their intracellular organelles have been reported to be less in numbers compared to the fibrous astrocytes (Miller and Raff 1984; Sofroniew and Vinters 2010; Liu, Wang et al. 2012; Tsai, Li et al. 2012).

Astrocytes have also been classified as type I and type II astrocytes. (Montgomery 1994). The type 1 astrocytes have been shown to be larger in size

with fewer processes as compared to the type 2 astrocytes. While both these type of astrocytes express glial fibrillary acidic protein (GFAP), type 2 astrocytes are also express A2B5 (an extracellular ganglioside) (Raff, Abney et al. 1983; Suarez and Raff 1989; Albrecht, Enterline et al. 2007; Rompani and Cepko 2010). Based on their anatomical features and regional distribution, there has been some correlation found between the two classifications; type 1 astrocytes have been found to have some similarity to protoplasmic astrocytes whereas type 2 astrocytes have been shown to resemble fibrous astrocytes (Raff, Miller et al. 1983).

1.5 Molecular signals responsible for specification of oligodendrocytes and astrocytes

As mentioned earlier neuronal stem cells, also known as neural precursors can become neurons or glial cells in the central nervous system depending on the molecular signals they receive. These signals such as sonic hedgehog (SHH) and fibroblast growth factor (FGF) have been identified to play role in the specification of oligodendrocytes (Chandran, Kato et al. 2003; Fogarty, Richardson et al. 2005). The role of bone morphogenetic proteins (BMPs), SHH, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) in restricting the lineage specification of the multipotent neural precursors towards astrocytes has been also well established (Fig.5) (Kessaris, Pringle et al. 2008; Agius, Decker et al. 2010; Rowitch and Kriegstein 2010).

Understanding of these signaling pathways has helped to elaborate the role of various transcription factors/protein complexes in regulation of transcription of genes (Turner 2002; Kouzarides 2007). Role of post-translational modifications on to the promoter of the target genes through histone modifying enzymes will help to provide more insight into the process as described in later sections. A more detailed view of common pathways involved in the specification of astrocytes below will aid in understanding the interplay between the signaling pathways and epigenetic modifications described in subsequent sections.

1.5.1 CNTF and LIF signaling pathway

Both CNTF and the LIF belong to the family of GP130 family of cytokines (Taga and Kishimoto 1997). LIF specifically belongs to the IL6 family of cytokines (Aghajanova, Skottman et al. 2006). LIF is known to play an important role in the proliferation and differentiation of the embryonic stem cells (Niwa, Burdon et al. 1998). CNTF and LIF initiate signaling upon binding to their receptors, the CNTFRα and LIFβ then recruit the co-receptor GP130 to form heterodimers leading to the phosphorylation and activation of the Janus kinase (JAKs) (Fig.2). Activated JAKs phosphorylate the transcription factor, signal transducer and activator of transcription 3 (Stat3). Activated Stat3, on dimerization, is translocated to the nucleus where it recruits the histone acetyl transferase (HAT) p300 to one of the binding sites on the GFAP promoter. The GFAP gene is one of the many astrocyte-specific genes critical for the proper differentiation of astrocytes (Darnell 1997; Levy and

Darnell 2002). Acetylation of histones on promoter has been shown to create changes to the chromatin structure there by providing access to the transcriptional machinery (transcriptional factors and non-histone protein co-activators) (Turner 2002; Kouzarides 2007). This further leads to the transcription initiation of target genes, astrocytic genes here as discussed in later sections.

1.5.2 BMP pathway (Bone morphogenetic proteins)

The BMPs belong to the transforming growth factor-β (TGFβ) family. They mediate their effect through type I and type II receptors which are members of serine threonine kinase family of proteins. Upon ligand binding, type 1 and type 2 receptor dimerize followed by tetramerization. The type 1 receptor then phosphorylates and activates the downstream signaling components, SMAD1, 5, and 8. The activated SMAD then bind to the co-SMAD (SMAD4) and the dimer is translocated to the nucleus where they cooperatively regulate gene expression with other transcription factors. Within astrocytes, SMADs, STAT3 and p300, cooperatively interact to upregulate the GFAP expression in astrocytes (Fig.2) (Ten Dijke, Goumans et al. 2002; Miyazono, Maeda et al. 2005; Kessaris, Pringle et al. 2008). Hence, both these pathways have at least one common meeting point, the GFAP promoter where they both bring in a HAT and activate the transcription of the genes for astrocytes. Both these pathways act parallel and in combination with one another to activate transcription factors and recruit co-activators that provide access to the GFAP promoter. Hence, the role of histone modifying enzymes (histone acetyl transferases and histone deacetylases)

during the transcriptional activation of astrocytic genes is crucial (Mabie, Mehler et al. 1999; Nakashima, Yanagisawa et al. 1999; Robel, Berninger et al. 2011).

1.5.3 Sonic hedgehog (SHH)

SHH belongs to the family of the hedgehog proteins and is known to play an important role in the patterning of the CNS, and in differentiation and proliferation of oligodendrocytes (Nery, Wichterle et al. 2001; Marti and Bovolenta 2002). There have been some reports that show SHH contributing to the development and proliferation of astrocytes as well (Garcia, Petrova et al. 2010). SHH has been documented through cortical cell culture studies to directly support the development of astrocytic precursors (Cahoy, Emery et al. 2008). Studies have also described the significance of SHH from retinal ganglion cells in development of optic disc and optic stalk neuroepithelial cells (Dakubo, Wang et al. 2003). Further SHH signaling from the retinal ganglion cells has been shown to stimulate the proliferation of both astrocytes and oligodendrocytes in the optic nerve (Wallace and Raff 1999). Previous work from our lab has shown that cooperation between the SHH and BMP7 pathway increases transcription of the *pax2* gene in type I astroyctes of the optic nerve by interfering with the binding of a transcriptional repressor, TLX, (Sehgal, Sheibani et al. 2009).

In vertebrates, the SHH signaling pathway is initiated by binding of SHH to the transmembrane receptor, patched (PTC). In the absence of SHH, PTC

inhibits smoothened (SMO), a seven transmembrane protein. This results in phosphorylation and modification of the Glioma (GLI) associated factors, zinc finger family of transcription factors, GLI2 and GLI3 by protein kinase A.(PKA) (Varas, Hager-Theodorides et al. 2003). Phosphorylated forms of these two are proteolytically cleaved to give rise to their repressor forms. On the other hand, SHH binding to PTC receptor releases the inhibition on SMO. PKA no longer phosphorylates GLI2 and hence it does not get converted to its repressor form, instead serves as an activator of transcription. Besides PKA other kinases, glycogen synthase kinase 3 β (GSK3β) and casein kinase, are also involved in this process of phosphorylation (Rubin and de Sauvage 2006). GLI1 have been shown to acts as a transcriptional activator only, whereas, GLI2 and 3 have been described to be modified as repressors or activators. Accumulation of GLI within the nucleus of the cells is further regulated by a multiprotein complex formed by suppressor of fused [Su (Fu)] which serves as an inhibitor of SHH signaling pathway (Fig.3) (Marti and Bovolenta 2002; Crompton, Outram et al. 2007). Studies showing increase in the level of GLI1 on application of SHH and knock down of the intermediate factors in the signaling pathway have suggested its role in changes to the development and proliferation of the astrocytes (Garcia, Petrova et al. 2010).

Hence, the CNTF, BMP, and SHH pathways have all been shown to play a role in lineage specification of neural precursors towards glial cells. Generation of mature and fully differentiated glial cells is a multistep process.

Many intermediate precursors have been identified to be formed during the process of differentiation (Rao 2004). For both oligodendrocytes and astrocytes a step wise process of differentiation has been identified as described in the next section.

1.6 Differentiation of astrocytes and oligodendrocytes

Precursor, immature perinatal and mature types astrocytes have been well established as shown in Fig.4 (Chu Hughesetal et al. 2001). These different stages can be distinguished from one another based on the proteins they express, their ability to further differentiate and their response to the growth factors. For example astrocyte precursor cells are identified by positive labeling for the A2B5 + and PAX2 +. Mature and well differentiated astrocytes are on the other hand identified by their expression of markers such as GFAP. Similarly different stages of differentiation have been identified for oligodendrocytes as well as shown in Fig.5 (Jackman 2000). Markers that help to identify these differentiating stages have also been identified. Oligodendrocyte precursor cells have been shown to express two specific markers, NG2, an integral membrane chondroitin sulfate proteoglycan and platelet derived growth factor receptor α (PDGFRα) (Rao and Mayer-Proschel 1997; Liu and Rao 2004).

Optic nerve, being a part of central nervous system, has always been an area of interest for studies related to glial cells; oligodendrocytes and astrocytes. Understanding the development of the optic nerve and various glial cell types

present has provided a platform to researchers for further exploring the mechanism involved in their differentiation and compare it with the one in the rest of the central nervous system. The development of the optic nerve and subtypes of glial cells present in the optic nerve will be explained in the next sections.

1.7 Development of the optic nerve

Literature has detailed explanation about how the development of the optic nerve takes place as described further Development of the eye has been described to begin from a single layer of neuroepithelial cells, called the eye field that lies in the center of the rostral part of the neural plate. This region has been documented to begin expressing molecules like PAX6, RX1, SIX3, LHX2, and many others which heralds the earliest stages of eye development (Zuber, Gestri et al. 2003; Lamb, Collin et al. 2007). As the neural tube is formed through the upward and inward folding of the neural plate the eye field is shown to be bisected into two optic grooves that lie to either side of the midline. As development proceeds the optic grooves continue to enlarge and evaginate, eventually forming the optic vesicles (Fig.6) (Lamb, Collin et al. 2007). The optic vesicles come into close proximity to a thickened region of the head ectoderm, called the lens placode, helping to initiate the development of the lens vesicle. The lens placode reciprocates by sending secreted signals fibroblast growth factors (FGFs) which interact with the optic vesicles, specifying the region that will give rise to the retina in the developing optic vesicles (Huang, Rajagopal et al. 2011). The optic vesicles invaginate to give

rise to a double layered optic cup. Out of this bilayered structure the inner layer becomes the neural retina while the outer layer is transformed into the retinal pigment epithelium (RPE) (Chow and Lang 2001). The invagination of the optic vesicles starts from the ventral side of the optic vesicle and proceeds dorsally such that a fissure forms in the ventral optic cup (Fig.7) (Lamb, Collin et al. 2007). This fissure, known as the choroidal fissure, eventually closes as the cells of the ventral optic cup proliferate and migrate into the area (Onwochei, Simon et al. 2000). It is the optic stalk through which the retinal ganglion cells axons exit the eye (Lamb, Collin et al. 2007). In the next section, the major types of glial cells in the optic nerve, oligodendrocytes, type I and type II astrocytes, will be discussed.

1.8 Glial cells in the optic nerve

The glial cells present in the optic nerve include type I and type 2 astrocytes and oligodendrocytes. Both mature type 1 and type 2 astrocytes are positive for the glial fibrillary intermediate filament (GFAP). Differences between the two types of astrocytes are; 1) type 1 astrocytes in the optic nerve are rat neural antigen 2 (Ran2) + and A2B5 - and type 2 astrocytes are Ran2 - and A2B5 +, 2) type 1astrocytes can be induce to proliferate in culture in the presence of the epidermal growth factors (EGF) and glial growth factors (GGF) unlike type 2 (Raff, Abney et al. 1983; Suarez and Raff 1989; (Lemke and Brockes 1984; Barres, Chun et al. 1989).

Like most cells, both the oligodendrocytes and astrocytes that populate the optic stalk/nerve have been shown to undergo a step-wise process in their differentiation (Rao et al. 2004). The type I astrocytes in the optic nerve originate from the neuroepithelial precursor cells (NEP) that comprise the optic stalk (Small, Riddle et al. 1987). These cells proliferate and give rise to a population of cells referred to as astrocyte precursor cells that appear producing astrocytes. Another group of progenitor cells, referred to as O2A progenitors, migrates from the lateral and medial ganglionic eminences of the diencephalon into the optic nerve, where they continue to proliferate and give rise to oligodendrocytes (Jackman, Ishii et al. 2009). When placed in vitro, these O2A progenitors can also give rise to type II astrocytes. There are conflicting reports in the literature as to whether O2A progenitors are capable of producing both astrocytes and oligodendrocytes in vivo (Levison and Goldman 1993; Rao 1999). Finally, results from Rompani and Cepko (2010) have shown a glial restricted progenitor from which both oligodendrocytes and astrocytes can be generated (Liu and Rao 2004; Kim, Park et al. 2006; Menn, Garcia-Verdugo et al. 2006; Rivers, Young et al. 2008; Zhu, Bergles et al. 2008; Jackman, Ishii et al. 2009). Retina also contains astrocytes, one of the three macroglail cells as described earlier and therefore an introduction to retina and its cell types including the müller glial cells will be discussed in the next section.

1.9 Mature retina and its cell types

The mature retina is made up of seven different layers: 1) retinal pigment epithelium (RPE) made up of the cuboidal cells forming the outermost layer, 2) the outer nuclear layer contains the cell bodies of the light transducing photoreceptors, 3) the outer plexiform layer is composed of synapses between the photoreceptors and the bipolar and horizontal cells, 4) the inner nuclear layer contains the bipolar cells and the inhibitory amacrine and horizontal cells, in addition to the Müller glial cells, 5) the inner plexiform layer comprised of synapses between the bipolar cells (inner nuclear layer) and the ganglion cells (ganglion cell layer), 6) the ganglion cell layer contains the ganglion cells and displaced amacrine cells and finally 7) the nerve fiber layer includes the fibers from the ganglion cells that leave the eye through the optic nerve and retinal astrocytes (Fig.8) (Adler 2000; Doh Hao et al. 2010). The three major types of glial cells in the retina are; the Müller glial cells, the retinal astrocytes and the microglia. Some species also have oligodendrocytes in the nerve fiber layer of the retina; however oligodendrocytes are not found in large majority of retinas (Czekaj, Haas et al. 2012). Müller glial processes span the entire width of the retina, comprising the outer limiting membrane near the photoreceptor cell bodies and the inner limiting membrane that forms the boundary between the retina and the vitreous. They arise from a common multipotent retinal progenitor that gives rise to both neuronal cell types as well as the Müller glial cells at different time points during the differentiation process. Retinal astrocytes do not originate within the retina but migrate in from the developing

optic nerve. Microglial cells arise from the mesenchymal cells outside the eye and migrate into the eye with the endothelial cells that will comprise the retinal vasculature eventually (Fletcher, Downie et al. 2010; Ma, Zhao et al. 2012; Nelson and Hyde 2012; Provis 2001).

Studies using genome wide profiling have shown an association between glial cell type lineage specification, differentiation and epigenetic regulation of the target genes with in telencephalon (Hatada, Namihira et al. 2008). Similarly results have also established role of epigenetic regulation in both neurogenesis and gliogenesis in central nervous system in general (Juliandi, Abematsu et al. 2010). However, the role of epigenetic regulation within the optic nerve is not understood.

1.10 Epigenetics

Epigenetics is defined as the “study of the heritable changes in gene expression that are not due to changes in DNA sequence” (Wolffe and Matzke 1999). Epigenetic alterations within the DNA and or the histone proteins in a cell have been shown to play a crucial role during the development and differentiation (Sharma, Kelly et al. 2010; Wang, Sorensen et al. 2012). Differentiation of cells is induced by the epigenetic changes in the chromatin and nuclear proteins within the nucleus of the multipotent stem cells as well as the intermediate progenitors (Schubeler, Francastel et al. 2000).

1.11 Chromatin and the histones

Chromatin, the nucleoprotein made of a histone octamer and DNA. Histones have been basically classified into five different families of proteins; H2A, H2B, H3 and H4 that form the core of the histone octamer and the linker histones, H1 and H5 that help to provide a higher order structure to the chromatin packaging. All these histones together with the 147 base pairs of DNA wrapped around the histones form the nucleosome (Fig.9) (Caterino and Hayes 2007). Chromatin has been divided into two main groups according to packaging and structural detail; 1) Euchromatin is the more loosely packaged DNA in the nucleus that represents genes that are actively being transcribed and 2) Heterochromatin is the more compact form of the nucleosome that represents genes that are not being actively transcribed (Fierz and Muir 2012).

Epigenetic alterations and or modifications of these histone proteins are responsible for bringing in changes to the organization of chromatin, thereby providing or restricting access of the transcription factors to cis-acting elements of target genes. There are several different ways identified in which histones can be modified.

1.12 Modifications of the histones

Post translational modifications of histones have been documented to be strongly influencing the transcription of several genes in eukaryotes. Of these

different types of modifications, methylation, phosphorylation, ubiquitylation and sumoylation, as well as ribosylation are the examples (Peterson and Laniel 2004). Histone modifications are likely to bring about distinct functional consequences to the chromatin. Histone modifications have been described to be associated with changes in the transcriptional activity of the genes. For example acetylation of H3K14 and H4K8 and phosphorylation at H3S10 position are all associated with transcriptional activation, while methylation at H3K9 and H3K27 are associated with transcriptional repression under most circumstances (Peterson and Laniel 2004; Fuks 2005).

The term ‘histone code’ refers to a particular combination of histone marks/modifications which, unlike the genetic code, may have different meaning depending on the context. For example, TAG always means STOP transcription as far as the gene code is concerned. However, in regard to the histone code/modifications methylation or acetylation of the lysine or arginine side chains of histones at the same position can act as a transcriptional activator or repressor under different situations depending on the gene (Jenuwein and Allis 2001; Agalioti, Chen et al. 2002; Turner 2002; Lachner, O'Sullivan et al. 2003; Peterson and Laniel 2004).

1.12.1 Histone acetylation

Histone acetylation is the reversible alteration at the N-terminus of a lysine rich histone that is controlled by the histone acetyltransferases (HATs) and

the histone deacetylases (HDACs) families of enzymes. Acetylation of these lysine residues leads to the conversion of the amine group into amides, thereby leading to the loss of the net positive charge on the histone tails that binds to the negatively charged phosphate group of the DNA, this loosening the bonds between the two forms a more open conformation of the DNA (Fig.10) (Korzus 2010). The open conformation of the DNA allows other transcription factors and transcriptional machinery access to the sites they must bind to in order to affect transcription. HDACs remove acetyl group from histone tails, generally resulting in chromatin condensation and an overall repression of transcription. While HATs, add an acetyl group to histone tail leading to open chromatin structure and increased transcriptional activity. The balance between the actions of these enzymes serves as a key regulatory mechanism for gene expression and therefore plays an important role in the cell type specification, differentiation and de-differentiation.

Although HDACs generally are thought of as being important for transcriptional repression, recent evidence suggests that they can also be involved in activation. The cell types that are derived from the stem cells of the nervous system are in part based on the epigenetic mechanisms leading to the reorganization of chromatin structure (Marks, Miller et al. 2003; Shabbeer and Carducci 2005; Wu and Sun 2006; Kiefer 2007; Sharma, Tomar et al. 2007; Korzus 2010).

The HDAC superfamily, based on sequence similarities has been divided into the following four classes; 1) Class 1 includes HDAC1, 2, 3, and 8, 2) Class 2 is further subdivided as Class 2a and 2b has HDAC4 through 7, 9, and 10 under, 3) NAD-dependent,Sir2-like deacetylases named sirtuins fall under this class and 4) Class 4 has HDAC11 (Fig.11) (Gray and Ekstrom 2001; Khochbin, Verdel et al. 2001; Kao, Lee et al. 2002; Kyrylenko, Kyrylenko et al. 2003; Gregoretti, Lee et al. 2004; Yang and Seto 2008), (See Fig.9) (Gao, Cueto et al. 2002; Ververis and Karagiannis 2012).

1.12.2 Histone methylation

Histone methylation is yet another post translational modification leading to epigenetic changes to the chromatin structure. Histone methyl transferases (HMTs) and lysine specific demethylases (LSD1) regulate the reversible methylation at the promoter associated CpG islands. Methylation of histone can serve as transcriptional activator or repressor depending on the lysine chain involved. DNA methylation has been shown to play important role in cell type specification and differentiation of glial cells (Kohyama, Kojima et al. 2008). Studies using HDAC inhibitors have shown the role of HDACS in regulating methylation marks (Sarkar, Abujamra et al. 2011). Hence HDACs do seem to help in histone methylation indirectly.

1.13 Histone modifications and transcription of genes

Gene regulation has been shown to be dependent on transcription factors and chromatin packaging. Transcriptional unit in eukaryotes is made up of enhancer, core promoter, also known as the transcription start site and initiator element. It is the core promoter that serves as the recruiting site for complex transcriptional machinery including several transcriptional factors (TFs), TFll D, TFllB and TFllA, transcription binding proteins (TBP) and RNA polymerase ll (RNA Pol ll) (Fig.12) (Fischle, Wang et al. 2003; Levine and Tjian 2003; Sekine, Tagami et al. 2012). Binding of the initiation complex serves to unfold the DNA and initiate transcription of the target genes. Modifications of histones cause changes to the chromatin structure and serve to recruit non histone complex proteins also known as chromatin remodeling complexes and coactivator complexes which further allows the binding of TFs and RNA Pol ll thereby regulating transcription of genes (Turner 2002; Kouzarides 2007). Studies have established that histone acetylation at H3K9/K14 further bring in SWItch/Sucrose nonfermentable (SWI/SNF) remodeling complexes along with the TFllD, transcription initiation factors, thereby regulating the initiation of transcription process for the target genes (Struhl 1998; Fukuda, Sano et al. 2006).

Transcription regulation by methylation of histones at different loci is yet another example of involvement of multiprotein complexes during the process. For example methylation of histone at H3K9 leads to the recruitment of

heterochromatin protein 1 (HP1) and SWI6, heterochromatin associated proteins. These protein complexes are known to recognize the methylated histone binding sites through their chromodomains thereby leading to changes in the chromatin packaging and preventing the access to the transcriptional factors (Jenuwein and Allis 2001; Rice and Allis 2001; Geiman and Robertson 2002). Studies using segments of mouse methyl CpG binding protein 2 (MeCP2) fused to Glutathione-S-transferase have shown role of DNA methylation and recruitment of HDAC complexes, mSin3A/HDAC, to the promoter of the target genes thereby leading to transcriptional silencing (Dobosy and Selker 2001).

1.14 Role of epigenetics in glial cell development

Cell-type specification and differentiation is controlled by epigenetic mechanism leading to the changes in chromatin structure and functions of many genes. HDACs have been well documented to play an important role in both neurogenesis and gliogenesis in ganglionic eminence and cortex-derived cultures in which HDACs were inhibited using inhibitors. Studies have also documented that HDACs are involved in specification and differentiation of oligodendrocytes (Marin-Husstege, Muggironi et al. 2002; Hsieh and Gage 2004; Shaked, Weissmuller et al. 2008). Inhibition/knock out of HDACs have shown to switch the fate between oligodendrocyte precursors and neurons (Liu, Han et al. 2007). Most of the work in literature discusses the role of HDACs in differentiation of neurons and oligodendrocytes with in the central nervous system in general. However not much is known about their role in differentiation

of glial cells in the optic nerve. In addition to histone deacetylation, are there other post translational modification also involved in transcriptional regulation does not seem to be clear. Whether the HDAC inhibition will lead to this fate switch between gliogenesis and neurogenesis in the developing optic nerve also has not been studied in detail yet.

Other investigators have hypothesized that neural stem cells and mature neurons maintain a state of global acetylation whereas the differentiated glial cells, astrocytes and oligodendrocytes, on the other hand maintain a state of global deacetylation (Hsieh et al. 2004). Based on our own observations we have put forth the following two-part working hypothesis: 1) Combinatorial role of specific HDACs are necessary for the differentiation of optic nerve astrocytes and oligodendrocytes and 2) inhibition of HDAC activity will block the fate of oligodendrocytes and astrocytes of the optic nerve. As a first step towards understanding the role of histone deacetylation in differentiation of astrocytes and oligodendrocytes with in the optic nerve, we examined whether the concept of a global state of deacetylation in differentiated cells holds true. Also to find answers to which of the classical HDACs are involved in the differentiation of glial cells, we characterized their spatial and temporal expression patterns in murine and chick optic nerve. These studies will lay the groundwork for determining which HDACs might be involved in the differentiation of optic nerve glia and will allow us to test the role of acetylation in glial cell differentiation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Animal husbandry and tissue collection

All animals used for this study were treated according to the ARVO statement for animal care. C57BL/6J mice were obtained from Jackson Labs and were housed in the Accreditation of Laboratory Animal Care (ALAC) accredited animal care facility in the School of Science on a 12 hours (hrs) light/dark cycle. For embryonic stages, mice were mated overnight and the females checked for vaginal plugs the next morning. The date the vaginal plug was detected was designated 0.5 days post coitum. At embryonic day 16 (E16), heads were removed and for postnatal stages, eyes were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C for 24 hours. Following fixation, eyes were rinsed twice in 1X phosphate buffered saline (PBS; potassium chloride 200 mg/L, potassium phosphate 200 mg/L, sodium chloride 8000 mg/L, and sodium phosphate 1150 mg/L), pH 7.5 and placed in a graded series of sucrose in 0.1 M phosphate buffer, starting at 5% and ending with 20%. Eyes were frozen in a 3:1 ratio of 20% sucrose in 0.1 M phosphate buffer to Optimal Cutting Temperature (OCT) solution and stored at -80°C until sectioned. Chicken eggs were ordered from University of

Michigan and stored at 16°C in a biochemical oxygen demand incubator (Jeio tech: IL-11A). Eggs were then moved to 38°C incubator (Kuhl, Flemington, NJ) and allowed to develop until E5, 8, or 18 and embryos (E5, E8) and dissected eyes (E18) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. E5 embryos were treated with 20% sucrose overnight while for E8 embryos and dissected E18 eyes were treated with a graded series of sucrose solutions from 5 to 20% in 0.1 M phosphate buffer, pH 7.4. Embryos and eyes were frozen in a 3:1 ratio of 20% sucrose in 0.1 M phosphate buffer to (OCT) solution and stored at -80°C.

2.2 Immunofluoroscence

Ten micron sections were cut with a Leica CM3050 S cryostat on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until used for immunohistochemistry. Slides were removed from the freezer, allowed to warm at room temp for 30-40 min, liquid blocker (Electron Microscopy) applied to the regions surrounding the sections, and sections post-fixed with 4% paraformaldehyde for 30 min at room temperature. Slides were washed in 1X PBS two minutes twice, incubated with methanol for 10 min. at room temperature and washed with 1X PBS for two minutes twice. Antigen retrieval was performed by incubating sections in 1.0% SDS in 1X PBS for 5 minutes at room temperature. Slides were washed for 5 minutes 3X at room temperature, followed by treatment with 1.0% sodium boro-hydrite for two minutes at room temperature to reduce auto fluorescence. After removal of

the sodium boro-hydride, sections were blocked with blocking solution (5% bovine serum albumin, 10% donkey serum, 0.25% triton X-100 in 1X Hanks balanced salt solution) for two hours at room temperature. Following removal of blocking solution, primary antibodies were added in their respective concentrations and incubated overnight at 4°C in a humid chamber. Dilutions and sources of each antibody can be found in Table I. In order to assess the specificity, antibodies were preabsorbed with 1.0 mg/ml of peptide or protein specified for each type of HDAC antibody we used for 1 hr prior.and immunofluorescence was performed as usual. For IgG controls, sections were incubated with 0.5 ug of purified IgG in 1 ml of blocking solution in place of the primary antibody and immunofluorescence performed as usual. Slides were then washed the next day in 1X PBS 10 min twice at room temperature and were then incubated with secondary antibodies (Alexa-fluor conjugated secondaries (Donkey anti rabbit 549) from Invitrogen and DyLight conjugated secondaries (Donkey anti goat 488 and Donkey anti mouse 546) from Jackson ImmunoResearch) dissolved in 1X PBS in 1:800 concentration for 1 hr in dark at room temperature. Secondary antibodies were removed by washing the slides in 1X PBS for 2 times 10 min each. Sections were mounted with Prolong gold anti fade containing DAPI. For the negative controls peptides corresponding to each HDAC were used to check the specificity of the HDACS as shown in Table 1. Immunolabeling was imaged using an Olympus Fluoview FV 10004 confocal microscope.

2.3 Real time-quantitative PCR (RT-qPCR)

Optic nerves were isolated from mice quickly following euthanasia and preserved in RNA later stabilizing agent (Qiagen; Cat # 76104) at -80°C until ready for total RNA extraction. Total RNA was isolated using High Pure RNA Tissue Kit (Roche; Cat # 12033674001) and prior to cDNA synthesis, the quality of the RNA to be used was checked. cDNA was synthesized through reverse transcriptase using iScript cDNA synthesis kit (Biorad; Cat # 170-8891). Real-time quantitative PCR reactions were performed on cDNA with Applied Biosystems Real Time PCR System (Model # 7300) using Power SYBR Green PCR Master Mix (Applied Biosystems; Cat # 4368706). The primer pairs used have been listed in Table 1. Efficiency of the primer sets was determined by the standard curve method, where efficiency,

$$E= (10(- 1/ (CT2 - CT1))- 1) X100$$

Microglobulin (B2M) was used as internal control for each cycle (Thal et al., 2008). The amplified samples were also checked to confirm if the amplification was of the right size. The change in the gene T expression levels was done using the The amplified samples were also checked to confirm if the amplification was of the right size. The change in the gene expression was tested using the delta delta CT method, where CT is the crossing threshold value,

ΔCT = CT Target gene – CT B2M and $\Delta\Delta CT = \Delta CT$ treated – ΔCT control

2.4 Statistical analysis

Using the unpaired t-test between the control and treated groups analyses of the RT-qPCR data was done statistically. SPSS software (IBM) and Excel 2010 (Microsoft) were used for this purpose.

2.5 SDS-PAGE and western analysis

Optic nerve samples were lysed using Radio-Immunoprecipitation Assay (RIPA) lysis buffer (5M Nacl, 1M Tris pH 8.0, 0.5 M EDTA, 5% Triton-X 100 supplemented with protease inhibitor cocktail (Roche; 04693116001) and 1 mM phenylmethanesulfonylfluoride (PMSF) on ice. Tissue lysates were centrifuged at 14000 rpm for 20 min at 4°C and supernatant was collected. Total protein concentration was determined using bicinchoninic acid assay (BCA) protein Assay reagent (Thermo Scientific). Proteins were resolved using SDS-PAGE (4-20% gradient gel), transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted using HDAC specific antibodies. The antibody concentration is specified in Table 2. The blots were probed for beta tubulin to check for equal protein loading. Densitometry analysis was performed using Image J 1.34 s software. Western data values were normalized with loading control and statistical comparisons were made by one way ANOVA with Bonferroni's post-test analysis using GraphPad Prism software (Version 5.0; GraphPad Software, Inc., San Diego, CA). $p<0.05$ was considered statistically significant.

2.6 Dissociation of glial cells from murine optic nerve

14 wks old C57BL/6J female mice were obtained from Jackson Labs. Optic nerves were dissected from optic chiasm to the optic nerve head. Small pieces of the dissected nerves were incubated in a solution containing DMEM with 0.02 M Hepes buffer and 0.25% trypsin (Sigma) in Tris-buffered saline (TBS), pH 7.7 with 0.02% collagenase (Worthington, Lakewood, NJ) for 20 min on a shaker at 37°C. This step was repeated for another 20 min. Nerve pieces were transferred to a solution containing trypsin in EDTA solution (SAFC Biosciences, Lenexa, Kansas) with 0.02% collagenase and placed on a shaker for 20 min at 37°C. Cells were then triturated 10-15 times using siliconized glass pipette in DNAse 0.4 µl/ml (Quiagen), trypsin inhibitor 0.05 mg/ml from Sigma and DMEM (Lonza). The resulting suspension was passed through a syringe with 25 gauge needle attached three times and filtered through a 0.45 µ mesh. Solution of 10% fetal calf serum (FCS) with sodium azide 0.2% in DMEM (Miller, David et al. 1985) was added to the final solution. A sample of the suspension was mixed with trypan blue and cells were counted using hemocytometer. Cells were diluted to 1.5 x 105 cells/ml and 600 µl was added to a cytospin and cells were spun onto vectabond coated slides at 8000 rpm for 2 min and imaged on Olympus FV1000 confocal microscope.

CHAPTER 3. RESULTS IN MICE

3.1 Co-localization of acetylated and methylated histone in murine optic nerve glial cells

In order to examine if the concept of global state of deacetylation in differentiated cells holds true, we co labeled murine optic nerve sections with markers for acetylated histone (H3K18) and methylated histone (H3K9) using triple label immunohistochemistry. Sections of E16, P5 and P30 stages mouse optic nerve were triple labeled with DAPI to label nucleus, acetylated histone specific to label histone 3 at lysine 18 loci (marker for transcriptionally active state) and methylated histone antibody that labels methylated histone 3 at lysine 9 (marker for transcriptionally inactive state) (Fig.14).

The analysis of the localization pattern for all the classical HDACs showed that the established belief that differentiated glial cells maintain a global state of deacetylation on differentiation does not seem to hold true. We did see glial cells showing expression of acetylated lysine with in their nucleus at all the three stages of optic nerve that we examined. There were also some cells that showed co-label for both the markers as shown by the arrow (Fig.14).

3.2 HDACs in developing murine optic nerve

3.2.1 mRNA Expression levels in the murine optic nerve glia

To determine if the large family of HDACs was expressed in the optic nerve, quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed on murine optic nerve samples for all the three stages. Three different stages of development were chosen for these studies; embryonic day 16 (E16) which denotes a period when astrocyte precursors are found in the optic stalk, postnatal day 5 (P5) when immature astrocytes and oligodendrocytes are found throughout the optic nerve, and P30 when optic nerve astrocytes and oligodendrocytes are mature (Dakubo, Wang et al. 2003; Petros, Williams et al. 2006; Pernet, Joly et al. 2008). RT-qPCR clearly indicated that HDACs 1-11 were all expressed during all stages of glial development examined in the murine optic nerve (Fig.16-20). Analysis was done in two ways. First, mRNA levels for all the HDACs were analyzed for all three stages relative to E16. Based on the level of expression of mRNA at P5 and P30 relative to E16 the HDACs were grouped into two as shown in (Fig.16 and 17). HDAC5, 9 and 11 were grouped separate from the rest of the classical HDACs as their level of expression was found to be lowest at P5 stage compared to the other two whereas all the other HDACs were found to have higher lever of expression at E16 in comparison to the other two stages. To directly compare the levels of mRNA for all the HDACs at a given stage, the data was also graphed so that the level of each HDAC mRNA was standardized to HDAC1 (Fig.18-20).

Comparatively, HDACs 5, 8, 9, and 10 were lower in levels than the other HDACs at E16, while HDAC 5, 8, 9, 10, and 11 were lower than the other HDACs at P5.

3.2.2 Patterns of HDAC localization in the developing optic nerve

The localization of each HDAC was addressed in this study using triple-label immunohistochemistry. The specificity of each antibody to be used was initially tested by western blot analysis of proteins isolated from E16, P5, and P30 mouse optic nerve. Each blot was also probed with β tubulin to normalize protein loading for subsequent densitometry readings (Fig.21). The antibodies used for these studies are included in Table 2. Unfortunately; many of the antibodies we tested appeared not to be specific for proteins of the predicted molecular weight using our protocols (Table 3).

To determine the patterns of expression for each HDAC in the developing optic nerve glia, sections of mouse optic nerve and optic nerve head were triple-labeled with DAPI to show nucleus, SOX2, which labels peripapillary and optic nerve glia (Stanke, Moose et al. 2010), and antibodies specific for each HDAC. The analysis of expression patterns in the murine optic nerve showed that there were 4 basic patterns into which we could group the HDACs; 1) predominantly nuclear with no to little cytoplasmic staining, 2) nuclear and cytoplasmic with both cytopasmic and nuclear label being apparent, 3) predominantly cytoplasmic with little to no nuclear label and 4) variable that showed differential expression

pattern depending upon stages examined (see Table 4). HDACs 1 and 2 were predominantly nuclear in localization in all the three stages of the murine optic nerve studied (Fig.23 and 24). HDACs 4, 6 and 11 showed both nuclear and cytoplasmic localization in all the stages (Fig.26, 28 and 32). HDAC5, 9 and 10 were predominantly found localized to the cytoplasm in all the three stages (Fig.27, 30 and 31). HDACs that showed variable response in expression pattern were 1) HDAC3 (Fig.25) predominantly cytoplasmic in E16 optic nerve section while cytoplasmic only in postnatal day 5 and 30 optic nerve sections studied and 2) HDAC8 (Fig.29) being predominantly cytoplasmic in localization at E16 and P5 stage and both nuclear and cytoplasmic in p30 optic nerve sections.

3.3 Expression of markers for glial cells in murine optic nerve

To prepare for the experiments in which HDAC activity will be inhibited to test their role in glial development, the number of astrocyte and oligodendrocyte precursors was quantitated. In order to get an estimate of O2A and astrocyte precursors the glial cells of the adult murine optic nerve were dissociated and attached to slides using a cytospin. Prepared slides were labeled for specific markers for the glial restricted precursors (GRPs) (NG2 -, PDGFRα - and A2B5 +), O2A precursors (NG2 +, PDGFRα + and A2B5 +) and astrocytes precursors (A2B5 +) (Liu and Rao 2004) (See Fig.43 as an example).

The quantitation of the labeled cells as shown in Fig.44 revealed that all the cell types were present. The glial cell precursors predominantly present were

O2A precursors (23%), astrocyte precursors/GRPs were found to be lowest in numbers (6%). 29% of the cells did not show label with any of the markers used for glial precursors and we assume that they may be either the mature glial cells types as they could not be identified in the absence of their specific markers.

CHAPTER 4. RESULTS IN CHICK

4.1 HDACs in developing chick optic nerve

4.1.1 Co-localization of acetylated and methylated histone in chick optic nerve glial cells

In order to examine if the concept of global state of deacetylation in differentiated cells holds true, we co labeled murine optic nerve sections with markers for acetylated histone (H3K18) and methylated histone (H3K9) using triple label immunohistochemistry. Sections from E5, E8 and E18 chick optic nerve were triple labeled with DAPI to show nucleus, acetylated lysine antibody to show acetylated histone 3 at lysine 18 position (marker for transcriptionally active state) and methylated histone antibody that labels methylated histone 3 at lysine 9 position (marker for transcriptionally inactive state) (Fig.15).

The analysis of the localization pattern showed, the common belief that differentiated glial cells maintain a global state of deacetylation does not seem to hold true. We did see glial cells showing expression of acetylated

lysine with in their nucleus at all the three stages of optic nerve that we examined. There were also some cells that showed co label for both the markers as shown by the arrow (Fig.15).

4.1.2 Patterns of HDAC localization in the developing optic nerve

To determine if there were species-specific differences in expression patterns of HDACs, murine patterns were compared with developing chick optic nerve. The localization of each HDAC was addressed in this study using triple-label immunohistochemistry. The specificity of each antibody to be used was initially tested by western blot analysis of proteins isolated from E5, E8, and E18 chick optic nerve (Fig.22). Each blot was also probed with β-tubulin which was used to normalize protein loading for subsequent densitometry readings. The antibodies used for these studies are included in Table 2.

To determine the patterns of expression for each HDAC in the developing chick optic nerve, sections were triple-labeled with DAPI to show the nucleus, SOX2, which labels peripapillary and optic nerve glia (Stanke, Moose et al. 2010), and antibodies specific for each HDAC. Similarly to the murine optic nerve, the localization patterns of HDACs were grouped into 4 basic patterns; 1) predominantly nuclear, HDACs with little to no cytoplasmic staining pattern, 2) nuclear and cytoplasmic were the HDACs showing both nuclear and cytoplasmic label, 3) predominantly cytoplasmic were the HDACs with little to no nuclear label and 4) variable that showed differential expression patterns depending

upon stages (see Table 5). HDAC1 and 2 were predominantly nuclear in localization in all the three stages of the chick optic nerve that we studied (Fig.33 and 34). HDAC3, 4, 5, 6, 8 and 11 showed both nuclear and cytoplasmic localization pattern (Fig.35, 36, 37, 38, 39, and 42). HDAC9 was predominantly cytoplasmic in localization in all the three stages (Fig.40). Variable expression pattern was observed for HDAC10 which was predominantly cytoplasmic at stage E5 and both nuclear and cytoplasmic in localization at E8 and E18 stage of optic nerve development (Fig.41).

4.1.3 Expression of markers for the glial cells in murine optic nerve

To prepare for the experiments in which HDAC activity will be inhibited to test their role in glial development, the number of astrocyte and oligodendrocyte precursors was quantitated. In order to get an estimate of O2A and astrocyte precursors the glial cells of the adult murine optic nerve were dissociated and attached to slides using a cytospin. Prepared slides were immunolabeled for specific markers for the glial restricted precursors (GRPs) (NG2 -, PDGFRα - and A2B5 +), O2A precursors (NG2 +, PDGFRα + and A2B5 +) and astrocytes precursors (A2B5 +) (Liu and Rao 2004) (See Fig.45 as an example).

The quantitation of the labeled cells as shown in Fig.46 revealed that all the cell types were present. The glial cell precursors predominantly present were O2A precursors (18.7%), astrocyte precursors/GRPs were found to be lowest

in numbers (5%). 56% of the cells did not show label with any of the markers used for glial precursors and we assume that that may be the mature glial cells or mesenchymal cells as we could not identify the mature cells yet.

CHAPTER 5. DISCUSSION

5.1 Summary

This study sought to determine the expression of classical HDACs during glial differentiation in mouse and chick optic nerve using RT-qPCR, western blotting, and immunohistochemistry. Our findings can be summarized as follows: 1) acetylation of histone 3 during optic nerve differentiation and co-localization of both acetylated and methylated histone within the nuclei of the differentiating glial cells indicate that both acetylation and deacetylation are required for astrocyte and oligodendrocyte development. 2) mRNA and protein for all of the classical HDACs were present at all of the developmental stages examined. 3) Majority of HDACs had similar patterns of mRNA and protein levels over time, with more present at earlier and less present at later stages of development. 4) The localization of the HDACs were grouped into four patterns; predominantly nuclear, predominantly cytoplasmic, both nuclear and cytoplasmic, and last a variable stage-dependent pattern. 1) HDACs 1 and 2 were predominantly nuclear throughout the optic nerve glial cell development in both murine and chick, 2) HDAC4, 6 and 11 in mouse and HDAC3, 4, 5, 6, 8 and 11 in chick were localized to nucleus as well as cytoplasm, 3) HDAC5, 9 and 10 in

mouse and HDAC9 in chick were localized predominantly to the cytoplasm, 4) variable pattern in localization were found for HDAC3 and 8 in murine optic nerve glial cells and HDAC10 in chick optic nerve glial cells during the different stages of development that were studied.

The HDAC localization patterns for chick and mouse were, not surprisingly, quite similar to one another. The HDACs which showed different patterns of localization in chick and mouse were 3, 5, 8, and 10. HDACs 3 and 8 showed relatively conservative changes; for example in chick HDAC3 was found in both nucleus and cytoplasm at E8 and E18 stage, whereas in mouse HDAC3 was found in both nucleus and cytoplasm at E16 stage in development. HDACs 5 and 10, on the other hand, showed non-conservative changes; both 5 and 10 were predominantly localized to the cytoplasm in mouse, but 5 was nuclear and cytoplasmic and 10 variable in the chick.

5.2 Cells in the developing optic nerve do not appear to be globally acetylated or deacetylated

Several studies have concluded that differentiating neurons are globally acetylated (Hsieh, Nakashima et al. 2004; Schwechter, Millet et al. 2007), while other studies have proposed that de-acetylation is the key to the differentiation of oligodendrocytes (Marin-Husstege, Muggironi et al. 2002; Shen, Li et al. 2005; Liu, Hu et al. 2007; Liu, Hu et al. 2008; Liu, Hu et al. 2009; Swiss, Nguyen et al. 2011; Conway, O'Bara et al. 2012). Our data showing co label for both

methylated and acetylated histone markers is consistent with the possibility of combinatorial role of both acetylation and deacetylation in glial cell differentiation with in the optic nerve. Methylated histone marker was used to label our slides in order to access the state of transcriptional repression as HDACs have been shown to be playing an indirect role in methylation of the histone and it was not possible for us to co label all the sections with 11 different HDACs.

5.3 Effect of HDAC inhibition on the differentiation of the glial cells in optic nerve

Studies have shown the role of HDACs in the differentiation of neurons and oligodendrocytes (Marin-Husstege, Muggironi et al. 2002; Siebzehnrubl, Buslei et al. 2007). Of all the classical HDACs, HDAC1, 2 and 11 have been shown to play significant role in the differentiation of oligodendrocytes (Liu, Hu et al. 2009; Ye, Chen et al. 2009). Inhibition of HDACs brings changes in the number of astrocytes (Marin-Husstege, Muggironi et al. 2002; Hsieh, Nakashima et al. 2004; Siebzehnrubl, Buslei et al. 2007). HDAC activity appears to play a role in cells arising from the ganglionic eminences of the telencephalon in which the HDACs act as a fate switch from neurons to astrocytes (Shaked, Weissmuller et al. 2008). However, the epigenetic role of the HDACs in the differentiation of astrocytes in the optic nerve is yet not fully understood. Some of our data is consistent with the idea that optic nerve astrocytes and oligodendrocytes might require deacetylation for their differentiation; we found there are seven of the classic HDACs (1, 2, 3, 6, 8, 10, 11) present in the nucleus in at least one or

more stages during the differentiation. However, our data are consistent with many more HDACs being involved in the process than HDAC1, 2 and 11 proposed by other investigators. In order for us to determine exactly which HDACs are involved in gliogenesis, much more needs to be understood concerning the gene targets and interacting partners for each HDAC.

5.4 Targets of different HDACs

HDACs do not come directly in contact with DNA; rather, a complex group of proteins recruit HDACs to specific cis-acting elements on the untranslated regulatory regions in the genome (Torchia, Glass et al. 1998). Transcription factors, co-repressors, and/or co-activators, are some important members of this complex protein machinery (Ballas and Mandel 2005). The repressor element transcription factor (REST-Co-REST) complex, a well-known suppressor for neuronal genes, is an example of such transcriptional regulation. REST, an important transcription regulator that interacts with repressor element 1 (RE1) sites on neuronal genes, serves as a binding site for Co-repressor (Co-REST) (Andres, Burger et al. 1999). Histone modifying enzymes, especially HDAC1 and 2 then bind to the repression domains present on Co-REST and are recruited to the specific loci on histone tails, thereby leading to the deacetylation of the lysine chains (Abrajano, Qureshi et al. 2009). We specifically focused on determining HDACs that were found in the nucleus at one or more stages of development to narrow down which HDACs may modify histones, and by extension play a role in gene regulation. HDACs, once known to target only the histone proteins, are

now known to interact with many non-histone proteins as well. They have been shown to interact with other proteins when present in the cytoplasm as well. Various proteins have shown a change in their structural and functional ability as a result of deacetylation by the HDACs. For instance, HDAC6 has been established to interact with cytoplasmic proteins like tubulin and chaperons like heat shock protiens (Hsp90), causing changes in the contractile functions of the muscles (Marks and Xu 2009). Similiarly, HDAC8 has been shown to deacetylate heat shock protein 20 (Hsp20) in the myometrium (Karolczak-Bayatti, Sweeney et al. 2011). HDACs also bring about changes to the proliferation and differentiation of cells by targeting transcriptional factors (p53, HIF1α, E2F, p21, c-Myc and Bcl-6), signaling mediators (STAT3, SMAD7), DNA repair enzymes (Ku70) and nuclear import proteins like importin-α7 (Dokmanovic, Clarke et al. 2007; Marks and Xu 2009).

The role of HDACs during the differentiation of neurons, astrocytes and oligodendrocytes has been tested using HDAC inhibitors such as trichostatin A (TSA). Studies have shown alteration in the expression of neuronal genes like Neurod D1, Hes1, Numb, astrocytic genes like BMP6, S100β and genes related to oligodendrocytes differentiation like PDGFRα, Sox6 and Hes5 (Swiss, Nguyen et al. 2011). Last but not least, HDACs have also found to effect renal development by regulation of transcription factors like PAX2, PAX8 and p21 (Rosenberg, Chen et al. 2011).

Studies suggesting the role of HDAC inhibitors (TSA, sodium butyrate, SAHA and Valproic acid) as treatment options for various cancers and diseases like Parkinsonism, Alzheimer and many others suggests that there is a wide area that still needs to be explored regarding the role of HDACs with various regulatory proteins and understanding the mechanism involved (Zarate, Singh et al. 2006).

5.5 HDACs as transcriptional activators

HDACs have been well established to play a role in transcriptional repression via deacetylation of histones, but studies related to gene regulation have now shown that this does not always hold true (Pazin and Kadonaga 1997; Nusinzon and Horvath 2005). HDACs have been shown to act as transcriptional activators under certain situations. Examples include: 1) HDAC1 regulation of the transcriptional machinery for the MMTV promoter (Smith 2008), 2) HDAC1 and 2 act as transcriptional activators for the B cell lymphoma 2 (bcl2) gene, a well-known anti-apoptotic gene (Duan, Heckman et al. 2005), and 3) HDAC6 upregulation of interferon β (IFNβ) (Nusinzon and Horvath 2006; Smith 2008). Hence, it is quite possible that some of the nuclear localized HDACs in our study may be acting as transcriptional activators.

5.6 Conclusions

All the classical HDACs are present in the glial cells, during different stages of development in both murine and chick optic nerve. Based on the localization

pattern we could categorize the classical HDACs into four different groups; predominantly nuclear, nuclear and cytoplasmic, predominantly cytoplasmic and variable. Six of the classical HDACs (1, 2, 3, 4, 6, and 11) are present in the nucleus in at least one or more stages during glial cell differentiation in murine optic nerve whereas nine of the classical HDACs (1, 2, 3, 4, 5, 6, 8, 10 and 11) have been found to be localized to the nucleus at one or more stages of glial cells development in chick optic nerve. Understanding the localization pattern of HDACs in optic nerve will help us to study their epigenetic role in the differentiation of the glial cell types in the optic nerve, an area that has not been yet fully explored.

In conclusion this project has provided an insight into which HDACs are nuclear in localization within optic nerve glia. It sets a platform for further research to determine the epigenetic role of these HDACs in the development of glial cell types. Further, our data also provide a baseline for understanding potential role of epigenetic changes that occur during disease processes such as glaucoma and diabetes.

5.7 Future directions

To further understand the role of HDACs during the glial cell differentiation in optic nerve we are focused on answering the following questions:

1) Which of the classical HDACs are playing a direct role in the differentiation of glial cells in the optic nerve?

2) What changes in the differentiation of oligodendrocytes and astrocytes in the optic nerve will take place when we inhibit HDAC activity?

3) Which genes do the various HDACs target during glial differentiation?

4) What happens to HDAC activity during diseases such as glaucoma and diabetes that affect the optic nerve?

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TABLES

Table 1 List of primers used in qPCR

Gene	Accession Number	Primer	Sequence	Product Length
HDAC1	NM_008228.2	Forward	TGGGGCTGGCAAAGGCAAGT	130
		Reverse	GACCACTGCACTAGGCTGGAACA	
HDAC2	NM_ 008229.2	Forward	CGTACAGTCAAGGAGGCGGCAA	97
		Reverse	TGAGGCTTCATGGGATGACCCTGG	
HDAC3	NM_010411.2	Forward	ACGTGCATCGTGCTCCAGTGT	150
		Reverse	AGTGTAGCCACCACCTCCCAGT	
HDAC4	NM_207225.1	Forward	AGCTCTGGCAACGTCAGCACT	114
		Reverse	AAGTGGGGCGACTGAGCCTTCT	
HDAC5	NM_ 001077696.1	Forward	ACGTAAATGTGGCGTGGACAGGA	147
		Reverse	TTCAACAGCATCAAACCCAGGCGGA	
HDAC6	NM_ 010413.3	Forward	TGGGGCTTCAAGG GCTGGATCT	148
		Reverse	TGCTCTCTGATGGC ATGGAGCC	
HDAC7	NM_001204275.1	Forward	GCTCAGCATGTGCATGTGGAACAC	132
		Reverse	TGAGAGCCTGGTGTGTCTGGCT	
HDAC8	NM_ 027382.3	Forward	ATGACACCAGTGGTCGGCAA	105
		Reverse	ACGAGCCGTGTTGGCAAGGTT	
HDAC9	NM_ 024124.3	Forward	TGCACCTTTGCCTCAGAGCACG	150
		Reverse	TGGCTGCCTGGTTGCTTCAGT	
HDAC10	NM_ 199198.2	Forward	TAGCAGCCAAACATGCCAAGCAGA	143
		Reverse	ATGCTCATAGCGGTGCCAAGAGAAA	
HDAC11	NM_ 144919.2	Forward	GCTGGGAAATGGGGCAAGGTGA	134
		Reverse	AGCTCGTTGAGATAGCGCCTCGT	
β2 micro globulin	NM_ 009735.3	Forward	TCGCGGTCGCTTCAGTCGTC	116
		Reverse	CATTCTCCGGTGGGTGGCGTG	

Table 2 List of antibodies used for western blots and immunofluorescence

Antibody	Source	Antibody Dilution		Peptide Supplier
		IHC	Westerns	
Rabbit Anti-HDAC 1	Abcam (Cambridge, MA)	1:300	1:2500	Abcam (Cambridge, MA)
Mouse Anti-HDAC 2	Abcam (Cambridge, MA)	1:350	1:2500	Abcam (Cambridge, MA)
Rabbit Anti-HDAC 3	Abcam (Cambridge, MA)	1:300	1:2500	Abcam (Cambridge, MA)
Mouse Anti-HDAC 4	21st Century (Marlboro, MA)	1:100	1:1000	21st Century (Marlboro, MA)
Rabbit Anti-HDAC 5	Abcam (Cambridge, MA)	1:100	1:500	Santa Cruz (Santa Cruz, CA)
Rabbit Anti-HDAC 6	Life Span Biosciences	1:250	1:500	Santa Cruz (Santa Cruz, CA)
Rabbit Anti-HDAC 8 (Cat # sc11405)	Life Span Biosciences	1:500	1:1000	Life Span Biosciences
Rabbit Anti-HDAC 9	Abcam (Cambridge, MA)	1:250	1:500	Abcam (Cambridge, MA)
Rabbit Anti-HDAC 10	Novus Biologicals (Littleton, Co)	1:100	1:500	Santa Cruz (Santa Cruz,
Rabbit Anti-HDAC 11	GeneTex (Irvine, CA)	1:100	1:500	Santa Cruz (Santa Cruz, CA)
Rabbit Anti-Acetylated	Abcam (Cambridge, MA)	1:100	N/A	N/A
Goat Anti-SOX2 (Cat # sc17320)	Santa Cruz (Santa Cruz, CA)	1:250	N/A	N/A
Mouse anti- tubulin	Sigma (St. Louis, MO)	N/A	1:1000	N/A
Mouse Anti-methylated histone	Abcam (Cambridge, MA)	1:300	N/A	N/A

Table 3 List of unsuccessful antibodies		
Primary Antibody	Catalogue No.	Source
HDAC 4	AP08440PU-N	Acris
HDAC 4	ab79521	Abcam
HDAC 4	HO163	Sigma
HDAC 4	PA1-25454	Thermo Scientific
HDAC 5	ab1439	Abcam
HDAC 5	2082	Cell Signalling
HDAC 5	AP02669PU-N	Acris
HDAC 5	PA1-14188	Thermo Scientific
HDAC7	3607-100	Bio Vision
HDAC7	CB-HDAC7	21st century
HDAC7	LS-C118482	Life Span Biosciences
HDAC7	IHC-00283	Bethyl Laboratories
HDAC7	LS-B7206	Life Span Biosciences
HDAC 11	ab18973	ABCAM
HDAC 11	07-1548	Millipore
HDAC 11 (Y-25)	sc-130776	Santa Cruz
HDAC 11 (N-term)	AP1111a	Abgent
Rabbit Anti-HDAC 11	Cat # PA5-11250	Thermo Scientific

Table 4 Localization of HDACs in murine optic nerve				
Stages	Predominantly nuclear	Nuclear and cytoplasmic	Predominantly cytoplasmic	Variable
E16	1 and 2	4, 6 and 11	5, 9 and 10	HDAC3 and 8 (Predominantly cytoplasmic)
P5	1 and 2	4, 6 and 11	5, 9 and 10	HDAC3 (cytoplasmic) HDAC8 (Predominantly cytoplasmic)
P30	1 and 2	4, 6 and 11	5, 9 and 10	HDAC3 (cytoplasmic) HDAC8 (Nuclear and cytoplasmic)

Table 5 Localization of HDACs in chick optic nerve				
Stages	Predominantly nuclear	Nuclear and cytoplasmic	Predominantly cytoplasmic	Variable
E5	1 and 2	3,4, 5, 6,8 and 11	9	HDAC10 (Predominantly cytoplasmic)
E8	1 and 2	3,4, 5, 6,8 and 11	9	HDAC10 (Nuclear and cytoplasmic)
E18	1 and 2	3,4, 5, 6,8 and 11	9	HDAC10 (Nuclear and cytoplasmic)

FIGURES

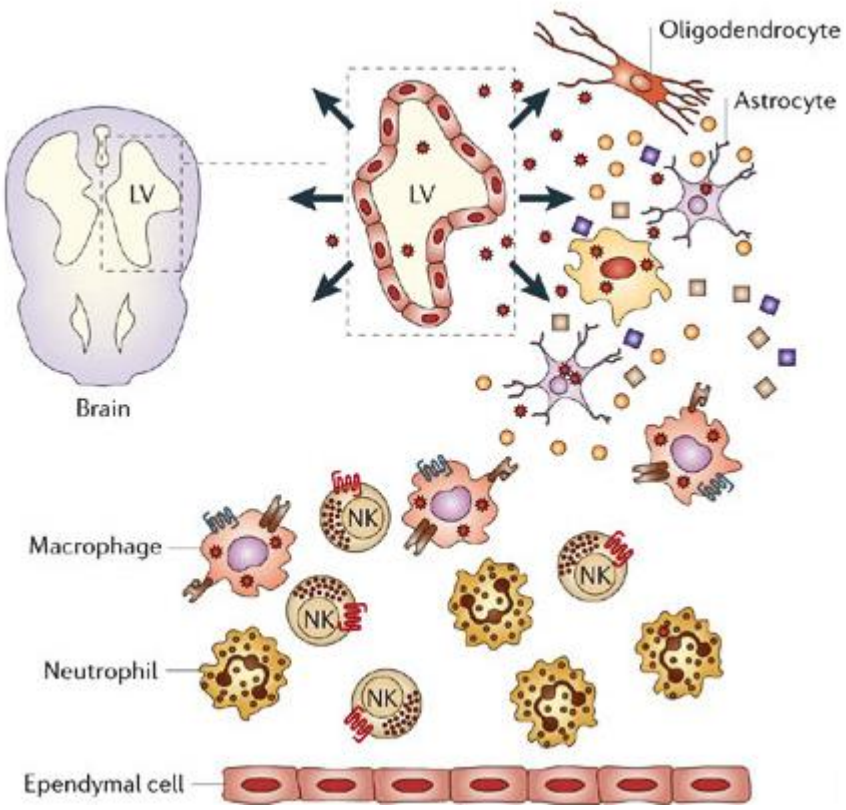

Fig.1 Glial cells in the central nervous system: Cells in the central nervous system are broadly grouped as neurons and the glial cells. Glial cells are further divided mainly into two groups; macroglia or microglia cells (Bergmann, Lane et al. 2006).

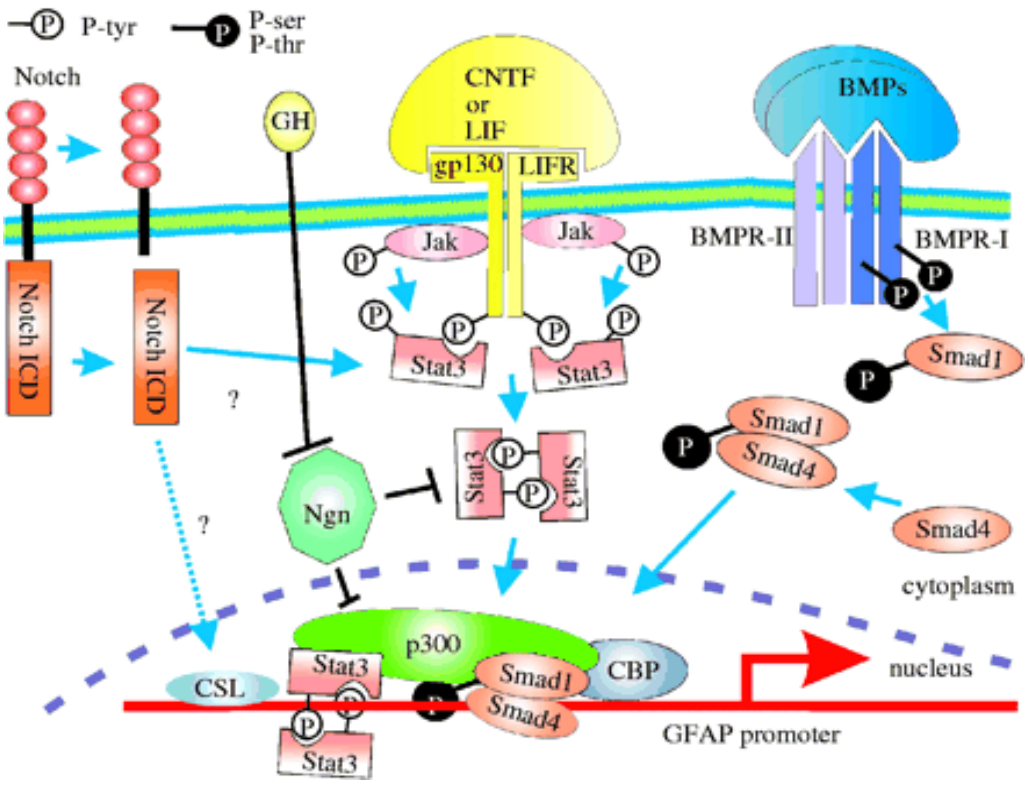

Fig.2 Specification of astrocytes: Both ciliary neurotrophic factor leukemia inhibitory factor and bone morphogenetic protein pathway function in parallel thereby inducing GFAP expression. This is thought to be a common mechanism by which most of the astrocytic genes might be regulated (Kessaris, Pringle et al. 2008).

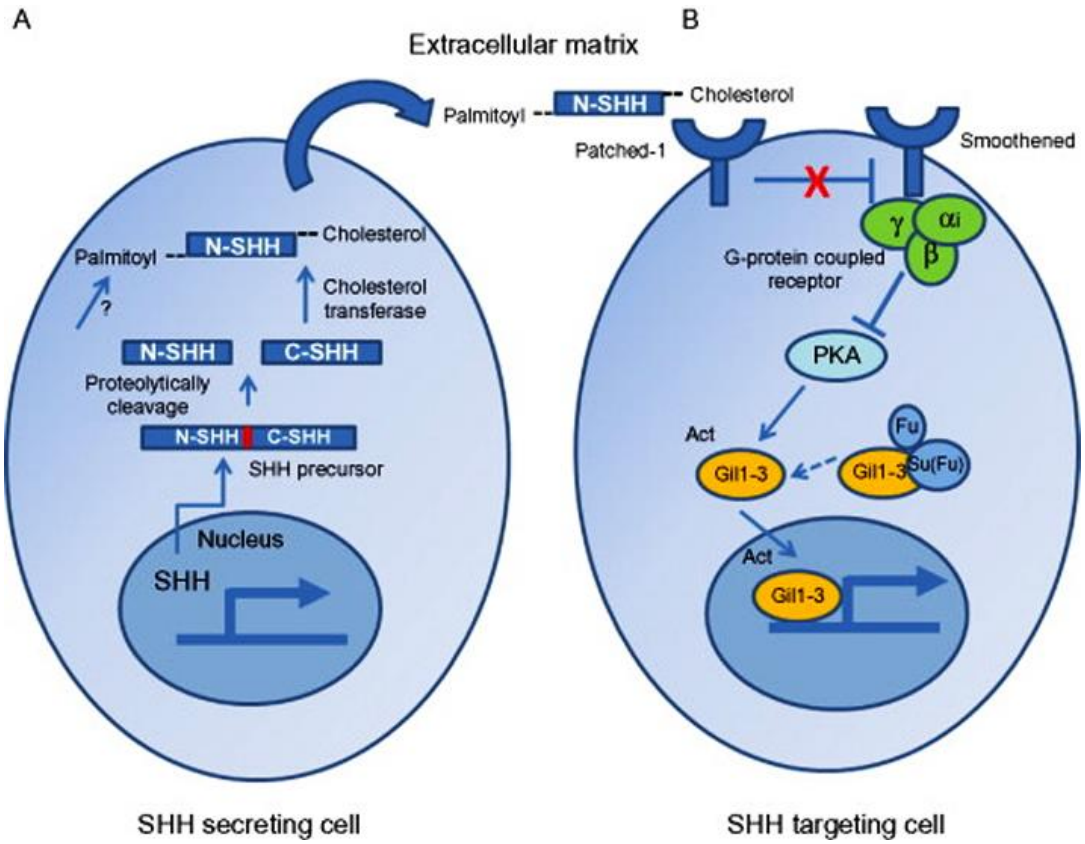

Fig.3 SHH signaling pathway: A-B show the SHH signaling pathway in vertebrates. (A) Precursor SHH is converted to N-SHH. It is N-SHH that is responsible for the signaling in vertebrates. (B) When SHH binds to PTC, the inhibition on SMO is released, this in turn activates GLI family of transcription factors by inhibiting phosphorylating activity of PKA. PKA along with fused (Fu) and [Su (Fu)] regulate the nuclear localization of GLI (Marti and Bovolenta 2002).

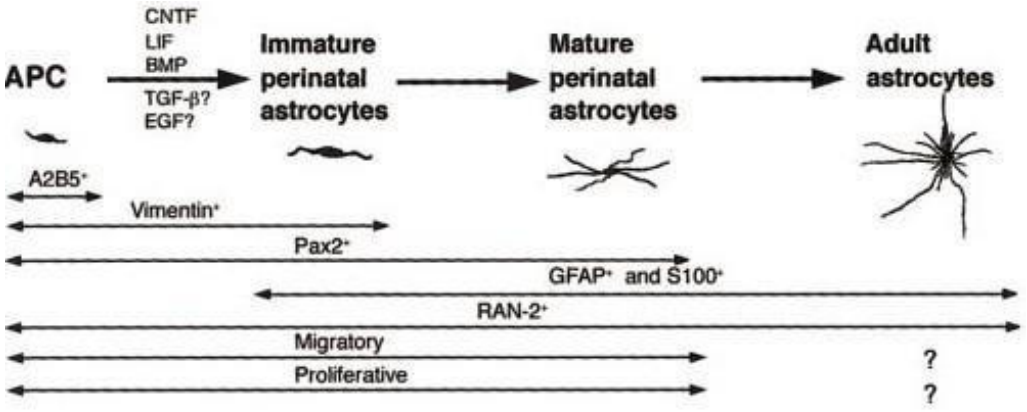

Fig.4 Differentiation of the astrocytic lineage cells in the optic nerve: Mature astrocytes are formed through progressive stages of differentiation. Three different stages of paired homeobox 2 (PAX2) positive astrocytic lineages have been described in the optic nerve; 1) Astrocyte precursor cells (APCs) are PAX2 +, vimentin +, and GFAP -, 2) Immature perinatal astrocytes are PAX2 +, vimentin +, and GFAP + and 3) Mature perinatal astrocytes are PAX2 +, vimentin -, and GFAP ++ (Chu, Hughes et al. 2001).

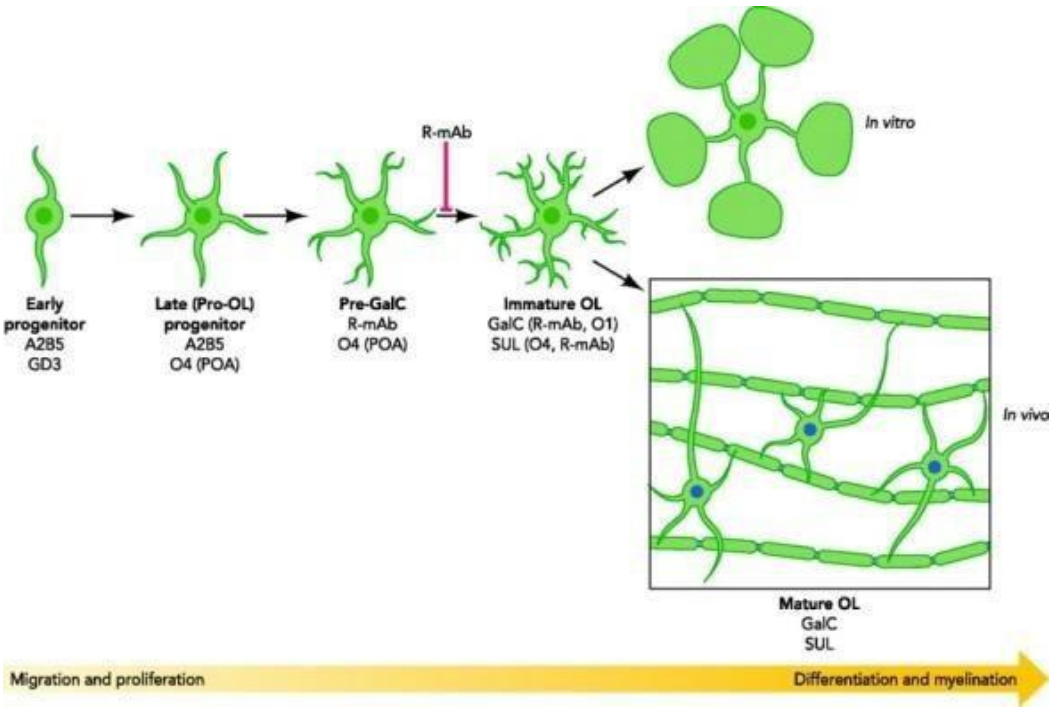

Fig.5 Stages of differentiation for oligodendrocytes: Oligodendrocytes, like astrocytes, also undergo process of differentiation through various stages of maturation. Specific markers have been established to identify the different stages of differentiation for oligodendrocytes, precursors, immature as well as mature (Jackman, Ishii et al. 2000).

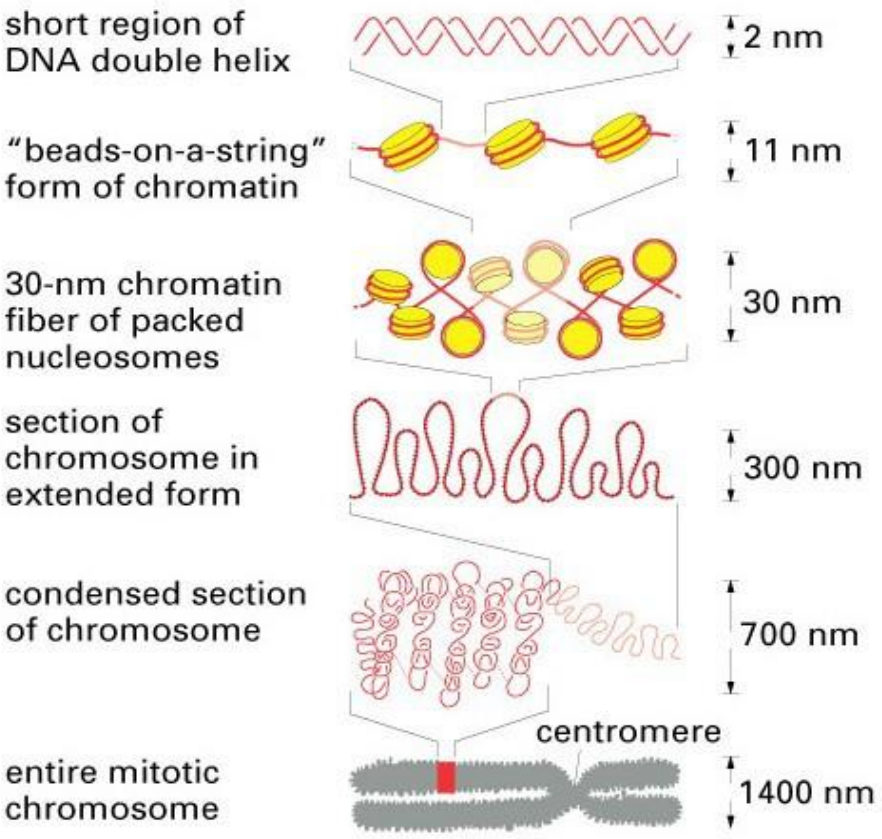

Fig.6 Chromatin structure and level of organization: Nucleosomes are composed of a core of the histone octamer, H2A, H2B, H3, and H4, with DNA being wrapped around the histones. DNA and protein inside the nucleus of the cell constitute the chromatin network (Essential cell biology 2nd ed, 2004).

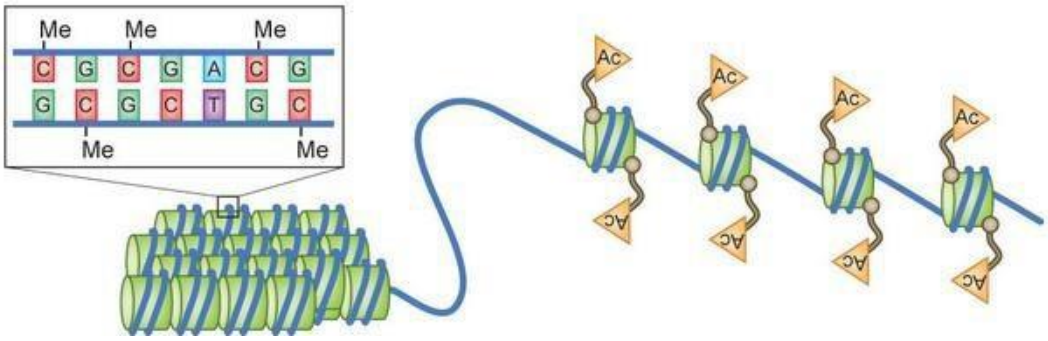

Fig.7 Histone acetylation and transcriptional activation: Histone acetylation and deacetylation is controlled by the histone acetyltransferase (HATs) and the histone deacetylase (HDACs) families of enzymes. Acetylation neutralizes the positive charge on the histones chains and decreases the ability of the histones to bind to DNA. This decreased binding allows to open up the DNA that provides access to the transcription factors to the sites they must bind to in order to affect transcription (Korzus 2010).

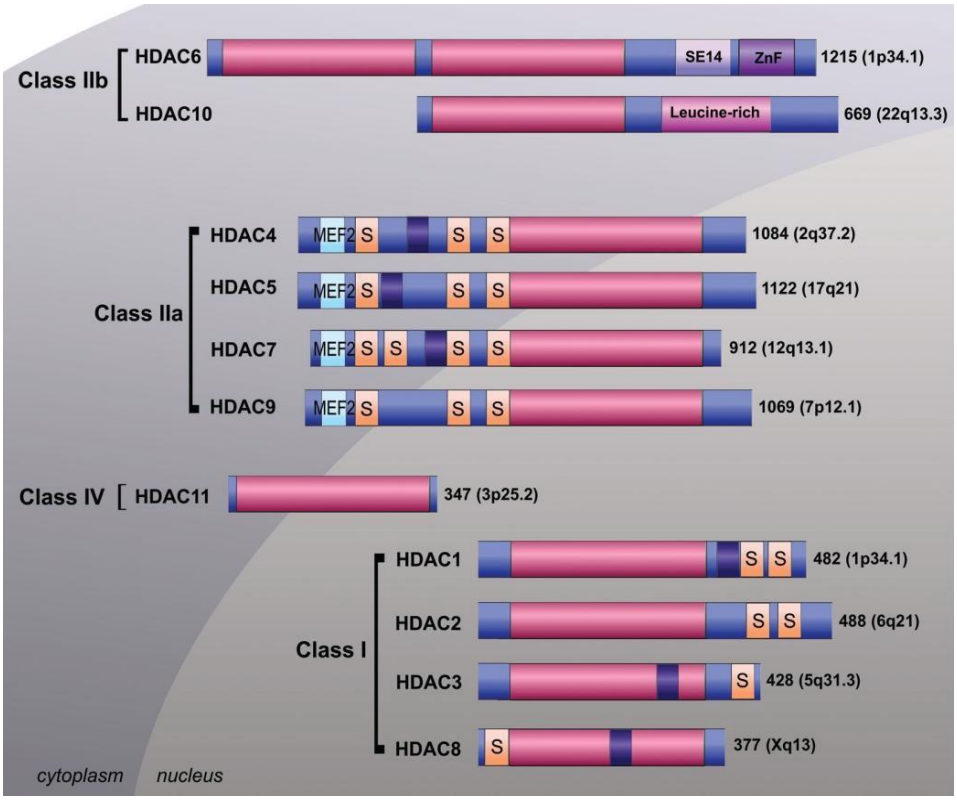

Fig.8 Classification of histone deacetylase enzymes: HDACs have been classified into four major groups based on their sequence similarities. The classical HDACs are categorized into class 1 (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9, and 10), and class IV (HDAC11) on the basis of their homology to yeast proteins. Class 3 in mammals includes the sirtunins and is also known as the atypical HDACs. In the figure DNA catalytic domains are shown in pink, the nuclear localization signal in purple,(Karagiannis and Maulik 2012).

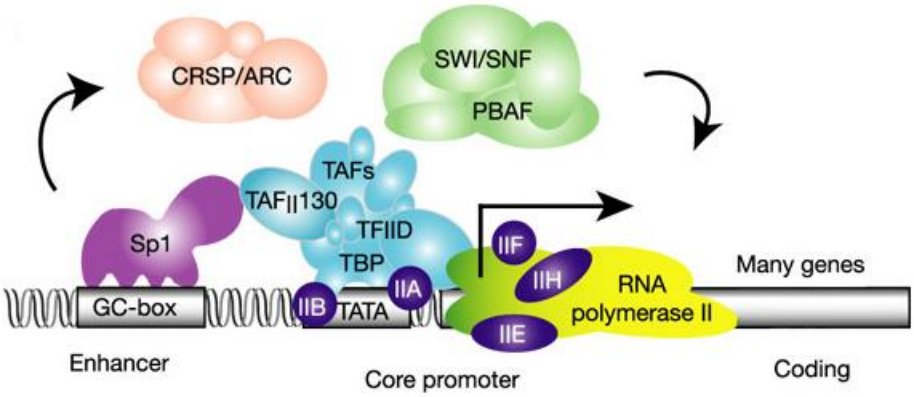

Fig.9 Transcription initiation complex: Transcriptional unit in eukaryotes is made up of enhancer, core promoter, also known as the transcription start site and initiator element. Transcription factors are involved in the initiation of gene transcription along with RNA polymerase II (Levine and Tjian 2003).

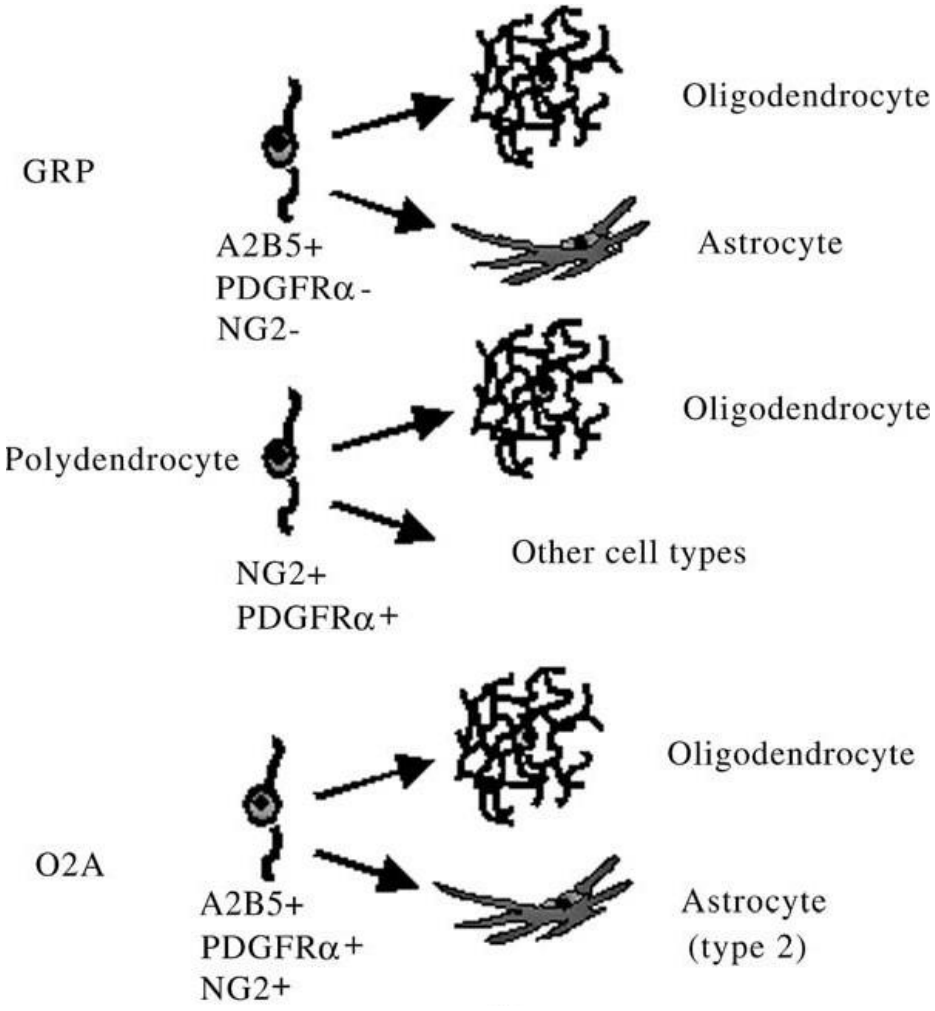

Fig.10 Expression of markers for the glial cells: Glial restricted precursors (GRPs) differentiate into either oligodendrocytes or astrocytes and are known to be positively stained for A2B5. Polydendrocytes can differentiate either into oligodendrocytes or other cell types and are stained positively with both NG2 and PDGFRα. O2A precursors are A2B5 +, NG2 + and PDGFRα + and can either give rise to both oligodendrocytes and type 2 astrocytes (Liu and Rao 2004).

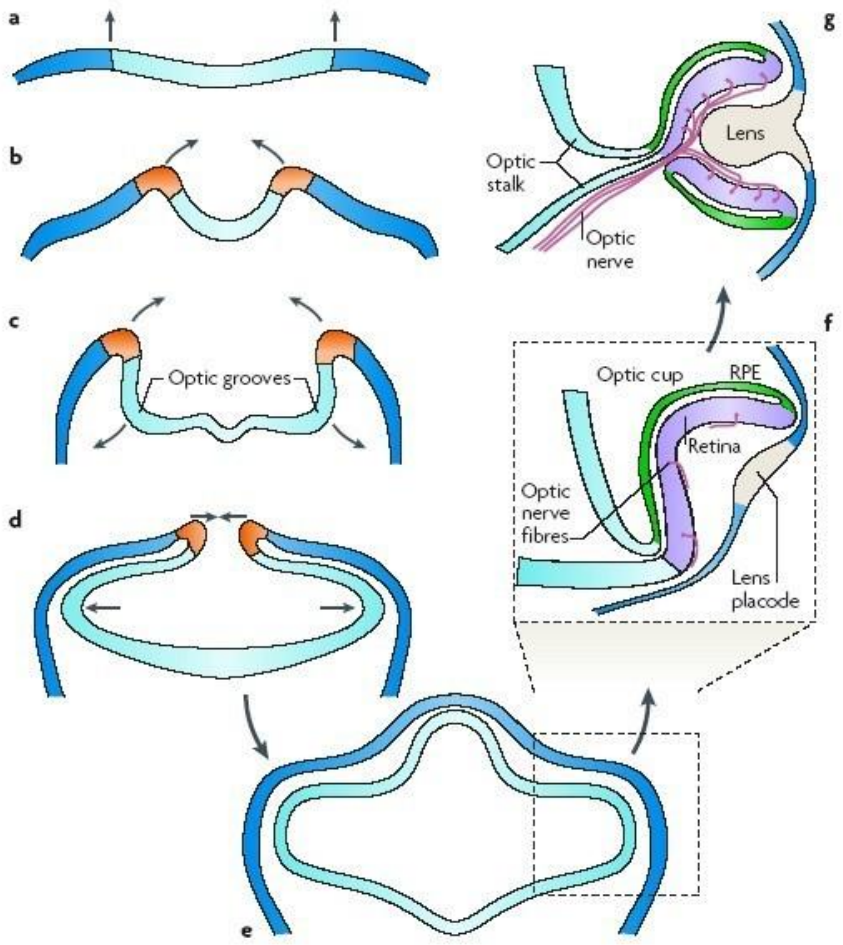

Fig.11 Vertebrate eye development: Developement of the eye takes place with the bifurcation of the eye field into two as the neural plate begins to form neural tube (a-d). As this is occurring, the two pits on either side now become optic grooves which further enlarge to form the optic vesicles (e). Optic vesicle further invaginate to form the double layered optic cup (g). The inner part of the optic cup becomes the multi-layered neural retina and the outer layer becomes the retinal pigment epithelium (Lamb, Collin et al. 2007).

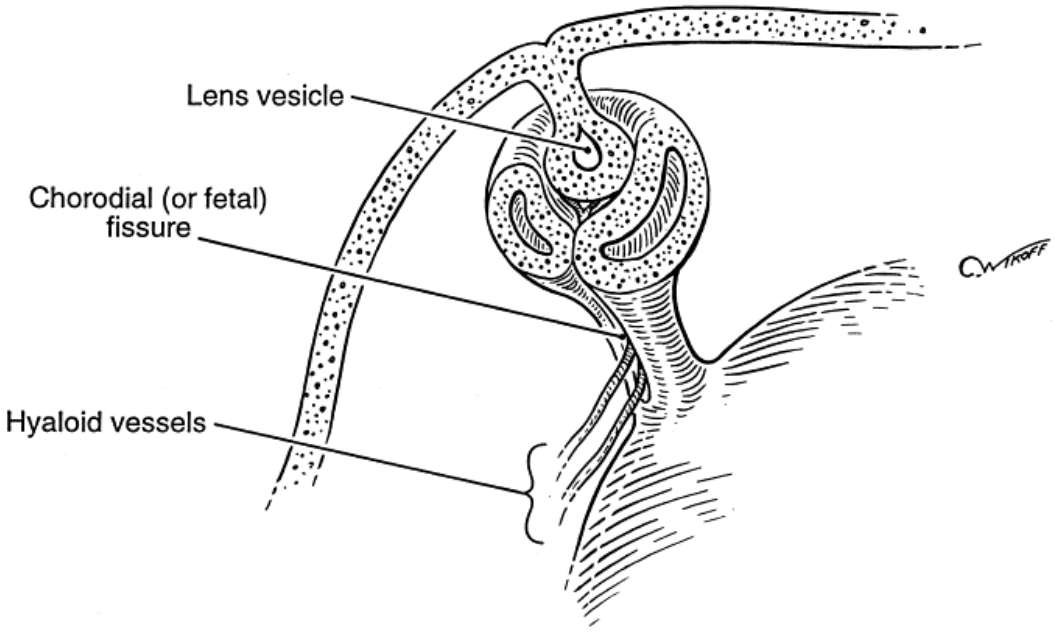

Fig.12 Margins of optic cup enlarging and sealing off at the choroidal fissure: The choroid fissure has been described to develop in the ventral optic cup due to the invagination of optic vesicle.It is the opening from where optic nerve is believed to exit the eye Hyaloid vessels run through this choroidal fissure. As development progresses the choroid fissure closes eventually as a result of continued proliferation of the two lips of the ventral retina adjacent to the fissure and eventual fusion of the two lips together to form the complete globe (Onwochei, Simon et al. 2000).

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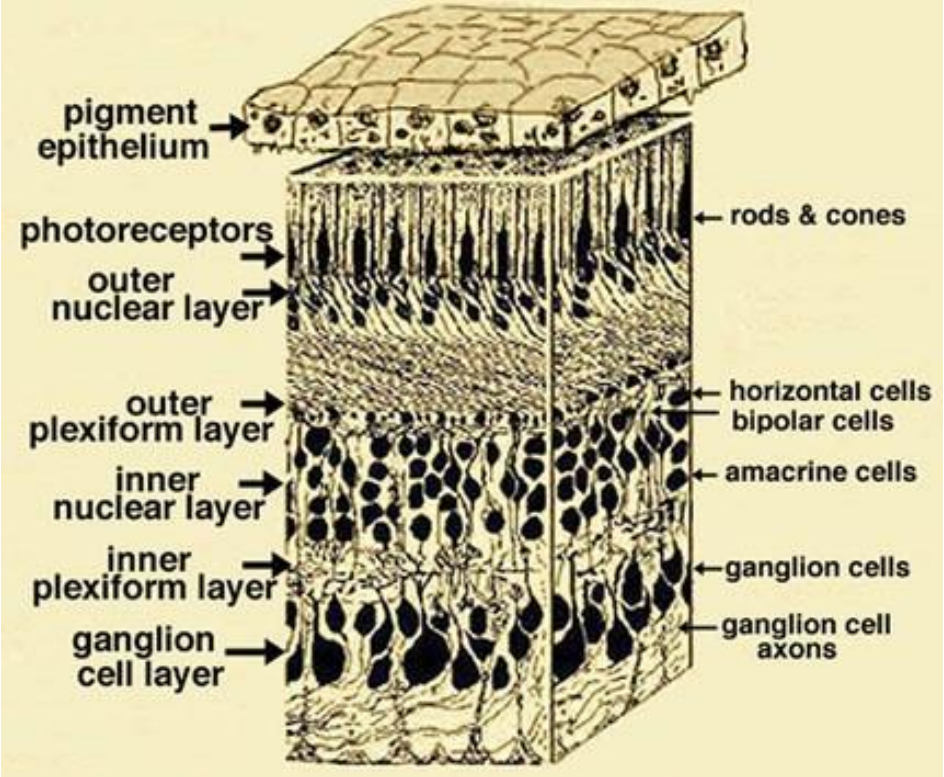

Fig.13 Mature retina and the retinal cell types: The retina has been shown to be made up of different layers; 1) the retinal pigment epithelium (RPE), 2) the outer nuclear layer contains the rod and cone photoreceptors, 3) the outer plexiform layer contains synaptic contact between the photoreceptors, bipolar cells and horizontal cells, 4) the inner nuclear layer contains amacrine cells, horizontal cells, bipolar cells and muller glial cells, 5) inner plexiform layer consists of synapses between the bipolar, amacrine and the ganglion cells, 6) ganglion cell layer contains the ganglion cells, misplaced amacrine cells and retinal astrocytes and 7) nerve fibre layer carrying axons from ganglion cells (Kolb 1995).

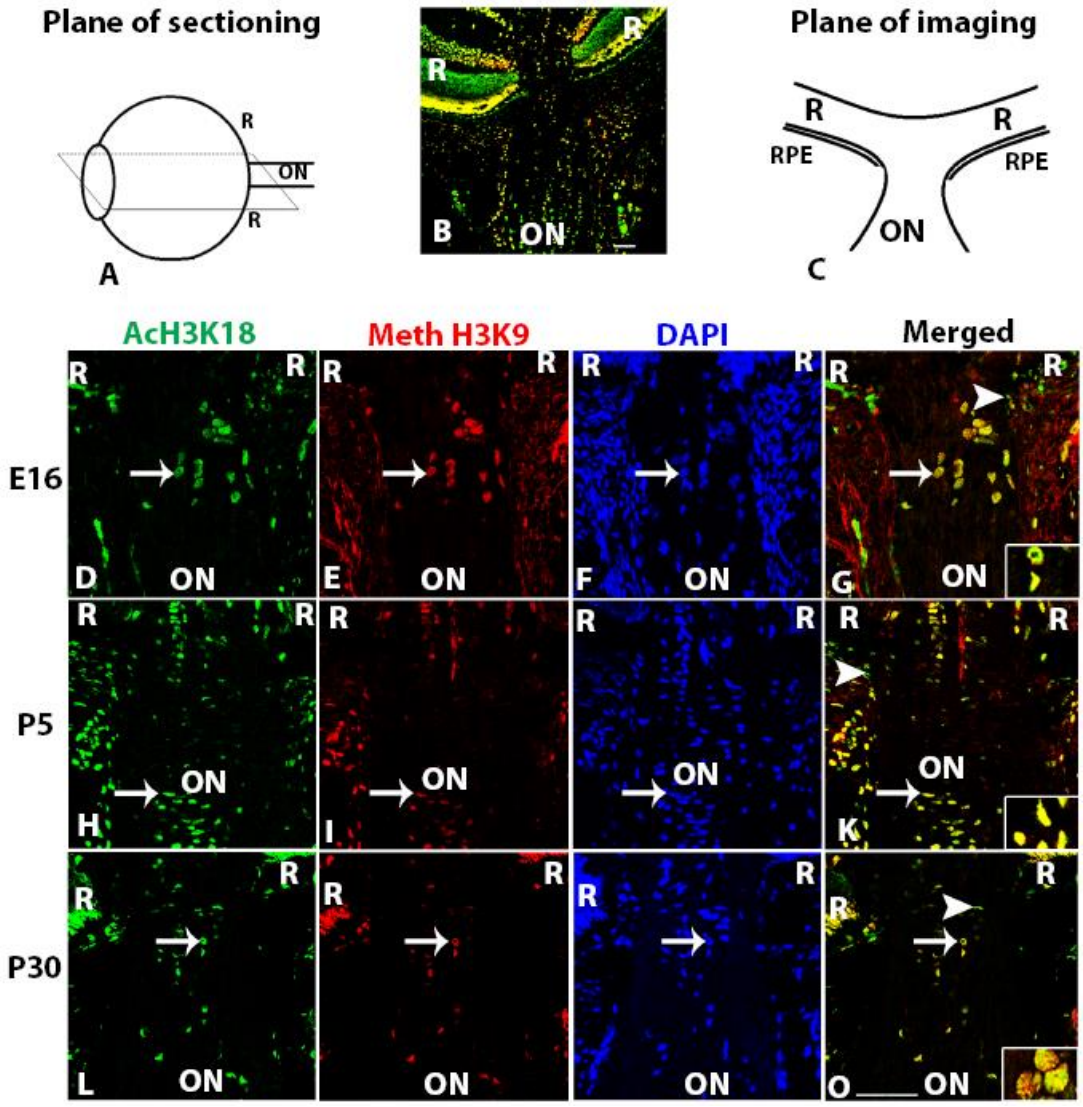

Fig.14 Histone acetylation and methylation in glial cells in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina (B) and plane of imaging to aid in orientation of the sections is shown in C. Drawing A shows the plane of sectioning for the optic nerve. Confocal imaging of AcH3K18, MethH3K9, and DAPI triple label of sections of murine optic nerve at E16 (D, E, F and G), P5 (H, I, J and K) and P30 (L, M, N and

O). Overlay of acetylated histone (green) and methylated histone marker (red) for all three stages (G, K and O) depicts glial cells during differentiation show localization of acetylated histone as well as methylated histone within their nuclei. Higher magnification views as shown in 60x magnification panels (insets G, K and O) show co-localization for both the markers. Arrows in panels D-G, H-K and L-O are pointing to the cells that show label for both the markers whereas the arrow heads are pointing to the cells that do not show a co label. Scale bars: (B) Bar = 50 µm, (G) Bar = 50 µm for images D-O.

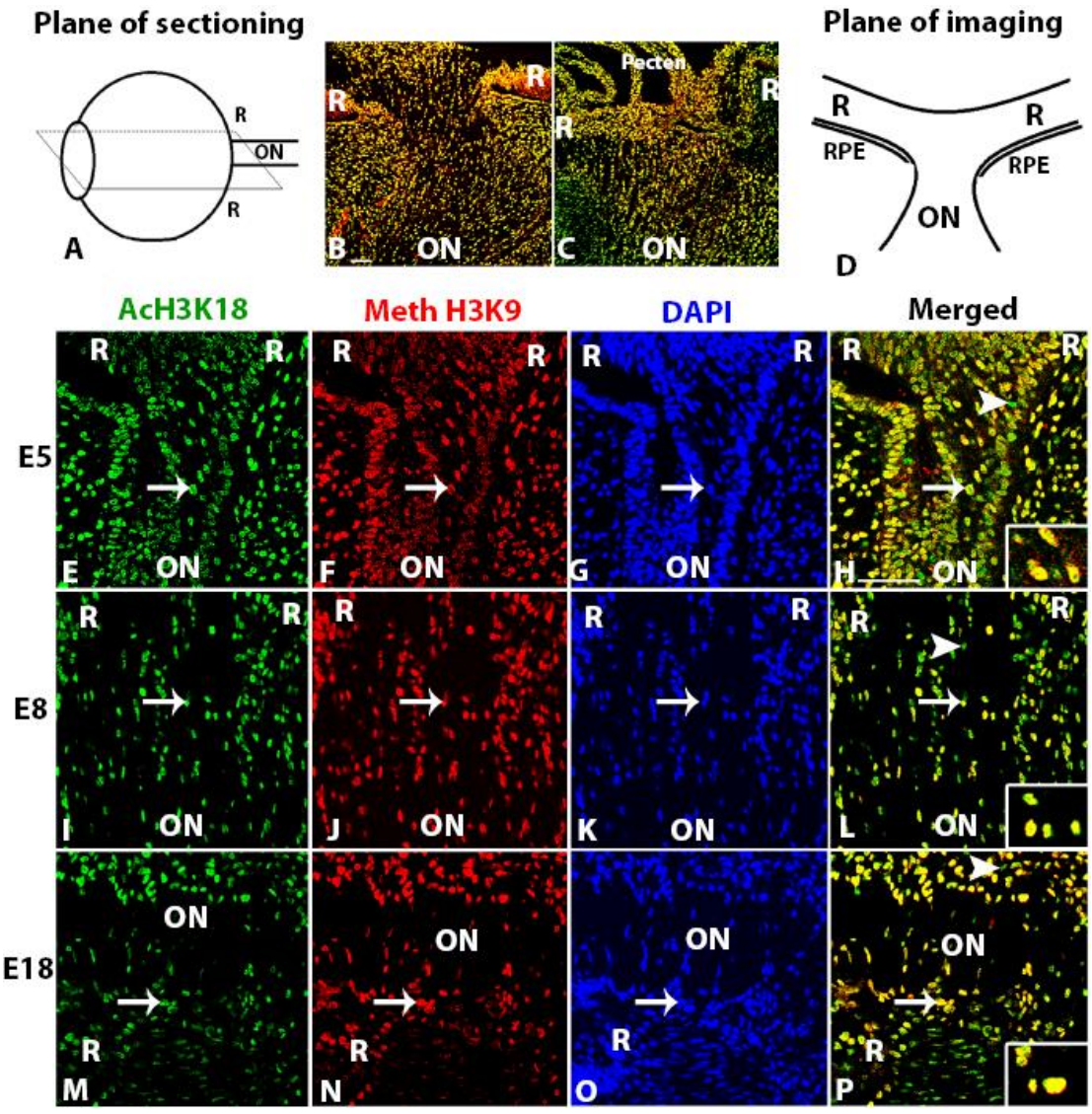

Fig.15 Histone acetylation and methylation in glial cells in chick optic nerve: Low magnification view of the E8 and E18 optic nerve, optic nerve head and retina (B) and plane of imaging to aid in orientation of the sections is shown in C. Drawing A shows the plane of sectioning of the optic nerve. Confocal imaging of AcH3K18, MethH3K9, and DAPI triple label of sections of murine optic nerve at E5 (D, E, F and G), E8 (H, I, J and K) and E18 (L, M, N and O). Overlay of

acetylated histone (green) and methylated histone marker (red) (G, K and O) shows that the glial cells during differentiation show localization of acetylated histone as well as methylated histone within their nuclei. Higher magnification views as shown in 60x magnification panels (insets G, K and O) show co localization for both the markers. Arrows in panels D-G, H-K and L-O are pointing to the cells that show label for both the markers whereas the arrow heads are pointing to the cells that do not show a co label. Scale bars: (B) Bar = 50 µm for images B-C, (G) Bar = 50 µm for images D-O. Scale bars: (G) Bar = 50 µm for images D-O.

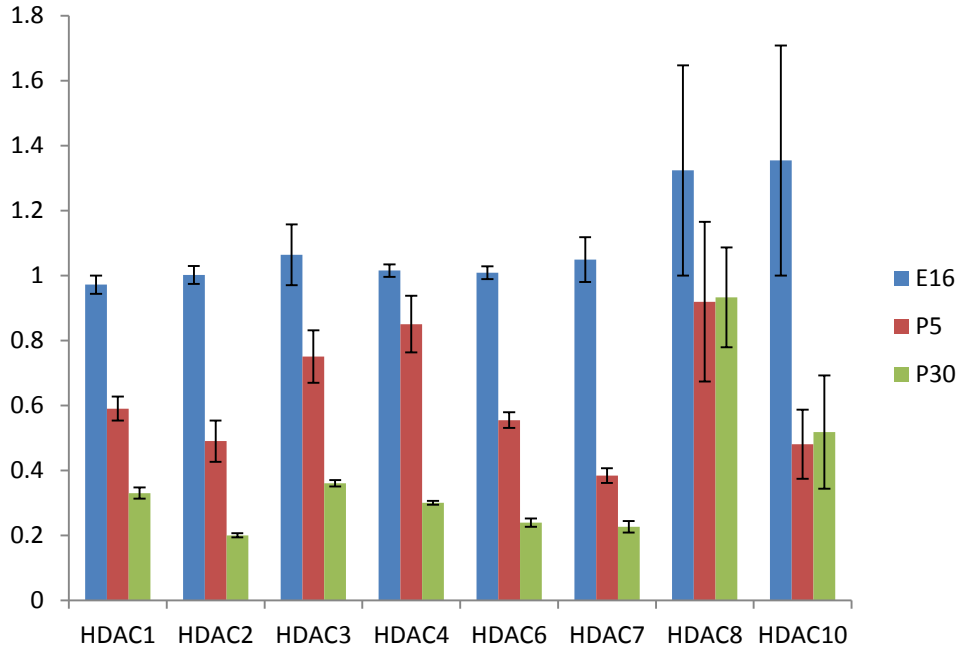

Fig.16 HDACs expression levels in murine optic nerve part A: qPCR analysis of the relative levels of HDACs expression in P5 and P30 murine optic nerve compared to the levels of the E16 murine optic nerve.

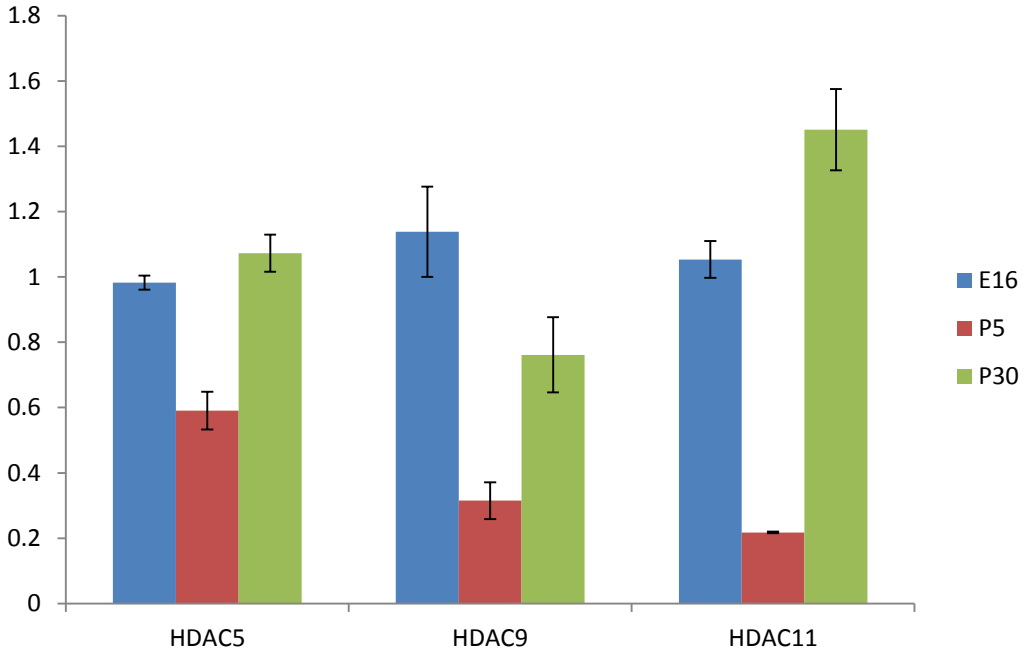

Fig.17 HDACs expression levels in murine optic nerve part B: qPCR analysis of the relative levels of HDACs expression in P5 and P30 murine optic nerve compared to the levels of the E16 murine optic nerve.

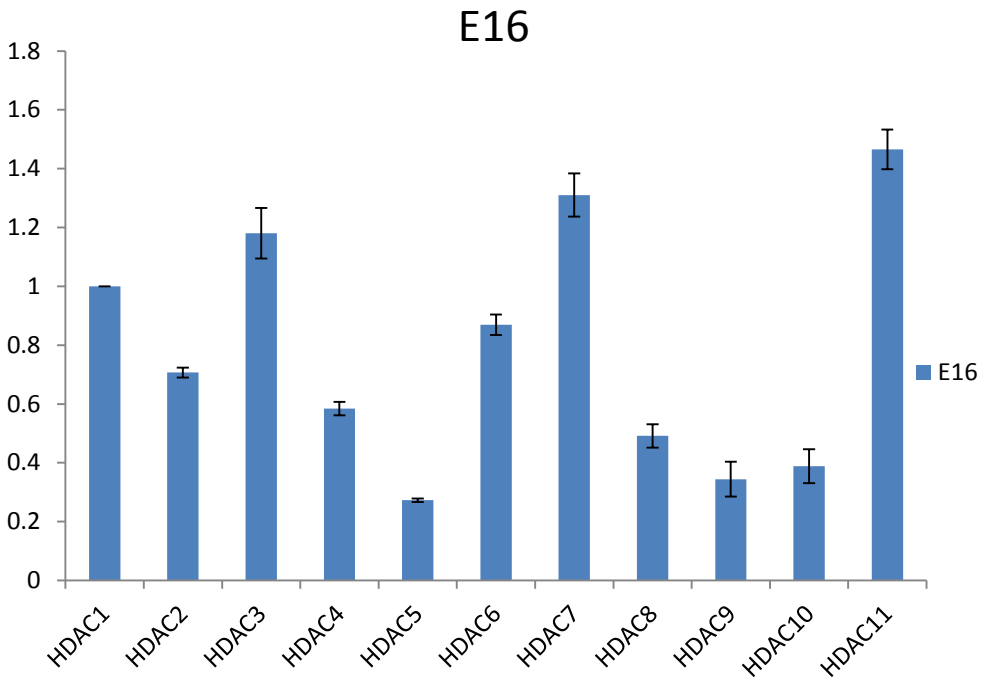

Fig.18 HDACs expression levels in E16 murine optic nerve: qPCR analysis of the relative levels of HDACs expression in E16 murine optic nerve compared to the levels of the HDAC1.

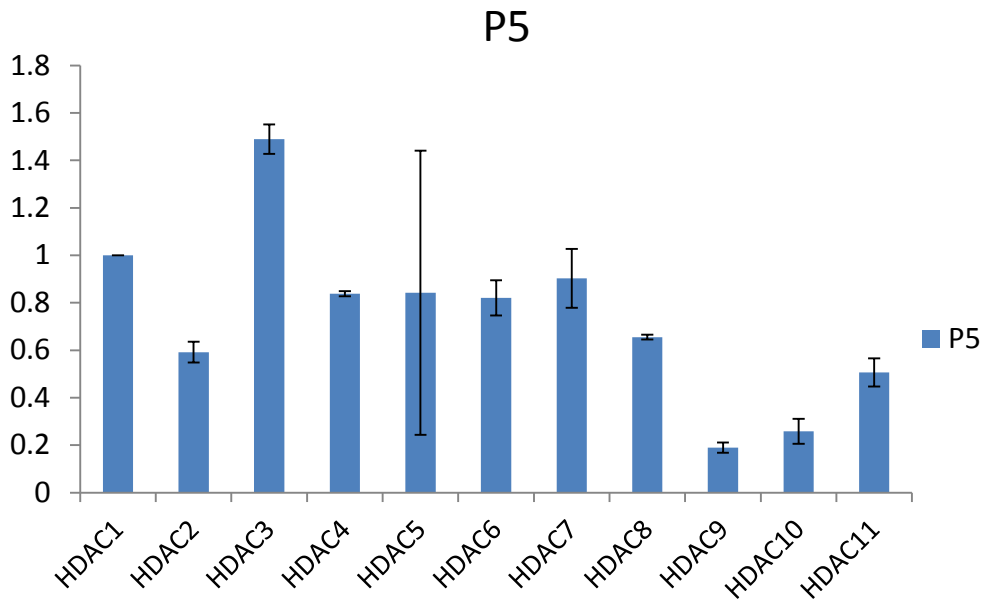

Fig.19 HDACs expression levels in P5 murine optic nerve: qPCR analysis of the relative levels of HDACs expression in P5 murine optic nerve compared to the levels of the HDAC1.

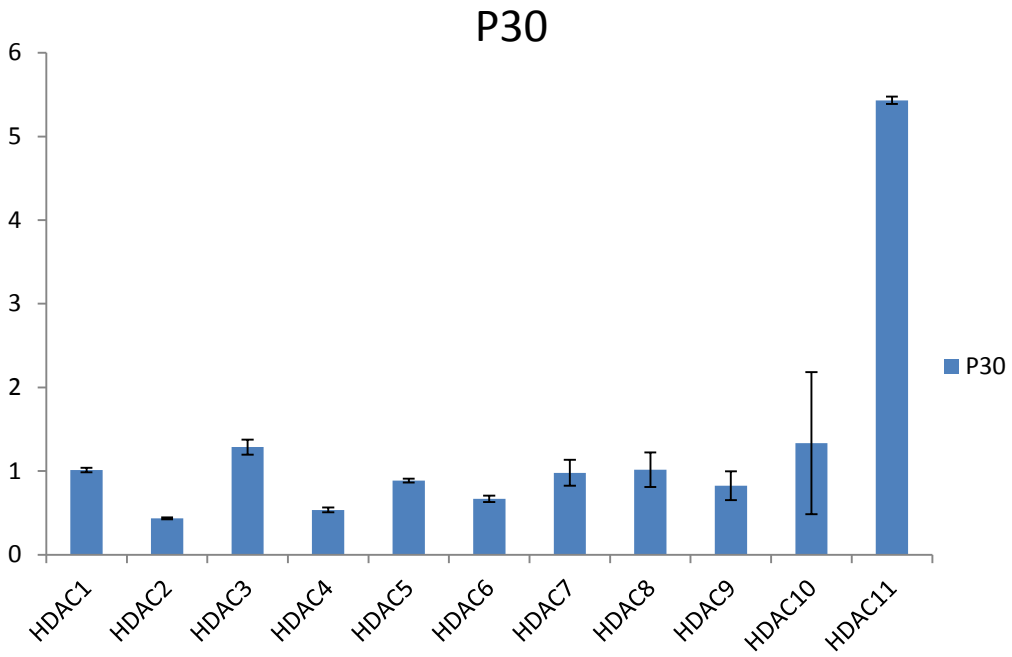

Fig.20 HDACs expression levels in P30 murine optic nerve: qPCR analysis of the relative levels of HDACs expression in P30 murine optic nerve compared to the levels of the HDAC1.

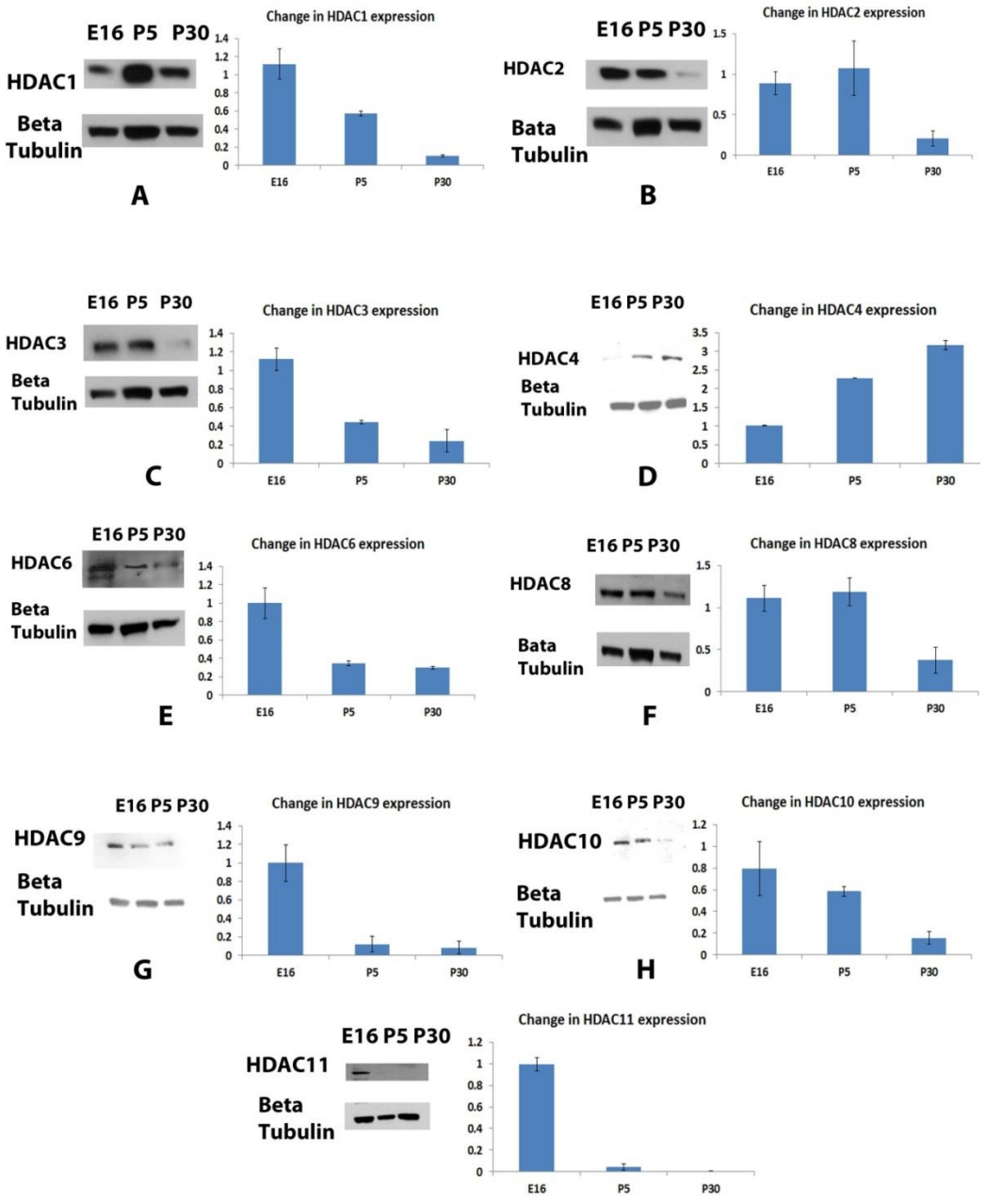

Fig.21 Expression of HDACs in murine optic nerve: Western blot analysis of HDAC proteins in E16, P5 and P30 murine optic nerve. Blots were been normalized to β tubulin. Densitometry shown is relative to E16 stage.

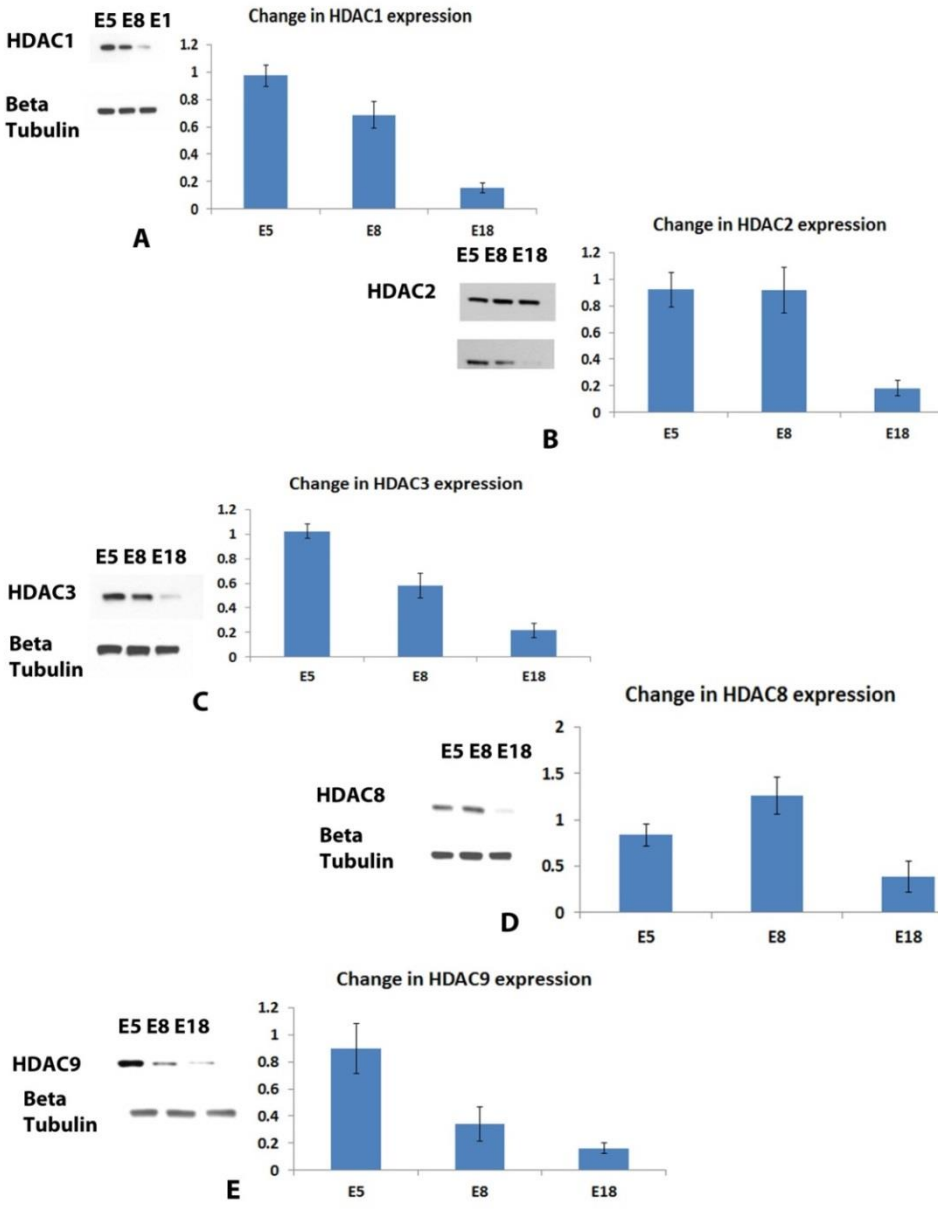

Fig.22 Expression of HDACs in chick optic nerve: Western blot analysis of HDAC proteins in E5, E8 and E18 chick optic nerve. Densitometric analysis of blots has been normalized to tubulin and graphs show values relative to E5 stage.

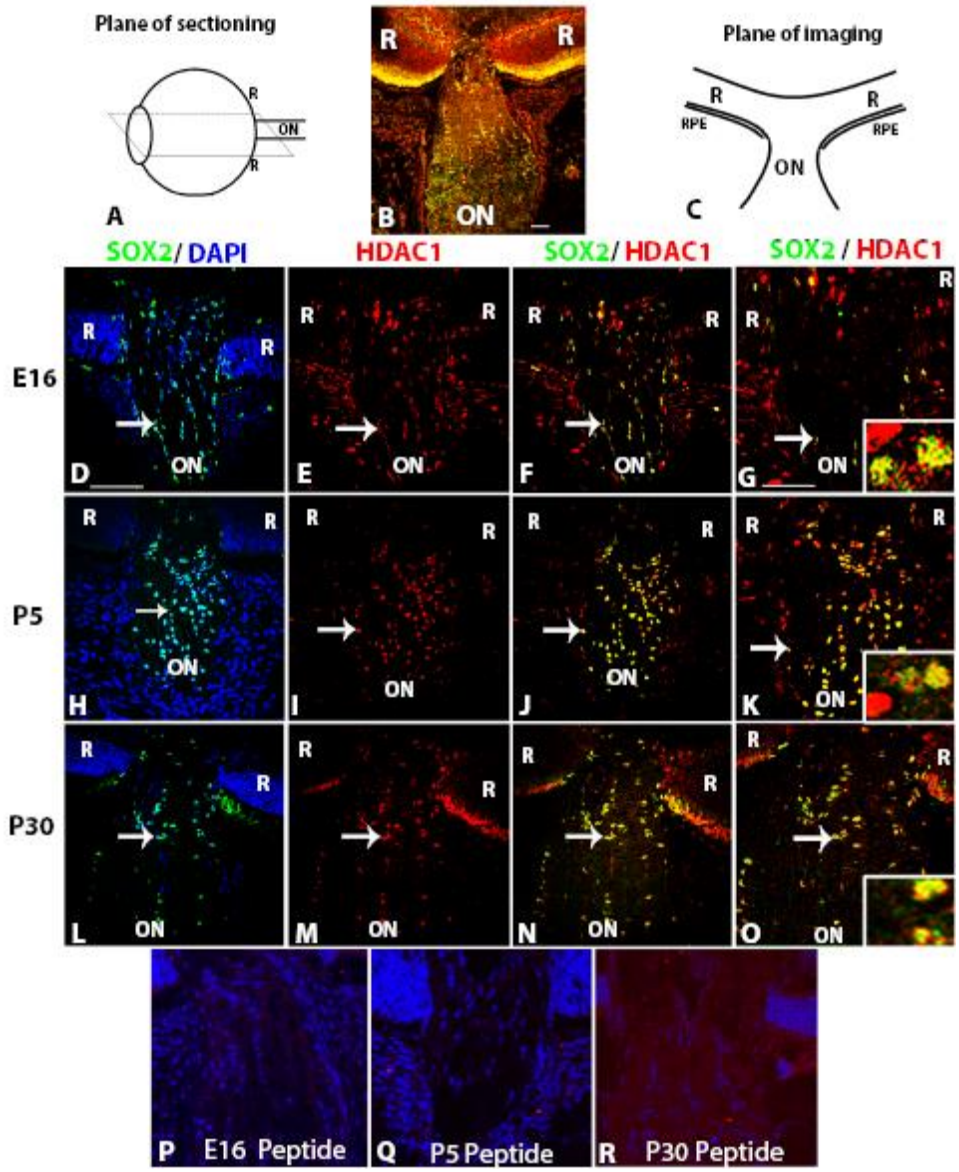

Fig.23 Localization of HDAC1 in murine optic nerve: Drawing in A depicts the plane of the sections through the optic nerve and optic nerve head. Low magnification of the optic nerve, optic nerve head and retina double-labeled for SOX2 (glia) and HDAC1 is shown in B to indicate the region which was imaged. A drawing depicting the region that was imaged each of the following

figures is shown in C. Confocal imaging of HDAC1, SOX2, and DAPI triple-label in murine optic nerve at E16 (D, E, F and G), P5 (H, I, J and K) and P30 (L, M, N and O). Each stage shows the overlay of SOX2 and DAPI to aid in orientation of the image (D, H, and L). Subsequent overlays did not include DAPI because it appeared to obscure some of the SOX2 and HDAC label. HDAC1 label is shown (E, I, M), while co-label of SOX2 and HDAC1 is shown at 40x (F, J, N) and 60x (G, K, O). Images taken at 60x also include higher magnification views (insets G, K, O). Arrows indicate nuclei that were co-labeled with SOX2 and HDAC1, arrow heads in higher magnification point to the cells that do not show co label. Sections from each stage were also incubated with antibody preabsorbed with peptide against which the antibody was raised to show antibody specificity (P, Q and R). Scale bars: (A) Bar = 50 µm (D) Bar = 50 µm for panels E-G, H-J and L-N (E) Bar = 50 µm for images G, K and O.

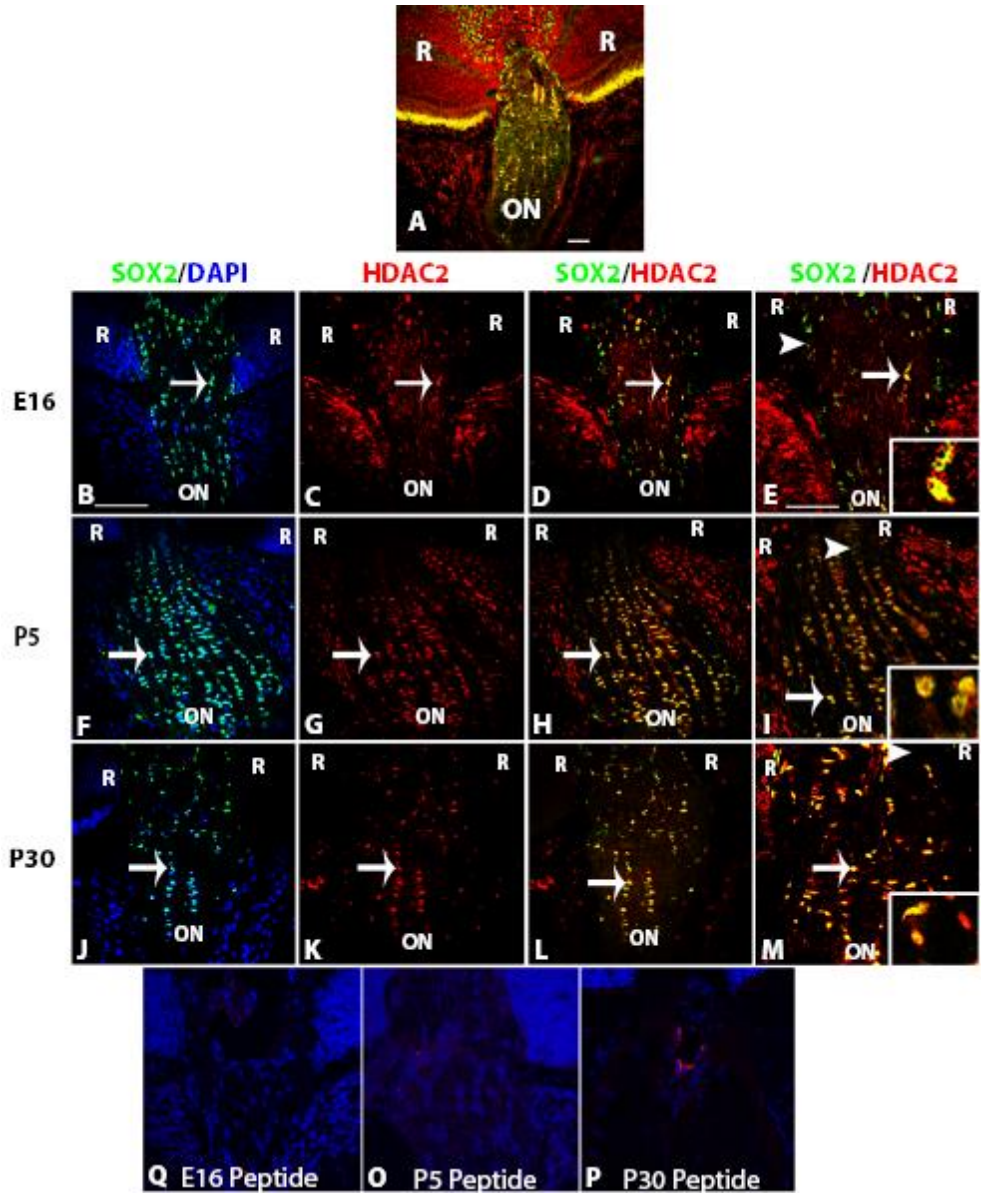

Fig.24 Localization of HDAC2 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A.Confocal imaging of SOX2, HDAC2, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown

to aid in localization of the optic nerve and optic nerve head (B, F, J). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC2 only label is shown (C, G, K) as well as SOX2 and HDAC2 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC2 is predominantly localized to the nuclei of optic nerve glia. Arrows indicate nuclei that are co–labeled whereas the arrowheads in higher magnication(E, I and M) point to the cells that do not show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC2 antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

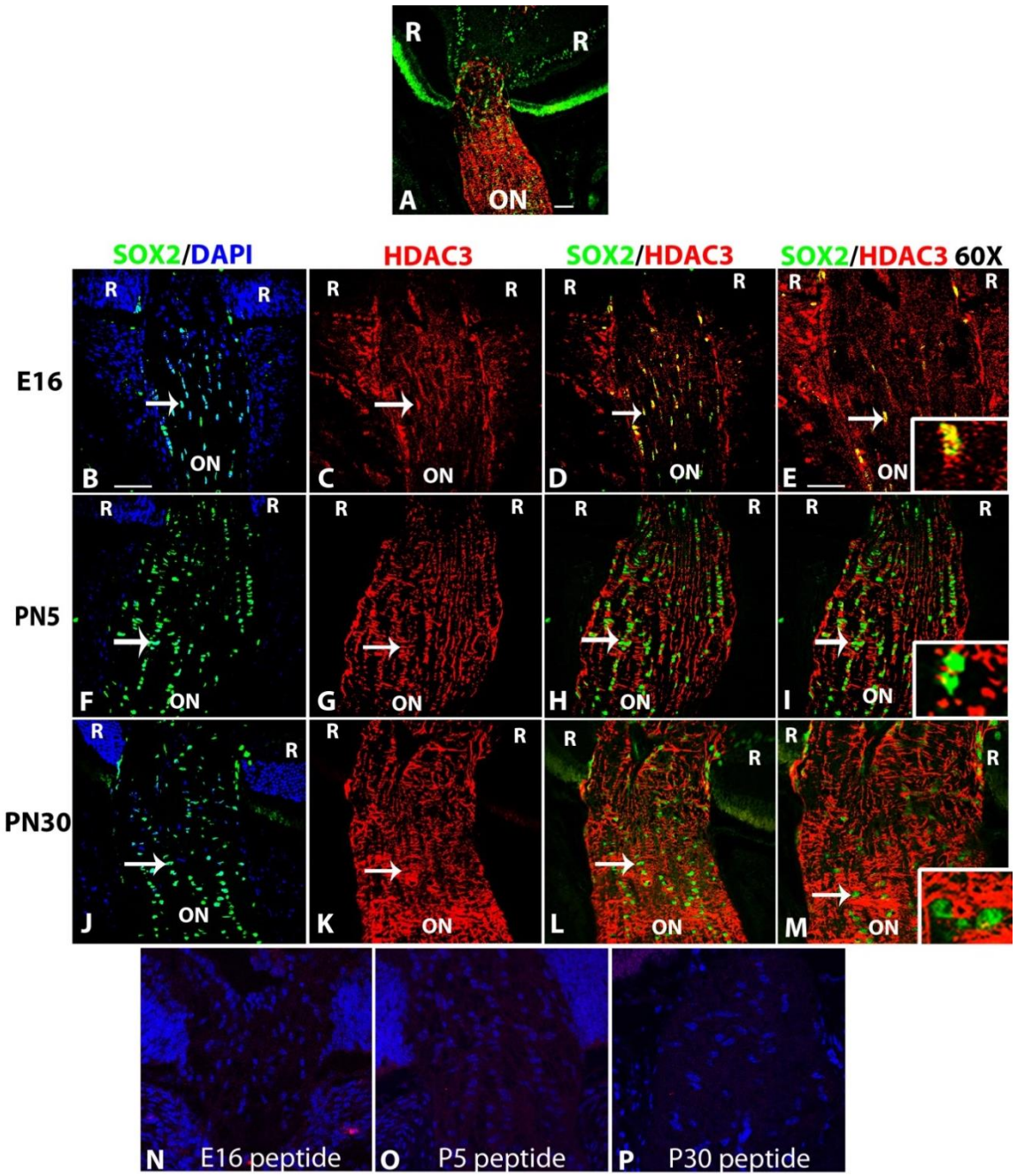

Fig.25 Localization of HDAC3 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A. Confocal imaging of SOX2, HDAC3, and DAPI triple

label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J).DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC3 only label is shown (C, G, K) as well as SOX2 and HDAC3 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC3 shows variable pattern, localized to the nucleus and cytoplasm of optic nerve glia at E16 as few cells show co-localization of HDAC3 and SOX2. In P5 and P30 optic nerve glia HDAC3 was localized predominantly to the cytoplasm. Arrows indicate nuclei that are co–labeled and arrow heads point to the cells that do not show co label. Peptide controls (N, O and P) for all three stages after preabsorption to verify the specificity of the antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm (B-D, F-H) and J-L (E) Bar = 50 µm for E, I and M.

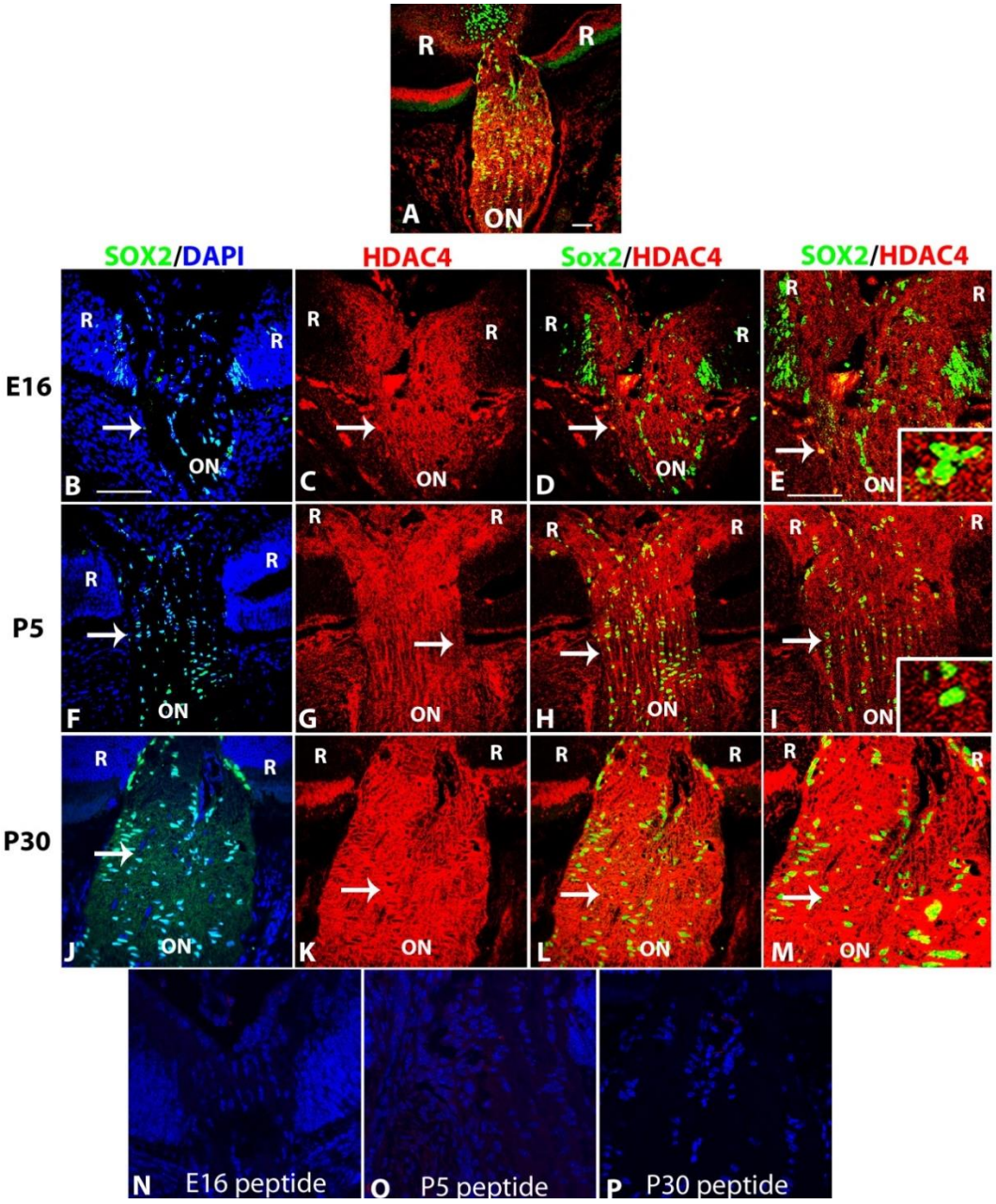

Fig.26 Localization of HDAC4 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A. Confocal imaging of SOX2, HDAC4, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G,H and

I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC4 only label is shown (C, G, K) as well as SOX2 and HDAC4 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC4 is localized to the nucleus as well as to the cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled and the arrowheads are pointing to the cells that do not show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC4 antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

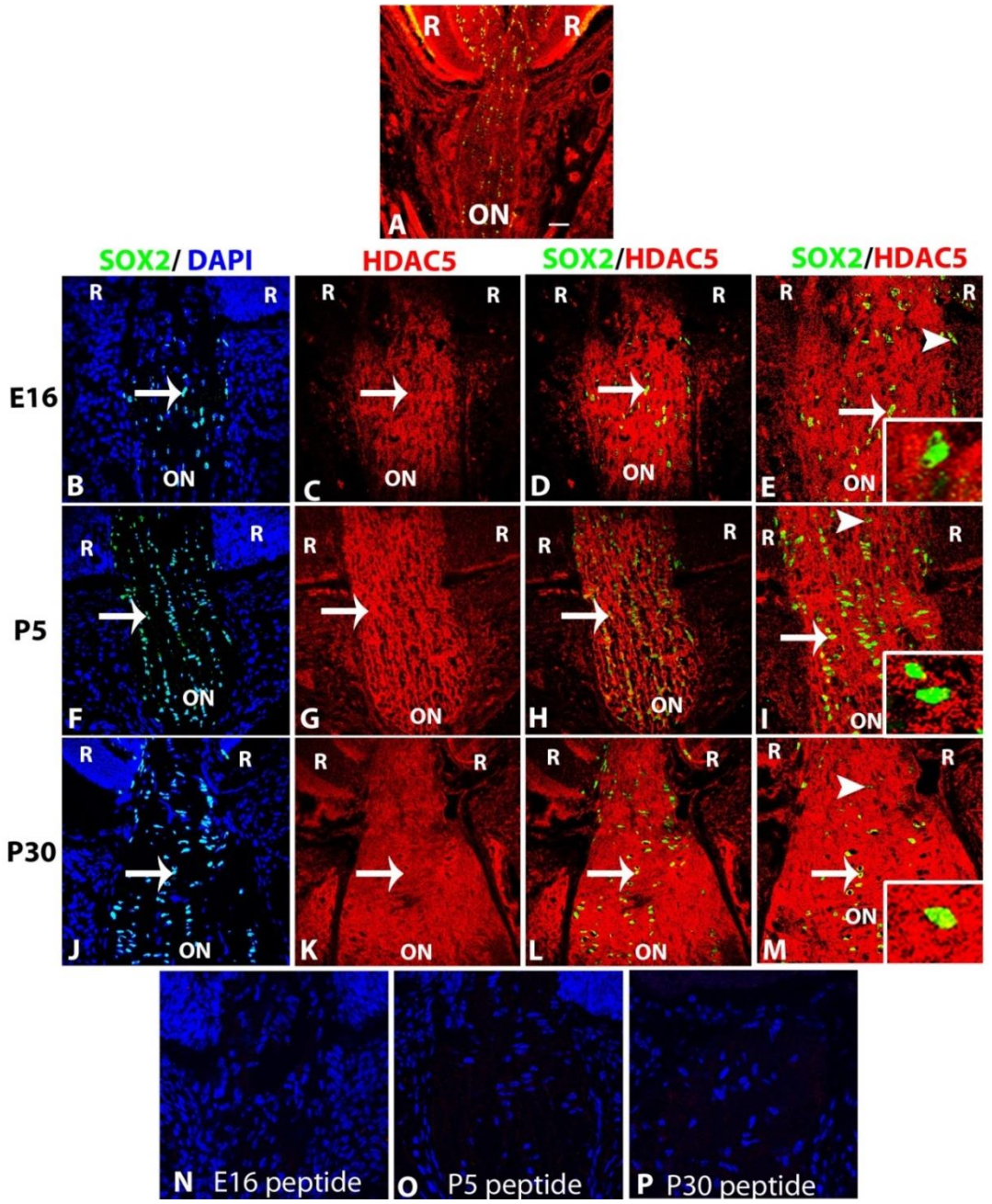

Fig.27 Localization of HDAC5 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A Confocal imaging of SOX2, HDAC5, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G,

H and I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC5 only label is shown (C, G, K) as well as SOX2 and HDAC5 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views in 60x magnification panels (insets E, I, M). Double-label shows that HDAC5 is localized predominantly to the cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled whereas the arrowheads point to the cells that do not show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for B-D, F-H and J-L (E) Bar = 50 µm for E, I and M.

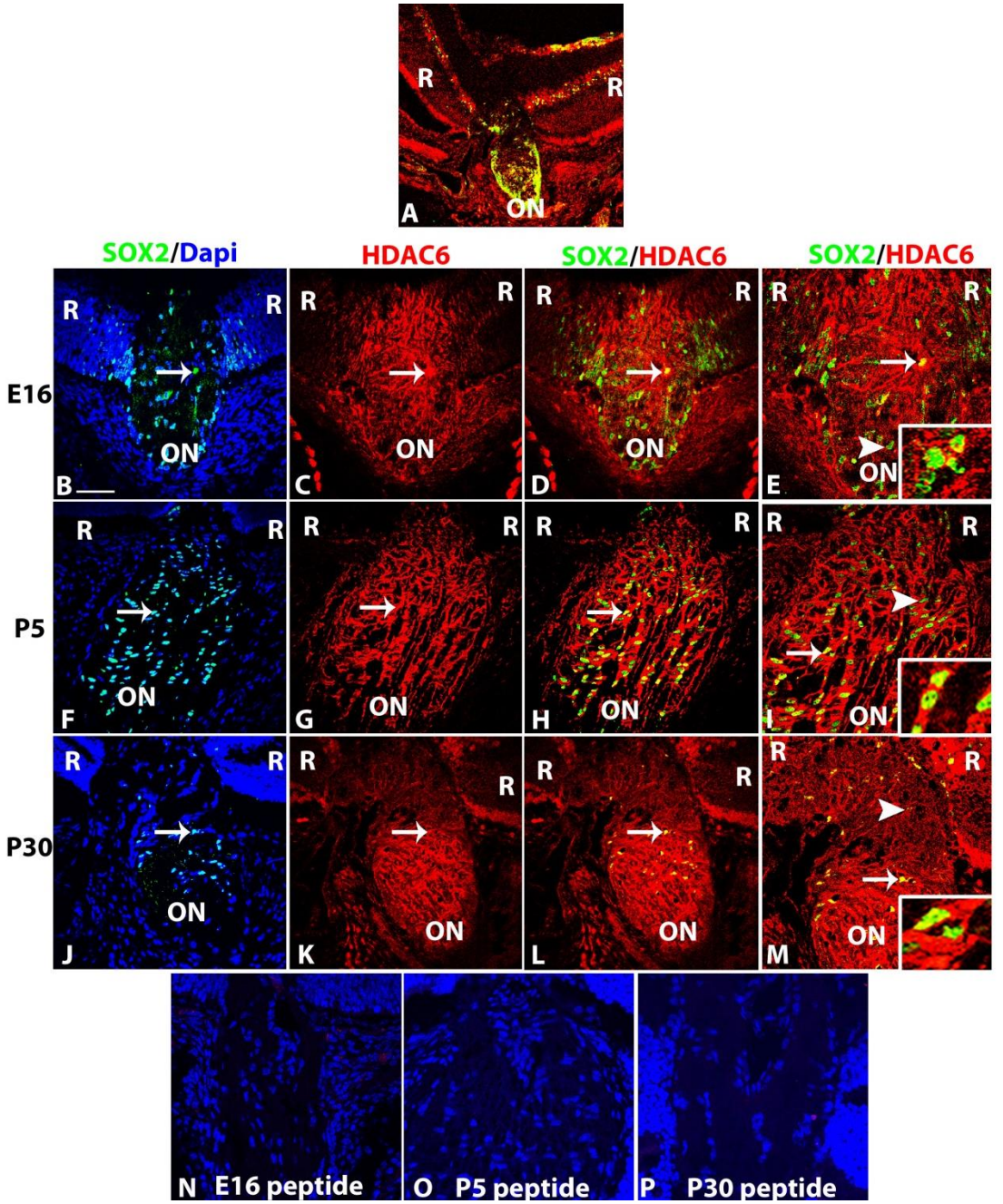

Fig.28 Localization of HDAC6 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A.Confocal imaging of SOX2, HDAC6, and DAPI triple

label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC6 only label is shown (C, G, K) as well as SOX2 and HDAC2 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC6 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing to the cells that do not show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC6 antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

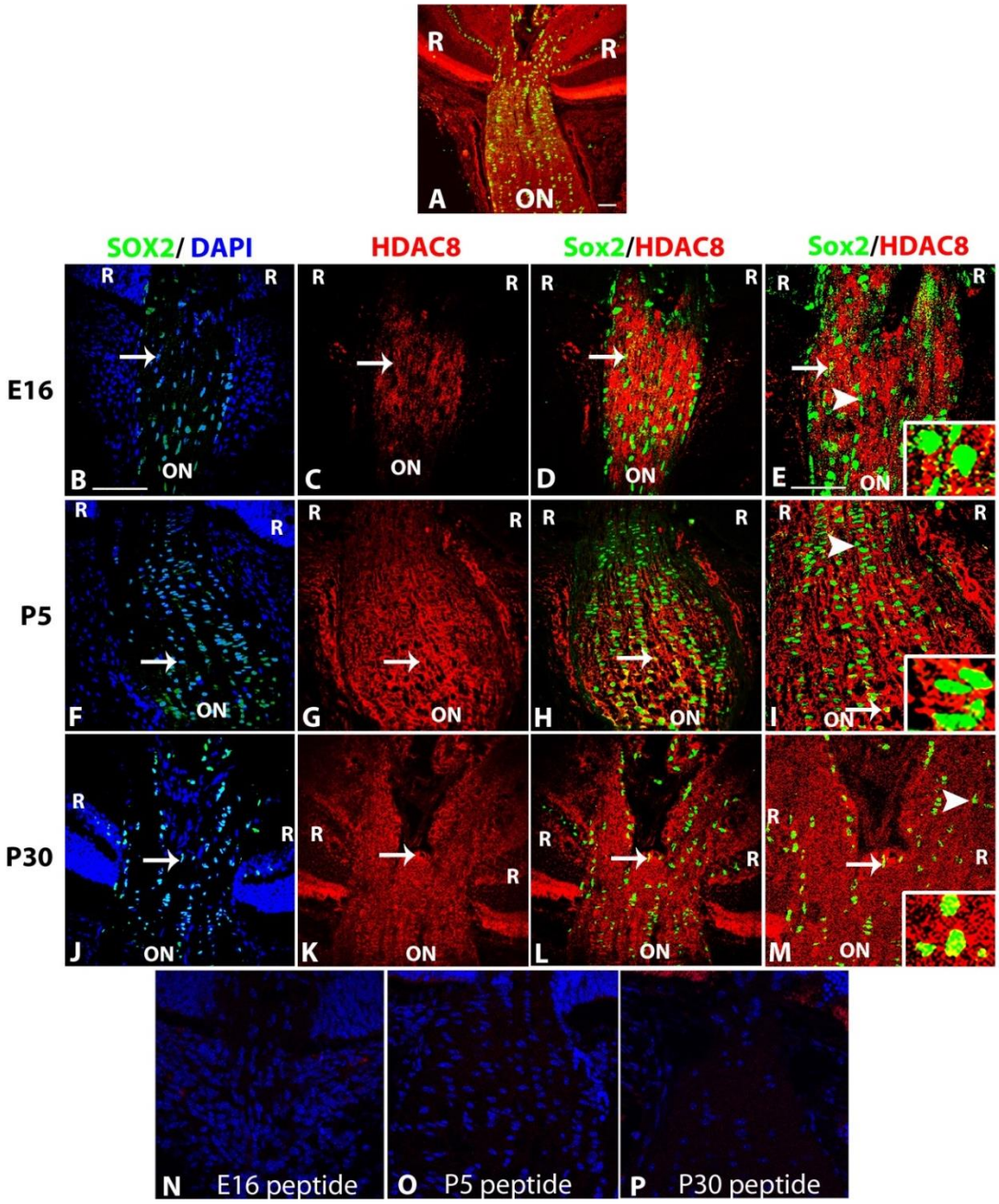

Fig.29 Localization of HDAC8 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A. Confocal imaging of SOX2, HDAC8, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and

I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J).DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC8 only label is shown (C, G, K) a s well as SOX2 and HDAC2 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC8 has a variable pattern of localization. It is localized predominantly to the cytoplasm of optic nerve glia at E16 and p5 stage and is localized to the nucleus as well as to the cytoplasm at P30 stage. Arrows indicate the cells that show the localization pattern clearly while the arrow heads point to the ones that donot show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

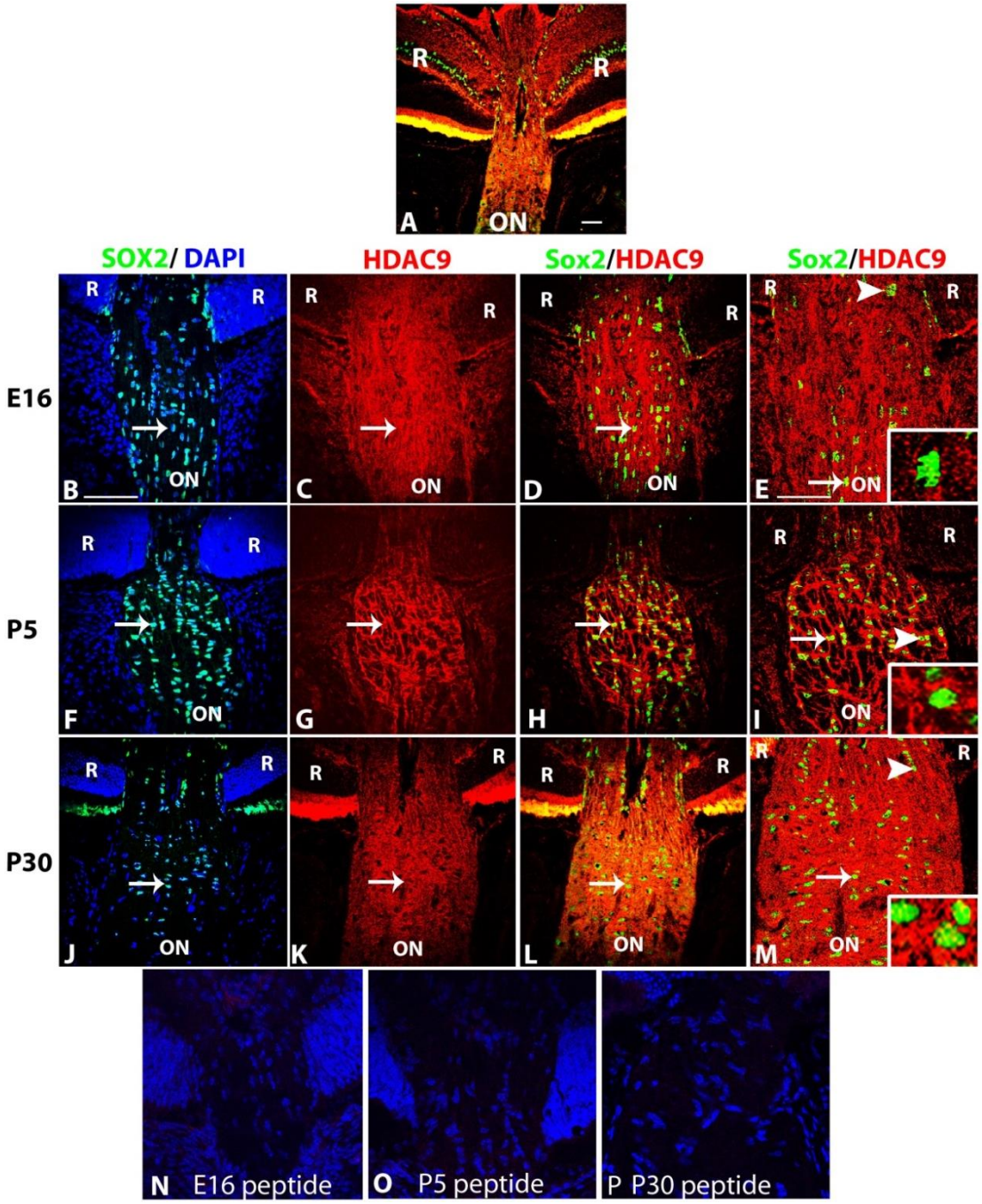

Fig.30 Localization of HDAC9 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A. Confocal imaging of SOX2, HDAC9, and DAPI triple

label of sections of murine optic nerve at E16 (B, C, D and E) , P5 (F, G, H and I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J). HDAC9 only label is shown (C, G, K) SOX2 and HDAC9 overlay at 40x (D, H, L) and 60x magnifications (E, I,M). Higher magnification views in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC9 is localized predominantly to the cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled whereas the arrowheads are pointing to the cells that do not show co label. Peptide controls (N, O and P) after pre absorption with the primary antibody to verify the specificity of the antibody. Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

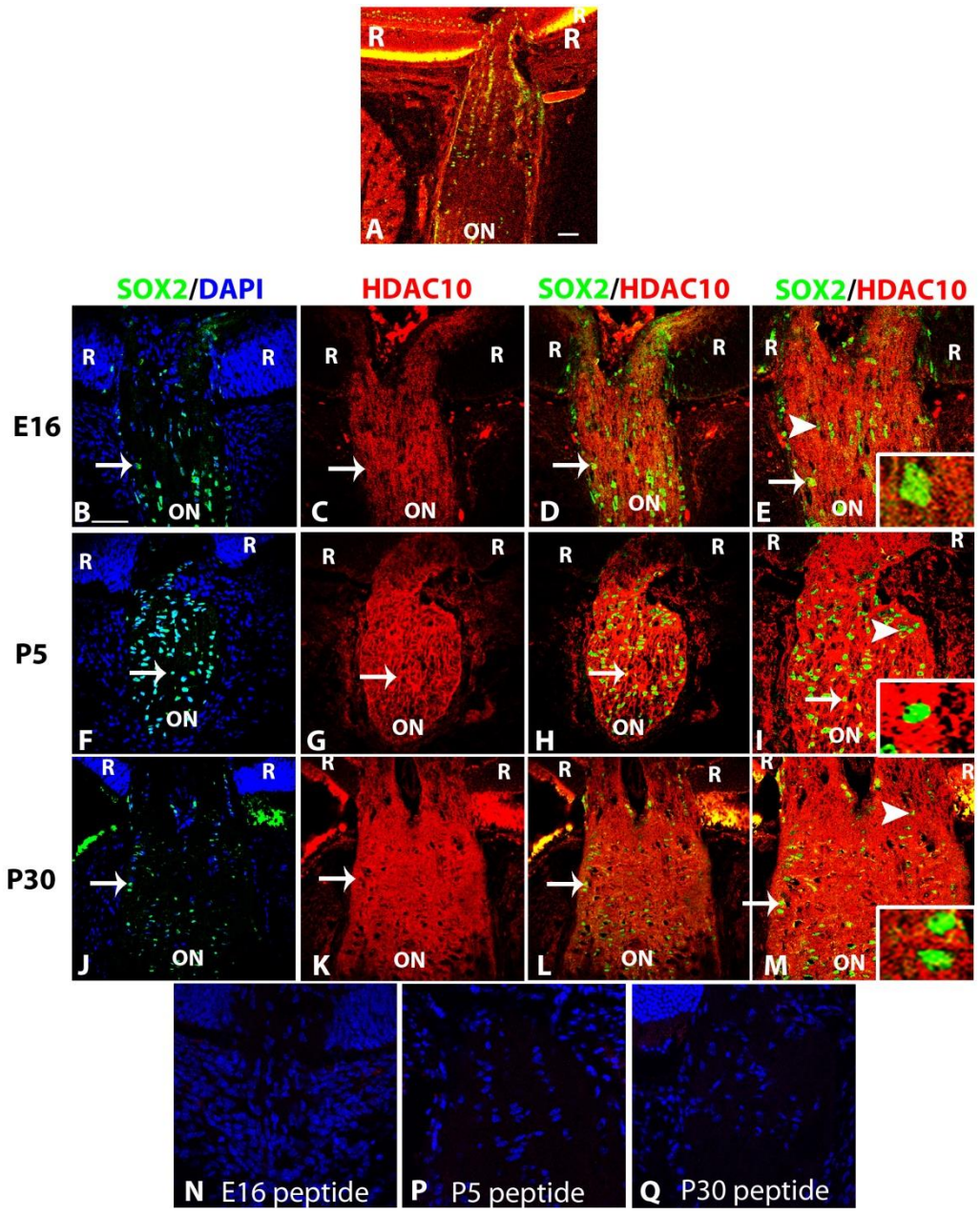

Fig.31 Localizationof HDAC10 in murine optic nerve: Confocal imaging of SOX2, HDAC10, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and I) and P30 (J, K, L and M). SOX2 and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve

head (B, F, J). HDAC10 only label is shown (C, G, K) Images showing SOX2 and HDAC10 colabel in lower magniication, 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC10 is localized predominantly to the cytoplasm of optic nerve glia. Arrows indicate that are co–labeled nuclei whereas the arrowheads are pointing to the cells that do not show co label. Peptide controls are shown in N, O and P to verify their specificity Scale bars: (A) Bar = 50 µm (B) Bar = 50 µm B-D, F-H and J-L (E) Bar = 50 µm E, I and M.

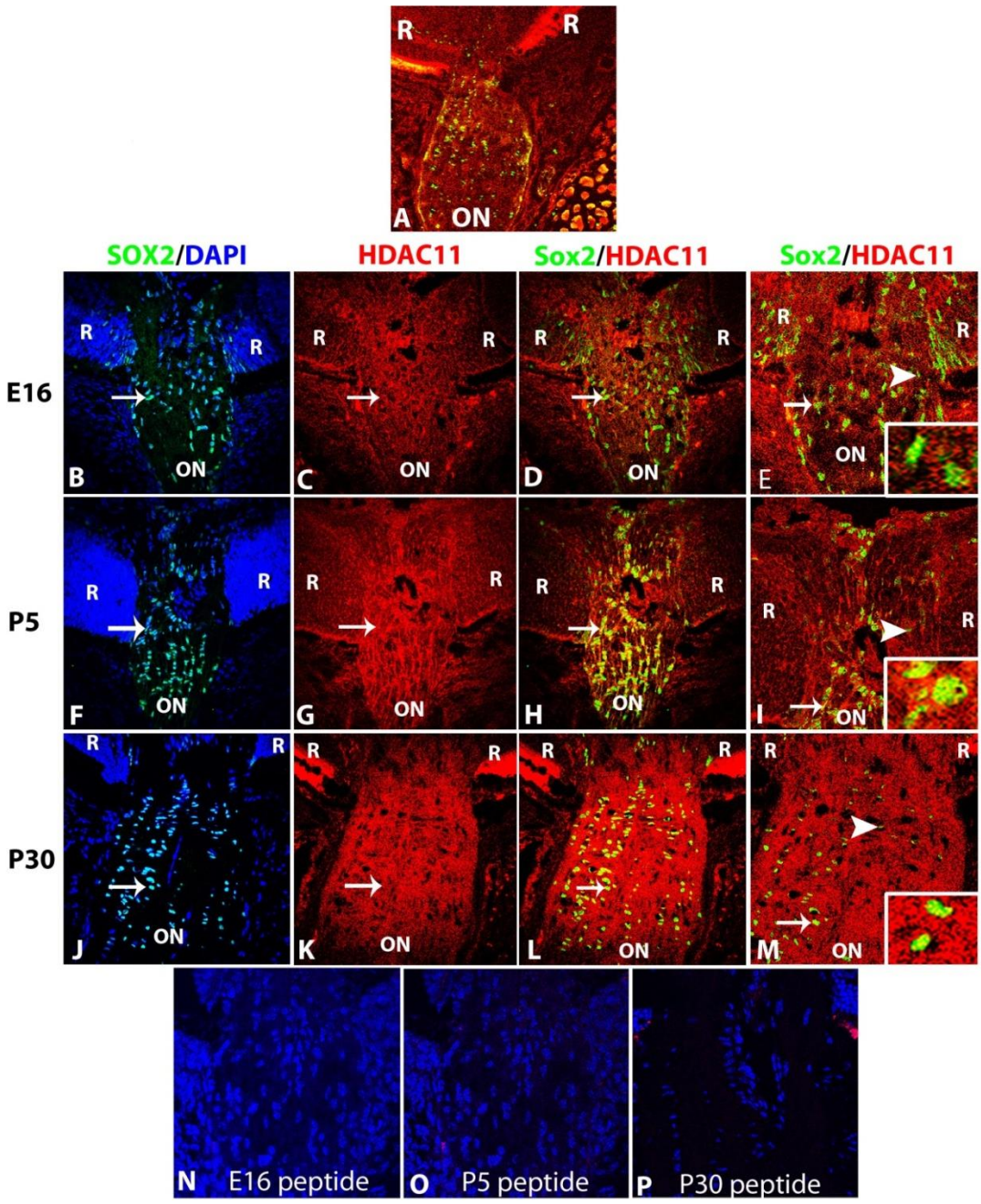

Fig.32 Localization of HDAC11 in murine optic nerve: Low magnification view of the P30 optic nerve, optic nerve head and retina to aid in orientation of the sections is shown in A.Confocal imaging of SOX2, HDAC11, and DAPI triple label of sections of murine optic nerve at E16 (B, C, D and E), P5 (F, G, H and

I) and P30 (J, K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (B, F, J). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC11 only label is shown (C, G, K) as well as SOX2 and HDAC2 overlay at 40x (D, H, L) and 60x magnifications (E, I, M). Higher magnification views are shown in 60x magnification panels (insets E, I, M). Double-label indicates that HDAC11 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are Co-labeled and arrow heads are pointing to the cells that do not show co label. Peptide controls (N, O and P) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC11 antibody. Scale bars: (A) Bar = 50µm (B) Bar = 50 µm for panels B-D, F-H and J-L (E) Bar = 50 µm for images E, I and M.

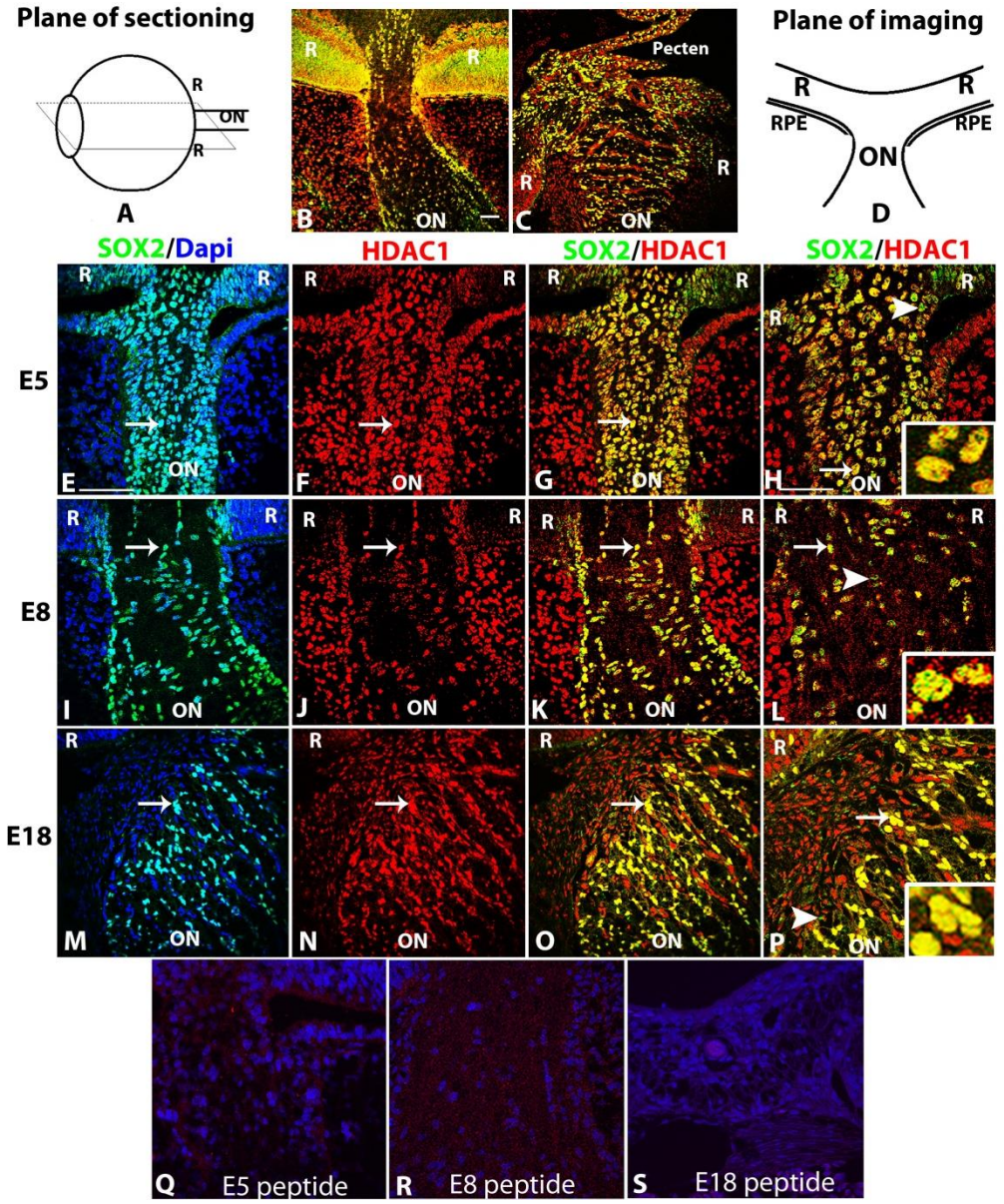

Fig.33 Localization of HDAC1 in chick optic nerve: Drawing A depicts the plane of the sections through the optic nerve and optic nerve head. D represents the plane of imaging showing the region that was imaged. Low magnification view of the E8 (B) and E18 (C) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image C

represents the vascularised part of the chick retina. Confocal imaging of SOX2, HDAC1 and DAPI triple label of sections of chick optic nerve at E5 (E, F and G), E8 (I, J and K) and E18 (M, N and O). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (E, I and M). DAPI is not included in all overlays as it appeared to obscure immunolabel. HDAC1 only label is shown (F, J and N) as well as SOX2 and HDAC2 overlay at 40x (G, K and O) and 60x magnifications (H, L and P). Higher magnification views are shown in 60x magnification panels (insets H, L and P). Double-label indicates that HDAC1 is predominantly localized to the nuclei of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing cells that do not show co label. Peptide controls (Q, R and S) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC1 antibody. Scale bars: (B) Bar = 50 µm for images B and C (E) Bar = 50 µm (E-G, I-K and M-O) (F) Bar = 50 µm for H, L and P.

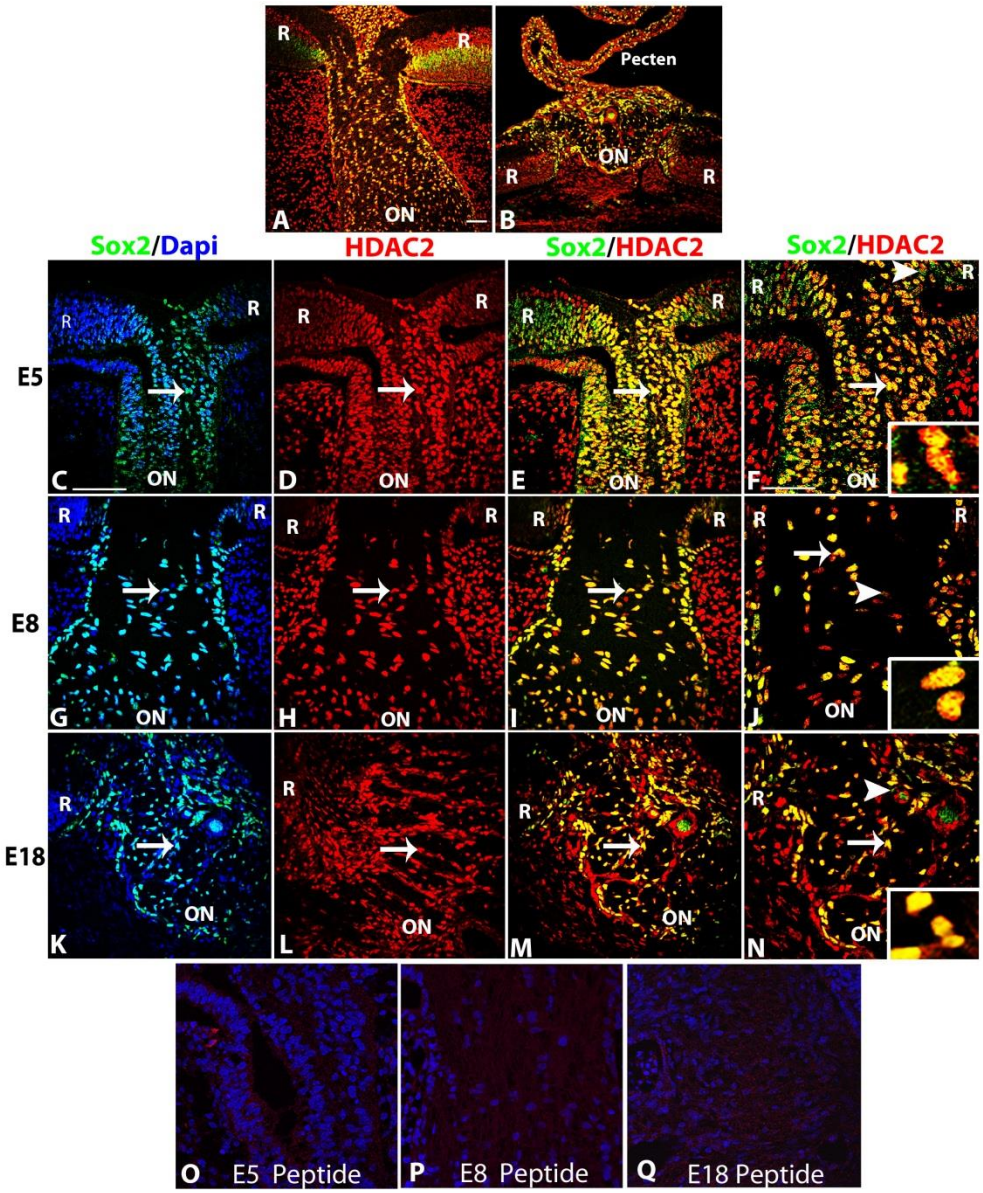

Fig.34 Localization of HDAC2 in chick optic nerve: Low magnification view of the ES (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC2 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H

and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays as it appeared to obscure some of the immunolabel. HDAC2 only label is shown (D, H and L) as well as SOX2 and HDAC2 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC2 is localized to the nuclei of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing to the cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC2 antibody. Scale bars: (A) Bar = 50 µm for A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for F, J and N.

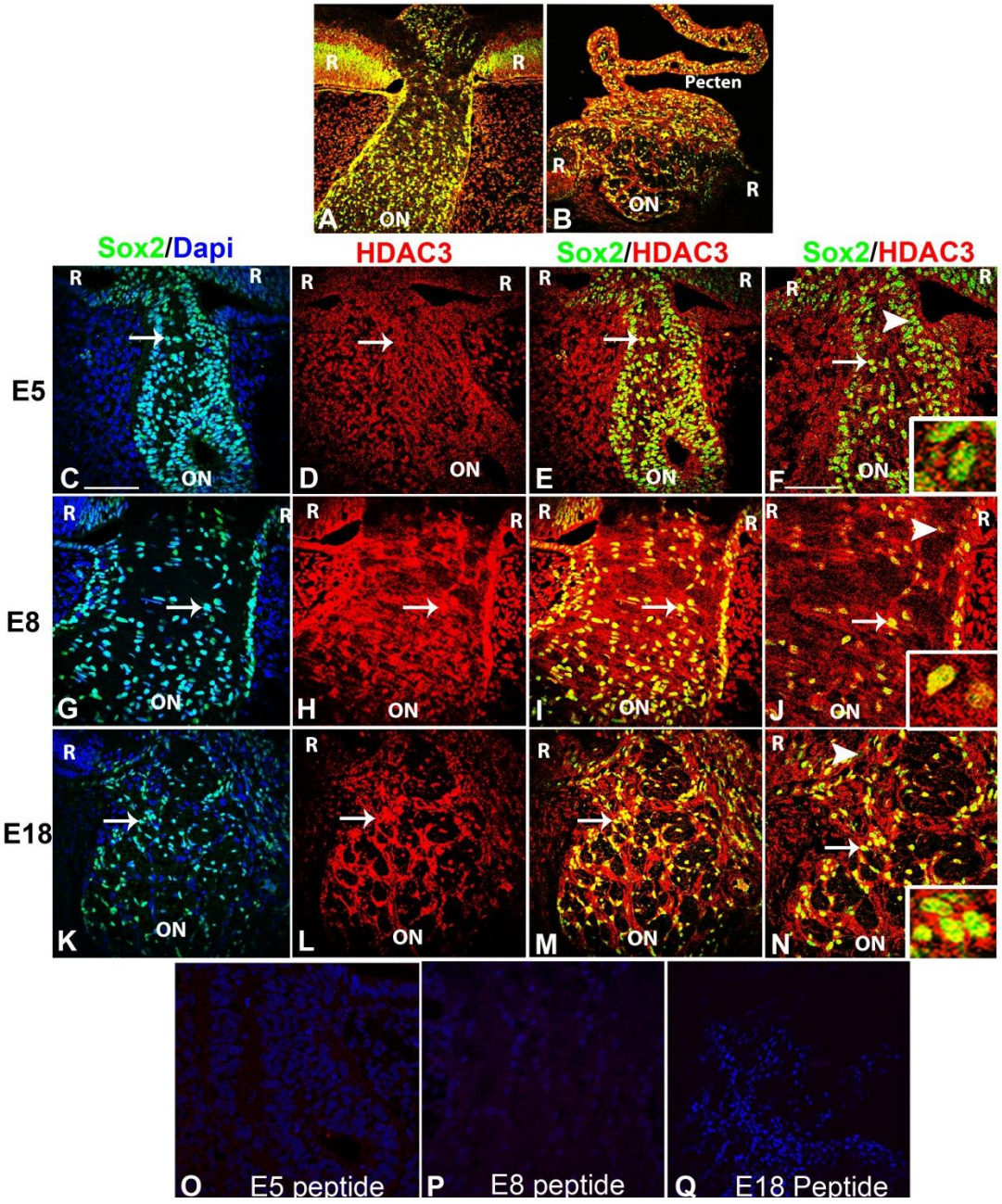

Fig.35 Localization of HDAC3 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC3 and DAPI triple

label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the immunolabel. HDAC3 only label is shown (D, H and L) as well as SOX2 and HDAC3 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC3 shows variable localization pattern. It is predominantly localized to the cytoplasm at E5 stage and to both nuclei and cytoplasm of optic nerve glia at E8 and E18 stages. Arrows indicate cells showing the localization pattern and arrow heads pointing to the cells that donot show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC3 antibody. Scale bars: (A) Bar = 50 µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

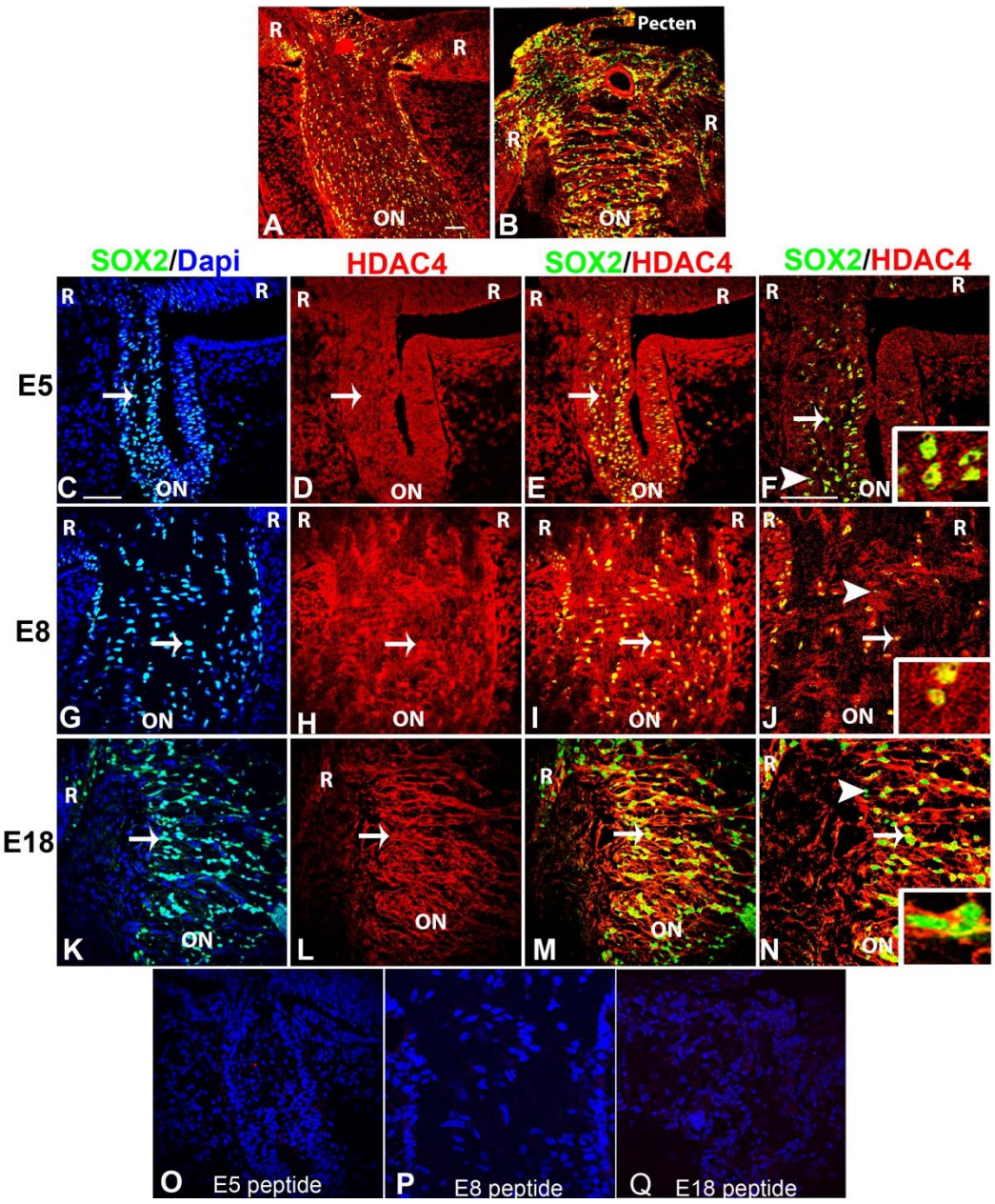

Fig.36 Localization of HDAC4 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC4 and

DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G,H and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the immunolabel. HDAC4 only label is shown (D, H and L) as well as SOX2 and HDAC4 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC4 is localized to the nucleus and cytoplasm. Arrows indicate nuclei that are co–labeled and arrow heads point towards the cells that do not show the co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC4 antibody. Scale bars: (A) Bar = 50µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

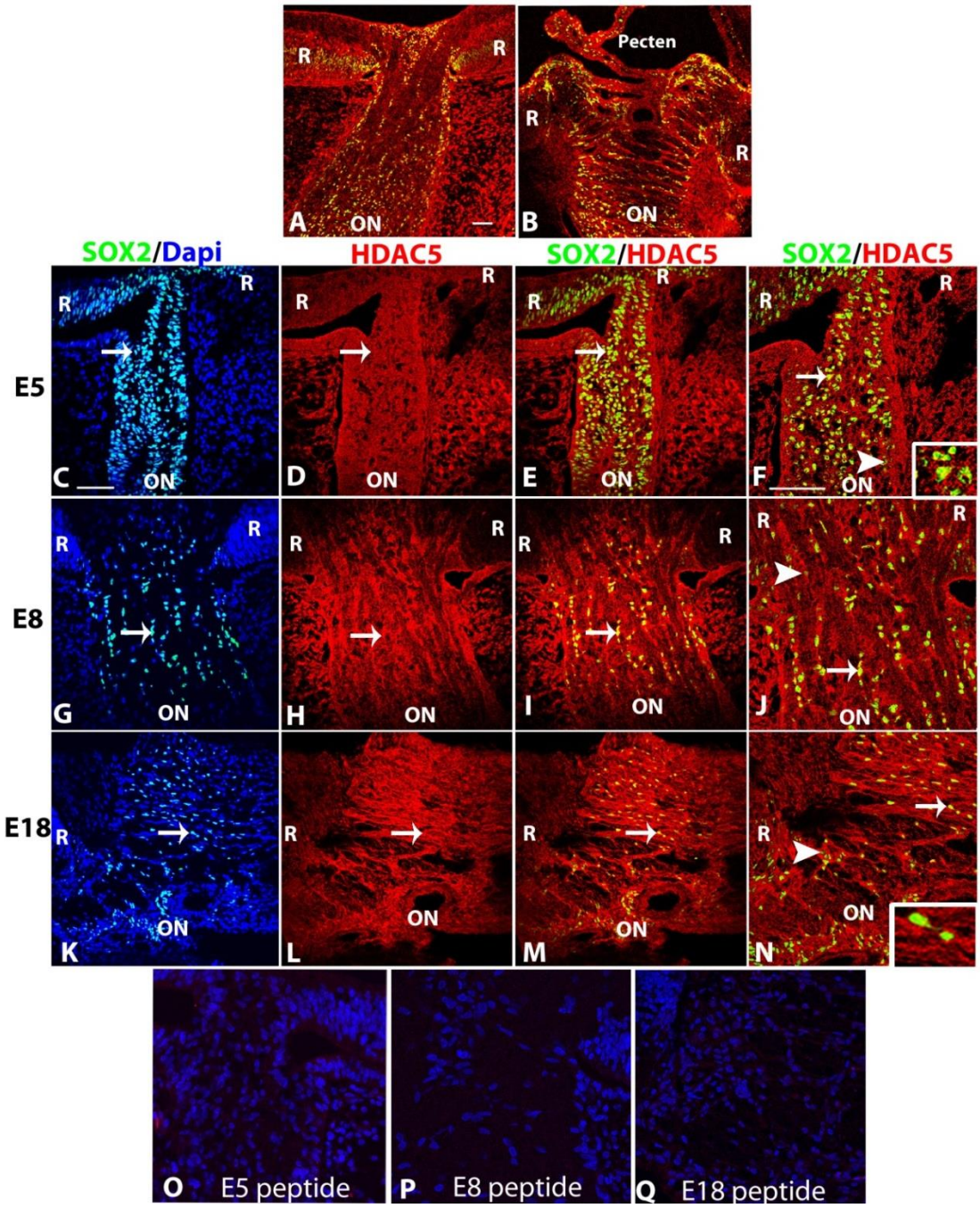

Fig.37 Localization of HDAC5 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized

part of the chick retina. Confocal imaging of SOX2, HDAC5 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H and I) and E18 (K,L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC5 only label is shown (D, H and L) as well as SOX2 and HDAC5 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F,J and N). Double-label indicates that HDAC5 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are co-labeled and arrow heads are pointing cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC5 antibody. Scale bars: (A) Bar = 50 µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

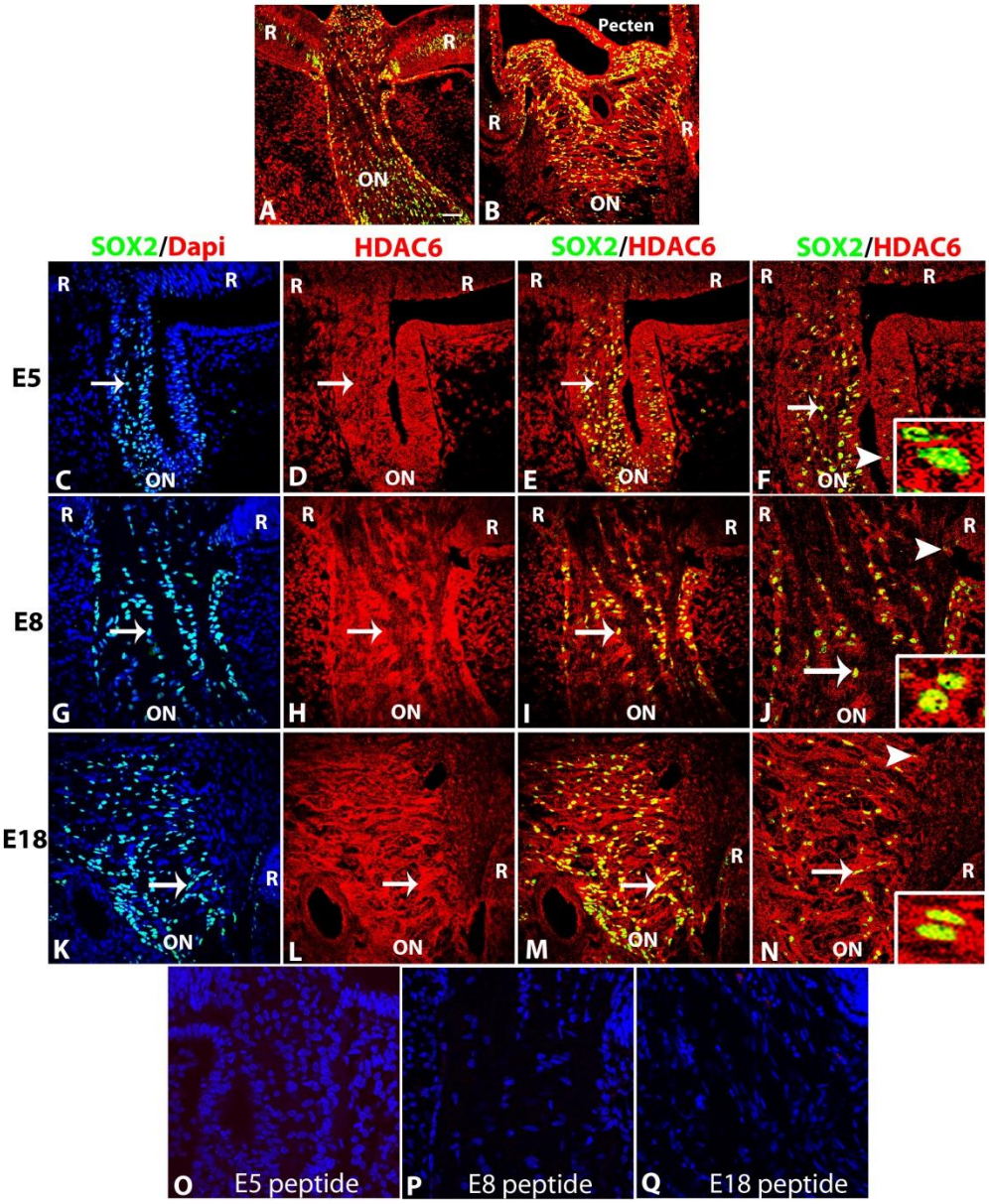

Fig.38 Localization of HDAC6 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC6 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H

and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC6 only label is shown (D, H and L) as well as SOX2 and HDAC8 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC8 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing to cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC8 antibody. Scale bars: (A) Bar = 50 µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

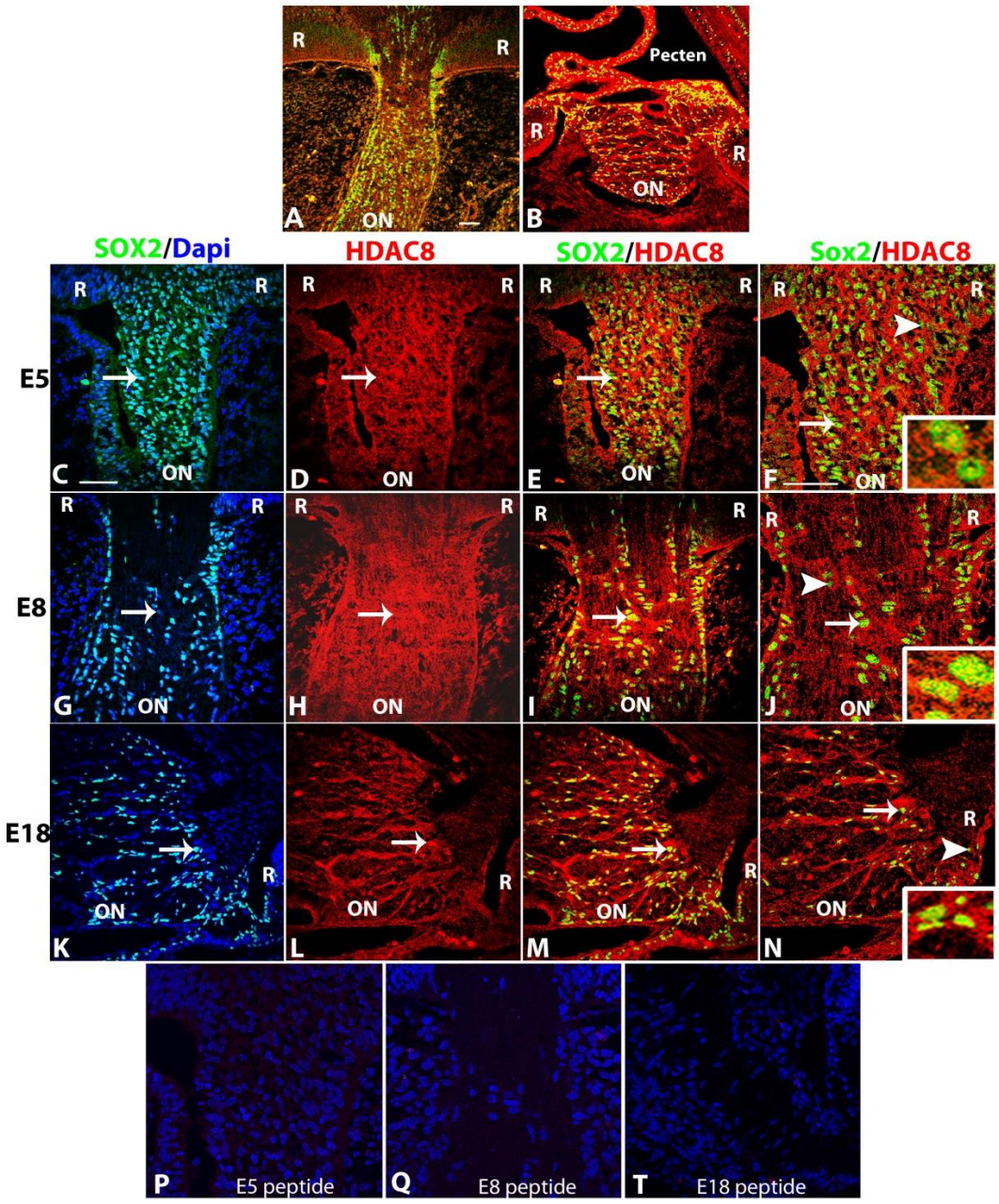

Fig.39 Localization of HDAC8 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC8 and DAPI triple

label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the other immunolabel. HDAC8 only label is shown (D, H and L) as well as SOX2 and HDAC8 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC8 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing to cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC8 antibody. Scale bars: (A) Bar = 50µm for images A and B (C) Bar = 50µm for panels C-E, G-I and K-M (F) Bar = 50µm for images F, J and N.

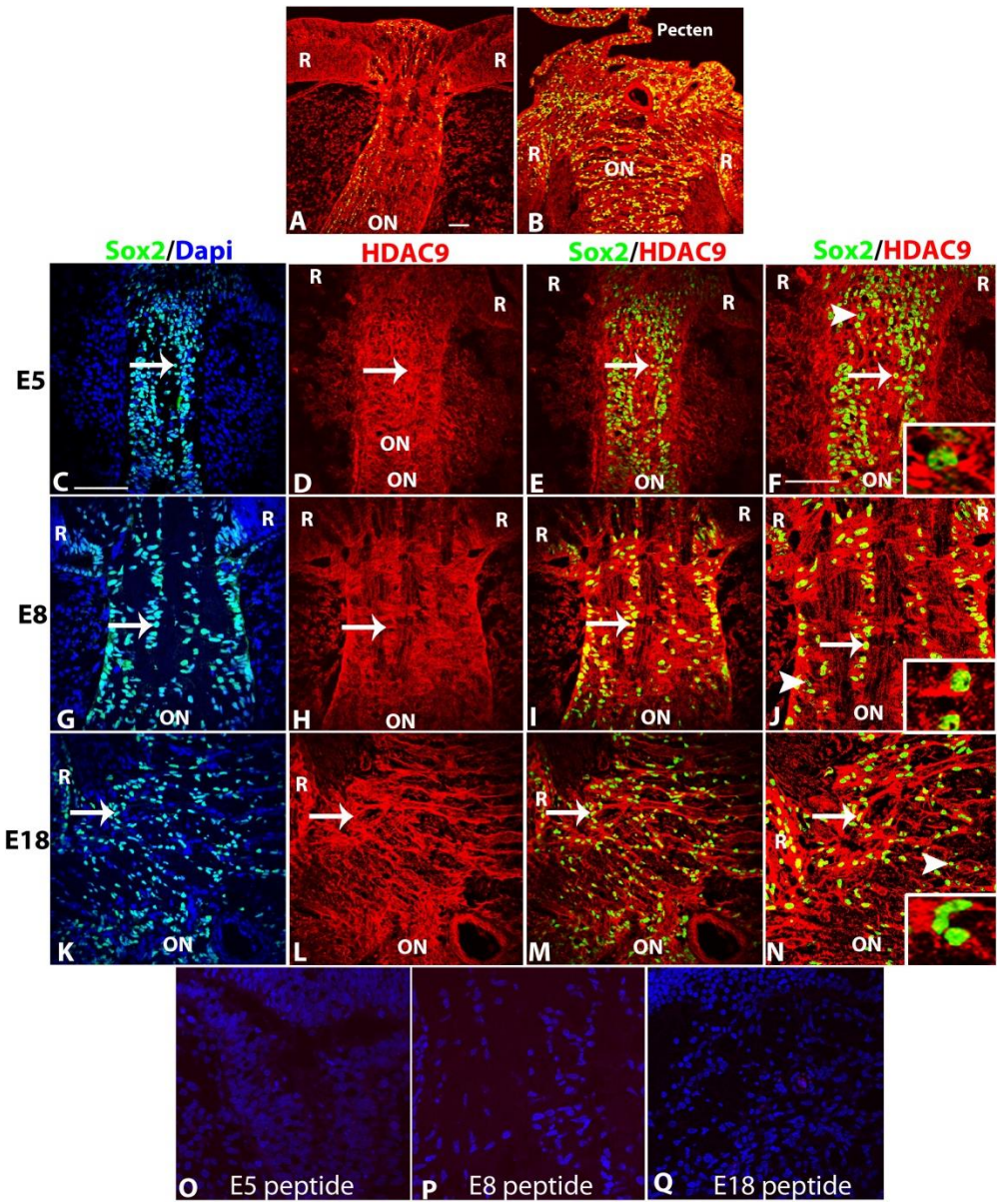

Fig.40 Localization of HDAC9 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularised part of the chick retina. Confocal imaging of SOX2, HDAC9 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H

and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). Pecten in image B represents the vascularisedpart of the chick retina. DAPI is not included in subsequent overlays because it appeared to obscure some of the immunolabel. HDAC9 only label is shown (D, H and L) as well as SOX2 and HDAC8 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC9 localized predominantly to the cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled whereas the arrowheads are pointing to the cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC9 antibody. Scale bars: (A) Bar = 50µm for images A and B (C) Bar = 50µm for panels C-E, G-I and K-M (F) Bar = 50µm for images F, J and N.

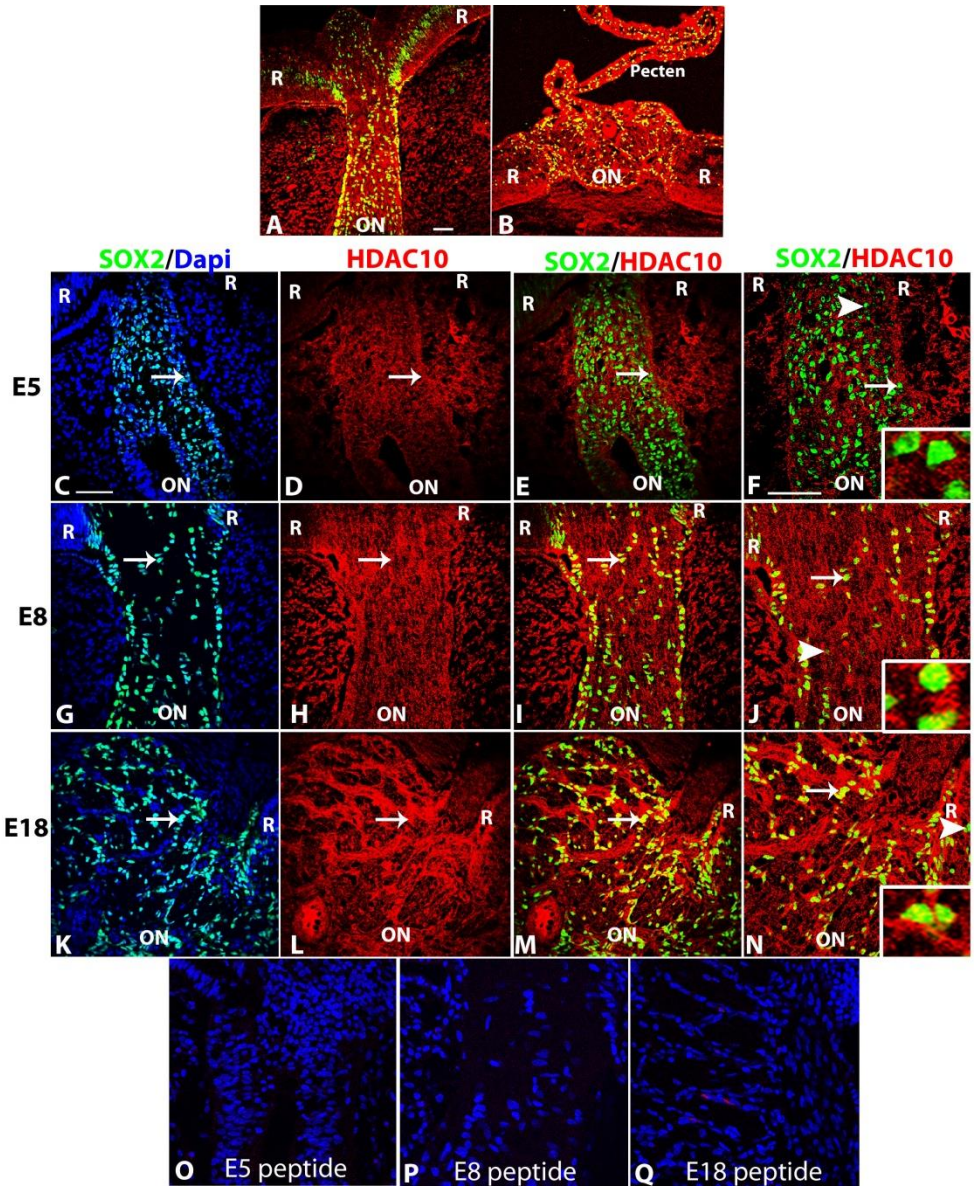

Fig.41 Localization of HDAC10 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularised Part of the chick retina. Confocal imaging of SOX2, HDAC10 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H

and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). DAPI is not included in subsequent overlays because it appeared to obscure some of the immunolabel. HDAC10 only label is shown (D, H and L) as well as SOX2 and HDAC10 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC10 has variable pattern of localization. It is present in the cytoplasm of optic nerve glia at E5 stage and is localized to nucleus and cytoplasm at E8 and E18 stage. Arrows indicate cells that show the pattern of localization and arrow heads are pointing to cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC10 antibody. Scale bars: (A) Bar = 50 µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

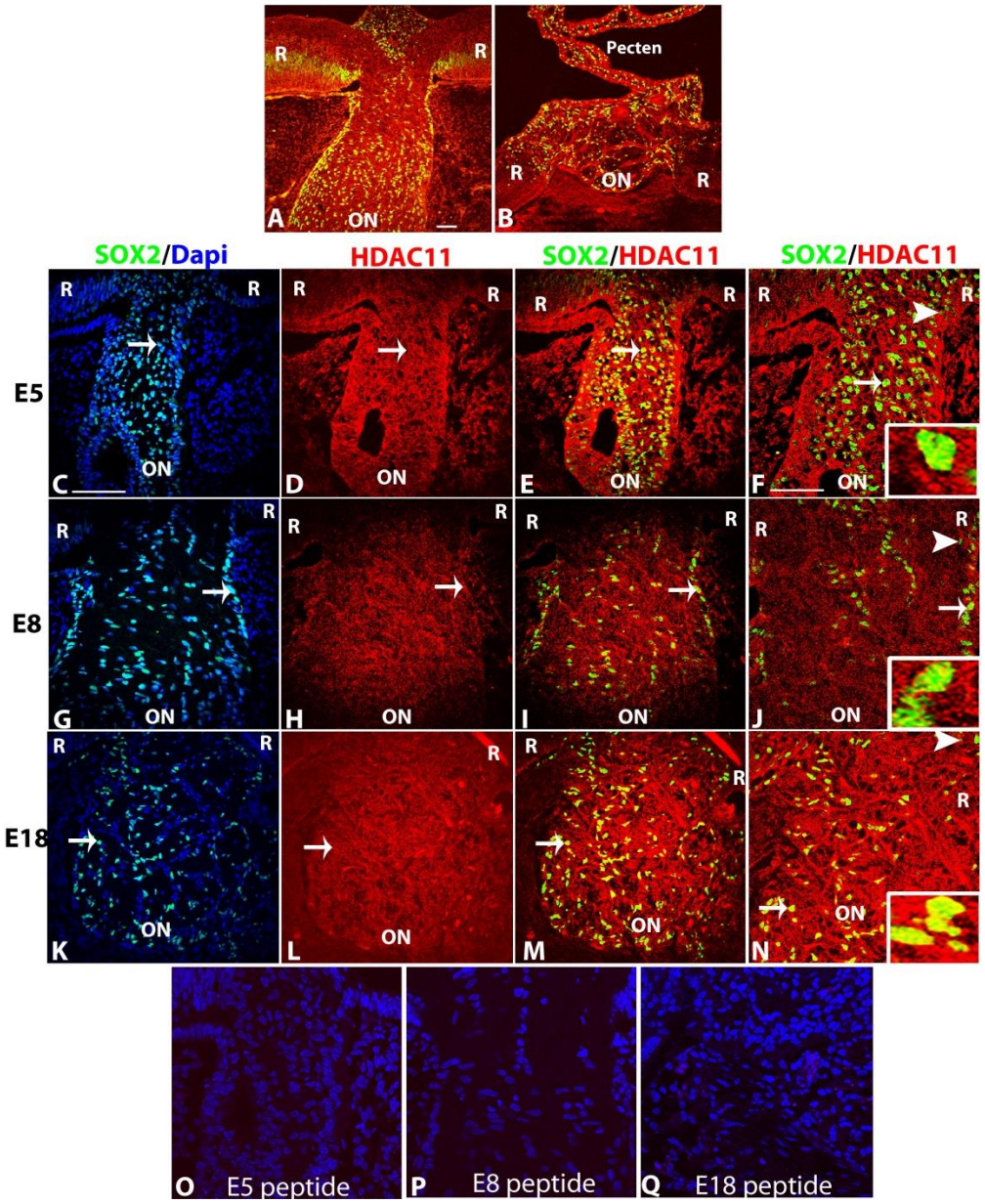

Fig.42 Localization of HDAC11 in chick optic nerve: Low magnification view of the E8 (A) and E18 (B) optic nerve, optic nerve head and retina is shown to aid in orientation of the sections. Pecten in image B represents the vascularized part of the chick retina. Confocal imaging of SOX2, HDAC11 and DAPI triple label of sections of chick optic nerve at E5 (C, D and E), E8 (G, H

and I) and E18 (K, L and M). Overlay of SOX2 (green) and DAPI (blue) is shown to aid in localization of the optic nerve and optic nerve head (C, G and K). Pecten in image B represents the vascularized part of the chick retina. DAPI is not included in subsequent overlays because it appeared to obscure some of the immunolabel. HDAC11 only label is shown (D, H and L) as well as SOX2 and HDAC11 overlay at 40x (E, I and M) and 60x magnifications (F, J and N). Higher magnification views are shown in 60x magnification panels (insets F, J and N). Double-label indicates that HDAC11 is localized to the nucleus and cytoplasm of optic nerve glia. Arrows indicate nuclei that are co–labeled and arrow heads are pointing to cells that do not show co label. Peptide controls (O, P and Q) for all the three stages were run after pre absorption with the primary antibody to verify the specificity of the HDAC11 antibody. Scale bars: (A) Bar = 50 µm for images A and B (C) Bar = 50 µm for panels C-E, G-I and K-M (F) Bar = 50 µm for images F, J and N.

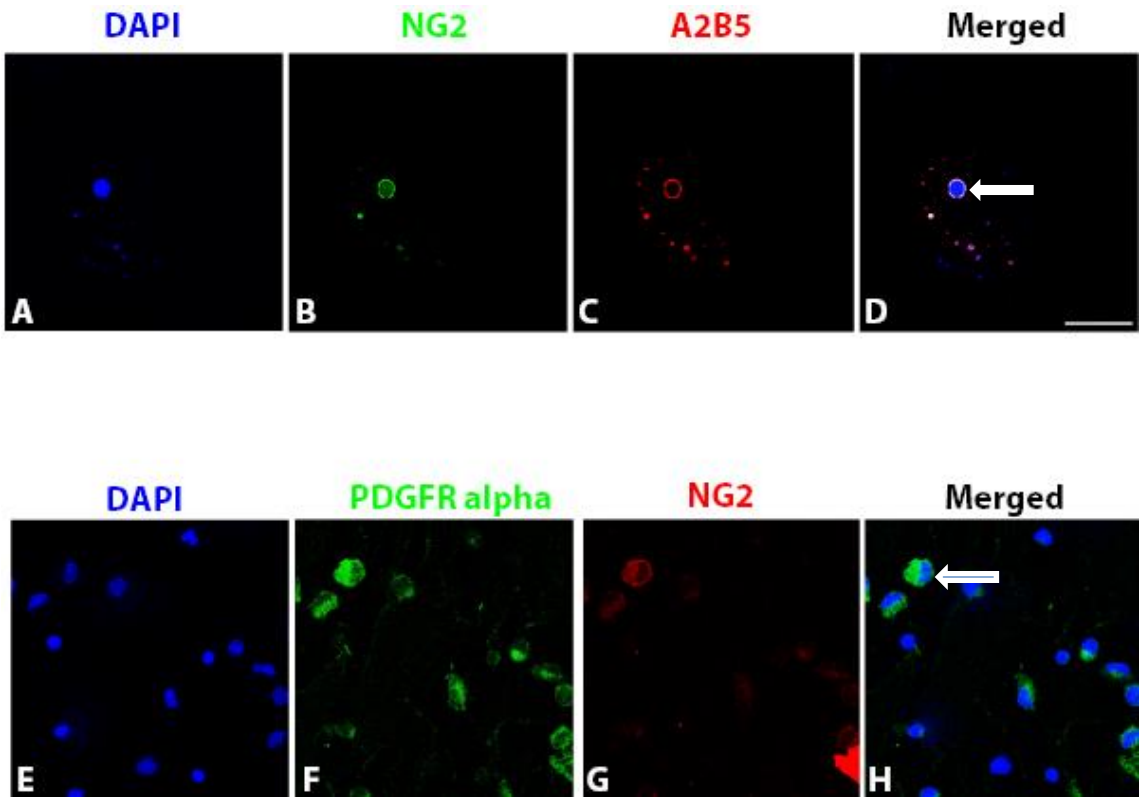

Fig.43 Expression of markers for the glial cells in murine optic nerve: Confocal images of dissociated cells from mature optic nerve double labeled for NG2 (B) and A2B5 (C). For panels A-D arrow depicts the O2Aprecursors which are NG2 (+) and A2B5 (+). For panels E-H the merged image (H) shows polydendrocytes that are NG2 (+) and PDGFRα (+). Scale bar: (D) = 50 μm.

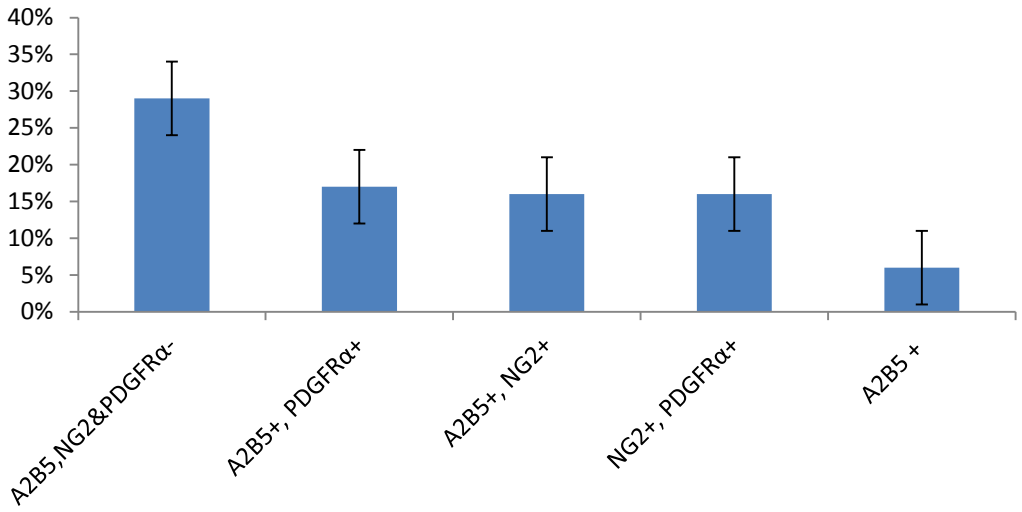

Fig.44 Quantitative analysis of the glial precursors in the adult murine optic nerve: The quantitation of the labeled cells revealed that all the cell types were present. The glial cell precursors predominantly present were O2A precursors (23%), astrocyte precursors/GRPs were found to be lowest in numbers(6%). 29% of the cells did not show label with any of the markers used for glial precursors.

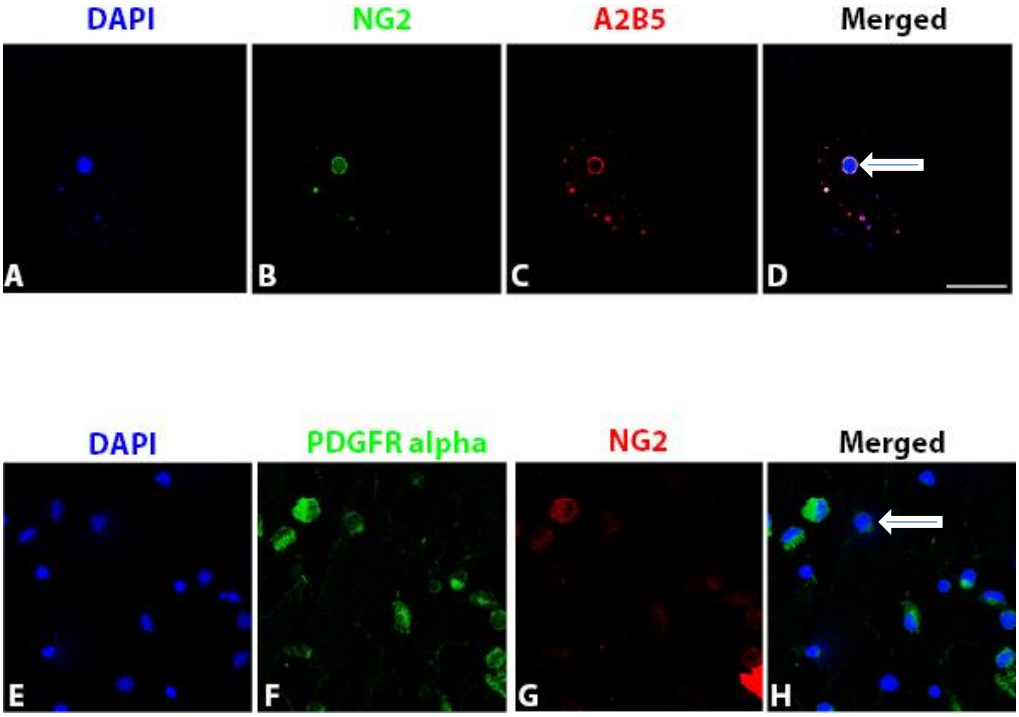

Fig.45 Expression of markers for the glial cells in chick optic nerve: Confocal images of dissociated cells from E18 chick optic nerve double labeled for NG2 (B) and A2B5 (C), PDGFRα (F) and NG2 (G). For the panels A-D merged image (D) shows the cell that is an O2A progenitor which is NG2 (+) and A2B5 (+). For the panels E-H, the merged image (H) shows polydendrocytes that are NG2 (+) and PDGFRα (+). Scale bar: (D) = 50 µm.

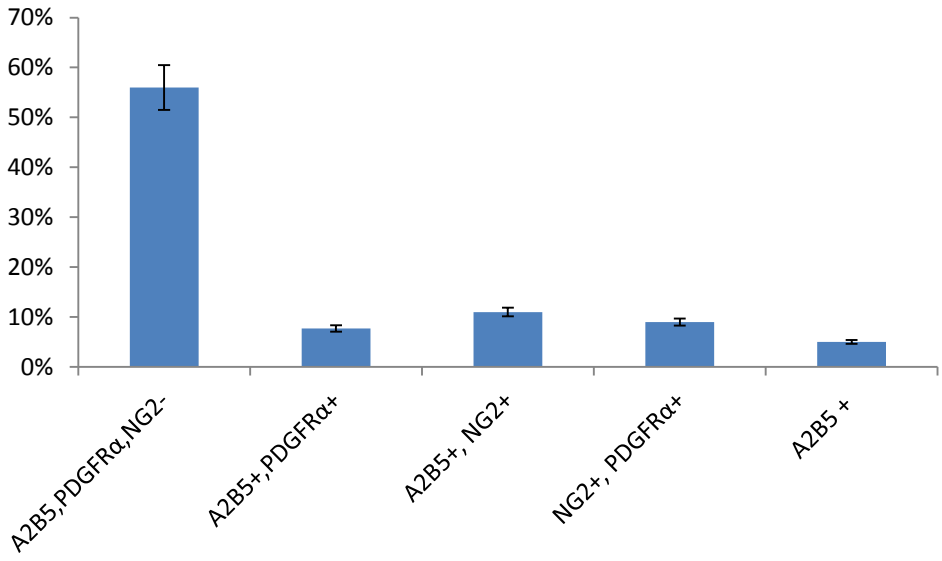

Fig.46 Quantitative analysis of the glial precursors in E18 chick optic nerve: The quantitation of the labeled cells revealed that all the cell types were present. The glial cell precursors predominantly present were O2A precursors (18.7%), astrocyte precursors/GRPs were found to be lowest in numbers(5%). 56% of the cells did not show label with any of the markers used for glial precursors.