Characterisation of the gene *PTPRS* in the development of the cerebral cortex

William Sherlock

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Supervised by Professor Antonella Riccio

MRC Laboratory for Molecular and Cell Biology

University College London

I, William Sherlock, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The cerebral cortex is a 6 layered structure, which is responsible for carrying out many of the higher functions of the brain including complex cognitive, sensory, emotional and motor tasks. During embryonic development, neuroepithelial stem cells (NSCs) or radial glia divide to produce post-mitotic neurons, which migrate radially to form the layers of the CP. We have previously shown that production of nitric oxide (NO) by neuronal nitric oxide synthase (nNOS) leads to S-nitrosylation of histone deacetylase 2 (HDAC2), thereby regulating the transcription of genes required for radial migration and cortical development. One such gene is protein tyrosine phosphatase receptor sigma (PTPRS), a membrane bound phosphatase, which acts as a receptor for chondroitin sulfate proteoglycans (CSPGs) and is associated with gross defects of several structures in the brain.

Here, I have examined the role S-nitrosylation of HDAC2 in regulating the expression of PTPRS and investigated the importance of PTPRS in the developing cortex. The expression of a HDAC2 construct which cannot be nitrosylated leads to downregulation of PTPRS in neurons and PTPRS is repressed in the cortices of nNOS knockout mice. To understand how PTPRS is involved in cortical development, I generated PTPRS conditional knockout mice in which I observed defects in radial neuron migration and cortical lamination. Enzymatic digestion of CPSGs in organotypic slice cultures show that CSPGs are also required for cortical neuron migration. Overall, this study identifies PTPRS as part of a regulatory mechanism involving NO and HDAC2, which is required for the radial migration of neurons and organisation of the cerebral cortex.

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Contents

Abbreviations

aRG	apical radial glia
Arp2/3	Actin-Related Proteins 2 and 3 complex
BAF	Brahma associated factor
BDNF	Brain-derived neurotrophic factor
BFPP	Bilateral frontoparietal polymicrogyria
bHLH	Basic helix-loop-helix family
BMP2/4	Bone morphogenetic protein 2/4
BP	Bipolar
Brm	Brahma
Cdc42	Cell division control protein 42 homolog
chABC	Chondroitinase ABC
CHD	Chromodomain helicase DNA-binding proteins
CGE	Caudal ganglionic eminence
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CNTN-1	Contactin 1
CP	Cortical plate
CR cells	Cajal-Retzius cells
CREB	cAMP-regulated element binding protein
CS	Chondroitin sulfate
CSPGs	Chondroitin sulfate proteoglycans
C6S	Chondroitin-6-sulfate
DCX	Doublecortin
DRF	Diaphanous-related formin
DRG	Dorsal root ganglia
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
Eph	Ephrin
EXT1	Exostosin Glycosyltransferase 1
IPCs	Intermediate progenitor cells
GABA	γ-aminobutyric acid
GAG	glycosaminoglycan
GAPs	GTPase-Activating Proteins
GDP	Guanosine diphosphate
GEFs	Guananine nucleotide exchanging factors
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate aspartate transporter
GTP	Guanosine triphosphate
GTPases	GTP hydrolysing enzymes
HAT	Histone acetyltransferase
HBSS	Hanks' balanced salt solution
HDAC	Histone deacetylase
HS	Heparan sulphate
HSPGs	Heparan sulfate proteoglycans
HS2ST	Heparan sulphate 2-O-sulphotransferase
HS6ST	Heparan sulphate 6-O-sulphotransferase
iNOS	Inducible nitric oxide synthase
INM	Interkenetic nuclear migration
IZ	Intermediate zone
LAR	Leukocyte antigen-related receptor
LIS1	Lissencephaly -1 gene
LIMK-1	LIM domain kinase 1
MGE	Medial ganglionic eminence
miRNA	Micro RNA
MLC	Myosin light chain
MP	Multipolar
MTOC	Microtubule organising centre
MZ	Marginal zone
NDE1	NudE Neurodevelopment Protein 1
NDEL1	NudE Neurodevelopment Protein 1 Like 1
NGF	Nerve growth factor
Ngn2	Neurogenin2
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP1	Neuropilin 1
NPCs	Neural progenitor cells
NuRD	Nucleosome remodeling deacetylase complex
PNS	Peripheral nervous system
PCM-1	Pericentriolar material 1
POA	Preoptic area
PP	Preplate
PTP	Protein Tyrosine Phosphatase
PTPRS	Protein tyrosine phosphatase receptor sigma
qPCR	Quantitative polymerase chain reaction
RELN	Reelin
RG	Radial glia
ROCK	Rho-associated protein kinase
RPTPs	Receptor-like protein tyrosine phosphatases
SDCCAG8	Serologically Defined Colon Cancer Antigen 8
SDF1	Stromal-derived factor-1
SNb/d	Segmental nerve b or d axons
SP	Subplate
SVZ	Subventricular zone
Trk	Tropomyosin-related kinase
VZ	Ventricular zone

1. INTRODUCTION

1.1 The architecture of the mammalian cortex

The roman physician Galen was the first to associate human cognition and willed action to anatomical structures of the body and specifically, the brain (Freemon, 1994). However, the importance of the cerebral cortex and the central role that it plays in cognition was not appreciated until much later when Thomas Willis described the expansion of the cortex during human evolution (Feindel, 1962). The first evidence that particular regions of the cortex were responsible for carrying out distinct functions came from the observation of clinical deficits in human patients suffering from brain damage. Lesions to specific areas of the left hemisphere were observed to affect language and cataloguing of the effects of various forms of epilepsy led to the creation of a map of areas of the cerebral cortex which controlled motor functions. Creating lesions to these areas in animals led to similar defects, while electrical stimulation caused movements in particular sets of muscles, thus confirming the existence of the motor cortex (Fritsch and Hitzig, 1870). These insights led to our current understanding of the cortex as the anatomical structure that allows us to produce a meaningful perception of ourselves and the world around us. It plays a vital role in higher brain functions, such as thought, consciousness, language, sensation and movement (Kwan et al., 2012a).

The human cortex consists of a sheet of tissue 3-4mm thick with a surface area of approximately 2600cm^2. It contains 27x10^9 neurons and a similar amount of glial cells (Mountcastle, 2003). Our modern understanding of the structure of the cerebral cortex and its division into functional regions is based on the study of cytoarchitecture(cellular composition). This began with the work of Theodor Meynert, who developed methods to stain and visualise cellular components of the cortex. In this way, he showed that different areas could be distinguished on the basis of their cellular composition. Maynert was the first to observe a region of the cortex, containing granule cells of increased size and density, which was later

identified as the primary visual cortex (Yarmohammadi et al., 2014). Shortly after, the motor cortex, which had previously been discovered by the use of electrical stimulation, was found to correlate with a cytoarchitecturally distinct area that contained a cluster of large neurons in the precentral gyrus (Betz, 1874).

A comprehensive study of the cytoarchitecture of the cerebral cortex was then carried out by Korbinian Brodmann between 1903 and 1908 (Brodmann, 1910). Herein, he described the laminar organisation of the cortex, dividing it horizontally into 6 layers, and identified over 40 distinct areas on the basis of how the cells of each layer were arranged in that region. Most of the cortex is organised in this 6 layered manner and is referred to as the neocortex or isocortex. However, variations on this organisation do exist. For instance, the allocortex, which contains the hippocampus and pyriform cortex, is composed of three or four layers in the adult. There is also a transitional region between the allocortex and the neocortex, which is referred to as the proisocortex. Although efforts have been made to refine this organisation and to base the various divisions on more objective measurements and functional imaging studies (Amunts et al., 2007), all architectural descriptions of the cortex still rely on discerning patterns of lamination in different cortical regions.

The layers of the cerebral cortex are composed of neurons, glia and fibers, all of which are organised into layers. Neurons located in these layers can be divided into two types: GABAergic inhibitory interneurons and glutamatergic excitatory neurons. Interneurons have sparsely spined dendrites and their axons project locally. They make up about 20-30% of all cortical neurons and play important roles in controlling cortical output and plasticity (Whittington and Traub, 2003). Glutamatergic neurons are further classified into stellate neurons and projection neurons, which are the most abundant class of neurons in the cortex. The uppermost layer I, also known as the molecular layer, contains very few neuronal cell bodies and is mostly made up of dendrites of neurons whose soma are located in lower layers. The next two layers of the cortex, layers II-III (collectively known as the supragranular

layers), consist of a mixture of stellate and pyramidal neurons, which project their axons to other areas of the cortex via the corpus callosum. Layer IV (granule layer) contains a large number of spiny stellate interneurons. In contrast, the deeper layers V-VI and the subplate (SP) contain very large neurons and small spindly pyramidal neurons, which project to subcortical structures (Shipp, 2007). Each of these layers is associated with unique patterns of gene expression and, thus, particular genes can be used as markers of cortical layers. Many of these genes are transcription factors that play important roles in determining the properties of the cortical layer in which they are expressed. Example of these genes include SatB2, which is expressed in upper layers and Tbr1, expressed in lower layers (Arlotta et al., 2005). Cortical layers have distinct functional attributes and the differences in their cytoarchitectural makeup reflect these functional differences. For instance, the large pyramidal neurons (Betz cells), located in layer V of the motor cortex, are connected to spinal motor centres of the corticospinal tract and are important for motor functions (Braak and Braak, 1976). Another example is the expansion of layer IV in the auditory, visual and somatosensory regions, which allows for a larger number of small granule neurons to be connected to sensory regions of the thalmus. Layer IV is so large in the visual cortex that it is further divided into sublayers 4A, B and C (Hubel and Wiesel, 1965).

Vertically, the cortex is organised into columns of cells that contain all the major neuronal types and are connected across layers by synapses. These structures are referred to as minicolumns and can be considered the basic functional units of the cortex. Each minicolumn is made up of about 80 to 100 neurons and contains all cortical cell types connected to each other vertically. In the visual cortex, simultaneous activation of neurons in a minicolumn corresponded to stimulation of the same receptive field (Mountcastle, 1957). Minicolumns are also thought to be organised into groups termed columns, which consist of a number of horizontally connected minicolumns that share physiological properties (Mountcastle, 1997). Minicolumns can also be distuinguished on the basis of differential gene expression (Kwan et al., 2012b).

1.1.1 The development of the cerebral cortex

The first events that lead to the development of the nervous system occur at gastrulation. During gastrulation, the embryo is divided into three germ layers, endoderm, mesoderm and ectoderm are formed. The endoderm, in the interior of the embryo gives rise to the gut, internal organs such as the liver, pancreas and lungs whilst the mesoderm forms the mesenchyme and notochord. The mesenchyme is the precursor of bone, connective tissue and cartilage, whereas the notochord is a cylindrical structure that later becomes the vertebral column. The notochord plays an important role in the induction of neural tissue. The mesoderm also forms musculature, the urogenital system and vasculature. Finally, the ectoderm on the outside of the embryo is the precursor of the epidermis and neural plate, which will go on to form the nervous system (Solnica-Krezel and Sepich, 2012). After gastrulation, the notochord induces the ectoderm overlaying it to become the neural plate (neuroepithelium). The neural plate then begins to fold inwards dorsally, with both ends eventually meeting and fusing to form the neural tube. Closure proceeds both caudally and rostrally from the site of initiation. Once complete, the neural tube separates from the rest of the ectoderm and the edges of what was once the neural plate become the neural crest. The neural crest gives rise to the peripheral nervous system and other tissues such as melanocytes, craniofacial cartilage and bone. In the neural tube, bulges now appear which are referred to as brain vesicles.

These vesicles represent the precursors of the main divisions of the brain and include the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). Adult features of the forebrain can start to be discerned in the prosencephalon at this point. It can be divided into the diencephalon, hypothalmus, retinae and telencephalon which can be subdivided into progressively more discrete regions as development proceeds. The cerebral cortex as well as the basal ganglia and olfactory bulb, is derived from the telencephalon. The process that results in the development of the cortex from the telencephalon (corticogenesis) is comparable in all mammals, however, our understanding of this process is mostly derived from work carried out in the developing rodent (Gilbert, 2013).

As corticogenesis begins, the wall of the telencephalon, from which the cortex will form, consists of a pseudo-stratified neuroepithelium made up of neural progenitor cells, (NPCs) from which all of the cells of the mature cortex will be derived. Each NPC extends a basal process to contact the pial surface and a smaller apical process that connects to the ventricular wall. Mitotic spindles of actively dividing cells are located at the ventricular wall. This produces an elongated morphology that spans the full extent of the cortical wall. Once this morphology is acquired, the cells are termed apical radial glia (aRG) and express a number of glia-associated markers, such as the astrocyte specific glutamate transporter (GLAST) and the glial fibrillary acidic protein (GFAP) (Doetsch, 2003) (Gotz and Barde, 2005). As they progress through the cell cycle, their nucleus moves in an apical to basal manner, in coordination with the cell cycle. During G1 the nucleus is moving to the basal surface of the neuroepithelium where the cell enters S phase. The cell is in G2 as the nucleus moves apically, and when it reaches the ventricular wall, the cell divides. This is known as interkinetic nuclear migration (INM) (Sidman et al., 1959) Over the course of development, the length of the cell cycle becomes progressively longer nearly doubling in duration over the course of cortical neurogenesis. This is due to an elongation in the length of the G1 phase (Miller and Kuhn, 1995).

Cortical aRGs undergo two different types of division, symmetric and asymmetric division. During symmetric division, two aRGs are produced, whereas asymmetric division results in one aRG and a post mitotic neuron or one aRG and one more fate restricted progenitor, such as an intermediate progenitor. Regardless of their individual fates, aRGs and their progeny undergo a strict programme of cell division, which results in the production of a defined number of neurons (Gao et al., 2014). It has been suggested that aRGs are not a

homogenous group as originally thought, and may possess distinct neurogenic potential. For example, CUX2, a transcription factor expressed in a subset of aRGs at E10, correlates with cells that will preferentially produce neurons destined for the upper layers of the cortex (Gil-Sanz et al., 2015). At E10, aRGs undergo several rounds of symmetric division, which results in the expansion of the pool of NPCs. As their proliferative potential diminishes (around E11-E12), they gradually switch to asymmetric division and post mitotic neurons begin to be generated. At E17.5, there is a transition from neurogenesis to gliogenesis and both astrocytes and oligodendrocytes begin to be produced at this point (Toma and Hanashima, 2015). These timings are, however, approximate and there are differences in the rate of maturation of different areas of the cortex with anterior and ventral regions beginning to develop slightly earlier than posterior and dorsal areas. This morphogenetic gradient results in differences of as much as a day or two of developmental progression with the dorsoventral lag being more pronounced than antereposterior one (Caviness et al., 1995).

The first post mitotic neurons to be generated migrate outwards towards the pial surface in a process called radial migration to form the preplate (PP). The area at the ventricular wall containing the proliferative aRGs is referred to as the ventricular zone (VZ) from this point onwards. The PP is made up of Cajal Retzius (CR) cells, which are large cells with long processes that extend parallel to the ventricular wall. Subsequent waves of neurons generated in the ventricular zone migrate into the PP, splitting it into the prospective subplate (SP) and marginal zone (MZ). The structure generated by these neurons moving into the space between the SP and MZ becomes the cortical plate (CP). The MZ will later become layer I, the first of the six layers of the mature cortex, while the CP will give rise to layers II to VI (Fig 1.1) (Ayala et al., 2007). The subplate is transient in nature and many of its neurons undergo apoptosis postnatally. The intermediate zone (IZ) is a cell sparse region that forms between the VZ and CP. Neurons destined for the CP must migrate through this area before reaching their final positions in the cortex and it will go on to contain the major efferent and afferent

axonal tracts, thus becoming the white matter of the developed cortex (Hoerder-Suabedissen and Molnár, 2015).

After the first post mitotic neurons have been generated, a secondary population of proliferative cells arises in the area between the VZ and IZ. This area is referred to as the subventricular zone (SVZ). Initially, the SVZ is thinner than the VZ but increases in size throughout embryonic development, reaching its peak during early postnatal development. The ventricular zone decreases in size until it begins to disappear late in gestation and this decrease in size is accompanied by a reduction in the rate of proliferation of progenitors in the VZ. Proliferation in the SVZ on the other hand increases dramatically during this time so that by E16, the majority of the VZ has exited the cell cycle, whereas 90% of cells are still actively dividing in the SVZ. (Takahashi et al., 1999). The SVZ persists into adulthood, where a residual population of progenitors remain mitotically active. It is sometimes referred to as the subependymal zone, as the cells are adjacent to the ependymal region (Gage, 2000) (Ma et al., 2009). The SVZ is subdivided into the medial ganglionic eminence (MGE), the lateral ganglionic eminence (LGE) and the caudal ganglionic eminence (CGE), each region containing progenitors that give rise to different populations of neurons and glia. The developing human cortex contains a further subdivision of the SVZ, called the outer SVZ. This region contains intermediate progenitors as well as large numbers of radial glia like progenitors, which lack a basal process. It is thought that the extensive expansion of the outer SVZ in humans is a key factor in generating the unique size and complexity of the human cortex (Hansen et al., 2010) (Dehay et al., 2015)

The order in which neurons destined for particular cortical layers are generated proceeds in a stereotypical fashion, with neurons destined to form the deepest layers of the cortex generated first, followed by later born neurons that will form more superficial layers. The CP is therefore, generated in a characteristic inside out manner (Berry and Rogers, 1965) (Angevine Jr. and Sidman, 1961).

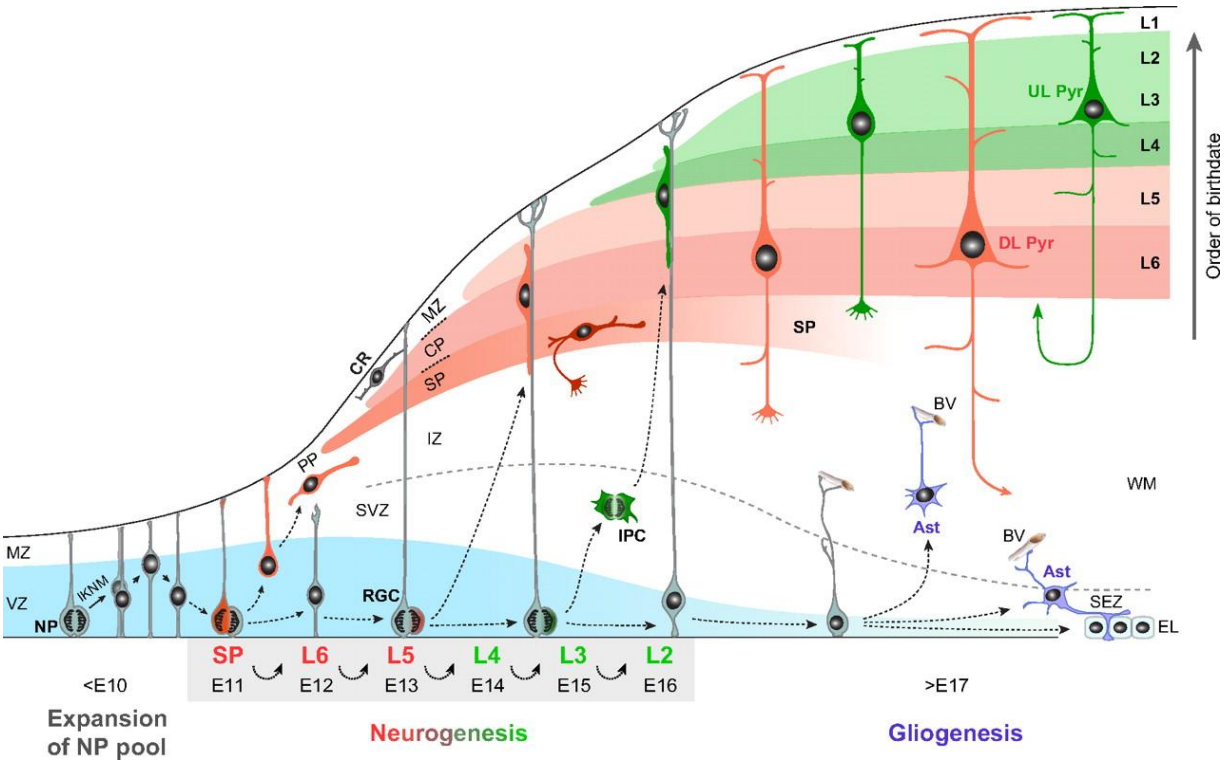

Fig 1.1 Schematic of projection neuron generation and migration in the mouse neocortex. Prior to the onset of neurogenesis, neural progenitors (NPs) in the ventricular zone (VZ; blue) of the developing neocortex divide symmetrically to expand the progenitor pool. From E11.5, NPs assume radial glial morphology and begin to divide asymmetrically to generate neurons. The first projection neurons migrate to the pial surface to form the preplate (PP). Additional incoming cortical plate (CP) neurons then split the PP into the marginal zone (MZ) and the subplate (SP). Thus, neurons destined for the SP are generated first followed by those destined for the deep layers (VI and V; red), and finally, those destined for the upper layers (IV, III and II; green). Some daughter cells of NPs become intermediate progenitor cells (IPCs), At the end of neurogenesis at E17.5, the radial scaffold is dismantled and NPs become gliogenic. BV, blood vessel; CR, Cajal-Retzius neuron; DL Pyr, deep-layer pyramidal neuron; IZ, intermediate zone; UL Pyr, upper-layer pyramidal neuron; astrocyte (Ast); radial glia cell (RGC); subependymal zone (SEZ); ependymal layer (EL). (Kwan et al., 2012c)

1.2 Neuronal migration

To reach their final location within the CP where they will terminally differentiate, neurons must migrate from the ventricular zone outwards towards the pial surface. In order to do this, neurons utilize a number of different migratory modes and transition between modes as they move through certain regions. In addition to this, different neuronal types use clearly distinct migratory routes and behaviours (Kwan et al., 2012a).

1.2.1 Migration of Cajal-Retzius cells

CR cells originate from multiple embryonic structures, including the cortical hem, the ventral hem, the caudomedial telencephalon and the thalamic eminence. They migrate to the cortex and enter perpendicularly to the direction of radial neuron migration and this is referred to as tangential migration (Meyer and Wahle, 1999); (Meyer et al., 2002); (Takiguchi-Hayashi et al., 2004); (Cabrera-Socorro et al., 2007); (Meyer, 2010). CR cells migrate in response to chemokine stromal-derived factor-1 (SDF1/CXCL12), a chemoattractant produced by the meninges (Paredes et al., 2006) (Borrell and Marín, 2006) and undergo a "surface-spreading" movement to cover the developing neuroepithelium. The spreading is dependent on contact repulsion, whereby repulsive Eph/ephrin interactions between CR cells lead to a dispersion across the cortical surface. (Villar-Cervillo et al., 2013).

1.2.2 Migration of interneurons

Interneurons are generated areas of the brain, which include the MGE, LGE, CGE and the preoptic area (POA) and begin to migrate at E11.5 (Gelman et al., 2009); (Anderson et al., 2001). They first undergo tangential migration in order to reach the cortex at which point they switch direction to radial migration and move into the CP (Anderson et al., 1997); (Parnavelas, 2000). The first cortical interneurons are generated in the MGE and migrate to the top of the PP and eventually contribute to the MZ. Between E13.5 and E15.5, a larger stream of interneurons migrates from the MGE and LGE migrate through the IZ before entering the CP. Later, interneurons enter the cortex via multiple routes, through the IZ and SVZ as well as the

SP and MZ (Marín and Rubenstein, 2001). Internuerons migrate to the cortex by extending multiple processes that sense migratory ques that stabilise processes orientated in the correct direction. Once directionality is obtained, the cell moves forward by first positioning the centrosome in the leading process and then moving the nucleus towards the centrosome. This pattern of nucleokinesis is repeated to generate directional movement (Bellion et al., 2005). The rules that determine laminar positioning of interneurons varies and is determined by their origin and birthdate. For instance, MGE and POA derived interneurons expressing somatostatin, parvalbumin or calbindin enter the cortex in an inside out manner, similar to projection neurons, occupying deeper layers first then populating upper layers. CGE derived calretinin expressing cells do the inverse, occupying outer layers first, then deeper. Other populations migrate to upper layers only or seems to distribute randomly (Rymar and Sadikot, 2007) (Guo and Anton, 2014).

It is believed that laminar positioning of interneurons depends on the area in which they are born rather than the timing of birth. For example, interneurons born in the CGE tend to locate to the superficial areas of the cortex independently of their time of birth. (Miyoshi and Fishell, 2011).

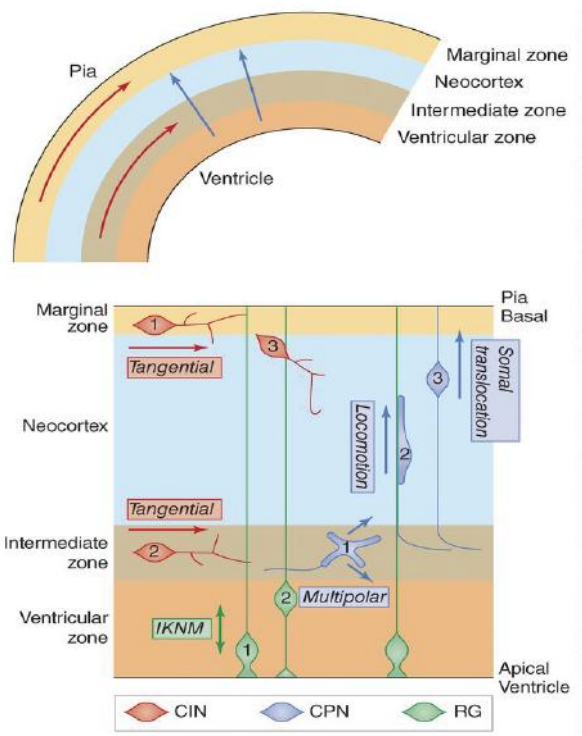

Fig 1.2 Modes of migration in the rodent brain

Transverse sections through the developing rodent brain (top). Top panels show the migration routes and bottom panels show the types of migration. The colors of arrows in the top panels correspond to the colors of cells in the bottom panels. Cortical interneurons (CINs, red) migrate tangentially along the MZ (1) and IZ (2) from their origins in the basal forebrain. Later they migrate into the cortical plate (3). Radial glia (RG, green) undergo interkinetic nuclear movement (IKNM), with mitosis apical (1) and S phase basal (2). Cortical projection neurons (CPN, blue) migrate through three phases: multipolar (1), locomotion (2), and somal translocation (3). Taken from (Cooper, 2013)

1.2.3 Migration of projection neurons

Numerous live imaging studies using labelled cells revealed that projection neurons use four modes of migration referred to as delamination, multipolar migration, locomotion and somal translocation. Delamination occurs when post-mitotic neurons and intermediate progenitors produced by asymmetric division of RGs leave the ventricular zone, assume a bipolar morphology and migrate to the subventricular zone (SVZ). During the second phase, cells acquire a multipolar morphology by extending and retracting numerous neurites. At this stage, cells move in an irregular way, often reversing their direction temporarily to migrate away from the CP before resuming their journey in the correct direction. Once they have reached the upper intermediate zone (IZ), cells orientate their Golgi and centrosome toward the CP and begin to protrude a single leading process towards the CP thereby reacquiring a bipolar morphology. This process is referred to as the multipolar to bipolar (MP to BP) switch. Early born projection neurons directly attach their leading process to the extracellular matrix of the pial surface and undergo terminal translocation during which the cell body is moved up the leading process and enters the CP where assumes its final position. As development proceeds and the CP is expanded, neurons are faced with a steadily increasing distance over which they must extend their leading process in order to reach the pial surface and initiate somal translocation. Later born neurons overcome this obstacle by introducing an extra mode of migration, termed locomotion. At the top of the IZ where early born neurons normally switch to somal translocation, later born neurons acquire a bipolar morphology and physically attach to RG fibres, which they then use as a guide in order to migrate into the CP (Rakic, 1972). Once the leading process has made contact with the pial surface, late born neurons switch to somal translocation to reach their final positions. The development of the cortex is therefore a very complex process, which requires that neurons undergo many changes of migration behaviour in a process that ultimately ensures the formation of its intricate cytoarchitecture (Cooper, 2013).

1.2.4 Role of the cytoskeleton during neuronal migration

Neurons migrate by extending a leading process, which in turn extends lamellipodia and filopodia. Lamellipodia and filopodia consist of filamentous acin (F-actin) stress fibres connected to substrates at points of contact termed focal adhesions. Cells begin forward movement by first creating a swelling in the leading process, and then moving the centrosome into the swelling. Once the centrosome reaches the leading process, bundles of microtubules connecting the centrosome to the nucleus coupled with dynein motors in the centrosome create the pulling power required to bring the nucleus into the leading process. Finally, the trailing processes at the rear of the cell retract as a result of actin and myosin action (Moon and Wynshaw-Boris, 2013). This remodelling of the cytoskeleton is dynamically regulated in order to produce the different modes of migration (Pilz et al., 2002). Regulators of cytoskeletal dynamics involved in this process such as Lissencephaly -1 (LIS1) were among the first genes to be linked to cortical malformations. Lis1 is a gene mutated in type I lissencephaly, which causes severe cortical malformations (Reiner et al., 1993). LIS1 associates with the microtubule organising centre (MTOC) in a complex that includes NDE1, NDEL1 and dynein where it regulates both cell division and the MP to BP transition. During migration, LIS1 co-ordinates centrosome and nuclear translocation, which allows the cells to enter into the CP (Moon et al., 2014). Coupling of the nucleosome and centrosome is required for cells to be polarized during directed migratory behaviours and for neurons to acquire bipolar morphology during the MP - BP switch (Sakakibara et al., 2014). Mutations in genes such as doublecortin (DCX), which stabilizes microtubules connecting the centrosome and nucleus are also associated with Lissencephaly and severe defects in radial migration (Horesh et al., 1999). Other proteins involved in this process include components of the perinuclear material (PCM) surrounding the centrsosome. These include γ-tubulin, PCM-1, pericentrin and ninein (Dammermann and Merdes, 2002). Serologically defined colon cancer antigen 8 (SDCCAG8) is required for this accumulation of PCM around the centrosome allowing the centrosome and the nucleus to be coupled (Insolera et al., 2014). Disruption of the PCM by mutation of SDCCAG8 or other PCM genes is associated with severe neurodevelopmental disorders

characterised by mental retardation, cognitive impairment, and seizures (Billingsley et al., 2012) (Otto et al., 2010).

1.2.5 Small GTPases

Small GTPases are a group of enzymes that hydrolyze guanosine triphosphate (GTP), converting it to guanosine diphosphate (GDP). They are collectively referred to as Ras superfamily GTPases and are subdivided into the Rho, Ras, Rab, Arf and Ran families. They act as molecular switches, cycling between GTP bound (active) and GDP (inactive) states in a manner regulated by guanine nucleotide exchanging factors (GEFs) and GTPase activating proteins (GAPs). GEFs promote activation of GTPases by inducing the exchange of GDP with GTP whereas GAPs enhance their enzymatic activity leading to hydrolysis of GTP and protein inactivation (Bos et al., 2007). In their active state small GTPases interact and activate downstream effectors that include serine/threonine kinases, scaffold proteins, lipid kinases, oxidases and lipases (Bishop and Hall, 2000). RhoA, Rac1 and Cdc42 regulate cell migration and structure by controlling polymerisation and bundling of actin in the cytoskeleton (Jaffe and Hall, 2005). They regulate polymerisation by controlling the action of actin filament capping, severing, and polymerisation proteins. Rac and Cdc42 stimulate actin polymerisation by activating the Arp2/3 polymerisation factors via the Wiskott-Aldrich syndrome family of proteins. (Millard et al., 2004). Rho proteins also stimulate polymerisation by activating formins such as the diaphanous-related formin (DRF), mDia1 (Zigmond, 2004). Cofilin on the other hand is an example of a protein that severs actin filaments. This either facilitates uncapping, which allows for subsequent filament extension or leads to the removal of actin monomers and filament disassembly. RhoA stabilises actin filaments by antagonising the action of cofilin. It

does this by activating the effector, Rho kinase (ROCK) which leads to phosphorylation of cofilin at serine 3 by LIM-kinase (LIMK-1) (Ohashi et al., 2000). The mechanisms by which Rho GTPases regulate actin organisation is less well understood and the best characterised example is the RhoA induced assembly of contractile actin:myosin filaments mediated by ROCK. ROCK phosphorylates myosin light chain (MLC) phosphatase which leads to cross-linking of actin filaments my myosin II (Riento and Ridley, 2003). The role of Rho family GTPases in cell migration is widely appreciated and more recently, numerous studies have shown that members of the family play diverse and critical roles in radial migration in the CNS (Govek et al., 2011).

Several members of the Rac family of GTPases are expressed in the nervous system but most studies have focused on the role of Rac1 in cortical development (De Curtis, 2007). Rac1 is required for the formation of the leading process during neuronal migration (Konno et al., 2005); (Kawauchi et al., 2003). Lack of Rac1 in the forebrain leads to a delay in cortical neuron migration. (Chen et al., 2007) (Kassai et al., 2008) (Yoshizawa et al., 2005). Knockout of Rac1 GEFs, STEF, Tiam1 or P-Rex1 also interferes with radial migration (Kawauchi et al., 2003), whereas electroporation of a dominant negative form of Rac1 (N17-Rac1) leads to much stronger defects of migration, with cells accumulating in the IZ (Kawauchi et al., 2003) (Konno et al., 2005) (Yang et al., 2012). Electroporation of a constitutively active Rac1 mutant (V12-Rac1) induces a similar phenotype illustrating the importance of maintaining tight control over the activity of small GTPase activation (Konno et al., 2005) (Yang et al., 2012). The fact that radial migration is not as strongly affected in Rac1 knockout mice could be due to an upregulation of other Rac proteins and small GTPases (Chen et al., 2007).

Rap1, is a Ras family GTPase and is required for the orientation of multipolar cells as they migrate through the IZ (Jossin, 2011). The activity of Rap1 is controlled by RapGEF1 and RapGEF2, which are both implicated in cortical development. In mice lacking RapGEF2 in the telencephalon, the PP fails to split and cells are unable to undergo the MP to BP switch (Bilasy et al., 2009) (Ye et al., 2014). RapGEF1, on the other hand, regulates somal translocation suggesting that the same GTPase can influence several aspects of cortical development and regulation of Small GTPase activity by GEFs and GAPs plays an important role in tailoring small GTPase activity to the developmental context (Ye et al., 2014). CDC42 regulates both proliferation of neural progenitors and radial migration. Conditional deletion of Cdc42 in the mouse forebrain using an $Emx1^{Cre}$ line leads to increased proliferation of basal progenitors (Cappello et al., 2006). Electroporation of a dominant negative Cdc42 (N17Cdc42) or a constitutively active Cdc42 (V12Cdc42) construct into migrating cortical neurons inhibited radial migration (Konno et al., 2005). Cdc42 promotes migration partly by interacting with Lis1, and mutations in Lis1 lead to decreased activation of both Cdc42 and Rac1 as well as improper localisation of the Cdc42 effector protein IQGAP1 (Kholmanskikh et al., 2003) (Kholmanskikh et al., 2006).

Rho proteins influence radial migration and RhoA is expressed in migrating cells. Inhibition of RhoA is necessary for the migration of cells through the IZ , whereas in cells located in the upper IZ , RhoA mediates MP to BP transition (Hand et al., 2005). Activation of RhoA and its downstream effector Rock leads to LIMK-dependent phosphorylation of cofilin, which stabilises actin microtubules. In contrast, unphosphorylated cofilin leads to disassembly of microtubules. Inhibition of LIMK or dephosphorylation of cofilin impair the dynamic assembly of the microtubule network that leads to defects in radial migration (Chai et al., 2016).

1.3 Transcriptional control of cortical development

During cortical development, the transcriptional landscape of neurons is constantly changing (Telley et al., 2016). Transcription factors play a key role in governing all aspects of cortical development. Neurogenin2 (Ngn2) is a pro-neural transcription factor critical for NPC differentiation into glutamatergic neurons that also regulates the MP to BP switch of cells that are about to enter the CP. Ngn2 influences MP to BP switch, at least in part, by increasing the expression of the Rho-GTPase Rnd2 (Heng et al., 2008). Reciprocal repression and activation of transcription factors ensures that gene expression is regulated both spatially and temporally. For instance, the zinc finger transcription factor RP58 is a target of NGN2 that is expressed in the CP at E15.5 and directly represses NGN2 and other IZ genes, such as RND2. Expression of both Ngn2 and RND2 must be inhibited in order to allow the MP to BP transition to occur and cells to migrate into the CP (Aoki et al., 1998) (Xiang et al., 2011) (Ohtaka-Maruyama et al., 2013). FOXG1 and Coup-tf1 are other examples of transcription factors required for the MP to BP switch. (Miyoshi and Fishell, 2012) (Alfano et al., 2011).

Cyclic-responsive element binding protein (CREB) is one of the most studied regulators of transcription in neurons, which mediates neuronal survival, growth and plasticity. Mice lacking CREB show severe defects in many tissues including the brain (Rudolph et al., 1998). It is essential for neuronal survival, and double knockout of CREB and the closely related protein CREM leads to widespread cell death (Mantamadiotis et al., 2002). It is expressed broadly during the development of the cortex, and CREB knockout mice displayed defects in radial neuron migration and sensory neuron development (Diaz-Ruiz et al., 2008); (Lonze et al. Neuron 2002). CREB binds to DNA at cAMP-response elements (CREs) located in regulatory regions controlling the expression of genes necessary for neuronal plasticity and development (Riccio et al., 2006) (Kornhauser et al., 2002). CREB is a major effector of neurotrophin signalling pathways including nerve growth factor (NGF) and brain derived neurophic factor (BDNF) amongst others (Lonze and Ginty, 2002). Neurotrophins control activation of CREB through multiple pathways including PI3K/Akt, Calcium Ras/ERK and nitric oxide. This leads

to phosphorylation of CREB on Ser133, which to the transcription of CREB responsive genes **(Fig.1.3)** (Mayr and Montminy, 2001) (Shaywitz and Greenberg, 1999).

1.3.1 Transcriptional control of laminar identity

In the CP, the characteristics of each layer is determined by the expression of combinations of layer specific transcription factors (Arlotta et al., 2005) (Heiman et al., 2008) (Telley et al., 2016). SOX5 is expressed in layers 5 and 6 and in the SP, and is required for neuronal differentiation. In the absence of SOX5, cells do not differentiate into lower layer neurons and do not project axons to the pons and spinal cord. They instead express ectopic layer markers, such as CTIP2. Moreover, cell migration to the lower layers is also affected (Kwan et al., 2008). Tbr1 is expressed in layer 6 neurons and Tbr1 knockout mice display defects in differentiation similar to Sox5 knockout mice. Neurons in layer 6 fail to acquire layer specific characteristics and the expression of Fexf2 and CTIP2 is increased. Fezf2 is a marker of layer 5 that regulates cell differentiation but not migration (Molyneaux et al., 2005). Upper layer neurons express a different set of transcription factors such as SATB2, Cux1/2 and Brn1/2. SATB2, is detected in layers 2 to 5 and controls multiple aspects of upper layer development by increasing the expression of other determinants such as Cux2, RAR-related orphan receptor beta (Rorb) and cadherin10 and at the same time repressing markers confined to other layers, such as CTIP2. Moreover, Satb2 regulates the projection of upper layers axons to the upper layers of the CP and $Satb2^{-/-}$ mice show a delay of neuronal migration (Britanova et al., 2008) (Alcamo et al., 2008).

1.3.2 Epigenetic regulation of cortical development

The transcription of genes in the developing cortex is also controlled on the epigenetic level, which refers to heritable changes in gene expression that are independent of the underlying DNA sequence (Wu Ct and Morris, 2001). In eukaryotes, DNA is packaged in units referred to as nucleosomes. Each one is made up 147bp of DNA coiled around a protein core of eight histones, consisting of pairs of the histones H2A, H2B, H3 and H4 forming a macromolecule known as chromatin (Richmond et al., 1997). The structure of chromatin is dynamically regulated and can either be in an open form which is permissive to transcription or in a compact state which leads to transcriptional repression (Patel et al., 2013). Chromatin structure is regulated by three mechanisms: post-translational modifications of the N-terminal tails of histones, methylation of cytosine residues in DNA or by ATP-dependant chromatin remodelling. These processes are controlled by large protein multisubunit chromatin remodelling complexes (Hargreaves and Crabtree, 2011); (Nord et al., 2015).

The N-terminal tail of histone proteins can be post-translationally modified by acetylation, methylation, phosphorylation, ubiquitylation, umoylationmethylation or ADP-ribosylation. Chromatin remodelling complexes catalyse both the addition or removal of these modifications. The Polycomb repressive 2 (PRC2) complex is an example of a polycomb-group protein complexes, which regulates the methylation state of histones. One of its subunits, EZH2 is a methyltransferase and results in the tri-methylation of lysine residue 27 on histone H3 (H3K27me3). In the developing cortex, EZH2 controls the proliferation of neural progenitors and is necessary for the MP to BP switch. Knockdown of EZH2 results in ectopic expression of proteins involved in the Reelin pathway (Zhao et al., 2015). Another histone methyltransferase, Prdm8, controls corticogenesis by forming a repressive complex with the basic helix loop helix transcription factor Bhlhb5, which recruits the complex to specific genomic loci. This complex is required for locomotion in the IZ and for the MP to BP switch (Inoue et al., 2014).

Acetylation of histones is catalyzed by histone acetyl transferases (HATs), which transfer an acetyl group (COCH3) from acetyl CoA to histone H3 or H4. HATs are composed of five families which include GNAT1, MYST, TAFII250, P300/CBP and nuclear receptor coactivators such as ACTR (Kuo and Allis, 1998). P300 and CREB binding protein (CBP) are widely expressed HATs, which are required for the development and normal functioning of the nervous system. Heterozygous mutations of CBP or mutations in P300 cause the neurodevelopmental disorder Rubinstein-Taybi syndrome (Petrij et al., 1995) This disease is characterised by mental impairment, growth delays, hypertelorism and microcephaly (Park et al., 2014). A CBP haploinsufficient mouse $CBP^{+/-}$ presents phenotypes comparable to the human syndrome and CBP has been shown to play a vital role in the proliferation and differentiation of NPCs (Lopez-Atalaya et al., 2011); (Wang et al., 2010).

ATP-dependent chromatin complexes use energy derived from ATP hydrolysis to alter the structure of nucleosomes and these complexes can be responsible for transcriptional repression or activation (Buscarlet et al., 2014). The brahma associated factor (BAF) complex otherwise known as the mammalian SWI/SNF complex contains an ATPase subunit (Brg-1 or Brm) associated with other subunits which modulate the activity of the core ATPase subunit as well as subunits responsible for recruiting the complex to DNA. BAF complexes are important regulators of differentiation throughout neural development and loss of BRG1 in NPCs prevents differentiation of both neurons and glia (Wu et al., 2007); (Matsumoto et al., 2006); (Yu et al., 2013).

Histone deacetylases are enzymes which act in opposition to HATs, catalyzing the removal of acetyl groups from histone tails. They are categorized into four classes: Class I (HDAC1, 2 3 and 8), class II (HDAC4, 5, 6, 7, 9 and 10), class III (sirtuins) and class IV (HDAC11) (Grunstein, 1997). HDACs are widely expressed in the nervous system and their expression is dynamically regulated in a tissue and stage specific manner. The class I HDACs, HDAC1 and 2 are highly homologous and functionally redundant enzymes expressed in the developing cortex. HDAC1 is predominantly expressed in neural progenitors and glia, whilst HDAC2 is restricted to post mitotic neurons and neuroblasts (MacDonald and Roskams, 2008) (Yoo et al., 2013). Mice lacking HDAC1 or HDAC2 in neural progenitors do not show any overt histological malformations in the cerebral cortex as deletion of single class I HDACs leads to upregulation of other members of the family (Lagger et al., 2002) (Montgomery et al., 2009). Deletion of both genes simultaneously, however, leads to serious abnormalities in the formation of many neural structures including the cortex suggesting that the proteins are acting redundantly (Montgomery et al., 2009). To overcome this and identify specific roles for each protein, knock-in mice expressing catalytically inactive forms of the enzymes were generated and these showed that the deacetylase activity of HDAC2, in particular, is required for the formation of many brain structures including the cortex (Hagelkruys et al., 2015).

HDAC1 and 2 can be incorporated into three chromatin remodelling complexes: Sin3a, NuRD and CoREST. CoREST and Sin3a are classical deacetylase complexes recruited to repressor element 1 (RE-1) sites by the RE-1 silencing transcription factor (REST). Recruitment of REST leads to repression of non-neuronal genes and the relief or REST mediated repression is associated with the onset of neuronal differentiation (Ballas et al., 2005) (Ballas and Mandel, 2005) Mice lacking REST die before birth and show widespread developmental defects (Chen et al., 1998). Knockdown of SIN3A specifically in mice led to defects in neurogenesis, differentiation and axon elongation and loss of function mutations in Sin3a have been linked to autism spectrum disorders (Witteveen et al., 2016). Knockdown of CoREST in migrating neuron has been shown to lead to defects in radial migration (Fuentes et al., 2012). The Nucleosome Remodelling Deacetylase (NuRD) complex uniquely combines ATP-dependent chromatin remodeling with histone acetylase activity. Whilst histone deacetylase activity is provided by HDACs, it's ATP dependent chromatin remodelling activity is due the presence of the chromodomain helicase DNA binding (CHD) proteins 3, 4 or 5. CHD5 is specifically expressed in the nervous system causes defects of neuronal differentiation and radial migration (Egan et al., 2013) (Nitarska et al, 2016 in press). Target specificity of the NURD complex is provided by interaction of the complex with transcription factors at specific genomic loci. In the cortex, NuRD interacts with SATB2 and is recruited to genes such as CTIP2, an event required for the production of upper layer projection neurons (Britanova et al., 2008) (Baranek et al., 2012).

The subunit composition of chromatin remodelling complexes is dynamically regulated during development leading to the assembly of distinct complexes with different activities and developmental roles. In the developing cortex the developmentally regulated expression of CHD3, 4 and 5 leads to the assembly of NuRD complexes with unique, non-redundant roles during cortical development. CHD4 is expressed first, in progenitors and regulates their proliferation but as development proceeds, CHD5 begins to be expressed in post-mitotic neurons and regulates the early stages of radial migration. Finally, CHD3 is expressed in the CP where it controls the late stages of neuronal migration and lamination. Each complex is recruited to and regulates the transcription of distinct set of neuronal genes (Nitarska et al., 2016 in press). There is a similar subunit switch in the BAF complex during neuronal development. As NPCs differentiate into post-mitotic neurons, the progenitor specific subunits BAF53B and BAF45A are replaced by BAF53B, BAF45B and BAF45C, whose expression is restricted to differentiated neurons. Preventing this switch by knockdown of neuron specific subunits and overexpression of the progenitor associated BAF45A leads to increased proliferation of NPCs (Lessard et al., 2007); (Wu et al., 2007). The subunit composition of BAF complexes can be controlled by post-translational modifications induced by extracellular signals including insulin which induces the phosphorylation of USF-1, leading to the exchange of USF-1 for BAF60c. This lipoBAF complex, then activates the transcription of lipogenic genes in the liver (Wang et al., 2013).

1.3.3 miRNA

Dicer is an RNase III ribonuclease required for the cleavage of miRNA precursors into mature miRNAs. In Dicer$^{-/-}$ mouse brains, there is an expansion of the lower layers and radial migration of cortical neurons is defective showing that miRNAs regulate several aspects of cortical development (Saurat et al., 2013) (McLoughlin et al., 2012). miR-9 and miR-132 are among the specific miRNAs that are known to regulate cortical development and do so by modulating the expression of the transcription factor Foxp2, which is prematurely expressed in Dicer$^{-/-}$ mice (Clovis et al., 2012). MiRNAs also regulate genes directly involved in migration such as DCX, which is regulated by miR-22 and miR-124 (Rago et al., 2014) and N-cadherin, which is regulated by a cluster of RNAs including miR369-3p, miR496 and miR543 (Volvert et al., 2014).

1.4 Extracellular signals that regulate cortical development

Extracellular signalling molecules provide vital positional and temporal information to the migrating cells, and many regulate cortical development by influencing the activity of small GTPases (Azzarelli et al., 2014a). Semaphorins are ligands for the plexin and neuropilin family of receptors, which provide both attractive and repulsive cues for outgrowing axons. Sem3A binds to neuropilin1 (NP1) and plexinA1, which leads to the activation of the Rho/Rock pathway via p190RhoGAP and reduces actin polymerisation (Brouns et al., 2000; Wu et al., 2005). Sem3A acts as a chemoattractant for projection neurons migration. Defects in radial migration are observed in NP1$^{-/-}$ mice, as neurons are unable to polarize in the IZ (Chen et al., 2008). Plexins bind to the Rnd family of small GTPases and are required for the fine-tuning of RhoA activation. Rnd2 regulates MP to BP switch, whereas Rnd3 influences locomotion in the CP. Rnd proteins inhibit the activation of Rho and binding of Plexin B2 to Rnd3 relieves this inhibition, allowing RhoA activation and locomotion. (Pacary et al., 2011) (Azzarelli et al., 2014b). Sem3A also regulates the migration of cortical interneurons. MGE derived interneurons, which express neuopilins, migrate to the cortex whereas those that do not enter

the straitum. This is due to a chemorepulsive effect of Sem3A and Sem3F expressed in the striatum which prevents neuropilin expressing cells from entering (Marín et al., 2001) (Nóbrega-Pereira et al., 2008).

1.4.1 ECM components are important signalling molecules

Naturally occurring reeler mice were first described in a landmark study as the first mutant with severe malformations of the cerebral cortex, impaired motor functions, ataxia and tremors (Falconer, 1951). Histological studies indicated that the PP failed to split resulting in accumulation of migrating neurons under the subplate and a characteristically inverted layering of the cortex (Sheppard and Pearlman, 1997). Reelin, the gene, affected in these mice was not cloned and mapped until many years later (D'Arcangelo et al., 1995). Reelin is a secreted ECM protein, which binds to the VLDR and ApoER2 receptors, inducing tyrosine phosphorylation of the adaptor protein Dab1 (Howell et al., 1999) (Andersen et al., 2002). VLDR and ApoER2 are expressed in migrating neurons in the IZ whereas reelin is produced by CR cells in the MZ (Franco et al., 2011) The importance of this pathway during cortical development was confirmed when it was shown that knockout of both reelin receptors or knockout of dab1 results in a phenotype similar to that observed in reeler mice (Trommsdorff et al., 1999) (Benhayon et al., 2003). Disruption of the reelin pathway leads to defects in somal translocation (Franco et al., 2011) This prevents the splitting of the CP by the earliest born neurons and the movement of later migrating neurons past early born cells by activation of Rap1, which leads to localisation of N-cadherin at the cell surface. (Franco et al., 2011). Another example of an ECM protein that regulates cortical development is collagen III which acts as a ligand for the G protein coupled receptor GPR56. Mutations of GPR56 have been found to cause bilateral frontoparietal polymicrogyria (BFPP), a cortical malformation caused by excessive migration of neurons, leading to a breach in the pial basal membrane and accumulate in ectopic structures outside the brain. GPR56 regulates the small GTPase RhoA via the downstream proteins Ga (12/13) (Singer et al., 2013).

1.4.2 Nitric oxide (NO) signalling in the developing cortex

Nitric oxide (NO) is a gaseous molecule produced via an enzymatic reaction that converts L-arginine to citrulline and is catalysed by nitric oxide synthases (NOSs) enzymes. In mammals, NOSs include endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). eNOS mainly produces NO in endothelial cells located in the inner lining of blood vessels where it regulates vasodilation (Förstermann and Sessa, 2012). iNOS is expressed in a range of cell types and produces NO in response to inflammatory stimuli (Xie et al., 1992) (Förstermann et al., 1998). Neuronal NOS (nNOS) is the primary source of NO in the nervous system and is specifically expressed in the developing cortex. nNOS expression first appears in the cortex at E12.5, is maintained through the height of neuronal migration and declines in the postnatal period. In the adult brain, nNOS is restricted to a subset of GABAergic interneurons, some cortical and hippocampal neurons and the cerebellum (Bredt and Snyder, 1994). The development of the cerebral cortex has been studied in hypomorphic mice carrying a partial deletion of nNOS, which results in 95% decrease of NOS activity in the brain. These mice display abnormal lamination with an expansion in the number of deeper layer neurons at the expense of the upper layers and defective neuron radial migration to the CP, which leads to the accumulation of multipolar neurons in the IZ and VZ (Nott et al., 2013). Although defects of neural structures are not observed in the adult, the mice display a number of neurological problems including impairment of learning and memory (Huang et al., 1993) (Pavesi et al., 2013) (Zoubovsky et al., 2011).

1.4.3 NO signalling regulates the expression of neuronal genes

NO signalling carries out its cellular functions via a number of mechanisms including post-translational modification of target proteins. S-nitrosylation is the addition of a nitrosyl group to the free thiols of cysteines and can affect the target proteins in many ways, including protein degradation, protein-protein interaction and sub cellular localisation. In *E. coli*, S-nitrosylation of the nuclear factor OxyR regulates it's affinity for DNA, thereby controlling the expression of OxyR target genes (Hausladen et al., 1996); (Seth et al., 2012). Since then, many other transcriptional regulators have found to be S-nitrosylated, including ABI5 (Albertos et al., 2015), AtMYB2 (Serpa et al., 2007) MEF2 (Okamoto et al., 2014) and MEF2C (Ryan et al., 2013). We have previously shown that in cortical neurons, HDAC2 is S-nitrosylated at cysteines 262 and 274. S-nitrosylation of HDAC2 disrupts binding to DNA, leading to increased histone acetylation and activation of gene expression. The changes in chromatin state associated with histone acetylation make the surrounding DNA more accessible to transcription factors and cofactors (Nott et al., 2008). Mutation of $Cys^{262/274}$ to alanines inhibits HDAC2 nitrosylation and expression of this construct in adult mice indicates that S-nitrosylation of HDAC2 regulates hippocampal plasticity (Gräff et al., 2014). In the developing cortex, neurons expressing the non nitrosylatable form of HDAC2 did not undergo the MP to BP switch and failed to migrate into the CP, displaying a phenotype similar to $nNOS^{-/-}$ mice. Although S-nitrosylation of HDAC2 regulates histone acetylation and transcription in cortical neurons, the target genes responsible for the migration defects are unknown. A genome-wide beadarray screen revealed that expression of HDAC2 mutated on $Cys^{262/274}$ induced widespread transcriptional changes of genes involved in cortical development, including the chromatin remodelling factor Brm which is required for radial migration (Nott et al., 2013).

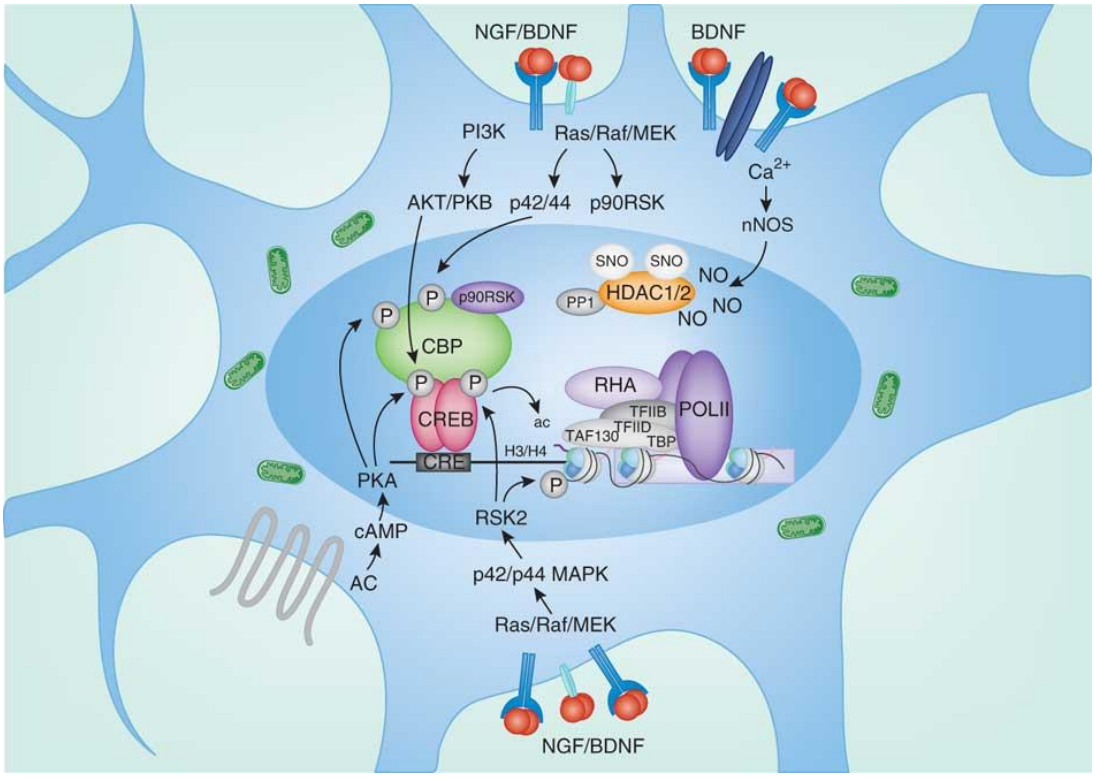

Figure 1.3 Binding of neurotrophins to Trk receptors initiates a number of signalling pathways that induce phosphorylation of CREB and CBP. BDNF-dependent activation of nNOS increases nuclear NO and triggers *S*-nitrosylation (SNO) of several nuclear proteins, including HDAC2. The dissociation of *S*-nitrosylated HDAC2 and the recruitment of phosphorylated CBP and transcription factors such as CREB lead to transcriptional activation. Both phosphorylation of histone H3 through RSK2 and CBP-dependent histone acetylation (ac) contribute to gene activation. AC, adenylyl cyclase; ac, acetyl. Taken from (Riccio, 2010).

1.5 PTPRS and LAR family phosphatases

The protein tyrosine phosphatase receptor sigma (PTPRS) was among the genes that were found to be transcriptionally regulated by S-nitrosylation of HDAC2. PTPRS (or PTPσ) is a receptor-type phosphatase (RPTP) of the Protein Tyrosine Phosphatase (PTP) family of proteins, which is expressed in the nervous system where it regulates axonogenesis and the formation of the cytoarchitecture of the brain (Meathrel et al., 2002). In total, there are 22 RPTPs all containing an extracellular domain of variable length, a transmembrane domain and a C-terminal catalytic cytoplasmic domain. They are further divided into 8 subtypes on the basis of structural similarities, which include the LAR (Type IIa) family (PTPRD, PTPRF and PTPRS). LAR phosphatases are characterised by three N-terminal immunoglobulin-like domains and a variable number of fibronectin III-like domains (Wang et al., 2003).

1.6.1 Role of RPTPs during development

The activity of tryosine protein kinases generally increases during development. Both kinases and phosphatases ensure that the balance between phosphorylation and dephosphorylation is tightly regulated in a tissue and stage specific manner (Dasgupta and Garbers, 1983). The first evidence that RPTPs play important roles in developmental processes was provided by a study showing that the chick homologue of the mammalian PTPRS CRYPa is expressed in growth cones (Stoker et al., 1995). Similarly, DLAR and other RPTPs are present in the growth cones of *Drosophila* motor neuron axons (Tian et al., 1991). Mutations of RPTPs disrupted axonal innervation of target tissues. When DLAR was mutated, segmental nerve (SN)b and SNd axons continued to grow past the perineal muscles, which they would normally innervate, instead following the path of the intersegmental nerve. DPTP69D and DPTP99A are also expressed in axons and mutations in these genes induces defects of axonal pathfinding. Combinations of DLAR, DPTP69D and DPTP99A mutations induced more severe defects than single mutations, indicating that RPTPs have partially overlapping functions (Krueger et al., 1996).

In mice, the LAR family of phosphatases regulate the development of many tissues, including the nervous system, craniofacial structures, mammary gland and genitourinary tract (Uetani et al., 2009) (Muise et al., 2007) (Stewart et al., 2013). All three members of the family are expressed in the developing mouse brain and regulate axon guidance and synapse formation (Kaufmann et al., 2002). In addition to their role in axon guidance, RPTPs also influence cell migration in the CNS. For instance, radial migration is disrupted in the hippocampus of mice lacking PTPRA and these animals perform poorly in cognitive tests (Petrone et al., 2003). PTPRA is also expressed in the developing retina and its inhibition in zebrafish induces early stage delay of retinal lamination. Although most defects were transient and eventually recovered, disorganisation of the amacrine and ganglion cell layers persisted. (Van Der Sar et al., 2002). PTPRS is expressed in the developing CNS and $PTPRS^{-/-}$ mice display defects in the cytoarchitecture of the hippocampus, cerebellum and cortex (Meathrel et al., 2002). They also have under-developed neuroendocrine system, pituitary defects and a reduced number of cholinergic neurons (Elchebly et al., 1999). Neurons derived from $PTPRS^{-/-}$ migrate migrate abnormally in neurosphere cultures *in vitro,* and defects in neuronal migration may be the cause of the observed structural defects in the brain (Kirkham et al., 2006).

1.6.2 PTPRS Signalling

LAR phosphatases function as receptors for proteoglycans. Proteoglycans are heavily glycosylated proteins that consist of glycosaminoglycan (GAG) side chains covalently attached to a core protein. GAGs are attached to their core protein through serine residues within the Ser-Gly-X-Gly motif. Proteoglycans can be located on the cell surface or be secreted to become part of the ECM. LAR phosphatases bind to two subtypes of proteoglycans, the chondroitin sulfate proteoglycans (CSPGs) and the heparan sulfate proteoglycans (HSPGs) (Aricescu et al., 2002); (Shen et al., 2009).

1.7 Role of proteoglycans during neural development

CSPGs are the prevalent type of proteoglycan present in the CNS and include aggrecan, versican, neurocan, phosphacan and brevican and regulate several aspects of neuronal development. Both CPSGs and HSPGs have been shown to regulate proliferation. In *C.elegans* early blastula cells, depletion of the chondroitin synthase, which is the enzyme responsible for the formation of GAG chains, or digestion of GAGs with chondroitinase ABC resulted in a failure of cytokinesis (Mizuguchi et al., 2003). In mice, the HSPGs Syndecan-1 and glypican-4 are expressed in the VZ and knockout of the HS sulfotransferase HS2ST, which is required for the synthesis of HS sidechains decreased proliferation in NPCs (McLaughlin et al., 2003).

During neuronal migration, CSPGs act as repulsive cues, forming barriers to migrating cells. Neural crest cells (NCCs) migrate along well defined routes in the head and trunk of the developing chick. Trunk derived NCCs travel through the developing dermis and the dorsal dermomyotome boundary along the dorsolateral path (Erickson et al., 1992). Chondroitin-6-sulfate (C6S) is detected along the dorsolateral path between the somite and the ectoderm where it forms a transient barrier to migrating neural crest cells. As C6S expression decreases, neural crest cells enter the dorsolateral path (Oakley and Tosney, 1991) (Oakley et al., 1994). Aggrecan is uniquely detected in areas that are not permissive to NCC migration. Ectopic administration of aggrecan interferes with NCC motility creating a barrier refractive to migrating cells. Conversely, when aggrecan is removed, NCCs invade areas that they would normally avoid (Kubota et al., 1999) (Perissinotto et al., 2000). There has been some controversy as to whether versican acts as an attractive or repulsive cue in NCC migration. Versican is expressed in tissues surrounding the migratory route where it may create a barrier that prevents NCCs from entering into these tissues (Henderson and Copp, 1997) (Landolt et al., 1995). Consistent with this hypothesis, versican expression is increased in Splotch mice, which carry a mutation of the Pax3 gene. In these mice, NCC cells fail to populate target tissues (Henderson and Copp, 1997). On the other hand, in the chick embryo,

NCCs move towards tissues expressing versican, perhaps indicating a permissive and/or attractive role in NCC migration (Perris and Perissinotto, 2000). Similarly, NCCs are drawn towards versican impregnated grafts in axolotl embyros. (Perissinotto et al., 2000). Cortical interneurons normally migrate from the MGE and CGE to the cortex, avoiding areas rich in CSPGs, such as the striatum. In this case, CSPGs act to form a barrier to migration and digestion of CSPGs allow interneurons to invade the striatum. Although, it has been suggested that in this context CSPGs may act in conjunction with SEM3A, it is not known how CSPGs repulse migrating cells and this effect could be mediated by LAR phosphatases (Zimmer et al., 2010). In the developing cortex, CSPGs are expressed in the developing PP and following its split continue to be expressed in the MZ and SP, which radially migrating cells must cross in order to enter the CP (Miller et al., 1995). Knockdown of the sulfotransferases uronyl 2-O-sulfotransferase (UST) or *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (4,6-ST), both of which are required for the synthesis of CSPGs prevents projection neuron from the moving from the IZ into the CP. It remains unclear however how knockdown of CSPG synthesising enzymes in migrating cells may induce these defects, given that CSPGs are normally expressed only in the SP (Ishii and Maeda, 2008).

1.7.1 Role of LAR phosphatases and proteoglycans in axon guidance

HSPGs were the first proteoglycans shown to interact with LAR phosphatases during axon guidance. Agrin, collagen XVIII and Syndecan all interact with PTPRS (Aricescu et al., 2002) (Johnson et al., 2006). HSPGs have long been known to have a role in axonal outgrowth. Application of exogenous HS or enzymatic digestion of HSPGs results in misdirection of growing axons. Similar effects were observed in pioneer axons of cockroach embryos and in retinal axons. (Wang and Denburg, 1992). *Xenopus* retinal ganglion cells treated with HS bypass the tectum, which they would innervate under normal circumstances (Chernoff, 1988). Mice lacking sulfotransferases HS 2-*O*-sulfotransferase (HS2ST) and HS 6-*O*-sulfotransferase (HS6ST) are unable to synthesise 2-O-sulfated or 6-0-sulfated HS moieties and HSPG signalling is compromised as a result. Axons of retinal ganglion cells in these mice are unable to navigate properly in the optic chasm and form disorganised bundles (Pratt et al., 2006). In the developing chick embryo, the growth-promoting action of HSPGs is mediated by the binding of HSPGs to PTPRS (Ledig et al., 1999).

CSPGs act as a repulsive directional cue for growing axons and inhibit neurite growth in several model systems (Snow et al., 1990a) (Fichard et al., 1991) (Verna et al., 1989)(Dou and Levine, 1994). CSPGs are expressed in tissues normally avoided by axons, such as the SP of chicken telencephalon and the roof plate of developing mouse spinal cord (Snow et al., 1990b). In zebrafish, enzymatic digestion of CS in the area surrounding the ventral motor nerves leads to inappropriate axonal branching whereas application of exogenous CSPGs induced axon truncation, indicating that CSPGs inhibit neurite extension. The expression of CSPGs is regulated during development, generating dynamic patterns of axon growth and innervation of target tissues appropriate to the developmental stage. In E12.5 mouse retina, CSPGs are expressed throughout the tissue. As the tissue matures, CSPGs are lost from the centre of the tissue and become progressively more peripheral until they are no longer detected around E17. Only at this point axons begin to grow in regions recently cleared of CSPGs, gradually filling more superficial structures. CSPGs inhibit retinal ganglion cell axon

growth and in the developing retina, enzymatic digestion of CSPGs leads to ectopic and precocious neurite formation. In these experiments, the inhibitory action of the CSPGs prevented axons from growing outwards and guiding them towards the optic nerve (Brittis et al., 1992). It was originally thought that CSPGs inhibited axon growth either by forming a physical barrier in the ECM and interfering with the action of adhesive molecules like laminin or by creating regions of negative charge (McKeon et al., 1995) (Olson, 2002). However, CSPG-mediated repulsion of growing axons is largely abolished when DRG neurons lacking PTPRS were grown on CSPG-rich substrates, indicating that the inhibitory effects of CSPGs on axon growth is at least in part mediated by PTPRS (Coles et al., 2011) (Brown et al., 2012) (Coles et al., 2011). PTPRS contains a conserved 24 amino acid intracellular wedge domain, a structural element, which is responsible for PTPRS binding to proteoglycans. Neurons exposed to a peptide mimetic of the PTPRS wedge domain that blocks binding to CSPGs were no longer sensitive to CSPG-induced inhibition of axon growth (Lang et al., 2015). In PC12 cells a number of compounds that interfere with the action of the D1 phosphatase domain were sufficient to rescue CSPG-dependent inhibition of neurite extension (Lee et al., 2016).

1.7.2 CSPGs and HSPGs signal through PTPRS via a shared site

CSPGs and HSPGs have mostly divergent roles during axon guidance, with HSPGs promoting axon growth and CSPGs inhibiting it. The effects of both proteoglycans are mediated by LAR phosphatases, particularly PTPRS. In agreement with the distinct functions of CSPGs and HSPGs, perturbation of HS or CS in developing *xenopus* embryos induces opposite effects. In the tectum, exposure to exogenous CS epitopes disperses axons away from their normal targets and removal of CS caused axons innervation of areas that they would normally avoid. This in contrast with the effect of HS, which induces axonal bypass of the tectum (Chernoff, 1988). Despite having such divergent roles in axon guidance, CSPG and HSPG both signal through PTPRS and do so by binding to the same site, located on the extracellular domain of the protein. It consists of a positively charged pocket of four lysines, which interact with the negatively charged GAG side chains of proteoglycans. Binding of HSPGs causes PTPRS clustering and oligomerisation on growth cones, whereas CSPGs have the opposite effect disrupting oligomerisation of the receptor (Coles et al., 2011).

1.7.3 Role of CSPGs in axon regeneration

Axons in the peripheral nervous system (PNS) have shown a tremendous capacity to regenerate, however in the CNS, they have a very limited regeneration potential. Although, axons are capable of growing long distances they are prevented from doing so by the non-permissive environment created after injury (David and Aguayo, 1981). This is due to the presence of growth-inhibiting molecules in the glial scar, which is formed in response to injury by reactive astrocytes, microglia, and oligodendrocyte precursor cells (Galtrey and Fawcett, 2007). After injury, these cells begin to express a number of ECM components including CSPGs and secrete them into the scar environment (McKeon et al., 1999). The ability of axons to regenerate inversely correlated with the abundance of CSPGs in the scar (McKeon et al., 1991). Reactive astrocytes are less effective in slowing axon growth when CS moieties

are enzymatically digested (Davies et al., 1999). During development, axons in the spinal cord lose the capacity to regenerate and this event correlates with progressively higher expression of CSPGs in the tissue (Pindzola et al., 1993). Chondroitinase ABC (chABC) is an enzyme derived from the bacteria *Proteus vulgaris* which cleaves CS-GAG sidechains from the CPSG core protein (Yamagata et al., 1968). Treatment of tissue derived from the glial scar with chABC allows axons projecting from cortical neurons to freely grow across it when transplanted into the cortex (Smith-Thomas et al., 1995). Treatment of spinal cord with chABC was sufficient to increase axon growth following CNS injury (Fouad et al., 2005)(Moon and Fawcett, 2001)(García-Alías et al., 2011). In injured spinal cord, degradation of CSPGs was associated with the restoration of axonal functions, including conduction through nerve fibres, locomotor function and proprioceptive behaviour (Bradbury et al., 2002) (Alilain et al., 2011) (Bartus et al., 2014) (García-Alías et al., 2011). A number of sulfotrasferases are required for the synthesis of GAG chains and the sulfotransferases C4ST, C6ST, and GalNAc4S-6ST are all induced in glial scars following injury (Properzi et al., 2005) (Karumbaiah et al., 2011) (Lin et al., 2011). Reduced expression of xylosyltransferase-1, an enzyme responsible for the first steps of GAG chains synthesis resulted in efficient growth of transplanted dorsal root ganglion (DRG) neurons in injured spinal cord (Grimpe and Silver, 2004). Mice lacking chondroitin sulfate *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1), an enzyme necessary for the synthesis of GAG chains, recover their motor functions faster than control mice, further confirming that inhibition of CSPG expression correlates with functional and anatomical recovery of CNS axons (Takeuchi et al., 2013)

Although CPSGs are considered inhibitory to axon outgrowth, there are a number of examples of CSPGs inducing neurite outgrowth. For instance CS-E has been shown to increase neurite extension in PC12 cells by facilitating the binding of neurotrophins to Trk receptors. Addition of CS-E was sufficient to induce NGF mediated phosphorylation of Akt and this effect was abolished following enzymatic digestion of CSPGs with ChABC or by blocking the binding of CS-E to its receptor Contactin 1 (CNTN-1) (Rogers et al., 2011) (Mikami et al., 2009).

1.7.4 PTPRS is a CSPG receptor during axonal regeneration

In the spinal cord, binding of CSPGs to PTPRS largely mediates CSPG-dependent inhibition of axonal regeneration. Peripheral nerves of $PTPRS^{-/-}$ mice regenerate at a faster rate than wild type controls, although axonal pathfinding was disorganised (McLean et al., 2002). Similar effects were observed following spinal cord injury in these mice where axons were observed to grow away from the site of injury following dorsal column crush (Shen et al., 2009) (Fry et al., 2010). A peptide mimetic of the PTPRS wedge domain, which disrupts the interaction of PTPRS with CPSGs increases axonal regrowth after injury. Mice given daily injections of the peptide showed improved recovery of locomotor function and balance (Lang et al., 2015).

1.7.5 Signalling pathways initiated by CSPGs

Since PTPRS and other LAR phosphatases are largely responsible for mediating the inhibitory effects of CSPGs on axon regeneration in the mature CNS, there has been a significant amount of interest in identifying the downstream signalling mechanisms by which they do so. Activation of RhoA in axons mediates the repulsive effects of many inhibitors of neurite outgrowth, including myelin-mediated repulsion of DRG axons in the retina, cortical neuron extension, and Sem3A repulsion of DRG outgrowth (Dontchev and Letourneau, 2002) (Zhang et al., 2011). This effect is mediated by activation of ROCK, a potent regulator of the actin cytoskeleton (Monnier et al., 2003) (Lehmann et al., 1999) (Jin and Strittmatter, 1997). RhoA is activated in response to injury in the spinal cord *in vivo* and inhibitors of RhoA facilitate axonal regeneration and functional recovery. Such inhibitors have been explored as potential therapeutic tools with encouraging results (Fournier et al., 2003) (Tanaka et al., 2004) (Chan et al., 2005). CSPGs induce activation of the RhoA by interacting with LAR and PTPRS and represents the main signalling pathway through which CPSGs are able to inhibit neurite growth (Borisoff et al., 2003) (Lehmann et al., 1999). Preventing the activation of RhoA is sufficient to overcome the inhibitory effects of CSPGs on axon outgrowth. (Dergham et al., 2002) (Monnier et al., 2003) (Dyck et al., 2015).

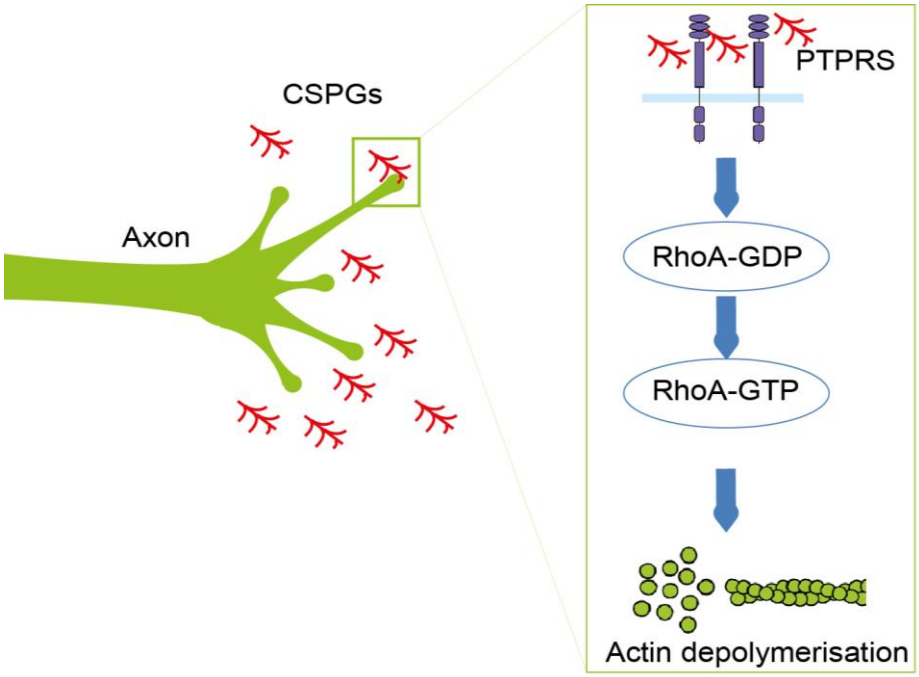

Fig 1.4 CSPGs and their receptors. PTPRS, a member of the LAR subfamily of receptor protein tyrosine phosphatase, is expressed in growth cones and acts as a receptor for CSPGs. Binding of CSPGs to PTPRS leads to activation of RhoA and subsequent actin depolymerisation.

1.8 RPTP substrates

Cadherins and catenins are the principle components of adherens junctions, which provide mechanical adhesion between cells, and are far the best-characterised substrates for LAR family phosphatases. (Karisch et al., 2011). They must be kept in a dephosphorylated state in order to maintain the integrity of junctional complexes. For instance phosphorylation of E-cadherin or VE-cadherin leads to disassembly of junctions (Fujita et al., 2002), (Potter et al., 2005). PTPU is an example of a PTP that maintains adherens junctions in a dephosphorylated state. It binds to cytoplasmic domains of VE-cadherin and p120catenin and inhibition of PTPU interferes with the formation of cell junctions, leading to increased permeability of endothelial layers (Brady-Kalnay et al., 1995) (Zondag et al., 2000). Interestingly, loss of PTPU also disrupts adherens junction formation in cancer cells but a form of PTPU, in which the phosphatase domain has been inactivated was unable to restore them, suggesting that this PTP is necessary for the formation of adherens junctions but the mechanism is independent of its role as a phosphatase (Hellberg et al., 2002). DEP-1 and a related phosphatase VE-PTP also associate with VE-cadherin, resulting in dephosphorylation of VE-cadherin (Holsinger et al., 2002) (Takahashi et al., 1999). They are necessary for the adhesive properties of VE-cadherin containing adherens junction complexes. However a phosphatase-dead VE-PTP does not rescue adherens junctions, suggesting that at least in some cases, the phosphatase activity is necessary for its functions (Nawroth et al., 2002). PTPs are not receptor type phosphatases but also regulate cell adhesion through cadherins. Examples include the regulation of N-cadherin by PTP1B, which promotes binding to β-catenin and localisation of the complex to the cell membranes (Xu et al., 2002). Another PTP, Shp-2 specifically interacts with β-catenin and the loss of Shp-2 leads to the increased phosphorylation of β-catenin and loss of the integrity of monolayers in endothelial cells.

1.8.1 PTPRS and Cadherins

PTPRS interacts and dephosphorylates the N-cadherin/β-catenin complex and both proteins can be co-immunoprecipitated with PTPRS in neurons. Axons of DRG cells derived from PTPRS$^{-/-}$ mice exhibit overgrowth and increased branching. Blocking N-cadherin with an inhibitory peptide or interfering otherwise with its function by lowering calcium levels was sufficient to restore normal levels of axonal growth (Siu et al., 2007). In the gut, E-cadherin/β-catenin complexes are necessary for maintaining the integrity of the epithelium. In this tissue, PTPRS dephosphorylates E-cadherin and the subsequent loss of adherens junctions in PTPRS$^{-/-}$ mice leads to ulcerative colitis. Importantly, SNPs flanking exon of the PTPRS gene are associated with the same disorders in patients (Muise et al., 2007).

1.8.2 GEFs/GAPs are substrates for PTPRS

PTPRS regulates the activity of small GTPases by controlling the action of GEFs and GAPs. p250GAP is dephosphorylated by PTPRS which increases its activity leading to, attenuation of rac1 activity. Expression of p250GAP or PTPRS was sufficient to inhibit rac1 in PC12 cells (Chagnon et al., 2010). Another PTP, PTP20 dephosphorylates p190RhoGAP (Shiota et al., 2003). This protein regulates the activation state of RhoA and is required for several aspects of cortical development (Brouns et al., 2000). Finally, LAR regulates the GEF TRIO (Debant et al., 1996). TRIO has GEF activity towards both Rac and Rho proteins and is an important regulator of neurite outgrowth in cortical neurons. Perturbing the phosphorylation state of trio interferes with its ability to regulate axonogenesis (DeGeer et al., 2013).

2. AIMS

The aim of this study is to investigate the regulatory mechanisms which control the expression of PTPRS in the developing cortex and how PTPRS influences cortical development.

In order to do this, I will address two main questions

1) Is PTPRS regulated by S-nitrosylation of HDAC2?

2) What is the role of PTPRS and its associated signalling pathways during corticogensis?

3. RESULTS

3.1 S-nitrosylation of HDAC2 regulates the expression of PTPRS expression in the developing cortex

We previously performed an *in vivo* bead-based microarray screen to identify the transcriptional targets of S-nitrosylated HDAC2 in the embryonic cortex (Nott et al., 2013). *In-utero* electroporation was used to electroporate NPCs of E14.5 embryos with pCIG constructs encoding either $HDAC2^{C262A/C274A}$ or $HDAC2^{WT}$. After 18 hours, the cortices of these mice were dissociated and cells FACs sorted. mRNA extracted from GFP positive cells was used to carry out a genome wide bead array screen. We applied a 1.6 fold cutoff in order to identify the genes that were most differentially expressed between the two conditions. In this way, we identified a list of 23 genes that were transcriptionally inhibited in neurons electroporated with $HDAC2^{C262A/C274A}$. Many genes identified by the screen regulated neuronal development, and the chromatin remodeler Brm was found to be necessary for neural radial migration **(Fig.3.1.1)**, (Nott et al. 2013). Whilst it is known that S-nitrosylation of HDAC2 regulates the chromatin structure of migrating neurons via such genes, whether this mechanism also regulates the transcription of genes directly involved in cortical development is unknown. I decided to investigate the role of PTPRS in cortical development, as it was one of the genes identified by the screen that was strongly inhibited by S-nitrosylation of HDAC2 and is a known regulator of neuronal migration and axon guidance (Coles et al., 2011).

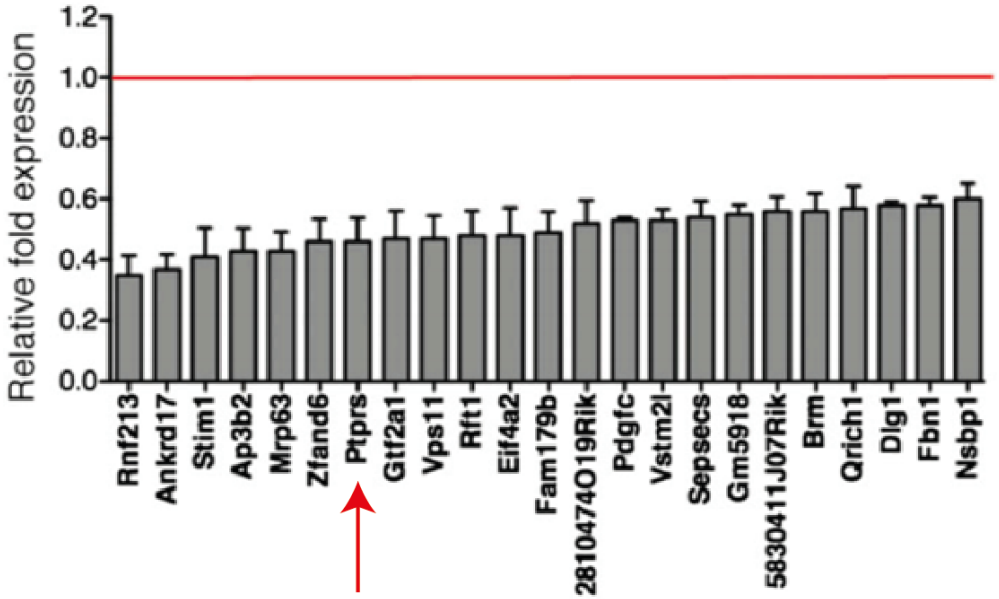

Figure 3.1.1 Identification of genes regulated by S-nitrosylation of HDAC2.

Shown are the 20 transcripts with the highest fold decrease of expression in HDAC2$^{C262/274A}$ versus HDAC2^{WT} electroporated neuronal cells *in vivo*. Fold expression is displayed relative to HDAC2^{WT}. Figure adapted from (Nott et al., 2013)

PTPRS is regulated by S-nitrosylation of HDAC2

The bead-array analysis revealed that the PTPRS transcript decreased over 1.6 fold in neurons expressing HDAC2$^{C262A/C274A}$ compared to HDAC2^{WT} and was amongst the most highly differentially regulated transcripts identified (**Fig3.1.1, arrow)** (Nott et al., 2013). I first confirmed that PTPRS transcripts are indeed downregulated upon expression of non-nitrosylatable HDAC2. To examine the effect on the endogenous transcript, I used PC12 cells, as they are easily transfected with high efficiency (>80%). To test whether S-nitrosylation of HDAC2 was necessary to regulate PTPRS expression, cells were cotransfected with pCIG constructs encoding either HDAC2$^{C262A/C274A}$ or HDAC2^{WT}. SiRNA targeting endogenous HDAC2 was used to ensure that the endogenous protein did not compensate for the effects of the mutagenised constructs. It should be noted that both HDAC2 constructs are expressed at comparable levels under these conditions (Nott et al., 2008). The levels of PTPRS mRNA were measured 48 hours later by quantitative real time polymerase chain reaction (qRT-PCR). Upon expression of HDAC2$^{C262A/C274A}$ in PC12 cells, I observed that PTPRS transcript levels were significantly reduced **(Fig.3.1.2).**

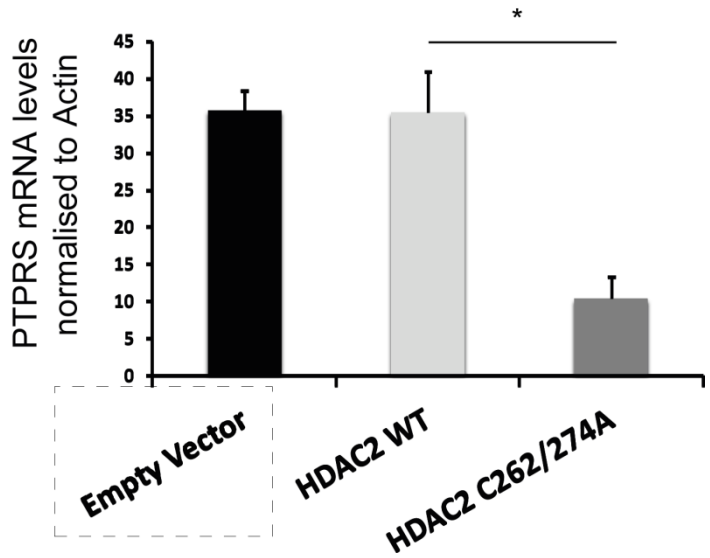

Figure 3.1.2 PTPRS is regulated by S-nitrosylation of HDAC2 in PC12 cells

Relative expression of PTPRS mRNA transcripts in PC12 cells transfected with HDAC2 siRNA and cotransfected with $HDAC2^{C262A/C274A}$ or $HDAC2^{WT}$ constructs. Cells were harvested and RNA extracted 48 hours after transfection. One way anova *p<0.05 . One way ANOVA N=3.

Regulation of PTPRS by S-nitrosylation of HDAC2 occurs at the TSS.

To identify the genomic region of the PTPRS locus that may be regulated by S-nitrosylation of HDAC2, I used of a luciferase reporter assay. The region immediately surrounding the PTPRS transcriptional start site (TSS, -948 to +140) was cloned from rat genomic DNA and fused to a luciferase reporter construct. In initial experiments, I confirmed that the PTRPS-promoter was sufficient to drive the expression of firefly luciferase when transfected in primary cortical neurons. When neurons were transfected with HDAC2 siRNA alongside constructs expressing either siRNA-resistant plasmids encoding $HDAC2^{C262A/C274A}$ or $HDAC2^{WT}$,

luciferase activity driven by the PTPRS promoter was significantly reduced in cells transfected with HDAC2$^{C262A/C274A}$, demonstrating that the region immediately surrounding the PTPRS TSS is sufficient to mediate the repressive effects of the HDAC2$^{C262A/C274A}$ (**Fig. 3.1.3)**. Because we and others have shown that S-nitrosylation of HDAC2 regulates the expression of genes involved in neural development and plasticity, it is conceivable that, in wild type conditions, S-nitrosylation of promoter-bound HDAC2 induces its dissociation from the PTPRS TSS leading to an up-regulation of the gene (Nott et al., 2013) (Gräff et al., 2014).

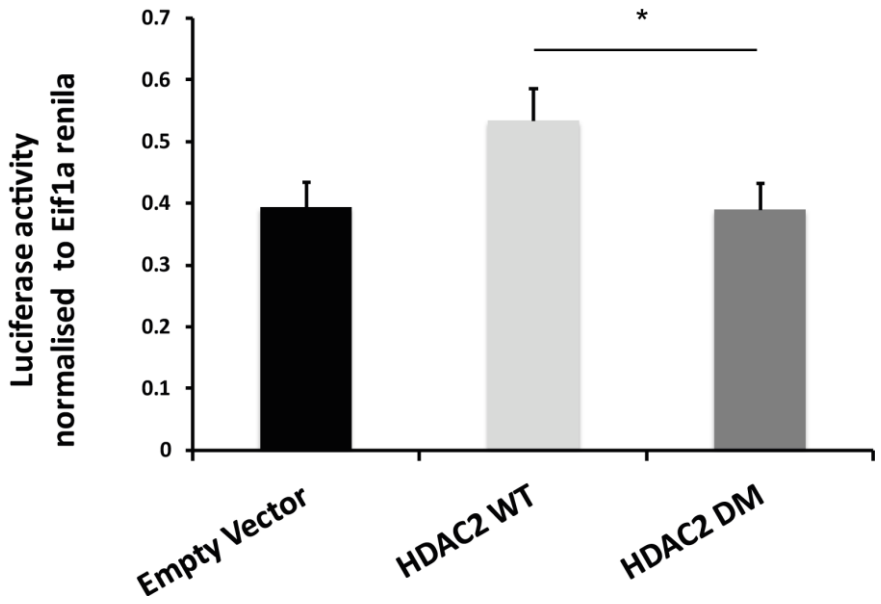

Figure 3.1.3. The PTPRS promoter is regulated by S-nitrosylation of HDAC2. Luciferase activity driven by the PTPRS promoter was measured in cortical neurons transfected with HDAC2 siRNA and either empty vector, HDAC2^{WT}, or HDAC2$^{C262/274A}$ expressing plasmids. In each case, luciferase activity levels were normalised to RPL11. Shown are averages and SEM, N=3 One way ANOVA *P<0.05.

The ability of the HDAC2$^{C262/274A}$ to inhibit PTPRS transcription could depend on the binding of HDAC2 to genomic regulatory elements that regulate the expression of PTPRS. Alternatively, it may be due to a secondary effect, for example through the regulation of another gene. To determine whether HDAC2 directly binds to the PTPRS locus *in vivo*, I performed a chromatin immunoprecipitation (ChIP) assay on E15.5 mice cortices using an HDAC2-specific antibody. qRT-PCR of PTPRS promoter revealed that HDAC2 is recruited to this region. **(Fig 3.1.4)**. A gene free region of chromosome 1 that does not recruit HDAC2 was used as a negative control region (Wang et al., 2009). Hence, HDAC2 binds to the PTPRS promoter *in vivo*.

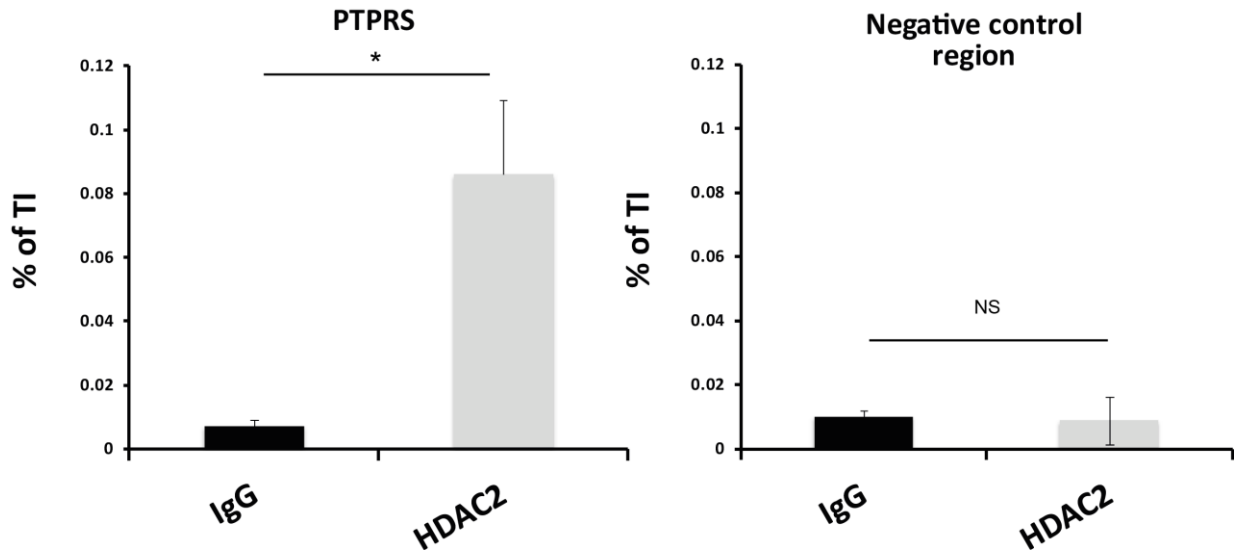

Fig.3.1.4 HDAC2 binds to the PTPRS promoter *in vivo*

ChIP assessing the occupancy of HDAC2 at the TSS of PTPRS in E15.5 dissected mouse cortices. Shown is the enrichment of immunoprecipitated genomic DNA for the HDAC2 antibody compared to the control IgG. A gene free region of chromosome 1 is used a negative control region. The abundance of genomic fragments detected in each condition is expressed as a percentage of total input (TI). Shown are averages and SEM. Student's t-test N=4 *p<0.05

Expression pattern of PTPRS in vivo

NO signalling acts as a positive regulator of activity induced genes in mature cortical neurons through S-nitrosylation of HDAC2, which relieves HDAC2 mediated repression of NO target genes. During development, NO signalling leads to upregulation of genes required for radial migration. To investigate whether NO signalling regulates the expression of PTPRS *in vivo,* I examined the pattern of the PTPRS expression in the cortex and correlated this with nNOS at different developmental stages. At E12.5, as post mitotic neurons begin to be generated, PTPRS is expressed in cells forming the nascent PP **(Fig.3.1.5.a)**. As development progresses and neuronal migration reaches its peak (E15.5), PTPRS becomes restricted to migrating cells in the IZ, and in the upper IZ **(Fig.3.1.5.e)**., Once this pattern of expression is established it is maintained throughout cortical development **(Fig.3.1.5.e,i)**. Importantly, PTPRS and nNOS are largely co-expressed in the developing cortex **(Fig.3.1.5)**, which is consistent with the idea that nNOS is a positive transcriptional regulator of PTPRS in this context. Both PTPRS and nNOS are strongly expressed at the top of the IZ where PTPRS, nNOS and HDAC2 are co-expressed **(Fig 3.1.6.e-h)**.

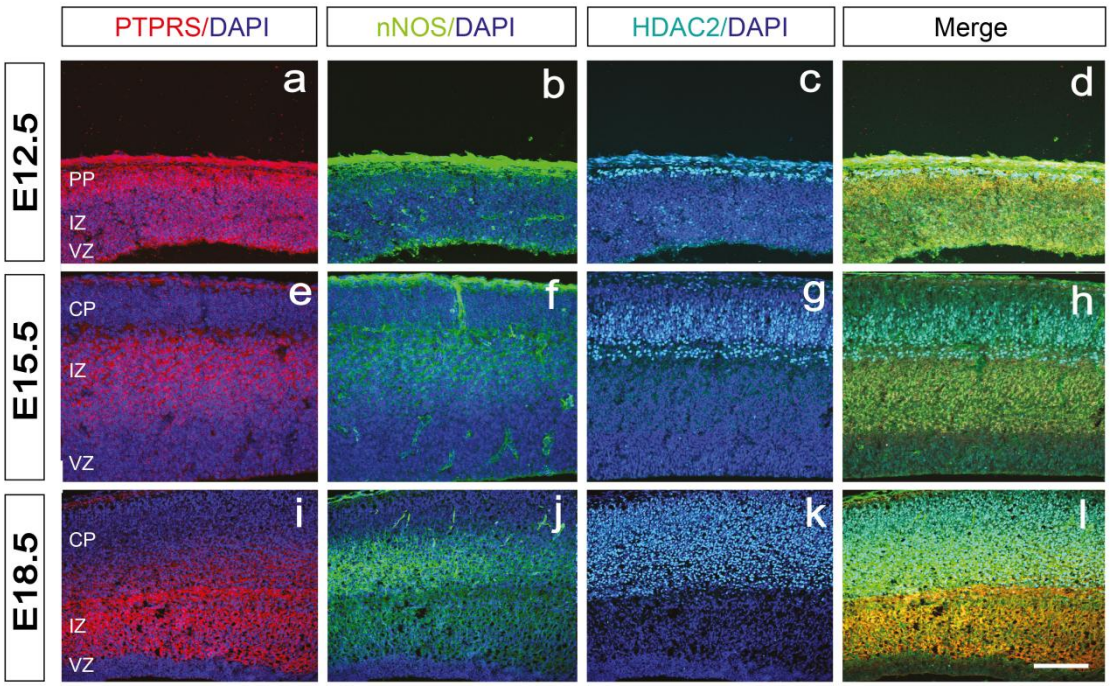

Fig.3.1.5 Expression of PTPRS, nNOS and HDAC2 in the developing cortex.

Confocal images of mouse cortices stained with antibodies for PTPRS (a,e,i), nNOS (b,f,j) and HDAC2 (c,g,k) and all 3 channels merged (d,h,l) at the developmental timepoints E12.5 (a-d), E15.5 (e-h) and E18.5 (i-l). Sections were co-stained with DAPI. Images are shown at 20X magnification. Scale bar 100µM.

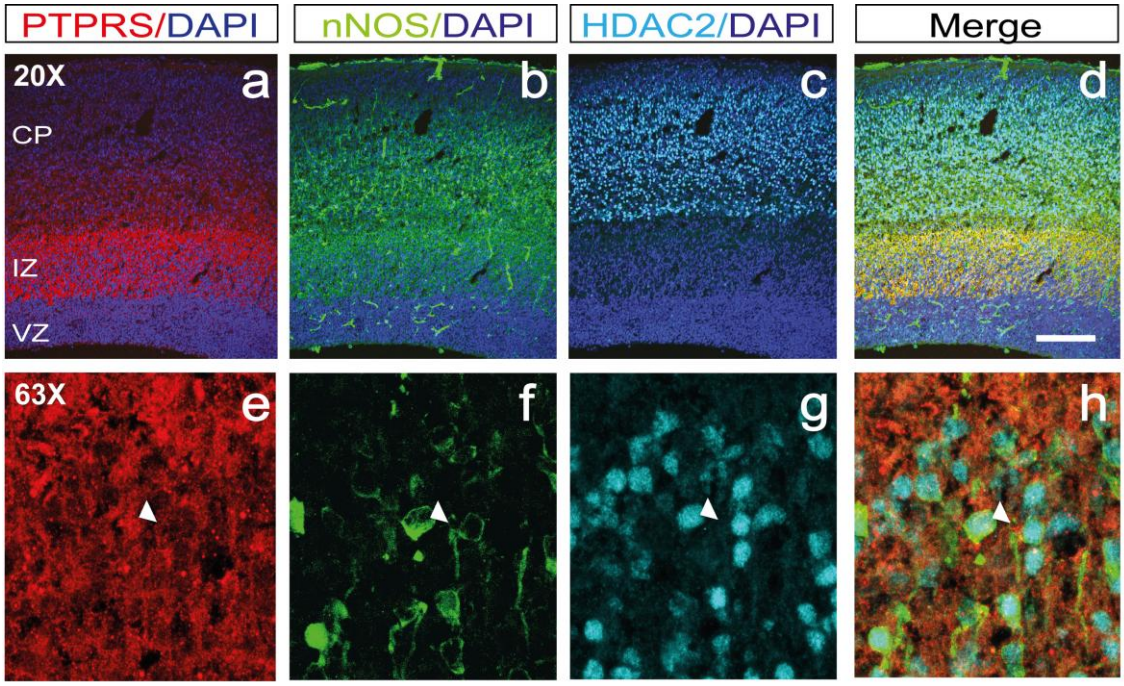

Fig.3.1.6 PTPRS, nNOS and HDAC2 are co-expressed in the developing cortex. Sections of tissue stained with antibodies for PTPRS (**a,e**), nNOS (**b,f**) and HDAC2 (**c,g**) and merged (**d,h**) at E17.5. Sections were co-stained with DAPI. Images are shown at 20X (**a-d**) and 63X magnification (**e-h**).

PTPRS is transcriptionally regulated by NO signalling *in vivo*

As PTPRS is co-expressed with nNOS and HDAC2 in the developing cortex **(Fig.3.1.6)** and S-nitrosylation of HDAC2 controls the transcription of PTPRS *in vitro* **(Fig.3.1.3)**, I next investigated whether PTPRS is transcriptionally regulated by nNOS *in vivo* by measuring the levels of PTPRS in the developing cortex of $nNOS^{-/-}$ mice by RT-qPCR. In these mice, nNOS transcript isoforms containing exon 2 are deleted, which results in a 95% decrease in NOS activity in the adult brain. RNA was extracted from E15.5 $nNOS^{-/-}$ cortices and PTPRS transcript levels were quantified relative to the housekeeping gene ribosomal protein (RPL11). I observed that PTPRS expression was reduced in $nNOS^{-/-}$ cortices by nearly 50%, when compared to wildtype **(Fig.3.1.7)**. The extent of this down-regulation is similar to the fold change observed in cells expressing $HDAC2^{C262/274A}$ in both the bead array screen **(Fig.3.1.1)** and subsequent validation **(Fig.3.1.2)**.

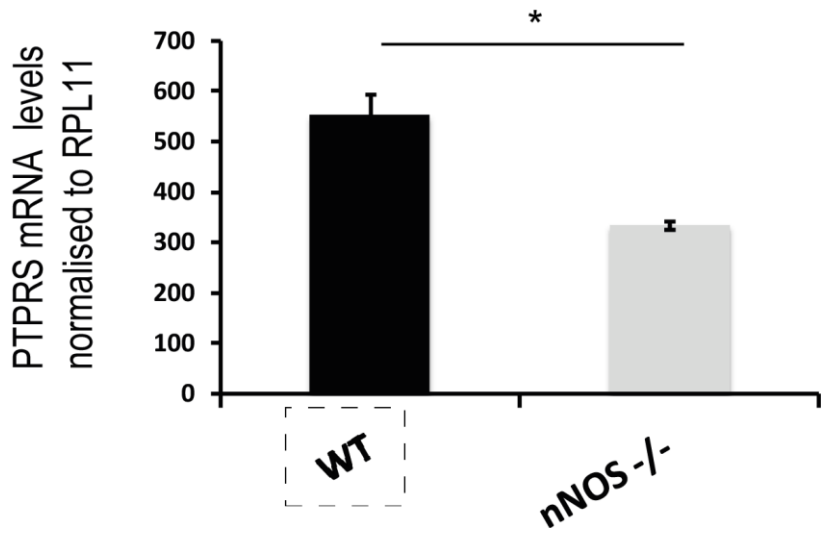

Fig 3.1.7 PTPRS expression is decreased in the cortex of nNOS$^{-/-}$ mice

RNA was extracted from E15.5 cortices of wild type and nNOS$^{-/-}$ mice and qRT-PCR was carried out to quantify the levels of PTPRS mRNA. mRNA levels are calculated relative to the housekeeping gene RPL11. N=3 Student's t-test **p<0.01.

PTPRS is bound by CREB *in vivo*

S-nitrosylation of nuclear proteins has been shown to regulate the occupancy of transcription factors at genomic loci of genes regulated by NO signalling (Kim et al., 2002) (Marshall and Stamler, 2001). Importantly, S-nitrosylation of HDAC2 regulates the binding of CREB at CRE elements in response to BDNF stimulation (Riccio et al., 2006) (Nott et al., 2008). A similar mechanism may influence the expression of CREB-dependent genes *in vivo* during development. I first investigated whether the PTPRS promoter contained CREB binding sites. CREB binds to canonical CREB responsive elements (CREs) as well as some non-canonical sites such as half-CRE sites (Montminy et al., 1986) (Short et al., 1986). When analysed using transcription factor binding site prediction tools, I found that the PTPRS locus

contains a canonical CRE site located just downstream of the TSS (+9), in the region that I previously identified as bound by HDAC2. To determine if this CRE was bound by CREB *in vivo* I performed ChIP on E15.5 cortices and found that CREB was detected at this CRE **(Fig.3.1.8)**. A gene free region of chromosome 1 which does not bind CREB was used as a negative control region (Kim et al., 2010). Thus, CREB may represent a transcriptional regulator of PTPRS in migrating neurons.

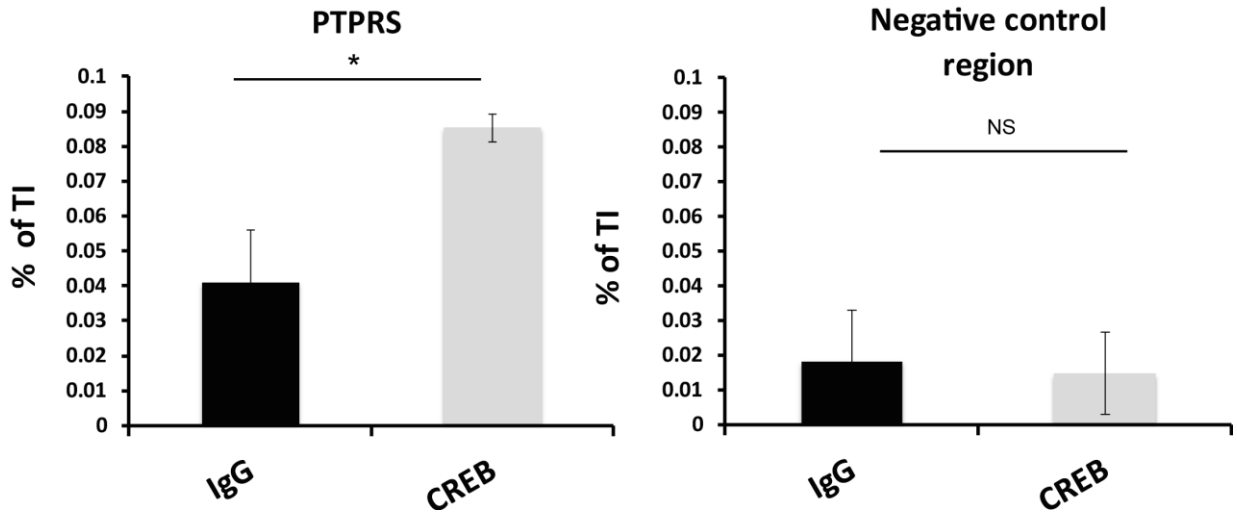

Figure 3.1.8 CREB binds to PTPRS *in vivo*

ChIP of CREB at the TSS of PTPRS in E15.5 cortices. Shown is the enrichment of immunoprecipitated genomic DNA for the CREB antibody compared to control IgG. A gene free region of chromosome 1 is used a negative control region. The abundance of genomic fragments is expressed as a percentage of total input (TI). N=3 Student's t-test *p<0.05

Summary

These results show that PTPRS is regulated transcriptionally by NO signalling in the developing cortex and that this is occurring via HDAC2 occupancy at the PTPRS promoter. I have also identified CREB as a transcriptional activator present at the PTPRS promoter that may be downstream of this important signalling mechanism. In order to further examine the role of NO signalling in regulating HDAC2 bound to the PTPRS TSS, I am in the process of performing ChIP experiments in order to assess the acetylation and occupancy of CREB at the PTPRS TSS in nNOS knockout mice.

3.2 Role of PTPRS in the development of the cerebral cortex

I next asked if PTPRS was required for the development of the cerebral cortex, focusing in particular on radial neuron migration and cortical lamination. PTPRS knockout mice display a variety of defects of neural development including gross abnormalities in the formation of CNS structures, such as the cerebellum, the hippocampus and the cerebral cortex (Elchebly et al., 1999) (Meathrel et al., 2002). PTPRS regulates axon guidance and neuronal migration in a variety of contexts (Henderson et al., 1997) (Coles et al., 2011) and neurons derived from PTPRS knockout mice migrate abnormally when grown from neurospheres *in vitro* (Kirkham et al., 2006). However, it is not known if PTPRS is required for radial migration *in vivo.* To address this question, I used the *in utero* electroporation technique, which allows labelling of migrating neurons in the developing cortex with fluorescent probes. E14.5 embryos in pregnant mice are surgically exposed and DNA is injected into the lateral ventricles. Electrodes are positioned laterally so that progenitors lining the ventricle wall are electroporated with GFP expressing plasmids **(Fig.3.3.1a)**. Embryos are implanted back into the pregnant female, who is returned to her cage, allowing the embryos to develop further. Since post-mitotic cells produced by electroporated progenitors are labelled with GFP their survival and migration can be assessed by examining the distribution of GFP labelled cells at various time points. Coupling this technique with shRNA expressing plasmids or CRE-recombinase based systems allows perturbation of gene expression in a spatially and temporally specific manner and to study the effects of such perturbations on neuronal development (Lo Turco et al., 2009).

To investigate whether PTPRS is required for radial migration, E14.5 cortices were electroporated either with an shRNA targeting the PTPRS transcript or scramble shRNA control. Migrating neurons generated at E14.5 reach their position in the upper layers of the cerebral cortex at E18.5 (Hand et al., 2005). When harvested and stained for GFP at E18.5, 78% of neurons electroporated with the control shRNA reached the CP. However, knockdown of PTPRS completely abolished the ability of neurons to reach the CP (Fig.3.3.1C), and led to accumulation of electroporated cells in the IZ (Fig.3.3.1). These data indicate that PTPRS is required for neuronal migration from the IZ into the CP.

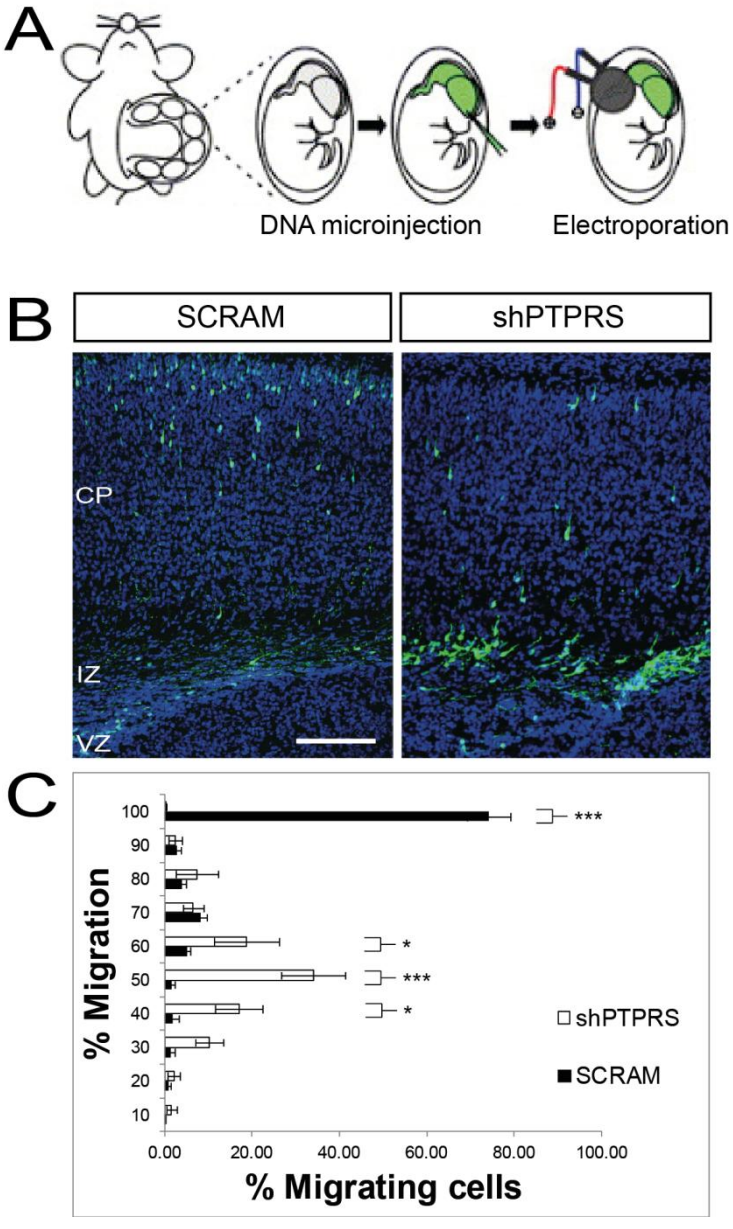

Fig 3.2.1 PTPRS inhibition leads to defects of radial neuron migration

Analysis of radial migration in cortical neurons 4 days after *in utero* electroporation performed at E14.5 using plasmids expressing GFP and PTPRS short hairpin RNAs or scramble controls (SCRAM). A) Schematic illustration of the *in utero* electroporation technique. B) Representative images show cortices harvested at E18.5 and immunostained for GFP. C) Number of GFP positive neurons detected in the cortex and expressed as a percentage of the distance migrated from the VZ to the pial surface. Student's t-test *P<0.05, **P<0.01, ***P<0.001. Scramble shRNA n=5 embryos. PTPRS shRNA n=6 embryos. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.

Generation and analysis of PTPRS conditional knockout mice

Given these encouraging preliminary results, I decided to investigate the effects of PTPRS on the development of the cerebral cortex by generating a conditional knockout mouse. A conventional $PTPRS^{-/-}$ mouse was previously generated, which exhibits defects in the development of neuroendocrine system, in particular the development of the pituitary gland. These defects could indirectly affect the development of the cerebral cortex (Elchebly et al., 1999) (Batt et al., 2002). To circumvent this issue, a mouse carrying a conditional allele for PTPRS was generated by the Knock-Out Mouse Project (KOMP) as part of the European Conditional Mouse Mutagenesis project (EUCOMM Project CSD76529). These mice were generated so that Exon 4 of PTPRS was flanked by two LoxP sites ($PTPRS^{fl/fl}$). Expression of Cre recombinase in cells containing this allele results in excision of DNA between loxP sites, deleting this critical exon, leading to a frameshift mutation **(Fig 3.2.2)**. The protein produced by the resulting allele is then degraded, probably via the nonsense mediated decay pathway (Skarnes et al., 2011).

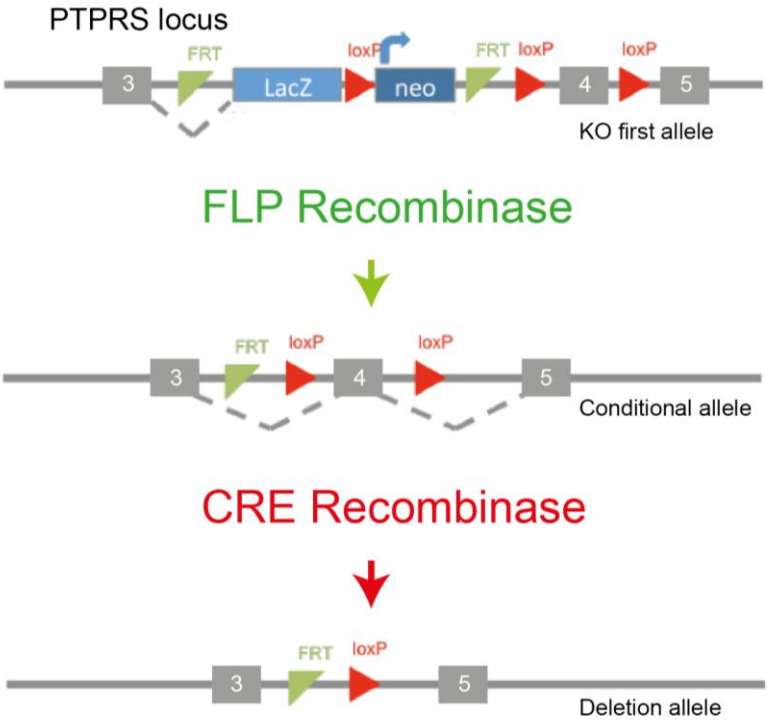

Fig 3.2.2 Strategy for the generation of conditional knockout mice

The 'knockout-first' allele generated by the KOMP consortium consists of an IRES:lacZ cassette and a *neo* cassette inserted in an intron within the PTPRS gene. FLP recombinase excises the region between FRT sites to eliminate the neo cassette. LoxP sites are located either side of exon 4 of PTPRS. Cre recombinase expression in specific cell types deletes exon 4, generating a frameshift mutation and subsequent nonsense-mediated decay of PTPRS.

CRE-mediated deletion of PTPRS *in vivo*

To delete PTPRS in migrating neurons, E14.5 PTPRS$^{fl/fl}$ embryos were *in utero* electroporated with a pCIG2-GFP plasmid expressing Cre recombinase under the control of the CAG promoter, which efficiently drives gene expression in neurons, or a pCIG2-GFP control plasmid, and embryos were harvested at either E17.5 or E18.5. At E17.5, a time point at which neurons are still in the process of migrating across the cortex, the number of neurons which have entered the CP in pCIG2-Cre expressing mice was strongly reduced (42%) compared to pCIG2-GFP controls **(p<0.01, Fig.3.2.3C)**. At E18.5 however the reduction in the number of neurons that reached the CP in pCIG2-Cre expressing cells compared to those expressing the control plasmid was reduced to 31% **(p<0.01, Fig.3.2.3C)**, suggesting that the migration defects may partially recover at later developmental stages. This observation is consistent with the phenotype of PTPRS$^{-/-}$ mice that show reduction of the size of the cortex, hippocampus and cerebellum of postnatal pups, which are absent in adult mice (Meathrel et al., 2002). Other non neural defects such as delayed myelination and lung development are also rescued as PTPRS$^{-/-}$ mice approach adulthood (Elchebly et al., 1999).

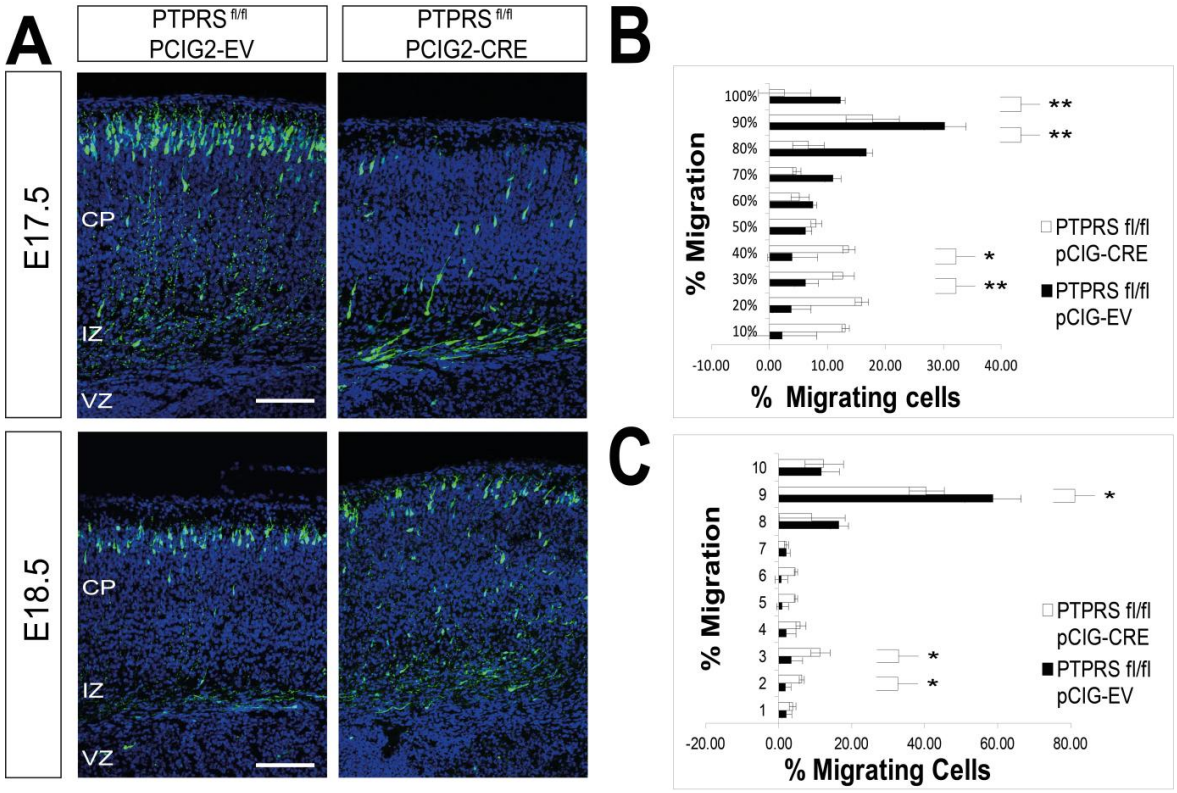

Fig 3.2.3 PTPRS knockdown leads to defects in radial neuron migration.

A). Representative images show PTPRS$^{fl/fl}$ cortices *in utero* elecroporated at E14.5 with pCIG2-Cre plasmid, or pCIG2-GFP control plasmid, and analysed at E17.5 (B) or E18.5 (C). (B and C) The distance travelled by GFP positive neurons is expressed as a percentage of the distance migrated from the VZ to the pial surface. Student's t-test *P<0.05, **P<0.01. PTPRS$^{fl/fl}$ For embryos harvested at E17.5, pCIG2-GFP n=6 embryos. PTPRS$^{fl/fl}$ pCIG2-Cre n=8 embryos. For embryos harvested at E18.5, pCIG2-GFP n=5 embryos. PTPRS$^{fl/fl}$ pCIG2-Cre n=7 embryos.

To generate mice that lacked PTPRS in the nervous system, I crossed PTPRS$^{fl/fl}$ mice with Nestin-Cre and NEX-Cre lines to generate PTPRS$^{fl/fl}$ Nestin-CRE and PTPRS$^{fl/fl}$ NEX-Cre knockout lines. Nestin-Cre transgenic mice express Cre recombinase under the control of the rat nestin promoter. In this line, Cre is primarily expressed throughout the nervous system from E11 (Tronche et al., 1999). The NEX-Cre line expresses Cre driven by regulatory elements of the NEX gene, a neuronal gene expressed in the cortex and hippocampus from E11 (Goebbels et al., 2006). Both lines will produce a knockdown of PTPRS in the cerebral cortex, however in contrast to Nestin, NEX-Cre lines express Cre specifically in post-mitotic pyramidal neurons and is not detected in proliferative neural precursors (Goebbels et al., 2006). As a result, comparing the phenotype of PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ NEX-Cre mice provides a useful way of differentiating phenotypes caused by knockout of PTPRS in progenitors from those caused by absence of PTPRS in post-mitotic neurons. To confirm that PTPRS is deleted in these two lines, I carried out immunofluorescence analysis of the developing cortex of PTPRS$^{fl/fl}$ Nestin-Cre, PTPRS$^{fl/fl}$ NEX-Cre and PTPRS$^{fl/fl}$ control mice. PTPRS is completely absent in PTPRS$^{fl/fl}$ Nestin-Cre brains and no residual expression is observed at E15.5 or E18.5 **(Fig.3.2.4)**. In PTPRS$^{fl/fl}$ NEX-Cre mice, the expression of PTPRS also appears to be abolished at E15.5 although some residual PTPRS expression was observed in the subplate at E18.5 **(Fig.3.2.4)**. This could be explained by the presence of other cell types at this time point such as glia or interneurons, which do not express NEX. Due to the apparent knockdown of the protein in neural tissues, both transgenic lines were considered a suitable model system to study the role of PTPRS.

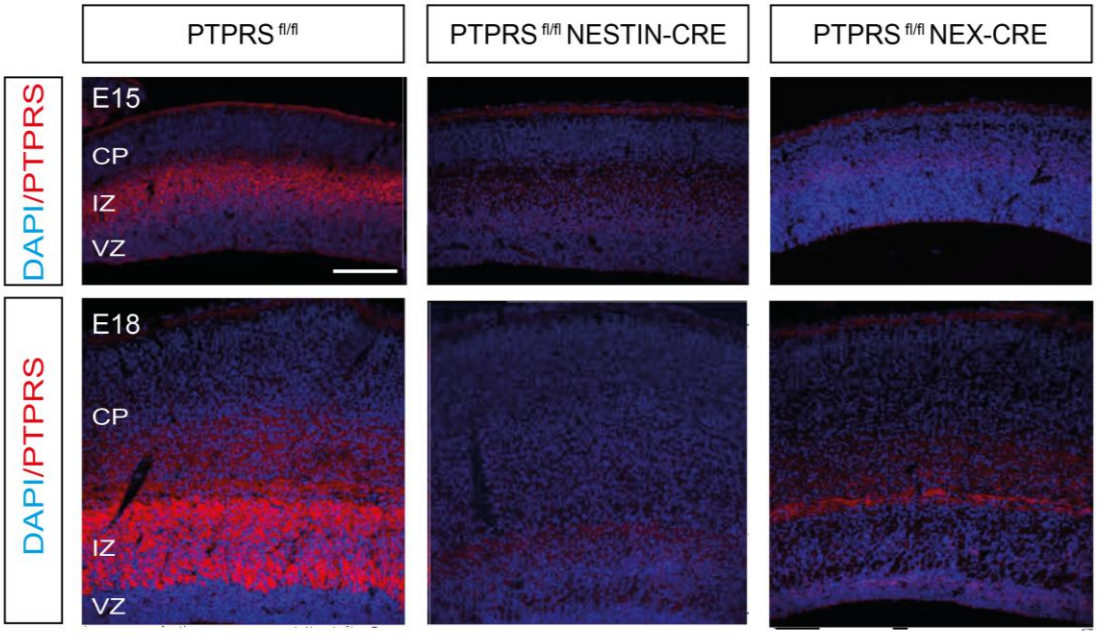

Figure 3.2.4 Knockout of PTPRS in the developing cortex of PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ NEX-Cre mice. Immunostaining of PTPRS$^{fl/fl}$ and control cortices shows that PTPRS is expressed in the IZ at E15.5 and E18.5. Expression of PTPRS is abolished in cortices of PTPRS$^{fl/fl}$ Nestin-Cre mice at both time points. In PTPRS$^{fl/fl}$ NEX-Cre animals, immunoreactivity for PTPRS is absent at E15.5. At 18.5, a band of residual expression of PTPRS is present in the subplate.

Deletion of PTPRS causes radial migration defects

To examine radial neuron migration in PTPRS$^{fl/fl}$, PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ NEX-Cre knockout animals, E14.5 cortices were electroporated with GFP and neurons that had migrated into the CP at E17.5 were quantified **(Fig.3.2.5A)**. In both PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ NEX-Cre embryos I observed a striking reduction in the number of cells that migrated from the IZ into the CP, compared to PTPRS$^{fl/fl}$ controls **(Fig.3.2.5)**. The reduction was similar in the two lines, with a 41% reduction of GFP positive neurons reaching the CP in PTPRS$^{fl/fl}$ NEX-Cre embryos **(Fig.3.2.5B)** and 49% reduction in PTPRS$^{fl/fl}$ Nestin-Cre **(Fig.3.2.5D)**. As NEX regulatory elements drive the expression of Cre in post-mitotic neurons only, the defect in migration observed in these mice cannot be due to deletion of PTPRS in

neural progenitors. However, we cannot fully exclude the possibility of non-cell autonomous effects on progenitors arising from the loss of PTPRS in post-mitotic neurons. The extent of the reduction in both conditions is similar to the 42% reduction observed when PTPRS$^{fl/fl}$ cortices are electroporated with pCIG2-Cre plasmid **(Fig.3.2.4b)**. Taken together, these results confirm that PTPRS is required for cortical neurons migration from the IZ into the CP.

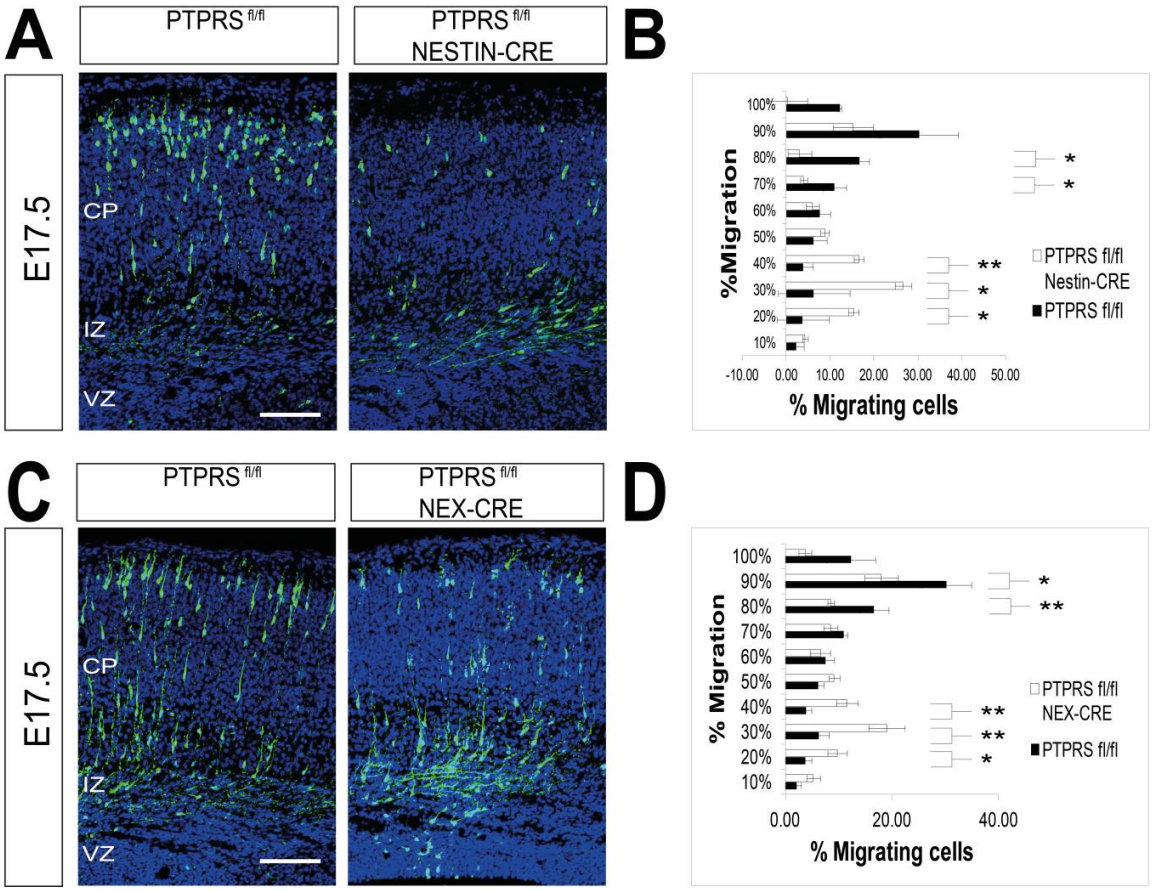

Fig 3.2.5 PTPRS knockdown leads to defects in radial neuron migration.

A) Analysis of radial migration in PTPRS$^{fl/fl}$ Nestin-Cre (A and B) and PTPRS$^{fl/fl}$ NEX-Cre (C and D) mice compared to littermates (A and C) Representative images of cortices harvested at E17.5 and stained with GFP. (B and D) Quantification of GFP positive neurons in different regions of the cortex expressed as a percentage of the distance migrated from the ventricle to the pial surface. Student's t-test *P<0.05, **P<0.01. PTPRS$^{fl/fl}$ n=6 embryos. PTPRS$^{fl/fl}$ NEX-Cre n=7 embryos. PTPRS$^{fl/fl}$ Nestin-Cre n=4 embryos.

Cortical lamination is altered in PTPRS knockout cortices

In nNOS$^{-/-}$ mice, radial migration defects are accompanied by defects in cortical lamination The lower layers of the cortex, marked by the layer specific transcription factor Tbr1, are expanded at the expense of upper layers marked by SATB2, which show a corresponding decrease in size (Nott et al., 2013). In PTPRS$^{-/-}$ mice, a reduction in the size of the cortex is observed in addition to disorganisation of the upper layers (Meathrel et al., 2002). Defects of radial migration are often accompanied by aberrations of cortical layering in situations where the migration of neurons destined for a particular layer is affected, leading to an accumulation of neurons in inappropriate positions and the formation of ectopic layers (Kwan et al., 2012a). Although PTPRS$^{-/-}$ mice display neuronal defects, the lamination of the cerebral cortex has not been studied in depth (Wallace et al., 1999). To assess lamination in the cortex of PTPRS conditional knockout mice, I stained E18.5 cortices with layer-specific transcription factors and quantified the cells expressing the markers. SATB2 is a marker of upper cortical layers predominantly expressed in layers II and III and to a less extent, in layers IV and V (Alcamo et al., 2008; Britanova et al., 2008). Tbr1 is a lower layer marker expressed mainly in layer V, VI, and the subplate, however it can be also detected in layers II and III (Hevner et al., 2001). CTIP2 is also a lower layer marker, expressed in a more superficial pattern in layers IV and V (Arlotta et al., 2005). In PTPRS$^{fl/fl}$ Nestin-Cre brains, there was a significant increase in the number of cells expressing Tbr1 when compared with PTPRS$^{fl/fl}$ control embryos **(24%, p<0.01; Fig.3.2.6A)**. However, there was no difference in the number of cells expressing SATB2, suggesting that upper layers are unaffected in these mice **(Fig.3.2.6.B)**. I next quantified the number of cells expressing CTIP2, a marker of layer V detected also in a subset of layer IV neurons. There was no difference in CTIP2 expression pattern between the PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ controls **(Fig.3.2.6C)**. Taken together, this data suggests that the increased number of Tbr1 positive cells is due to an expansion of the deepest layers (Layer VI and subplate) of the cortex, which express Tbr1 but not the more superficial marker, CTIP2. Indeed, the number of cells expressing only Tbr1 (Tbr1+/CTIP2-) was 42% higher in PTPRS$^{fl/fl}$ Nestin-Cre cortices compared to PTPRS$^{fl/fl}$ **(Fig.3.2.6)**.

The same markers were studied in cortices of PTPRS$^{fl/fl}$ NEX-Cre mice. The results obtained were similar to those found in PTPRS$^{fl/fl}$ Nestin-Cre mice with a significant increase of Tbr1 positive cells (21%) in PTPRS$^{fl/fl}$ NEX-Cre mice compared to PTPRS$^{fl/fl}$ **(Fig.3.2.7B)**. This was accompanied by a slight increase in the number of cells expressing CTIP2 **(Fig.3.2.7C)** and an increase (35%) of Tbr1+/CTIP2- cells **(Fig.3.2.7E).** Similar to PTPRS$^{fl/fl}$ Nestin-Cre mice, there was no difference in the number of cells expressing SATB2 **(Fig 3.2.7D)**. The fact that the lamination defect observed in cortices of PTPRS$^{fl/fl}$ NEX-Cre mice resembles the phenotype observed in PTPRS$^{fl/fl}$ Nestin-Cre mice suggests that the defect is not due to an effect on progenitors but to the lack of PTPRS in post mitotic neurons.

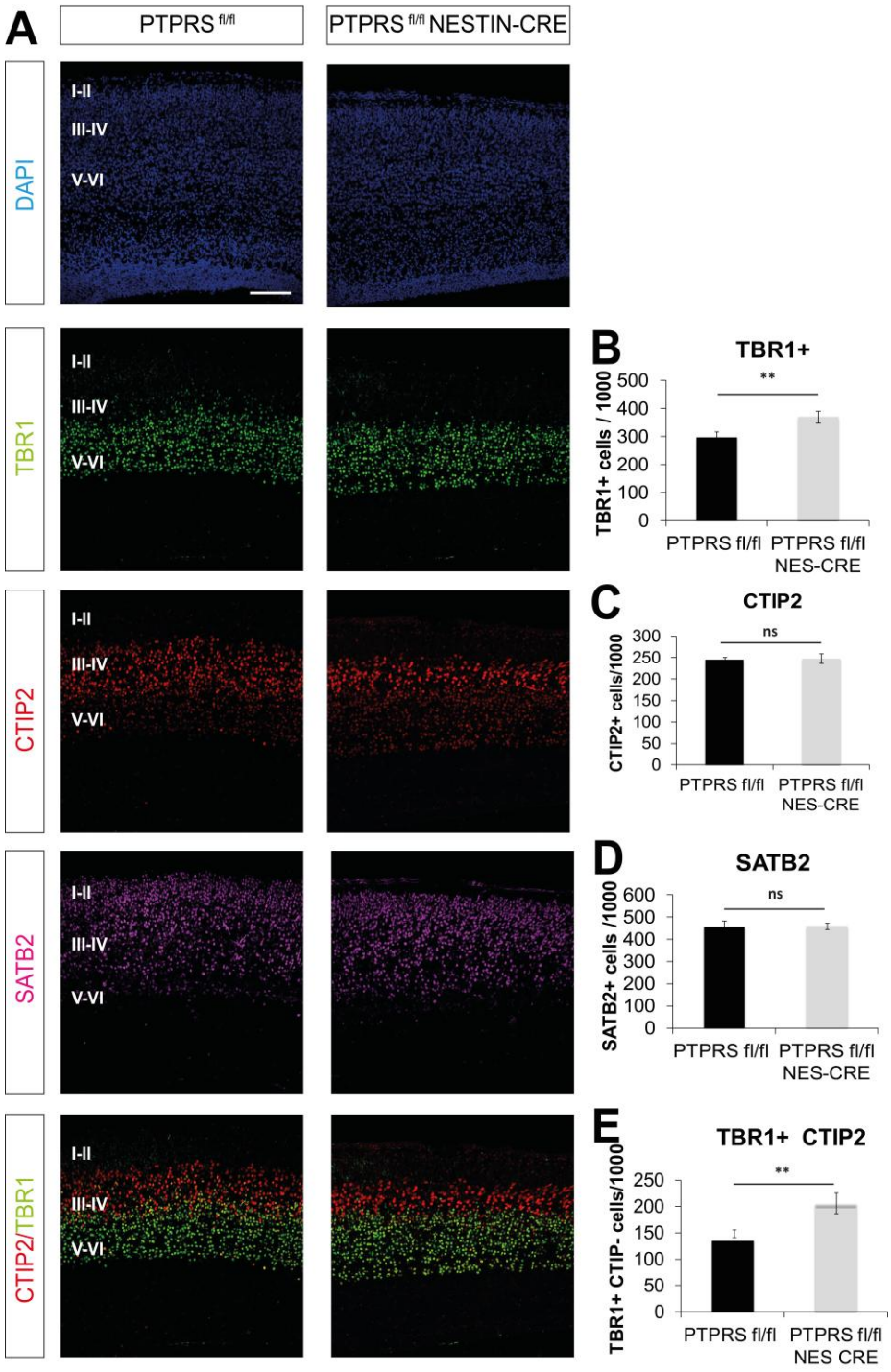

Fig (3.2.6) Lamination of the cerebral cortex is affected in PTPRS$^{fl/fl}$ Nestin-Cre cortices.

A) Representative images of PTPRS$^{fl/fl}$ Nestin-Cre or PTPRS$^{fl/fl}$ cortices stained with Tbr1, CTIP2, and SATB2, expressed in layers II/III. B) Quantification and statistical analysis of cells expressing for Tbr1, C) CTIP2 , D) and Satb2 in PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ cortices. E) The number of Tbr1+ CTIP- cells is increased in the PTPRS$^{fl/fl}$ Nestin-Cre brains *p<0.05 **p<0.01. Students t-test. PTPRS$^{fl/fl}$ Nestin-Cre n=7, PTPRS$^{fl/fl}$ n=6

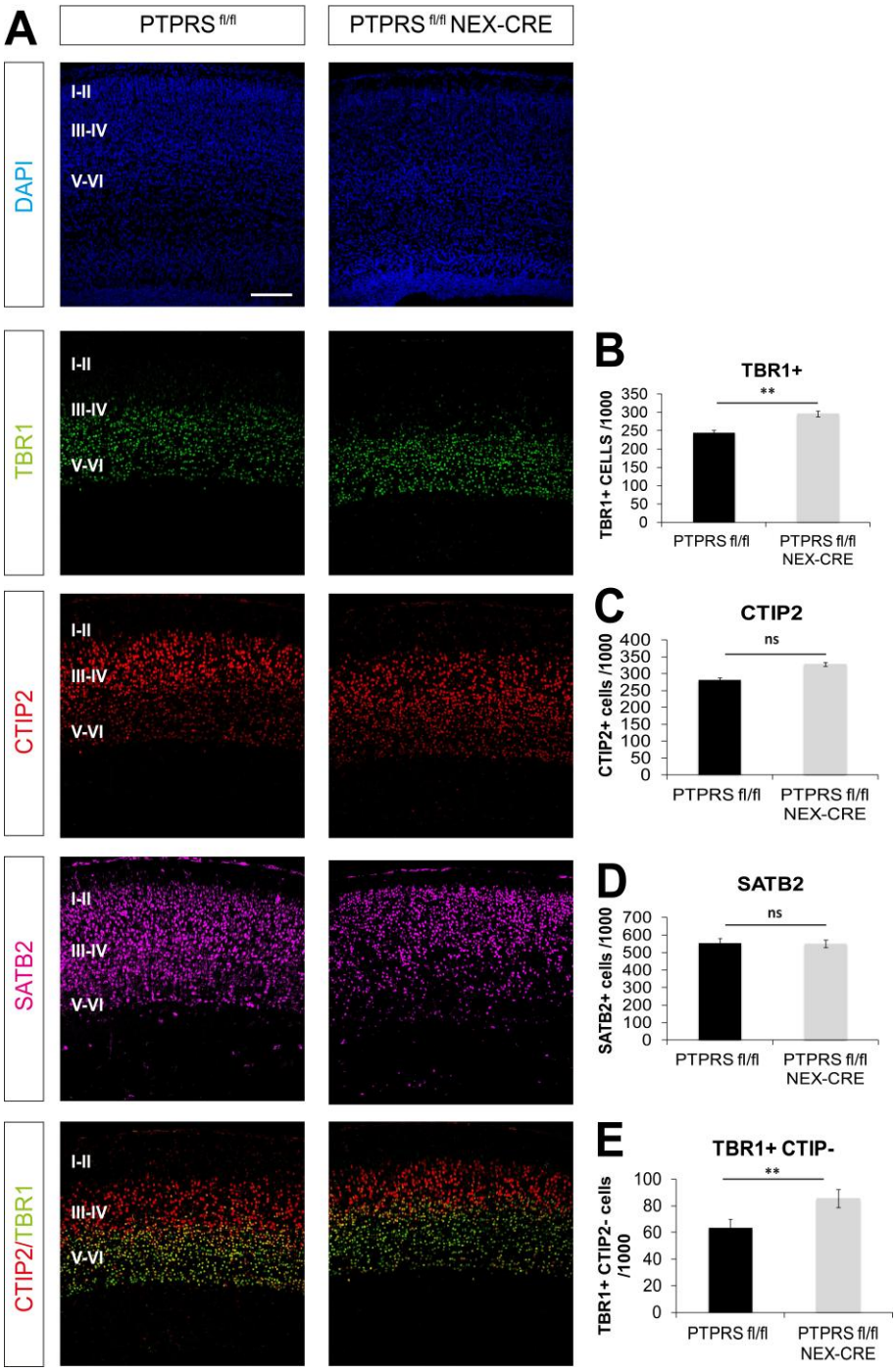

Fig (3.2.7) Lamination of the cerebral cortex is affected in PTPRS$^{fl/fl}$ NEX-Cre cortices. A) Representative images of PTPRS$^{fl/fl}$ Nex-Cre and PTPRS$^{fl/fl}$ cortices stained with the layer specific transcription factors Tbr1, CTIP2, and SATB2. B) Quantification and statistical analysis of B) Tbr1, C) CTIP2 and D) Satb2 show that the number of cells expressing Tbr1 is expanded in PTPRS$^{fl/fl}$ Nestin-Cre cortices in comparison to litter matched PTPRS$^{fl/fl}$. E) Number of Tbr1+ CTIP- cells is increased in PTPRS$^{fl/fl}$ Nestin-Cre compared to controls *p<0.05 **p<0.01. Students t-test. PTPRS$^{fl/fl}$ Nex-Cre n=9, PTPRS$^{fl/fl}$ n=8

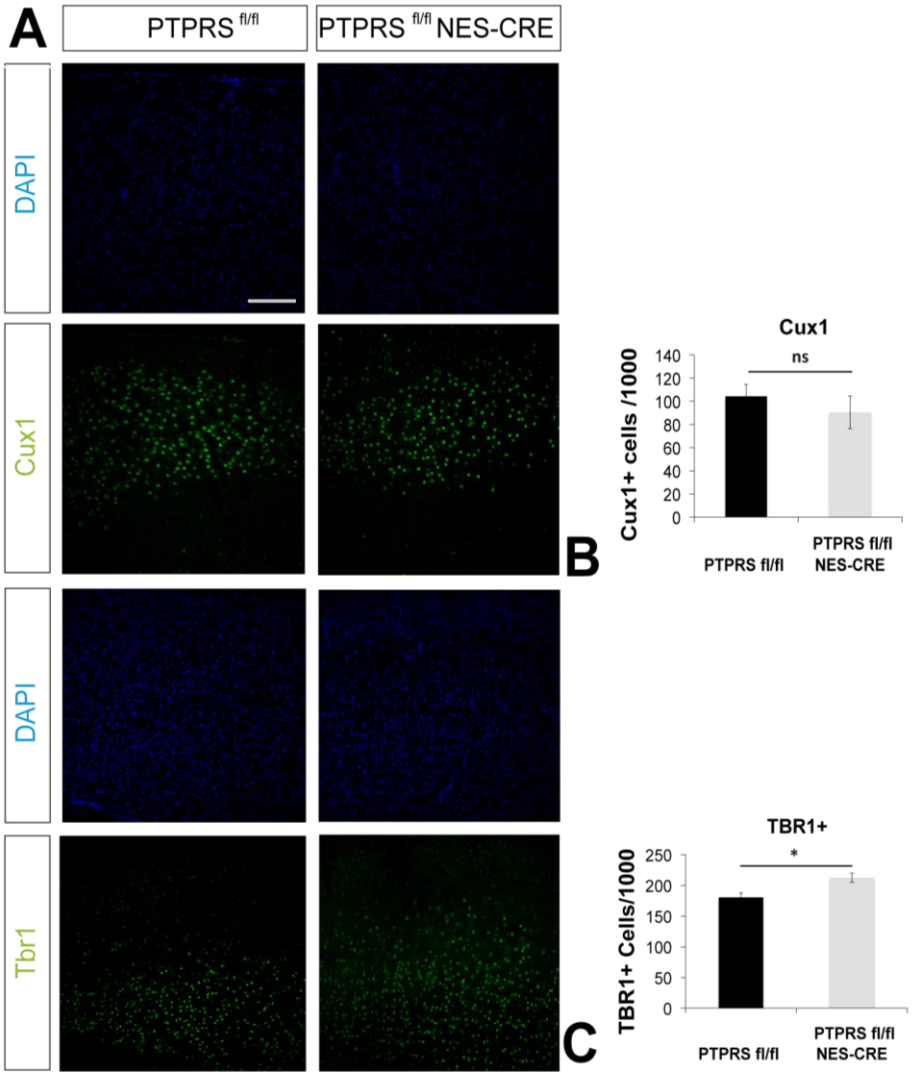

Fig (3.2.8) Lamination of the cerebral cortex is affected in PTPRS$^{fl/fl}$ Nestin-Cre cortices at P30. A) Representative images of PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ cortices stained with the layer specific transcription factors Cux1 and Tbr1. Quantification and statistical analysis of B) Cux1 and C) Tbr1 show that the number of cells expressing Tbr1 is expanded in PTPRS$^{fl/fl}$ Nestin-Cre cortices in comparison to litter matched PTPRS$^{fl/fl}$ embryos. The number of Cux1+ is reduced but not significantly *p<0.05 **p<0.01. Students t-test. PTPRS$^{fl/fl}$ Nex-Cre n=4, PTPRS$^{fl/fl}$ n=4

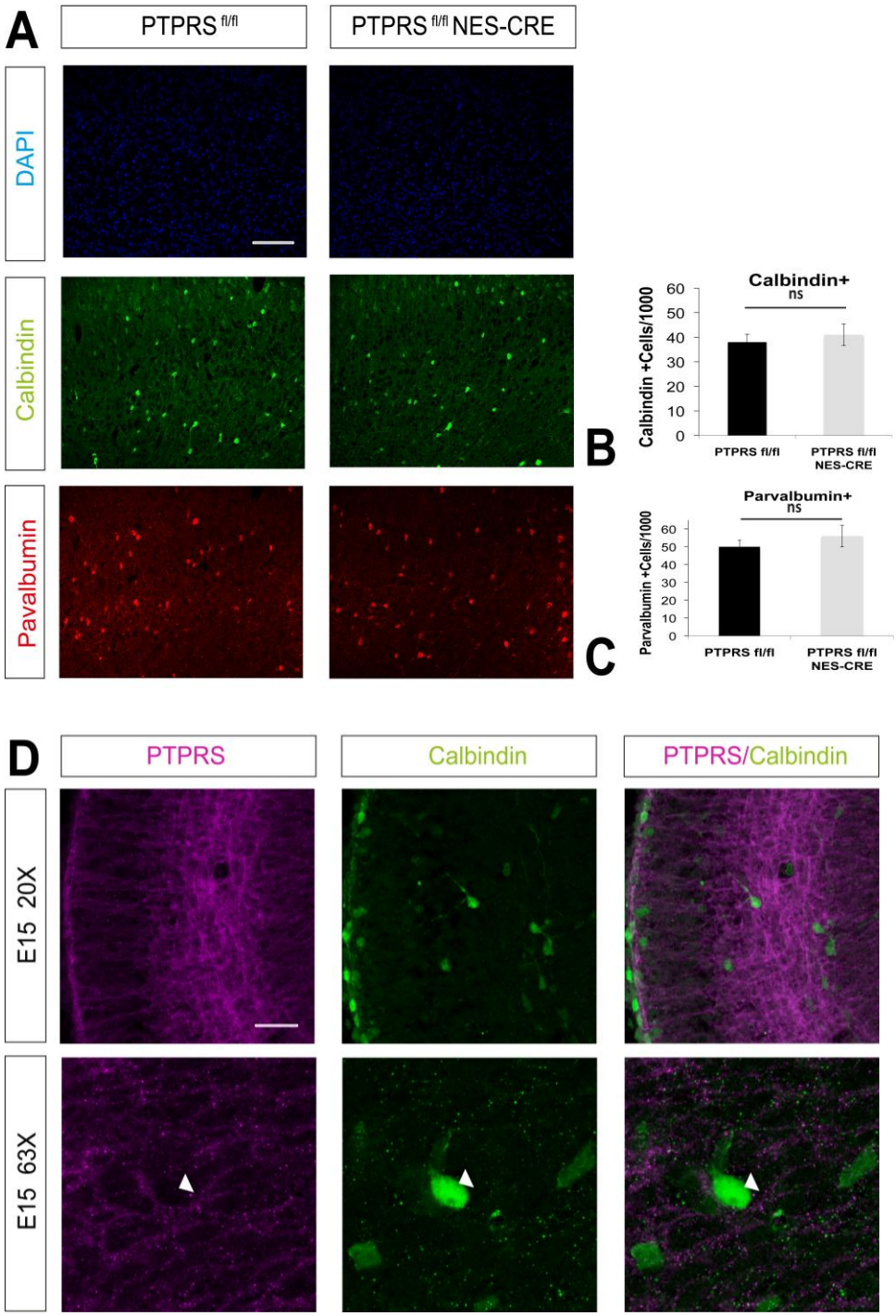

Fig (3.2.9) Analysis of interneuron populations in PTPRS$^{fl/fl}$ NES-Cre cortices. A) Representative images of PTPRS$^{fl/fl}$ Nestin-Cre and PTPRS$^{fl/fl}$ cortices stained with the interneuron markers Calbindin and Parvalbumin. B) Quantification and statistical analysis of B) Calbindin, and C) Parvalbumin expressing populations of interneurons show that the numbers of cells expressing both markers in unchanged in PTPRS$^{fl/fl}$ Nestin-Cre cortices in comparison

to litter matched PTPRS$^{fl/fl}$. D) E15 WT cortices stained with PTPRS and the interneuron marker Calbindin shows that PTPRS does not appear to be expressed in Calbindin+ interneurons at this timepoint. *p<0.05 **p<0.01. Students t-test. PTPRS$^{fl/fl}$ Nex-Cre n=4, PTPRS$^{fl/fl}$ n=4

The same markers were studied in cortices of PTPRS$^{fl/fl}$ NEX-Cre mice. The results obtained were similar to those found in PTPRS$^{fl/fl}$ Nestin-Cre mice with a significant increase of Tbr1 positive cells (21%) in PTPRS$^{fl/fl}$ NEX-Cre mice compared to PTPRS$^{fl/fl}$ **(Fig.3.2.7b)**. This was accompanied by a slight increase in the number of cells expressing CTIP2 **(Fig.3.2.7c)** and an increase (35%) of Tbr1+/CTIP2- cells **(Fig.3.2.7e).** Similar to PTPRS$^{fl/fl}$ Nestin-Cre mice, there was no difference in the number of cells expressing SATB2 **(Fig 3.2.7d)**. The fact that the lamination defect observed in cortices of PTPRS$^{fl/fl}$ NEX-Cre mice resembles the phenotype observed in PTPRS$^{fl/fl}$ Nestin-Cre mice suggests that the defect is not due to an effect on progenitors but to the lack of PTPRS in post mitotic neurons.

Finally, I assessed the lamination of the cortex in PTPRS$^{fl/fl}$ Nestin-Cre mice at P30 when the cortex has fully matured. The results obtained were similar to those found in these mice at earlier timepoints with a significant (27%) increase in the numbers of Tbr1+ cells in PTPRS$^{fl/fl}$ NEX-Cre mice compared to PTPRS$^{fl/fl}$ controls **(Fig.3.2.8c)**. This was accompanied by a slight decrease in the number of cells expressing Cux1 (9%) although this decrease did not reach significance **(Fig.3.2.7B).** Similar to PTPRS$^{fl/fl}$ Nestin-Cre mice, there was no difference in the number of cells expressing SATB2 **(Fig 3.2.7d)**. The fact that the lamination defect observed in cortices of PTPRS$^{fl/fl}$ NES-Cre mice are preserved in mature cortices suggests that defects in lamination do not seem to recover later in life.

As I observed defects in the distribution and migration of neurons in the developing cortex, I next analysed several populations of interneurons in PTPRS$^{fl/fl}$ Nestin-Cre mice in order to rule out the possibility that they are directly affected by a loss of expression of PTPRS or indirectly as a result of knocking out PTPRS in neurons. In order to do this, I stained P30 cortices with markers of different populations of interneurons and quantified the number of cells. Different , non-overlapping populations of GAGAergic interneurons present in the mature cortex express distinct sets of calcium binding proteins such as Calbindin and parvalbumin, which can be used as markers of these populations (Celio, 1990) (Tóth and Freund, 1992). When I quantified the numbers of calbindin and Parvalbumin positive cells In PTPRS$^{fl/fl}$ Nestin-Cre brains, I found no differences in the number of cells expressing either marker when compared to WT controls **(Fig.3.2.9.b) (Fig.3.2.9.c)**. Furthermore I could not detect the expression of PTPRS in calbindin positive cells in the cortex at E15 when PTPRS is strongly expressed in neurons in the developing cortex **(Fig.3.2.9.d)**

Summary

My findings demonstrate that PTPRS is necessary for radial migration of postmitotic neurons from the IZ into the CP. This is accompanied by an expansion of the deepest layers of the cerebral cortex whilst more superficial layers are unaffected. Altogether, these data indicate that PTPRS plays a key role in the development of the cerebral cortex.

3.3. CSPGs is a ligand of PTPRS in the developing cortex

CSPGs are expressed in the developing cortex and are considered markers of the subplate, separating the CP from the IZ (Miller et al., 1995). However, whether CPSGs or their binding to PTPRS regulate corticogenesis or cortical neuron migration remains unknown. I performed a series of immunostainings to compare the expression patterns of PTPRS and CSPGs in the developing cortex. I observed that PTPRS is expressed in migrating neurons within the upper IZ and in neurons traversing the subplate, however, its levels decrease as migrating cells enter the CP. At the height of radial migration at E16.5, CSPGs are expressed in the subplate and the MZ **(Fig.3.3.1)**. Therefore, PTPRS-expressing neurons first encounter CSPGs in the upper IZ as they reach the subplate, Both PTPRS and CPSGs are coexpressed in the subplate **(Fig.3.3.1)**. The expression pattern is conserved throughout the cortical development and is also observed in other laminar structures of the brain such as the hippocampus (data not shown).

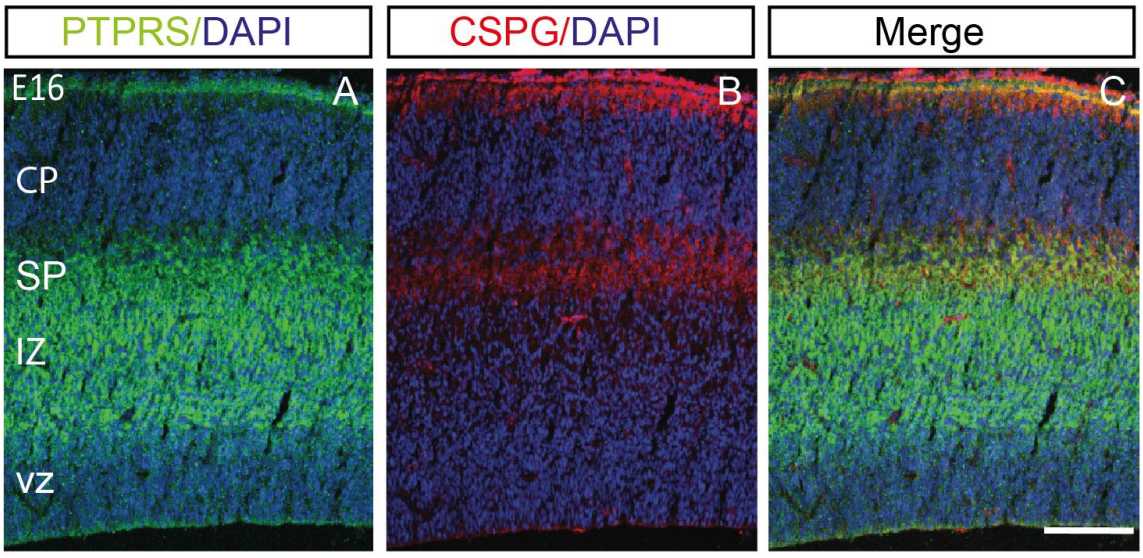

Fig.3.3.1 Expression of PTPRS and CPSGs in the developing cortex.

Confocal images of E16.5 cortex stained with PTPRS (green, A) and CSPG (red, B) antibodies or merged images (yellow, C). All sections were co-stained with DAPI. Scale bar 100µM.

Cells lacking PTPRS fail to migrate past CSPGs in the subplate

When PTPRS expression is inhibited in migrating cells by electroporation of PTPRS shRNA, migrating neurons were unable to cross the subplate and enter the CP **(Fig.3.2.1)**. This may be due to compromised PTPRS-CSPG signalling in these cells. In order to assess this possibility, I first performed an immunostaining to ascertain where these cells arrest their migration in relation to the CSPGs in the cortex. Brains were electroporated at E14.5 and 4 days later, cell migration was assessed by quantifying the numbers of GFP positive cells, which had migrated past CSPGs expressed in the subplate. Whereas the majority of cells electroporated with control shRNA crossed the region of the subplate expressing CPSGs and reached the CP, the majority of cells lacking PTPRS halted their migration upon encountering CSPGs **(Fig.3.3.2)**. Hence, PTPRS is required for migration of cortical neurons past areas which express CSPGs.

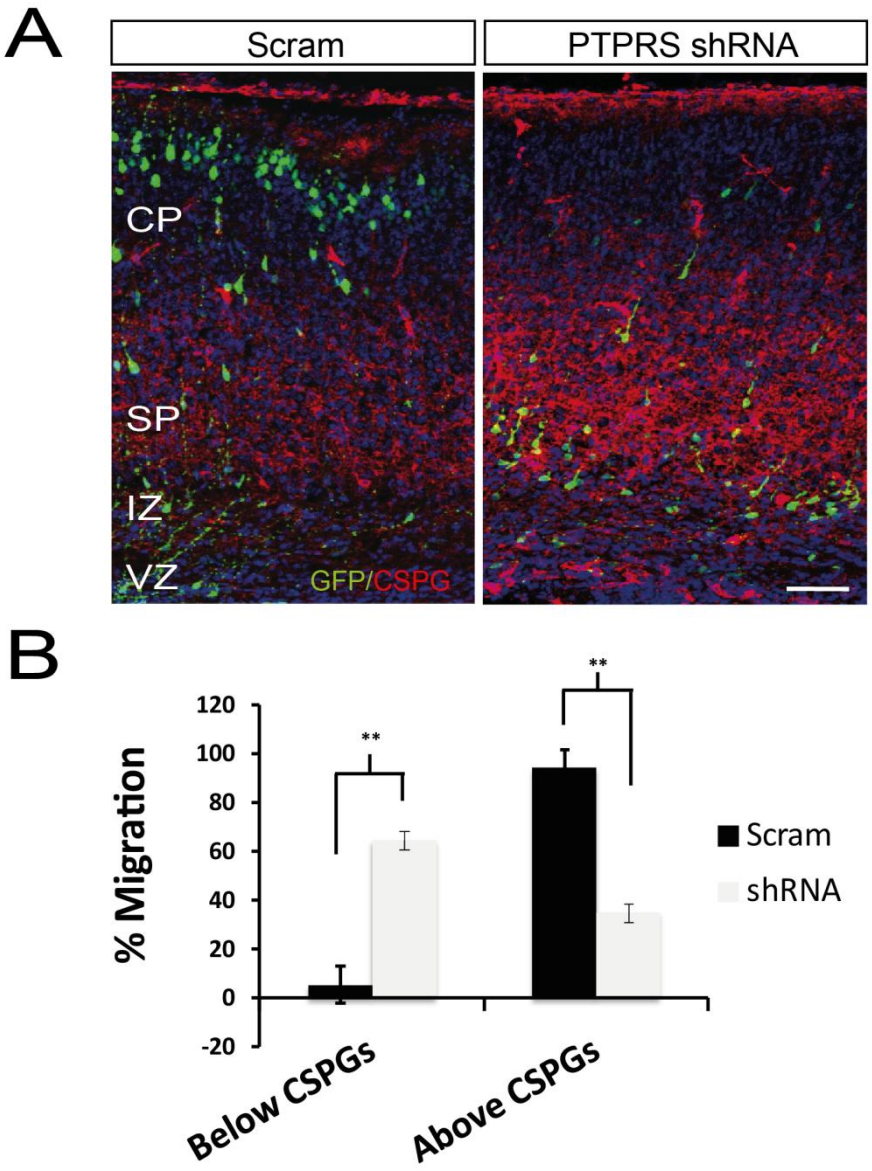

Fig 3.3.2 Expression of CPSGs in cortices electroporated with PTPRS shRNA.

A. Confocal images of CSPG and GFP antibody stainings of E14.5 brains *in utero* electroporated with PTPRS shRNA or scrambled shRNA. B. Quantification of GFP positive cells that have migrated past CSPGs. N = 5 embryos, shCTL, N = 6 embryos. Student's t-test **p<0.01. Scale bar = 100μM

CSPGs are required for radial neuron migration.

To investigate whether CSPGs themselves are necessary for radial neuron migration, chABC was used to digest CPSGs in cortical slices. chABC (2U/ml) was applied to cortical slice cultures harvested from E14.5 embryos. After 24h, slices were fixed and stained for CSPGs. Treatment with chABC abolished the expression of CSPGs in the cortex when compared to control slices treated with vehicle only **(Fig.3.3.3)**, Thus, I concluded that this model system, represents a useful tool to assess the role of CPSG in the developing cortex.

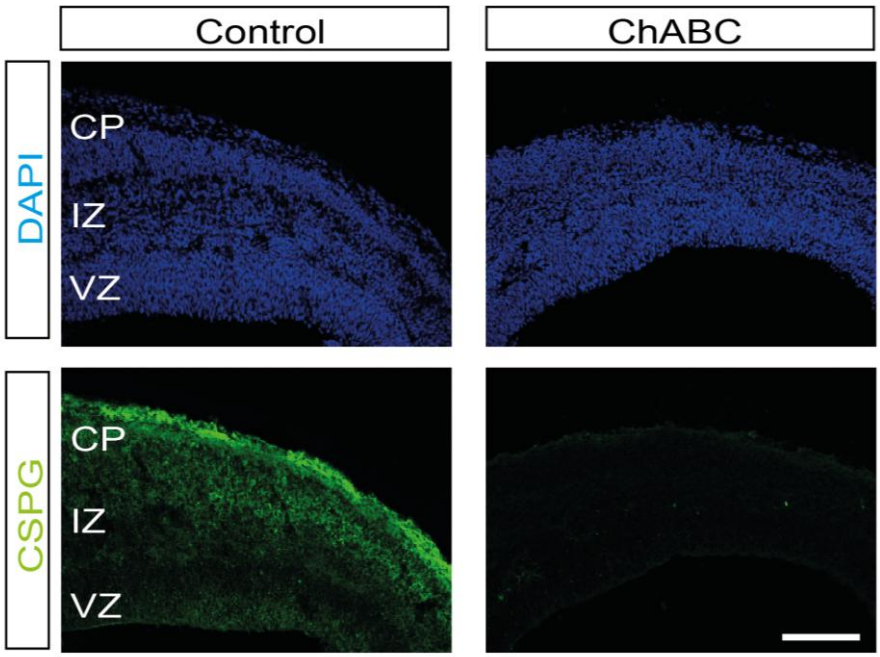

Figure 3.3.3 ChABC treatment of cortical slice cultures

CSPG staining in brain slice harvested from E14.5 WT embryos treated either with chABC or vehicle (Control). n=3.

Cortical slices were acutely dissected from brains of E14.5 mice embryos, electroporated with pCIG2-GFP, and immediately after electroporation treated with either chABC (2U/ml) or vehicle. Slices were cultured for 4 days, and stained with GFP to visualize electroporated cells. Cells in control slices migrated into the CP, as expected, whereas neurons in slices treated with chABC showed severe migration defects, with a higher number of cells unable to reach CP **(Fig.3.3.4)**. These cells accumulated in the IZ , suggesting that CSPGs expressed in the subplate are required for migration of neurons out of the IZ and into the CP. Hence, in cultured brain slices, CSPGs are required for radial neuronal migration.

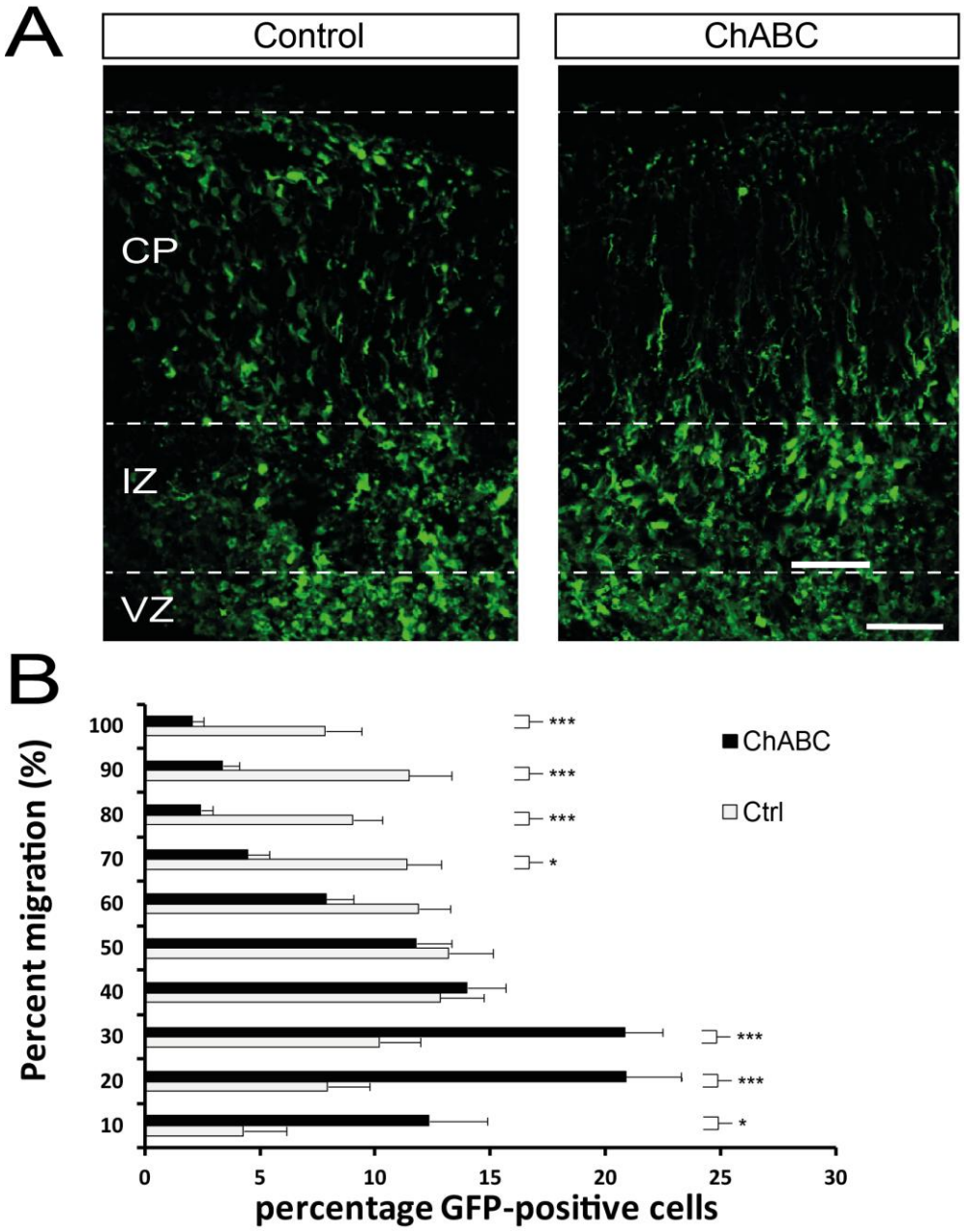

Figure 3.3.4 Enzymatic digestion of CSPGs leads to defects in radial migration. Analysis of radial migration in cortical slices harvested from E14.5 WT embryos, *ex vivo* electroporated with GFP and treated with chABC or vehicle (Control) for 4 days. **A**) Representative images of sections stained with GFP antibody. **B**) Quantification of GFP positive neurons with distance travelled expressed as a percentage of the total distance from the ventirical wall to the pial surface. Average and SEM are shown. n= 21 Student's t-test *p<0.05, ***p<0.001.

Expression of activated RhoA in the developing cortex

Binding of LAR family phosphatases to CSPGs induces a switch of RhoA from the inactive GDP-bound form to the active RhoA-GTP (Wu et al., 2013). Activation of RhoA is required for cells migration into the CP (Hand et al., 2005). RhoA could therefore be an important downstream effector of PTPRS and CSPGs during neural radial migration. To determine whether CSPGs activated RhoA, I performed immunostainings using an antibody that specifically targets the active, GTP-bound form of RhoA, and compared the expression of active RhoA with CSPGs. RhoA-GTP was expressed in migrating neurons in the subplate as they were entering the CP. This pattern of expression is remarkably similar to that of CSPGs, suggesting that cortical neurons migrating through CSPG rich regions activate Rho as they enter the CP.

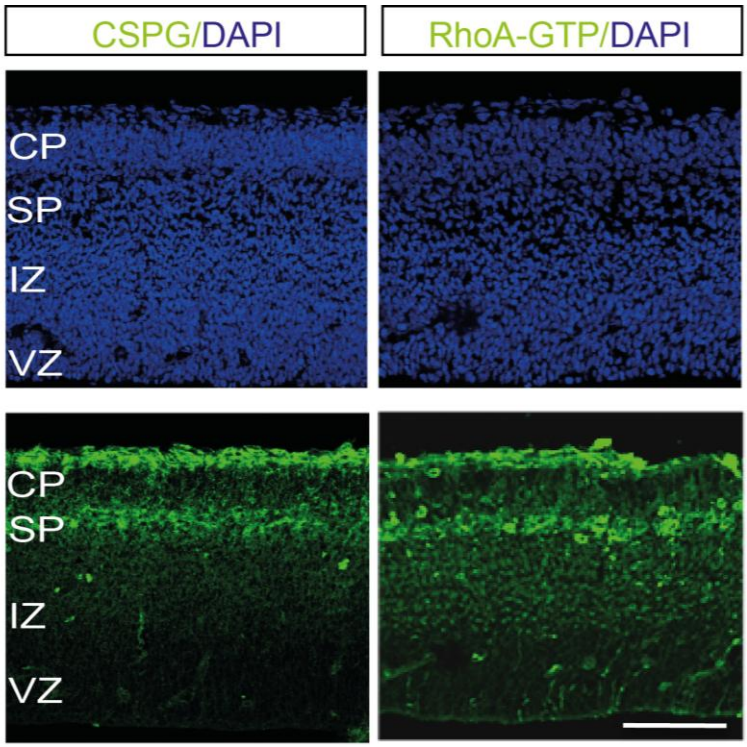

Fig 3.5 Expression of activated RhoA in the developing cortex

E16.5 cortical sections immunostained for RhoA-GTP or CSPG and co-stained with DAPI. Separate slices where stained for CSPG and RhoA-GTP due to the fact that antibodies were raised in the same species. Images are shown at 20X magnification. Representative images from n=3 embryos per staining.

Summary

My results demonstrate that PTPRS expressing cells migrate towards the CP, before encountering a band of CSPGs in the subplate. Migrating cells lacking PTPRS are unable to migrate past this point, as interaction of PTPRS with CSPGs is necessary for cells migration towards the CP. When migrating neurons come into contact with the CSPGs in the subplate, RhoA becomes activated. It is therefore possible that PTPRS-dependent activation of RhoA in this region allows the migration of neurons into the CP **(Fig.3.3.4)**.

4. DISCUSSION

The human brain is arguably the most complex physical structure known with the cerebral cortex alone containing as many as 100 billion neurons (Edelman, 1992). The formation of a properly organised cortex depend on the regulated action of thousands of neuronal genes that are co-ordinated on several levels. Environmental signals provide neurons with positional and temporal information, which must be converted into a programme of gene expression that determines cellular behaviour. Neurons generated from the progenitor pool in the ventricular zone migrate radially into the CP, where they form connections with other neurons. Genetic mutations or environmental factors that disturb this process lead to severe neurodevelopmental defects (Cugola et al., 2016) (Reiner et al., 1995). In addition, migratory defects are increasingly being linked to complex neuropsychological conditions such as autism, psychosis and bipolar disorder (Millar et al., 2000) (Goh et al., 2007) (Reiner et al., 2015).

My findings demonstrate that PTPRS is regulated by S-nitrosylation of HDAC2 and importantly, its expression is decreased in nNOS knockout mice. I also showed that PTPRS plays a pivotal role in cortical development and is required for neuron radial migration. Moreover, CSPGs, which act as ligands for PTPRS are required for radial neuron migration. Taken together these findings suggest that S-nitrosylation of HDAC2 regulates an important and novel signalling pathway required for cortical neuron migration.

PTPRS is regulated by S-nitrosylation of HDAC2

Our lab had previously shown that NO signalling is required for neuronal migration and proper lamination of the CP (Nott et al., 2013). An important mechanism of NO signalling during development is through S-nitrosylation of proteins, which in the case of HDAC2 leads to transcriptional derepression of developmental genes (Nott et al., 2013). In this study, I have confirmed that S-nitrosylation of HDAC2 controls PTPRS transcription **(Fig.3.1.1); (Fig.3.1.2)**. Importantly, PTPRS expression was also inhibited in nNOS knockout mice, showing that NO signalling regulates the expression of PTPRS *in vivo* **(Fig.3.1.7)**. The extent of the downregulation of PTPRS transcripts in nNOS cortices was comparable to that observed in our screen to identify genes regulated by S-nitrosylation of HDAC2 as well as subsequent confirmation *in vitro. This* shows that PTPRS is transcriptionally regulated by S-nitrosylation of HDAC2 *in vivo* as well as *in vitro*.

I have shown that HDAC2 binds directly to the promoter of PTPRS *in vivo* **(Fig.3.1.4)**. The transcriptional effects of S-nitrosylation of HDAC2 were reproduced in primary cortical neurons expressing a luciferase reporter plasmid whose activity was driven by a 1054bp PTPRS promoter region containing this site **(Fig.3.1.3)**. An intriguing possibility is that NO signalling regulates PTPRS by modulating the binding of HDAC2 to the PTPRS locus at this site. NO signalling has been shown to lead to S-nitrosylation of nuclear factors and regulates the binding of these proteins to DNA. This was first demonstrated for the bacterial transcription factor OxyR, for which stress-dependent S-nitrosylation of two key cysteines located within the DNA binding domain increased its affinity to chromatin (Kim et al., 2002). S-nitrosylation of DNA binding proteins may increase or decrease affinity for DNA. For instance, S-nitrosylation of NF-kB has been shown to promote the expression of NF-kB induced genes (Marshall and Stamler, 2001). Conversely, S-nitrosylation of the transcription factors AP1, MEF2C, and AtMYB30 reduces the ability of each protein to bind DNA (Tavares et al., 2014) (Brendeford et al., 1998). Our lab has shown that S-nitrosylation of the histone deacetylase HDAC2 reduces its interaction with chromatin. In mature cortical neurons, NO is produced in neurons in

response to extracellular stimuli and subsequent S-nitrosylation of HDAC2 leads to dissociation of HDAC2 from target gene promoters and transcription of activity regulated genes (Nott et al., 2008). It would be intriguing to assess whether binding of HDAC2 to the PTPRS promoter is regulated by NO signalling. A ChIP of HDAC2 on the PTPRS promoter in nNOS knockout and wild type cortices will allow us to determine whether binding of HDAC2 at the PTPRS locus is regulated by NO. S-nitrosylation may also affect the enzymatic activity of proteins (Whalen et al., 2007) (Gu et al., 2002) (Uehara et al., 2006), although data from our laboratory indicate that S-nitrosylation does not affect the acetylase activity of HDAC2 (Nott et al., 2008)

HDAC2 regulates transcription as a component of large multisubunit chromatin regulating complexes. Interestingly, components of the HDAC2-containing regulatory complexes Sin3a, CoRest and NuRD were found to be S-nitrosylated in a recent screen carried out to identify S-nitrosylated proteins in cortical neurons (JGS and AR, manuscript accepted). Since all three complexes have been implicated in radial neuron migration, it is conceivable that S-nitrosylation controls the expression of migratory genes via modulation of the function of one or all of these complexes (Fuentes et al., 2012); (Egan et al., 2013); (Witteveen et al., 2016). Interestingly, post-translational modifications can regulate protein complexes by regulating their subunit composition. For instance, phosphorylation of BAF60c causes it to translocate to the nucleus, forming the lipoBAF complex and subsequent activation of lipogenic genes (Wang et al., 2013) (Lessard et al., 2007). Intriguingly, recent work from our lab has shown that composition of the NuRD complex is regulated during cortical development, generating functionally distinct complexes, which participate in different stages of corticogenesis (Nitarska et al., 2016 in press). Ongoing work is aimed at determining whether S-nitrosylation is involved in the regulation of NuRD complex subunit composition and function during development. Whether NuRD is regulating PTPRS expression is currently unknown. Indeed, further experiments are required to determine which particular HDAC2-containing complexes influence transcription of PTPRS.

Chromatin modifying complexes sculpt the epigenetic landscape of cells, through epigenetic modifications that either facilitate or block other factors accessing target genes (Turner, 2000). Following stimulus-induced S-nitrosylation of HDAC2, there is an increase in acetylation and transcription of CREB dependent genes (Nott and Riccio, 2009) (Riccio et al., 2006). In agreement with this, treatment of neurons with BDNF induces generation of NO and increased binding of CREB to target genes (Riccio et al., 2006) (Nott et al., 2008). BDNF is an important regulator of migration in the developing brain (Huang and Reichardt, 2001) (Borghesani et al., 2002). BDNF and its receptors are expressed in the developing cortex and are implicated in cortical neurons (McCarthy et al., 2011) (Polleux et al., 2002) (Snapyan et al., 2009) (Grade et al., 2013) (Medina et al., 2004). Here, I have shown that the PTPRS locus contains a canonical CRE and that this site is bound by CREB *in vivo* **(Fig.3.1.6)**. Since CREB is an important regulator of cortical development (Diaz-Ruiz et al., 2008), S-nitrosylation of HDAC2 may control the expression of genes required for radial neuron migration by regulating the occupancy of CREB at target gene promoters. The expression of PTPRS in $CREB^{-/-}$ mice should be assessed in order to assess this possibility. Similarly, S-nitrosylation of HDAC2 may also control the occupancy of other pro-neural transcription factors such as nuerogenin2 and neuroD family transcription factors, which regulate the expression of migratory genes in the developing cortex (Heng et al., 2008).

Could NO signalling regulate neuronal migration and cortical development by controlling the levels of PTPRS in the cortex? I have demonstrated that PTPRS is transcriptionally inhibited in cortices of $nNOS^{-/-}$ mice **(Fig.3.1.7)**. Since loss of PTPRS leads to defects in migration and expansion of deep layer neurons as was previously observed in $nNOS^{-/-}$ mice, it would be interesting to determine if rescuing the levels of PTPRS in migrating neurons of $nNOS^{-/-}$ mice is sufficient to revert any of these defects.

PTPRS and cortical development

Previous studies have shown that neurons derived from PTPRS knockout mice migrate abnormally *in vitro* and exhibiting defects in neurite outgrowth (Kirkham et al., 2006). Here, I have demonstrated that PTPRS is required for the migration of neurons from the IZ into the CP *in vivo* **(Fig.3.3.1, Fig.3.3.4 and Fig.3.3.5)**. I came to this conclusion using a number of different approaches including shRNA-mediated inhibition and CRE-mediated deletion of PTPRS in migrating neurons. Importantly, I observed similar effects on migration in $PTPRS^{fl/fl}$ Nestin-Cre mice and $PTPRS^{fl/fl}$ Nex-Cre mice. This discrepancy between the shRNA and Cre mediated knockdowns could be due to off-target effects associated with shRNA knockdown and may target other LAR family phosphatases such as PTPRD and PTPRF. When the expression of these genes was assessed during corticogenesis, PTPRF was not expressed at E14.5 but some staining for PTPRD was detected in the VZ (Sahin and Hockfield, 1993). It is also possible that Cre-loxP recombination may result in a less efficient removal of PTPRS protein early in neuronal migration than knockdown by shRNA. If the PTPRS protein is particularly stable, it may still be present for a time after removal of the critical exon. However, staining for PTPRS at E15 suggests, that at least by this time, PTPRS is not present. In these experiments, I electroporate at E14.5 so staining for PTPRS in knockout animals at this timepoint would eliminate the possibility that PTPRS protein is present during the migration of these cells. Another reason that may explain why the phenotype associated CRE-mediated knockout of PTPRS is less severe than PTPRS shRNA is that other LAR phosphatases may functionally compensate for the lack of PTPRS. In support of this, previous studies have shown that LAR family of phosphatases acts in a redundant manner during development (Stewart et al., 2013). In order to address this possibility, I will assess the expression of PTPRF and PTPRD in the cortices of wild type and PTPRS conditional knockout mice. I also observed that migration defects in neurons lacking PTPRS were less severe at 4 days after electroporation than at 3 days **(Fig.3.2.3)**. A recovery of other developmental defects in mature PTPRS mice has been observed previously and it is possible that developmental upregulation of other LAR family phosphatases at later developmental time points is

responsible for this (Elchebly et al., 1999). The migratory phenotype observed in PTPRS conditional knockout mice may recover further after birth and the phenotype observed in PTPRS conditional knockout mice could represent a slowing of migration rather than a complete halt. This should be assessed by examining the position of electroporated neurons lacking PTPRS after birth and in adult mice. Delayed radial migration is associated with altered patterns of neuronal activity during early circuit development in FMRP knockout mice. More moderate defects in migration such as these may underlie mental deficits associated with this and other neurodevelopmental disorders such as autism spectrum disorders (La Fata et al., 2014).

Analysis of $PTPRS^{fl/fl}$Nestin-Cre mice and $PTPRS^{fl/fl}$Nex-Cre cortices did not uncover a significant reduction of the size of the brain in these mice **(Fig.3.2.4)**, as previously observed in $PTPRS^{-/-}$ mice (Meathrel et al., 2002). What sets my study apart from these is the use of a CRE-recombinase based system to generate conditional knockout mice lacking PTPRS specifically in the nervous system. Thus, it is likely that many of the structural defects previously observed in the brains of $PTPRS^{-/-}$ mice are indirect consequences of defects in other tissues such as the neuroendocrine system (Elchebly et al., 1999). Lamination of the cortex of $PTPRS^{fl/fl}$Nestin-Cre mice and $PTPRS^{fl/fl}$ Nex-Cre was disrupted and this analysis revealed an expansion of the lower cortical layers in both transgenic mouse models. In $nNOS^{-/-}$ mice, expansion of deep layer neurons is coupled with defects in radial migration and a corresponding decrease in the size of the upper layers, which is due to defects in the migration of upper but not lower layer neurons (Nott et al., 2013). However, in the cortex of PTPRS conditional knockout mice, I did not observe any difference in the expression of the upper layer marker SATB2 **(Fig.3.2.6); (Fig.3.2.7)**. I observed similar results when I carried out the same analysis at P30 with an increase in the numbers of Tbr1+ cells accompanied by a small but insignificant decrease in the numbers of Cux1+ cells **(Fig.3.2.8)**. SATB2 and Cux1 are strongly expressed in the upper layers of the CP but other markers expressed in specific layers may reveal more differences in lamination not visible using these markers (Ferrere et al., 2006). The expression of such markers should also be carried out in order to examine the

lamination of these specific layers. An alternative explanation for the observed expansion of the lower layers could be that deletion of PTPRS leads to altered proliferation of neuronal precursors. However this is unlikely as I observed this expansion of the lower layers in $PTPRS^{fl/fl}$Nex-Cre mice **(Fig.3.2.7)**, in which, PTPRS is knocked down in post-mitotic neurons only. This suggests that the expansion of the lower layers is not due to an effect on proliferative cells. In agreement with this, previous studies have shown that NPCs and progenitors derived from $PTPRS^{-/-}$ mice showed no defects in proliferation and generated cells of different neuronal lineages normally (Kirkham et al., 2006). It is still possible that deletion of PTPRS in post-mitotic neurons has an indirect effect on proliferation. Deletion of both PTPRS and PTPRF affects mandibular cell proliferation due to impairment of the Wnt signalling pathway (Stewart et al., 2013). To rule out the possibility that proliferation of NPCs or progenitors is altered in PTPRS conditional knockout mice, the proliferation rates of NPCs should be assessed *in vivo.*

CSPGs are required for cortical development

I demonstrated that CSPGs, which act as ligands for PTPRS, are required for migration of neurons from the IZ into the CP **(Fig.3.3.4)**. This is in agreement with previous studies, which have implicated CSPGs in radial neuron migration. For instance, knockdown of the sulfotransferases UST and 4,6-ST, which are required for the synthesis of CSPGs causes an impairment in the MB to BP switch (Ishii and Maeda, 2008). However, CSPGs are not expressed in migrating neurons and it is unclear how knockdown of these sulfotransferases in migrating cells might disrupt CSPGs, which are mostly expressed in the subplate (Miller et al., 1995). My results provide the first direct demonstration that GAG sidechains of CSPGs expressed in the embryonic subplate are required for cells to enter the CP. Altered CSPG signalling may therefore play a role in neurodevelopment disorders, in which neuronal migration is affected. In agreement with the expression of the proteoglycan NGC/CPSG5 rescues migration defects observed in mice lacking PHF6, a gene associated with x-linked intellectual disability. (Zhang et al., 2013).

CSPGs stimulate activation of RhoA by signalling through PTPRS and this pathway plays an important role in providing directional cues to migrating axons (Monnier et al., 2003). I have shown that the active GTP bound version of RhoA is expressed in the same area of the cortex as CSPGs during development **(Fig.3.3.5)**. Previous studies assessing the expression of active RhoA in the cortex using a FRET imaging system reported a similar pattern of expression in migrating neurons in the upper IZ and the SP (Azzarelli et al., 2014b). Importantly, preventing the activation of RhoA leads to a defect in the MB to BP switch (Hand et al., 2005) (Azzarelli et al., 2014b). Excessive RhoA activity also leads to an arrest of migration at an earlier stage before cells enter the IZ highlighting the importance of tightly regulated RhoA activity during radial migration (Hand et al., 2005) (Britz et al., 2006) (Britz et al., 2006). A recent study has shown that although $RhoA^{-/-}$ mice display defects in radial migration, neurons derived from these mice migrate normally in a wild type background (Cappello et al., 2012). Since other Rho proteins, including RhoA, B and C, are expressed in the developing cortex, they could compensate for the lack of RhoA in these neurons (Olenik et al., 1999). My results demonstrate that both PTPRS and CSPGs are required for cells to migrate into the CP and that RhoA is activated as PTPRS expressing migrating neurons encounter CSPGs in the subplate. An intriguing possibility is that CSPG signalling through PTPRS is responsible for activation of RhoA, which in turn triggers the MB to BP switch, allowing cells to migrate past the subplate **(model in Fig 5.1)**.

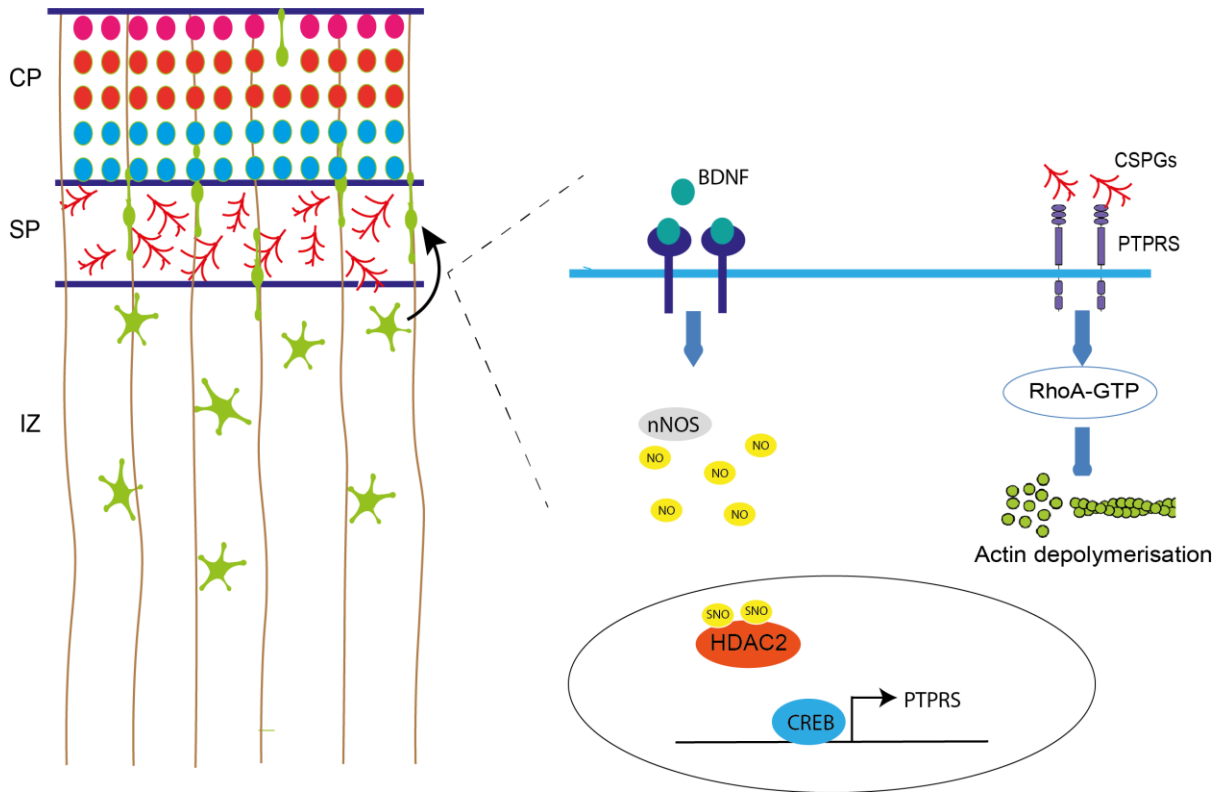

Figure 5.1 Model of PTPRS and CSPG signalling in cortical neuron migration

Extracellular stimulus leads to S-nitrosylation of HDAC2 and upregulation of PTPRS. Cells expressing PTPRS (green) radially migrate before encountering a band of CSPGs in the SP. Upon CSPG binding to PTPRS, this induces activation of RhoA in these cells. Activated RhoA signalling then allows movement of cells from the intermediate zone (IZ) into the CP.

ECM molecules including proteoglycans such as CSPGs regulate many aspects of the development of the nervous system including migration and lamination (Franco and Müller, 2011). Neurons undergoing radial migration utilize different migratory modes to reach their final positions and areas where neurons undergo switches in migratory mode, such as in the embryonic subplate and MZ, are rich in ECM molecules (Miller et al., 1995). Neurons express receptors which bind ligands in the ECM and this has been shown to trigger transitions in migratory modes (Franco and Müller, 2011). For instance, migrating neurons express the transmembrane receptors VLDLR and ApoER2 which bind reelin expressed in the developing MZ and this induces somal translocation (Franco et al., 2011). Collagen III regulates the termination of migration via it's receptor GPR56 and may do this by regulating RhoA activity in the cortex (Luo et al., 2011) (Singer et al., 2013). The expression of CPSGs in the embryonic cortex is restricted to the subplate, and marks the boundary between the IZ and the CP (Fig.3.3.1) (Miller et al., 1995). The SP initially acts as a barrier to neurons migrating in a multipolar fashion through the IZ. Once they have reached the SP, migrating neurons must undergo the multipolar to bipolar switch in migratory mode, in order to move through the SP and into the CP (Heng et al., 2013). Because of this pattern of expression, CSPGs are ideally placed to provide a positional cue to migrating neurons expressing PTPRS as they reach the subplate, and PTPRS/CSPG signalling may be involved in triggering the MP to BP switch (Fig.4.1). In future, a more detailed examination of cell morphology and the migratory dynamics of cells lacking PTPRS or CPSGs should be carried out in order to assess this possibility.

HSPGs are ligands for PTPRS, however they regulate neurogenesis rather than migration in mice. Exostosin glycosyltransferase 1 (Ext1) is a glycosyltransferase required for the synthesis of HSPGs. Mice lacking Ext1 in the nervous system are microcephalic due to defects in neurogenesis (Inatani et al., 2003). Although knockdown of the Ext1 homologs rib-1 and rib-2 in C.*elegans* leads to disruptions in neuronal migration, mice lacking Ext1 do not display defects in lamination or migration. (Inatani et al., 2003). Mice lacking the enzyme heparan sulphate 2-O-sulphotransferase (Hs2st), a sulphotransferase which is also required for the synthesis of HSPGs have a phenotype similar to the $Ext1^{-/-}$ mice (McLaughlin et al., 2003) (Pratt et al., 2006).

In many cases, LAR family phosphatases exert their physiological roles by regulating components of the cadherin-catenin complexes (Fujita et al., 2002). A recent study has shown that PTP1B regulates radial migration by facilitating the formation of complexes containing cadherin 2 (CDH2), CDH4, α-catenin and β-catenin (Martinez-Garay et al., 2016). Furthermore, there is loss of E-cadherin and β-catenin containing adherens junctions in $PTPRS^{-/-}$ mice, leading to defects in the integrity of epithelia in the gastrointestinal tract as a result (Muise et al., 2007). Moreover, PTPRS dephosphorylates N-cadherin (Siu et al., 2007). In the embryonic cortex, N-cadherin is expressed in the IZ and is involved in the MB to BP switch (Shikanai et al., 2011) (Jossin, 2011) (Stewart et al., 2013). Disruption to the integrity of adherens junctions in the developing cortex has been shown to lead to delayed neuronal migration (Dudok et al., 2013). Future work should assess the phosphorylation state of cadherins and catenins in the cortices of PTPRS knockout mice. It would also be intriguing to examine the integrity of adherens junctions in these mice to provide further insight into the role of PTPRS in corticogenesis.

Potential role of PTPRS in the pathogenesis of neurodevelopmental disorders

The identification of the genetic basis of neurodevelopmental disorders represents a potential approach to develop new therapeutic strategies for human diseases such as autism. Many migratory defects lead to heterotopia, a state in which some neurons migrate abnormally whilst other neurons in the same regions migrate normally (Spalice et al., 2009). This observation suggests that the environment could still support migration if the stopped or slowed cells could be induced to migrate. Knockdown of DCX *in utero,* in the rat cortex leads to defects in neuronal migration and patterning. One important study has shown that this can be corrected by reexpression of DCX during a critical time period in the second postnatal week. This was even sufficient to reduce seizure risk associated with these malformations. This finding suggests that certain neuronal migration can be corrected upon manipulation of gene expression after birth. (Manent et al., 2009). In addition, mice lacking MECP2, a HDAC2 interacting protein, leads to the early onset developmental disorder, Rett syndrome. Restoration of MECP2 expression in immature and adult mice leads to the recovery of neurological deficits showing that such interventions can not only correct structural defects but also lead to functional recovery (Guy et al., 2007). Beneficial effects can also be achieved pharmacologically. Mice lacking the phosphatase PTEN are considered a model for focal cortical dysplasia (FCD), which is due to hyperactive mTOR signalling (Kwon et al., 2003). Treatment with the mTOR inhibitor rapamycin led to a reduction in the number of hypertrophic neurons (Ljungberg et al., 2009). Strikingly, mutations in LAR phosphatases are associated with multiple neuropsychiatric disorders and PTPRS itself has been linked to autism (Malhotra et al., 2011) (Elia et al., 2010) (Pinto et al., 2010). Interestingly, the identification of the ligand binding site on PTPRS has led to the development of a number of pharmacological modulators of PTPRS ligand binding (Lang et al., 2015). Currently, these inhibitors are being explored as a tool to encourage axonal regrowth in the spinal cord (Lang et al., 2015) and their potential use for neurodevelopmental disorders has not yet been explored. In addition to this, inhibition of HDACs has been shown to have therapeutic effects in models of neurodevelopmental disorders including Fragile X syndrome and Friedreich's ataxia (Rai et al.,

2008) (Chiurazzi et al., 1999). Of note, the nitrosylated cysteines on HDAC2 are specific to class I HDACs (Nott et al., 2008), which may facilitate the design of a new class of specific inhibitors of HDAC activity. Targeting these cysteines pharmacologically may provide a way in which to target HDAC activity at promoters of developmentally-regulated genes.

The discovery of reeler mutant mice led to the identification of the first signalling pathway that regulates neuron radial migration (Falconer, 1951). More recently, the role of ECM proteins such as reelin and their associated receptors in triggering key developmental decisions has deepened our understanding of the importance of these proteins during development and their relevance for neurodevelopmental disorders. My findings provide new insight into the signalling events that co-ordinate the expression of these pathways during development. Understanding the developmental roles of migratory genes such as PTPRS and how they are regulated by S-nitrosylation of HDAC2 open exciting opportunities for new therapeutic interventions for neurodevelopmental disorders.

5. MATERIALS AND METHODS

Cell Cultures

Embryonic cortical neurons were obtained from E15 embryos. Cortices were dissected on ice in HBSS buffer (1xHBSS, 2.5mM Hepes pH 7.4, 30 mM D-glucose, 1 mM CaCl2, 1 mM MgSO4, 4 mM NaHCO3). They were then incubated at 37°C for 25 minutes in digestion buffer (82mM Na2SO4, 30mM K2SO4, 6mM MgCl2, 0.25mM CaCl2, 0.001% Phenol Red, 0.126mN NaOH) supplemented with 200Units of Papain solution and washed 4 times in plating medium (MEM supplemented with 10% FBS, 5% horse serum and 1mM glutamine). The neurons were then dissociated by pipette passed through a cell strainer (40µm BD falcon) to remove clumps. An aliquot of cells was stained with Trypan blue solution and counted. Cells were then plated at a density of 7 x 10^5 neurons per well on 12 well Nunc dishes coated, which had previously been coated with 40µg/ml poly-D-lysine (Sigma) and 2µg/mlLaminin (BD bioscience). After 4 hours, plating medium was replaced with Neurobasal medium supplemented with 1 x B27 supplement (Thermo Fisher Scientific).

PC12 cells were cultured on nunc dishes (Thermo Scientific) in DMEM medium (Life Technologies) supplemented with 10% FBS and 5% HS. Cells were passaged twice weekly.

Transient transfection of cultured cells

All transfection were carried out using Lipofectamine® 2000 (Invitrogen, UK). For luciferase experiments, primary cortical neurons were transfected after 2 days in culture. OptiMEM containing 0.4µl lipofectamine 2000, 400ng DNA plasmids and HDAC2 siRNA (1mM) was incubated for 20 minutes temperature. The plating medium was removed, the OptiMEM:DNA mix applied and the cells incubated at 37°C for 2 hours. After incubation, the original plating media was replaced. For qPCR experiments, PC12 cells were transfected using lipofectamine 2000 as described previously. The protocol was modified as follows. Cells were transfected at 40/50% confluency. The incubation with the OptiMEM:DNA mix was carried out for 4 hours and replaced with fresh culture medium.

RNA isolation and Reverse transcription

For extraction of RNA from embryonic brains, Trizol reagent (Invitrogen) was added to dissected cortices and the tissue disrupted using a tissue homogenizer. For PC12 cells, Trizol was added directly to cells in nunc dishes. The suspension was centrifuged for 10 minutes at 14,000rpm at 4°C. The supernatant was removed and incubated at 30°C for 5 minutes. 200µl of chloroform was added to the cells, the samples shaken, then incubated at 30°C for 5 minutes before being centrifuged at 14,000rpm for 15 minutes at 4°C. The aqueous phase was removed. For samples derived from tissue, an additional round of purification with 200µl chloroform was performed. 500µl of isopropanol was added and incubated at room temperature for 10 minutes before being spun at 14000rpm for 10 minutes at 4°C. the pellet was dried and resuspended in DEPC treated water. DNase treatment was carried out for 30 minutes with 1µl DNase (Roche) at 25°C. Phenol/chloroform:isoamyl reagent (Sigma) was added to each sample in a 1:1 ratio. Samples were shaken and centrifuged at 14,000rpm for 5 minutes at 4°C. The aqueous phase was transferred to a new tube. 0.1 Volumes of 3M Na-O-Ac and 2.5 volumes of 100% ethanol were added and the mixture incubated at -20°C overnight. The samples were then centrifuged for 30 minutes at 14,000 rpm for 5 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged again at 14,000 rpm for 5 minutes at 4°C. The pellet was dried and re-suspended in DEPC treated RNase free water.

Reverese Transcription

1µg of Total RNA was used for reverse transcription. Random hexamer (50ng/µl) was added and this was incubated at 25°C for 20 minutes. This was then incubated for 1 hour with 50 units of Supersript III reverse transcriptase (Invitrogen). One reaction without enzyme was used as a control to assess the efficiency of DNase treatment. The sample was then treated with RNase for 30 minutes at 37°C. This reaction was then diluted 1:10 for use in subsequent reactions.

Quantitative real-time PCR

PCR reactions were carried out in 20µl reactions containing 10µl DyNAmo Flash qPCR reagent (Thermo Scientific), 0.5µM forward and reverse primers. Reactions were performed using a Mastercycler ep realplex (Eppendorf). Serial dilutions of a purified PCR product was used as a template to generate standard curves. Each reaction was run in triplicate with the following qRT-PCR conditions: Initial denaturation 95°C for 7 minutes, followed by 40 cycles of denaturation 95°C for 10s, annealing 25 sec at 60°C and 8 seconds of reading fluorescence. Finally a melting curve is carried out between 60 and 98°C to assess the specificity of the qPCR reaction. A linear regression analysis of the standard plot is used to calculate the abundance of DNA in the sample.

Chromatin immunoprecipitation assay

Embryonic cortices were fixed using 1% formaldehyde in PBS for 1 hour. This reaction was stopped using glycine at a concentration of 125mM. The tissue was disrupted using a tissue homogeniser. The sample was centrifuged at 2000g for 5 minutes and the pellet resuspended in PBSi (PBS supplemented with 1:100 protease inhibitor cocktail (Sigma P8340), 1:1000 phosphatase inhibitor cocktail II (Sigma P5726), 1:1000 phosphatase inhibitor cocktail III (Sigma P0044), 1mM PMSF (Sigma)). The cells were centrifuged at 2000g, rinsed twice in

cold PBSi. Sample were then resuspended in 300µl of cell lysis buffer (0.1% SDS, 0.5% Triton x-10, 20mM Tris-HCL pH 8.1 and 150mM NaCl) and incubated on ice for 20 minutes. Nuclei extraction was carried out by centrifugation at 1000g at 4°C for 5 minutes. The nuclei were resuspended in 300µl nuclei lysis buffer (50mM Tris-HCl pH 8.1, 100mM NaCl, 10mM EDTA, 1% SDS) and incubated for 20 minutes on ice. The samples were then sonicated in a biorupter (Diagenode) using the following settings: 30 pulses of 30 seconds duration at 30 second intervals. An aliquot of this sample is removed, the DNA isolated and run on a gel to assess fragmentation. Samples are sheared to a size between 200 and 400bp. The samples are centrifuged at 13,000g for 10 minutes at 4°C and the supernatant removed to another tube. 100µl Protein A-Sepharose beads (GE Healthcare) are added and rotated for 1 hour at 4°C. The samples are centrifuged at 1000g and the supernatant transferred to a new tube. 10% of the sample is set aside as a total input. Samples containing 10µg of DNA are adjusted to 500µl using lysis buffer and immunoprecipiatation carried out overnight with 3-5µg antibody. The following antibodies were used: CREB1 rabbi monoclonal (New England Biosciences 9197s), HDAC2 mouse monoclonal (Abcam ab12169), Ms Histone H3 (Abcam ab10799), Rb IgG (Sana Cruz sc-2027), Ms IgG (Santa Cruz sc2025). 80µl of Protein A-Sepharose were added and rotated at 4°C for 1 hour. The samples were centrifuged at 1000g for 5 minutes to collect the beads and the beads underwent the following 10 minute washes in the following buffers which are all supplemented with 1:100 protease inhibitor cocktail (Sigma P8340), 1:1000 phosphatase inhibitor cocktail II (Sigma P5726), 1:1000 phosphatase inhibitor cocktail III (Sigma P0044), 1mM PMSF (Sigma): 2 washes lysis buffer (0.1% SDS, 0.5% Triton X100, 2mM EDTA, 20mM Tris-HCl pH8.1, 150mM NaCl), 1 wash LiCl buffer (10mM Tris-HCl pH8.1, 1mM EDTA, 0.25M LiCl, 1% DOC, 1% NP40), 2 washes TE (10mM Tris-HCl pH 8.1, 1mM EDTA). After the final wash, the beads were vortexed twice for 15 minutes at room temperature in elution buffer (0.1 M NaHCO3 pH 8.0, 1% SDS) to elute the DNA. 10µl of 5M NaCl was added and Crosslinking reversed overnight at 65°C. DNA from samples and total input were purified using PCR purification columns (Qiagen). Purified DNA was then subjected to qPCR to quantify the abundance of particular fragments.

Luciferase assay

Primary cortical neurons were transfected with the PTPRS promoter reporter plasmid and an Eif-Renilla reporter plasmid. 2 days after transfection, cells were harvested. Luciferase and Renila activity was assessed using Dual-Glo Luciferase assay system according to the manufacturers instructions. A Tecan infiniteF200 plate reading machine was used to measure luminescence. Luciferase levels were normalized to Renila levels.

Immunohistochemistry

Embryonic brains were fixed in 4% paraformaldehyde (PFA) at room temperature for 1 h and cryoprotected in 30% sucrose overnight at 4 °C. Brains were frozen in optimal cutting temperature (OCT) medium and 10- μM sagittal sections were collected using a Leica vibratome. Sections were then blocked/permeabilized in PBS (0.5% triton, 10% goat serum or 10% donkey serum) at room temperature for 30 min. Sections were incubated in primary antibodies overnight at 4 °C in PBS and 1% BSA and secondary antibodies (1:300) with DAPI (1:1,000) at room temperature for 90 min. Sections were washed with PBS and then mounted using Flouromount-G (SouthernBiotech). Primary antibodies used were mouse nNOS (Santa Cruz sc5302), rat Ctip2 (Abcam, ab18465), CSPG [CS-56] (ab11570), PTPRS (R&D Systems af3430), RhoA-GTP (NewEast Biosciences 26904), SATB2 (Abcam ab51502), TBR1 (Abcam ab31940) Mouse and rabbit secondary antibodies were AlexaFlour conjugated (Invitrogen). Images were acquired on an SP5 confocal microscope (Leica) using LAS AF software, and images were processed using Fiji software

Ex-vivo Electroporation

Electroporation of progenitors lining the ventricular wall was carried out by injecting pCIG2 plasmid DNA at a concentration of 1 µg/µl with 0.5% Fast Green using a Picospritzer III (General Valve) microinjector into the lateral ventricles of isolated E14.5 embryonic mouse heads that were decapitated and placed in complete HBSS. Electroporations were performed on the whole head with gold-coated electrodes (GenePads 5 × 7 mm BTX; Figure S1) using an ECM 830 electroporator (BTX) and the following parameters: four 100ms long pulses separated by 100ms long intervals at 50V. Immediately after electroporation, the brain was extracted and vibratome sectioned at 250 microns (LEICA VT1000S) with special care toward the integrity of the pial surface. The resulting slices were cultured on semi-permeable organotypic membranes in neurobasal medium supplemented with B27 (Thermo Fisher Scientific) at 37°C. For digestion of CSPGs, 01 Unit/ml chABC was added to the culture medium. The culture medium was replaced with fresh medium after 1 day. Slices were harvested and fixed in 4% PFA overnight. They were cryopreserved, cut into sections and stained with GFP (Abcam ab13970)

In-utero Electroporation

Pregnant E14.5 mice were anesthetized with 3% isofluoran 1 vol% O2 , and anesthesia was maintained with 0.5 - 1.5% isofluoran / 1.5 vol% O2 /min. The uterine horns were exposed by midline laparotomy and kept hydrated with warm sterile PBS. Embryos were injected with 1 - 2 µl of plasmidic DNA at a concentration of 1-3µg/µl and 0.5% Fast Green using heat pulled glass capillaries (bevelled at 30º with an inner diameter of appr. 75-100 µm). Progenitors were electroporated with pCIG2-GFP, pCIG2-CRE, pSUPER-SCRAM or pSUPER-shPTPRS (Kindly provided by professor Toshihide Yamashita). Tweezertrodes were positioned outside of the uterine sac with the positive electrode positioned by the ventricular wall to be electroporated. 5 electric square wave pulses (30V, 50ms duration, 1s interval) were applied across the brain using a BTX ECM 830 electroporator (BTX Harvard Apparatus). The uterine horns were replaced inside the abdomen and the abdominal muscle and skin sutured shut. Post surgery, mice were kept in a recover chamber at 29ºC until mobile. Embryos were harvested at 3 or 4 days post electroporation. The brains were dissected in ice cold PBS and fixed in 4% PFA overnight. The brains were cryopreserved, cut into sections and stained with GFP (Abcam ab13970)

Quantification of Migration

Images of electroporated cortices were run through a Bandpass Filter to segment and isolate cell-sized shapes. The image was then thresholded and segmented into radial regions of interest where individual cell positions along the radial axis was recorded relative to the distance between the ventricle and the pial surface. Cell coordinates were recorded using ImageJ's Analyze Particles feature. These coordinates were imported into Excel along with the top (pial) and bottom (ventricle) boundaries coordinates obtained using ImageJ's Path Writer plugin. From these top and bottom boundaries, 200 boundary points were taken at regularly spaced intervals leading to a sampling of approximately 10 microns wide. All of this processing was done using an Excel macro.

nNOS mouse line

nNOS homozygous null mice or wild-type control embryos were intercrossed to generate null or control litters respectively. Embryos were collected at E15.5 for qPCR experiments

PTPRS mouse lines

PTPRS $^{fl/fl}$ mice were bread with Nestin-Cre and NEX-Cre lines to generate PTPRS $^{fl/fl}$ Nestin-Cre and PTPRS $^{fl/fl}$ NEX-Cre lines respectively.

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