S-Nitrosylation of the NuRD Complex in Cortical Neurons

Sarah Grace Aldous

Riccio Lab
MRC Laboratory for Molecular Cell Biology

A thesis submitted for the degree of
Doctor of Philosophy
University College London
October 2018
Declaration

I, Sarah Grace Aldous, 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 Nucleosome Remodelling and Histone Deacetylation (NuRD) complex is a chromatin remodeling complex that couples ATP-dependent nucleosome sliding with histone deacetylase activity. Despite playing an essential role during neuronal differentiation and cortical development, the mechanism regulating NuRD complex formation and specificity remains unknown. The aim of my project was to investigate whether S-nitrosylation of the NuRD subunits affects their function. S-nitrosylation (SNO) is the addition of a nitrosyl (NO) group to cysteine thiols. Both NO signalling and specific SNO-protein modifications are known to regulate the expression of genes necessary for neuronal development and migration. A recent screen in our laboratory aimed at identifying S-nitrosylated nuclear proteins in cortical neurones revealed that most subunits of the NuRD complex undergo S-nitrosylation. I investigated the functional relevance of this modification for the NuRD subunit Retinoblastoma binding protein 7 (RBBP7) and identified the nitrosylated cysteine residue. My data indicate that S-nitrosylation of Cys^{166} regulates RBBP7 interaction with the Chromodomain helicase DNA binding protein 4 (CHD4) subunit and promotes activity-dependent dendritic growth. I also found that the three ATPase CHD3, CHD4 and CHD5 are targets of S-nitrosylation. I have also characterised a lysine containing motif required for specific target S-nitrosylation. Overall my work demonstrates the widespread role of S-nitrosylation in regulating nuclear functions and provides the first evidence that NO links extrinsic signals to NuRD-dependent chromatin modifications.
Impact Statement

My research into the S-nitrosylation of the NuRD chromatin remodelling complex will add to the field’s understanding of the widespread importance and regulation of this modification. The experimental characterisation of a lysine constraining motif will contribute to the knowledge of what guides SNO specificity and aid further investigations by our lab and other researchers into the S-nitrosylation modification. Furthermore, aberrant S-nitrosylation of key proteins is implicated in the pathology of Alzheimer’s and Parkinson’s disease. It is possible that SNO-NuRD is also dysregulated in these conditions and given that S-nitrosylation affects NuRD function my work will give insight into this potential role in disease, and thus provide another avenue to explore in building our information regarding neurogenerative diseases. Importantly, my findings into how a chromatin remodelling complex is regulated will increase our understanding of brain development. NuRD is chromatin remodelling complex with a key role in gene regulation during cortical development. The identification of S-nitrosylation as a mechanism that could regulate NuRD function will provide more detail into the molecular mechanisms of corticogenesis. The correct formation of the cortex is essential for proper embryonic development and for adult brain function. Defects in corticogenesis contribute to behavioural and memory disorders, and the dysregulation of chromatin remodellers are also implicated in these conditions. My data can contribute to our awareness of how defects in chromatin complex regulation can have a long-lasting impact on development and brain function.
Acknowledgements

I am sincerely grateful to my supervisor Antonella Riccio for the opportunity to undertake my PhD in her lab, and for all the support and guidance throughout the project.

I am also indebted to Dr Jacob Smith, for teaching me the trials and tribulations of the Biotin Switch Technique and for being a great mentor and collaborator throughout this project.

I am deeply grateful to all the lab members, past and present who made the Riccio lab such a fantastic environment throughout my PhD; Dr. Catia Andreassi, Dr. Hamish Crerar, Dr. Luca Crepaldi, Dr. Emily Brookes, Dr. Justyna Nitarska, Dr. Jacob Smith, Dr. Cristina Policarpi, Dr. William Sherlock and Sarah French. It has been a joy and a privilege to work alongside you and I have learnt so much from you all. Special thanks as well to Lucie Van Emmenis, Emily Brookes and Hamish Crerar for generously reading and editing drafts of this thesis.

Thank you to my LMCB family for your support throughout my PhD, I am very lucky to have such a wonderful group of peers. I am also grateful to my flatmates, Darryl and Tom for helping me throughout all aspects of this PhD.

I am endlessly grateful to Alice and James, my incredible parents, and to my friends for all your fantastic support and encouragement over the past four years, I truly could not have done it without you.
Table of Contents

Abstract .......................................................................................................................... 3
Impact Statement ............................................................................................................ 4
Acknowledgements ........................................................................................................ 5
Table of Figures ............................................................................................................. 8
List of Tables .................................................................................................................. 10
1. Introduction ............................................................................................................. 12
  1.1 Structure and Development of the Mammalian Cortex .......................................... 12
    1.1.1 Generation of Cortical Layers ............................................................................ 15
    1.1.2 Regulation of Layer Formation ........................................................................ 18
    1.1.3 Establishment of Cortical Layer Identity .......................................................... 22
  1.2 Epigenetic Mechanisms of Gene Expression ......................................................... 24
    1.2.1 Histone Modifications ...................................................................................... 26
    1.2.2 Nucleosome Remodelling .............................................................................. 32
    1.2.3 Chromatin Remodelling Complexes .............................................................. 37
    1.2.4 The NuRD Complex ...................................................................................... 39
  1.3 Nitric Oxide Signalling ......................................................................................... 46
    1.3.1 Synthesis of NO .............................................................................................. 47
    1.3.2 Protein S-Nitrosylation ................................................................................... 49
    1.3.3 S-Nitrosylation in Cortical Development ....................................................... 55
    1.3.4 S-Nitrosylation in Disease ............................................................................. 59
  Aims ............................................................................................................................. 61
2. Results ....................................................................................................................... 62
  2.1 NuRD subunits are S-nitrosylated ......................................................................... 62
    2.1.3 RBBP7 is S-nitrosylated at Cys\textsuperscript{166} ................................................. 66
    2.1.4 RBBP7 is a target of S-nitrosylation in rat cortical neurons ............................. 67
    2.1.5 RBBP4 is S-nitrosylated at Cys\textsuperscript{167} .................................................... 69
    2.1.6 RBBP4 is S-nitrosylated in cortical neurons .................................................. 70
    2.1.7. CHD3, CHD4 and CHD5 are targets of S-nitrosylation ................................. 72
    2.1.8. MBD3 is S-nitrosylated at two target cysteines .......................................... 74
    2.1.9. Identification of an S-nitrosylation motif ....................................................... 76
2.2 NO regulates NuRD subunit interactions

2.2.1 CHD3 interaction with RBBP7 increases with CysNO treatment

2.2.2 CHD5 interactions with HDAC2 and RBBP4 change with CysNO treatment

2.2.3 CHD4 interaction with RBBP7 increases with CysNO treatment

2.2.4 S-nitrosylation of RBBP7 at Cys166 regulates interaction with CHD4

2.3. NO and NuRD recruitment to target genes

2.3.1 CHD recruitment to target genes in E15.5 cortices

2.3.2 CHD recruitment to target genes in E18.5 cortices

2.4. NO and NuRD chromatin remodelling

2.5. S-nitrosylation regulates NuRD subunit function

2.5.1 Single knockdown of RBBP7 does not affect dendritogenesis

2.5.2 RBBP7 Cys166 is required for normal dendritogenesis in vitro

3. Discussion

4. Materials and Methods

4.1 Cell Culture

4.2 Animal Procedures

4.3 Preparation of Cortical Neurons

4.4 Biotin Switch Technique

4.5 Co-IP Protocol

4.6 Western Blotting

4.7 Dendritogenesis Assay

4.8 ChIP Protocol

4.9 DNaseI Hypersensitivity Assay

4.10 Cloning

4.11 Statistical Analysis

Reference List
Table of Figures

Fig.1.1 Schematic of mouse cortical development .................................................. 15
Fig.1.2: Schematic of neuron progenitor divisions .................................................. 17
Fig.1.3: Schematic illustrating chromatin accessibility .............................................. 25
Fig.1.4: Schematic illustrating writers, readers and erasers ..................................... 27
Fig.1.5: Schematic of principal nucleosome remodelling activities .......................... 33
Fig.1.6. Schematic of loop propagation model of nucleosome sliding ...................... 34
Fig.1.7: Schematic of the four main chromatin remodelling enzyme structures ......... 37
Fig.1.8: Schematic of principal subunits of the NuRD complex ............................... 40
Fig.1.9: CHD developmental stage and target specificity ........................................ 45
Fig.1.10: Domain structure and enzymatic activity of the nitric oxide synthase enzymes ........................................................................................................................................ 48
Fig.1.11. Generation of S-nitrosothiols ..................................................................... 50
Fig.1.12: nNOS expression during rodent cortical development ............................... 56
Fig.2.1: The Biotin Switch Technique ....................................................................... 63
Fig.2.2: RBBP7 can be S-nitrosylated at Cys$^{166}$ .................................................... 67
Fig.2.3: RBBP7 is S-nitrosylated in rat cortical neurons .......................................... 68
Fig.2.4: RBBP7 and RBBP4 sequence and structural similarity ............................... 69
Fig.2.5: RBBP4 can be S-nitrosylated at Cys$^{167}$ .................................................... 70
Fig 2.6: RBBP4 is S-nitrosylated in rat cortical neurons ......................................... 71
Fig.2.7: CHD3, CHD4 and CHD5 can be S-nitrosylated ......................................... 73
Fig.2.8: MBD3 can be S-nitrosylated at Cys$^{215}$ and Cys$^{266}$ ................................. 75
Fig.2.9: Motif-X analysis of S-nitrosylated cysteine containing peptides .............. 77
Fig.2.10: Characterisation of a SNO-Motif in MBD3 .............................................. 79
Fig.2.11: Characterisation of a SNO-Motif in HDAC2 ........................................... 80
Fig.2.12: S-nitrosylation regulates the interaction of RBBP7 with CHD3 ............... 82
Fig.2.13: Other NuRD subunits interactions with CHD3 do not change upon CysNO treatment ........................................................................................................... 83
Fig.2.14: S-nitrosylation regulates the interaction of CHD5 with RBBP4 and HDAC2 ........................................................................................................................................ 84
Fig.2.15. Other NuRD subunits do not change interaction with CHD5 due to CysNO treatment.................................................................85

Fig.2.16: S-nitrosylation regulates the interaction of RBBP7 with CHD4..................86

Fig.2.17. Other NuRD subunits do not change interaction with CHD4 with CysNO treatment..........................................................................................87

Fig.2.18: S-nitrosylation of RBBP7 at Cys\textsuperscript{166} promotes the interaction with CHD4................................................................................................................88

Fig.2.19: Analysis of CHD3, 4 and 5 binding to target genes in E15.5 cortex..........................................................................................................................90

Fig.2.20: Analysis of CHD3, 4 and 5 binding to target genes in E18.5 cortex..........................................................................................................................92

Fig.2.22: RBBP7 knockdown in Neuro-2a cells.....................................................97

Fig.2.23: RBBP7 knockdown and dendritogenesis analysis.................................98

Fig.2.24: Confirmation of RBBP4/7 double knockdown.........................................99

Fig.2.25: S-nitrosylation of RBBP7 promotes dendritogenesis in cortical neurons.................................................................................................................101

Fig.2.26: Sholl analysis for RBBP7/4 knock down and myc-RBBP7\textsuperscript{WT} or myc-RBBP7\textsuperscript{C166S} rescue experiments..........................................102

Fig.3.1 Schematic model of the SNO NuRD role in cortical neurons.................122
List of Tables

Table 2.1. NuRD subunits identified as S-nitrosylated ........................................62
Table 2.2. Expression vectors of the NuRD subunit ..................................................65
Table 4.1 Cortical neuron preparation buffers ..........................................................126
Table 4.2 BST buffers .................................................................................................128
Table 4.3 CoIP buffers .................................................................................................130
Table 4.4 ChIP buffers .................................................................................................133
Table 4.5. Target gene primers used in DNase and ChiP assays .................................135
Table 4.6. Primers used to generate overexpression constructs .................................136
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AP</td>
<td>Apical progenitor</td>
</tr>
<tr>
<td>Asc</td>
<td>Ascorbate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAF</td>
<td>Brahma-associated factor</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>bHIH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BP</td>
<td>Basal progenitor</td>
</tr>
<tr>
<td>BRM</td>
<td>Bhrama</td>
</tr>
<tr>
<td>BST</td>
<td>Biotin Switch Technique</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca2+/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Chromodomain helicase DNA-binding</td>
</tr>
<tr>
<td>ChiP</td>
<td>Chromatin immuno-precipitation</td>
</tr>
<tr>
<td>CoIP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CP</td>
<td>Cortical plate</td>
</tr>
<tr>
<td>CR</td>
<td>Cajal-Retzius</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic adenosine monophosphate response element-binding protein</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CysNO</td>
<td>Nitrosylated cysteine</td>
</tr>
<tr>
<td>Drp1</td>
<td>Dymamin-related protein 1</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>E9</td>
<td>Embryonic day 9</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelial derived relaxation factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>esBAF</td>
<td>Embryonic stem cell BAF</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FeNO</td>
<td>Iron nitrosyl species</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth Factor</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>GSNOR</td>
<td>S-nitrosoglutathione reductase</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293 large T antigen expansion</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KN1</td>
<td>First nNOS knockout mice</td>
</tr>
<tr>
<td>KN2</td>
<td>Second nNOS knockout</td>
</tr>
<tr>
<td>LSD1</td>
<td>Lysine specific demethylase</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl-CpG-binding domain</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Myocyte enhancer factor 2C</td>
</tr>
<tr>
<td>MMTS</td>
<td>Methyl methanethiosulfonate</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MTA</td>
<td>Metastasis associated protein</td>
</tr>
<tr>
<td>NAPDH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>nBAF</td>
<td>Neuronal BAF</td>
</tr>
<tr>
<td>NDMA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NEC</td>
<td>Neuroepithelial cells</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>npBAF</td>
<td>Neuron progenitor BAF</td>
</tr>
<tr>
<td>NPC</td>
<td>Neuron progenitor cells (cell culture system)</td>
</tr>
<tr>
<td>NuRD</td>
<td>Nucleosome Remodelling and Deacetylation Complex</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PMN</td>
<td>Post-mitotic neuron (cell culture system)</td>
</tr>
<tr>
<td>PRC2</td>
<td>Polycomb repressive complex 2</td>
</tr>
<tr>
<td>PSD93/95</td>
<td>Post synaptic density protein 93/95</td>
</tr>
<tr>
<td>RBBP4/7</td>
<td>Retinoblastoma-binding protein 4/7</td>
</tr>
<tr>
<td>RG</td>
<td>Radial glia</td>
</tr>
<tr>
<td>ROS/RNS</td>
<td>Reactive oxygen/nitrogen species</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble Guanylate cyclase</td>
</tr>
<tr>
<td>Sin3a</td>
<td>Mammalian Sin3a complex</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosylation modification</td>
</tr>
<tr>
<td>SNO-Cys</td>
<td>SNO-L-cysteine</td>
</tr>
<tr>
<td>SNO-protein</td>
<td>S-nitrosylated protein</td>
</tr>
<tr>
<td>SNORAC</td>
<td>S-nitrosothiol Resin-Assisted Capture</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
</tbody>
</table>
1. Introduction

During the formation of the neocortex, numerous transcriptional regulatory pathways control the timing of neuronal development generating the six-layered cortical structure (Molyneaux et al., 2007; Fernández, Llinares-Benadero and Borrell, 2016). One of these regulatory mechanisms involves changes of chromatin structure due to specific epigenetic modifications that in turn control neuronal gene expression, neuronal differentiation and radial migration (Martynoga, Drechsel and Guillemot, 2012). The activity of the chromatin remodelling complex Nucleosome Remodelling and Histone Deacetylation (NuRD) ensures correct formation of the cortical layers as NuRD regulates the expression of specific neuronal genes (Nitarska et al., 2016). Although NuRD binds extensively to mammalian chromatin, how target specificity is achieved remains unknown. A potential mechanism entails the regulated post-translational modifications of one or more NuRD subunits. S-nitrosylation is a post-translational modification, whereby nitric oxide (NO) groups covalently bind free cysteine thiols. The production of NO increases during cortical development and as such, S-nitrosylation of key proteins has an established role in neuronal and cortical development (Bredt and Snyder, 1994; Nott et al., 2008; Nott and Riccio, 2009).

1.1 Structure and Development of the Mammalian Cortex

The mammalian neocortex is the most evolutionarily recent area of the brain and is organised into specialised areas for somatosensory, visual and motor functions. The neocortex also regulates the production of language in Broca’s
area, and the understanding of language in Wernicke’s area. Additional areas for association functions allow the perception and integration of signals from other brain regions (Rakic, 1988; Finlay and Darlington, 1995; Purves et al., 2001). The cortex has undergone dramatic expansion during evolution with a one-thousand-fold increase in surface area between mice and human, and a two-fold increase in cortical thickness (Rakic, 1988; Chenn and Walsh, 2002). The increase in surface area coincided with the folding of the cortex surface, as the cortex is smooth in small rodents such as rats and mice and becomes folded in primates (Rakic, 1995). The cortex primarily comprises spiny excitatory pyramidal neurons, also known as projection neurons, which respond to the neurotransmitter glutamine, alongside a small subset of spiny stellate glutamergic neurons. These neurons have numerous branches or dendrites which connect to other neurons at synapses to integrate or send excitatory signals. Pyramidal neurons can either span small scale distances within the same cortical area, or cover larger distances to connect distant cortical areas to establish a neuronal network (Purves et al., 2001). Non-spiny, inhibitory GABAergic interneurons are another, distinct, type of cortical neuron that respond to the neurotransmitter gamma-aminobutyric acid (GABA). These neurons receive inputs from both other interneurons and the excitatory neurons, integrating the signal to inhibit further neuronal activity, and form local connections within the cortex (Jones, 1986). Interneurons are generated outside the cortex in the medial and caudal ganglionic eminences, and migrate tangentially into the developing neocortex, before reaching their ultimate location by migrating radially (DeFelipe and Fariñas, 1992; Anderson et al., 1997, 2002). In rodents, the cortex is organised
into six distinct layers containing numerous neuronal subtypes (Gilbert and Kelly, 1975; Brodmann and Garey, 2006) (Fig.1.1). The layers are formed in an 'inside out' manner such that the inner layers are generated first and the outer layers last (Angevine and Sidman, 1961). Progenitor cells reside in the ventricular zone and subventricular zone (VZ and SVZ, respectively) and produce waves of post-mitotic neurons in a sequential manner (Fig.1.1). The earliest born neurons migrate out from the SVZ and settle in the pre-plate prior to the next wave of neurons which splits the pre-plate to form the cortical plate (CP) (Caviness Jr., 1982). This event is followed by further waves of neuronal production, with new neurons migrating past the CP to settle above it and generating the deepest layer of the cortex (layers IV- VI). Later born neurons migrate past older neurons to form the upper layers of the cortex (layers II/III) (Angevine and Sidman, 1961). The most upper layer, layer I, consists of Cajal-Retzius (CR) cells which are formed within different progenitor zones, but are guided by the meninges into the marginal zone by CKCL12 chemokine signalling (Bielle et al., 2005; Borrell and Marín, 2006).
1.1.1 Generation of Cortical Layers

Cortical layer formation is regulated by several fundamental processes which include the expansion of the progenitor pool, the timing of the switch of progenitor cells from proliferative to neurogenic, the radial migration of the post-mitotic neurons to the correct layers for terminal differentiation, and toward the end of the neurogenic phase, the switch from neuronal to glial fate.

1.1.1.1 Expansion of the progenitor pool and neurogenic switch

In mice, the generation of the layered cortex starts early at the 9th day post-fertilisation (E9). It begins with the generation of neuroepithelial cells (NECs), from the neural plate. NECs are polarised stem cells and divide symmetrically to expand the stem cell pool, generating more daughter NECs. The symmetric
divisions depend on the polarisation of the NEC’s and their cell-cell adhesions (Chenn and Walsh, 2002). The pool increases in number and the layer of NEC progenitors expands laterally and radially to generate the ventricular zone, or VZ (Caviness and Takahashi, 1995; Huttner and Brand, 1997). This pool expansion is completed around E10.5 when the progenitor pool switches from proliferative symmetric divisions to neurogenic divisions (Fig.1.2). NEC asymmetric divisions in the VZ generate radial glia (RG) cells, and an intermediate progenitor named an apical intermediate progenitor (aIP). The RG cells initially divide symmetrically and then at the time of the neurogenic switch divide asymmetrically to generate a neuron and a basal progenitor (BP) cell (Gregg, Chojnacki and Weiss, 2002; Malatesta et al., 2003; Miyata, 2004). These BPs will move into the SVZ and subsequently divide symmetrically to generate two neurons (Noctor et al., 2004). The aIP remain in the VZ and divide to give two daughter neurons (Rakic, 1988; Gal, 2006). The first neurons in the embryonic cortex appear at around E11.5 and are generated from dividing aIPs, but as the RG and BP go through neurogenic divisions, sequential waves of neurons are generated, with distinct birth dates.
Fig.1.2: Schematic of neuron progenitor divisions. Symmetric and asymmetric divisions give rise to the progenitor cells and neurons. The NEC pool expands and then each NEC divides and differentiates to give two progenitors, apical intermediate progenitors (aIP) and radial glia (RG) which then divide to generate differentiated neurons (N) either directly or through generating another progenitor, the basal progenitors (BP). BPs divide, and the daughter cells differentiate into neurons.

1.2.1.1 Radial migration

Once generated, the neurons migrate radially out from the VZ and SVZ to form the distinct six-layered structure, with early born neurons generating the deeper layers and the later born neurons forming the outer layers of the cortex (Fig.1.1). There are two major modes of neuronal migration; neurons generated first normally migrate through somal translocation. In this mode of migration, a long leading process extends and attaches to the CP, leaving a short trailing process behind the cell body. The nucleus moves up through the cell toward the leading process and as a result the leading process becomes shorter, whereas a longer trailing process is formed behind the cell body (Nadarajah and Parnavelas, 2002). In contrast, later born neurons principally migrate through locomotion and are guided by radial glial cells. The locomotion process is more dynamic and requires a switch from a multipolar to a bipolar morphology before the neurons
migrate from the SVZ towards the CP. These cells have a free motile leading process and move along the glial fibres. Once they reach the uppermost layer of the cortex the leading process attaches to the marginal zone and the nucleus and cell body moves up through this radial process towards the marginal zone, in a similar mechanism to somal translocation (Nadarajah et al., 2001; Sekine et al., 2011).

Another key process in cortical layer formation is the production of astroglial cells. The RG undergo a switch from neurogenic to glial fate, and subsequently daughter cells differentiate into astroglial cells (Levitt, Cooper and Rakic, 1981). This stage takes place at around E17 and ensures an appropriate number of glial cells and astrocytes will be present in the post-natal brain. Furthermore, because the timing of this final switch marks the end of neurogenesis it plays a key role in regulating neuronal number (Schmid et al., 2003; Seuntjens et al., 2009).

1.1.2 Regulation of Layer Formation

The correct timing of layer development and cortical formation depends on both cell intrinsic and extrinsic mechanisms. Extrinsic signalling molecules including guidance cues and growth factors trigger intracellular signalling pathways that allow the appropriate cellular response, whereas intrinsic factors such as transcription factors cooperate with epigenetic mechanisms to control the expression of specific genes in progenitor cells and post-mitotic neurons.

1.1.2.1 Extrinsic mechanisms of cortical layer formation

An important neurotrophin involved in determining neuronal fate and cell specificity is brain-derived neurotrophic factor (BDNF). BDNF signalling is
initiated by BDNF binding to the tyrosine kinase receptor TrkB and regulates neuronal survival, development, and at later developmental stages, synaptic function (Reichardt, 2006). BDNF is expressed in neuron progenitors and influences neuronal migration by enhancing the expression of the neurogenic bHLH transcription factor Mash1 (Fukumitsu et al., 2006). The growth factor fibroblast growth factor (FGF), regulates Notch signalling and is necessary for the maintenance of the RG progenitor pool, for promoting RG identity and for controlling the timing of progression to neurogenesis (Rash et al., 2011). The importance of FGF is evident in the brains of mice lacking the FGF variant Fgf2, where a decreased number of both cortical neurons and glia is observed (Vaccarino et al., 1999). The Fgf10 ligand binds the receptor Fgfr2b and is expressed transiently in progenitors at the time of RG transition. Fgf10 drives the differentiation of NECs toward the production of RG (Sahara and O’Leary, 2009). FGF signalling is also required later in development for the switch from neuronal to glial fate, as it regulates astrocyte differentiation (Morrow, Song and Ghosh, 2001). Another important cell signalling mechanism is the Notch pathway, which plays a key role in regulating cell fate decisions. Notch signalling mediates the signalling between adjacent cells and both receptors and ligands are membrane bound. Once activated, the intercellular domain of the Notch receptor is cleaved and translocated to the nucleus, where it acts as a transcriptional regulator. In the progenitor population this event causes the transcriptional activation of neuronal genes required for progenitor maintenance, including Hes1 and Hes5 (Yoon and Gaiano, 2005). Later in development, Notch also plays a role in promoting NEC to RG differentiation (Gaiano, Nye and Fishell, 2000),
including the expression of brain lipid-binding protein which marks the onset of radial gliogenesis and is expressed in the RG scaffold that guides the migrating neurons (Anthony et al., 2005).

1.1.2.2 Intrinsic mechanisms of cortical layer formation

The activation of specific transcription factors (TFs) is a key event in all stages of cortical development as this regulates the expansion of the progenitor pool, cell fate decisions and the establishment of neuronal identity. The basic helix-loop-helix family (bHLH) of TFs are especially important as the onset of their transcription correlates with the initiation of lineage restriction (Nieto et al., 2001; Schuurmans et al., 2004). bHLH proteins Hes1 and Hes5 are downstream effectors of Notch and are expressed in neuronal progenitor cells, where they prevent premature neurogenesis by regulating both the NEC pool size and the timing of RG generation (Ishibashi et al., 1995; Hatakeyama, 2004). In addition, Wnt signalling induces neuronal differentiation by inducing the expression of the bHLH proteins Ngn2 and Mash1 in progenitor cells (Hirabayashi, 2004). Both these TFs regulate the neurogenic commitment of progenitor cells and SVZ maturation (Nieto et al., 2001; Chenn and Walsh, 2002). Loss of Ngn2 leads to changes of neuronal identity and disrupts the RG scaffold, causing earlier astrocytic fate decisions. Interestingly, later in development Ngn2 maintains VZ proliferation, illustrating how a TF can have multiple roles depending on the stage of development (Nieto et al., 2001). Tbr2 is another bHLH protein, expressed in the SVZ in the multipolar BPs and in early born neurons. Tbr2 is necessary for maintaining the BP pool size and to preserve the timing of neurogenesis. Inhibition of Tbr2 expression leads to a dramatic loss of BPs and premature
neurogenic differentiation, leading to a reduction in the number of neurons (Arnold et al., 2008; Sessa et al., 2008). Pax6 is expressed in the RG and is also essential for neurogenesis and for controlling the neurogenic commitment of the progenitors. Lack of Pax6 expression causes a decrease in the number of RG cells, loss of RG identity and a drastic reduction of the neurogenic potential (Walther and Gruss, 1991; Heins et al., 2002). In Small-eye mutant mice that lack Pax6 activity, the cortex shows an aberrant morphology due to changes in both cell adhesion and cell cycle regulation (Stoykova et al., 1997; Götz, Stoykova and Gruss, 1998). Pax6 has a number of downstream effectors and is bound to the promoters of a number of developmentally expressed genes in neural progenitors. Sox2, another pro-neurogenic factor is also found at these active promoters, indicating a cooperation between the two transcription factors in activating gene expression (Thakurela et al., 2016).

Another aspect to the regulation of cortical development involves the control of the cell cycle. When progenitors exit the cell cycle, they lose the proliferative potential and become neurogenic, which if instigated too early can deplete the progenitor pool and lead to a reduction in final neuronal number. Conversely if the switch to neurogenesis is delayed, the progenitor pool undergoes excessive expansion, leading to thickening of the VZ and SVZ and subsequently a distorted cortical morphology. Cyclins and cyclin dependent kinases (Cdk) such as Cdk4 and cyclinD1 determine cell cycle progression through the growth phase (G1). The timing and length of the G1 phase is instrumental in determining whether neural cells will proliferate or differentiate, as a longer G1 can switch BPs into neurogenic divisions (Lange, Huttner and Calegari, 2009). Cdk12 regulates
transcriptional elongation and is involved in the maintenance of the neural progenitor pool by ensuring DNA damage repair in the progenitors, and by regulating migration of later born neurons via the Cdk5 pathway (Chen et al., 2016). Wnt signalling and β-catenin also determine whether progenitor cells will divide or exit the cell cycle, thereby regulating the size of the progenitor pool. This event is mediated by changes to adherens junctions, which affect progenitor cell polarity and determine whether a cell divides either asymmetrically or symmetrically, ultimately affecting the neurogenic output (Chenn and Walsh, 2002).

The epigenetic state of chromatin also exerts an essential control on the expression of genes that regulate the progenitors cell cycle and neuronal development. In embryonic stem cells (ESCs) and NECs the chromatin surrounding most developmental genes is kept in a poised chromatin state with epigenetic marks associated with both transcription activation and inhibition (Bernstein et al., 2006). During neurogenesis key neural genes, such as Ngn1, become de-repressed and are transcribed, while non-neuronal lineage genes lose the poised state to become fully repressed. Similarly, once neurogenesis, terminal differentiation and the final glial fate switch has occurred, pro-neurogenic factors such as Ngn1 and Ngn2 become re-repressed again. Glial factors including members of the JAK/STAT pathway will in contrast lose repressive marks at the bivalent promoters and become activated (Hirabayashi et al., 2009).

1.1.3 Establishment of Cortical Layer Identity

Once they have reached the correct position with the cortical layers, post-mitotic neurons undergo terminal differentiation, which correlates with the
expression of specific transcription factors. These TFs determine the transcriptional output and the functional properties of neurons and are used experimentally as layer markers. Early born neurons, which form the deeper cortical layers will express the TF markers Ctip2 and Sox5 (Arlotta et al., 2005; Kwan et al., 2008). They are detected in the VZ progenitors as soon as neurons are generated from dividing progenitors, and their expression becomes restricted to the terminally differentiated neurons of the deeper layers. Sox5 controls the timing and differentiation of the next wave of neurons, and loss of Sox5 leads to premature differentiation of early born neurons (Lai et al., 2008). Mid-layer neurons express TF markers such as Brn2, which is required to initiate neuronal radial migration, ensuring that neurons reach the correct layers at the right time points (McEvilly et al., 2002). The later-born, upper layer neurons express Cux1/2, (Nieto et al., 2004) whereas Satb2 is detected in layers II/III. Satb2 regulates the levels of axon guidance molecules necessary to establish upper layers connections, and also represses Ctip2 signalling, ensuring that deeper layer identity is molecularly distinct from the upper layers (Alcamo et al., 2008). Upper layer neurons make both local and distant connections within the cortex whilst deeper layers such as layer IV, mostly project to the subcortical nuclei, including the thalamus. For this reason, upper-layer neurons are divided into corticothalamic and sub-cerebral neurons (Killackey et al., 1989; Arlotta et al., 2005). After the switch from neurogenic to glial fate, RG cells start to express specific astroglial markers such as glutamate aspartate transporter and glial fibrillary acidic protein, which correlates with the establishment of astrocytic identity (Levitt, Cooper and Rakic, 1981; Gal, 2006).
1.2 Epigenetic Mechanisms of Gene Expression

The term epigenesis was initially used to describe the mechanism by which cells with the same genome could give rise to distinct phenotypes without changing the DNA sequence (Waddington, 1956). This concept can also include the responsiveness of the genome to intracellular activity and changes to the extracellular environment. Moreover epigenetic traits may be inherited from mother to daughter cell or intergenerationally through meiosis (Bird, 2007; Berger et al., 2009). In post-mitotic neurons of the cortex epigenetic mechanisms specifically regulate transcription through modifications to the local chromatin structure.

Within the nucleus DNA is tightly packaged into chromatin fibres. The fundamental unit of chromatin is the nucleosome, a core particle consisting of approximately 147 bp of DNA wrapped around histone protein octamers (Olins and Olins, 1974; Richmond et al., 1984; Luger et al., 1997). Genome wide, areas of open chromatin with few nucleosomes form euchromatin, whereas the more condensed and nucleosome occupied stretches of DNA are known as heterochromatin (discussed in Passarge, 1979). At more local levels the accessibility of DNA to the transcriptional machinery is regulated by nucleosome positioning and chromatin structure. Promoters and genes can either be maintained in a repressed state by the presence of nucleosomes or can be made open and accessible by the sliding of nucleosomes (Fig.1.3).
Epigenetic changes to chromatin structure therefore can control gene expression (Berger et al., 2009). The most widely studied and best understood epigenetic changes are DNA methylation of cytosine bases, modifications to the histone proteins and nucleosome remodelling (Sneider and Potter, 1969; Grippo et al., 1987; Riggs, 1975; Bird and Sovthern, 1978). Expression of different histone variants that can be incorporated into the nucleosomes cores at specific points in the cell cycle or in certain cell types may also effect gene expression (Berger et al., 2009).

DNA can be methylated at position 5’ on cytosine bases – termed 5’-methylcytosine and this modification is chemically interchangeable with unmodified cytosine bases, as cytosine methylation has no impact on DNA structure (Doskočil and Šorm, 1962). DNA methylation occurs primarily at CpG sequences, where the cytosine is adjacent to a guanine base. The exception to this are the CpG islands, which are areas of concentrated CpG sequences near
to transcription start sites (TSSs) of genes, that remain mostly un-methylated (Doskočil and Šorm, 1962; Bird and Southern, 1978; Bird et al., 1985). CpG methylation is inherited by daughter cells and is associated with areas of gene repression and silencing (Bird, 1978). Early studies showed that CpG methylation is necessary for chromosome X inactivation, since demethylation reactivated genes present on the inactive X chromosome (Riggs, 1975; Jones et al., 1982).

1.2.1 Histone Modifications

There are four main histone proteins in the core nucleosome; H2A, H2B, H3 and H4 (Kornberg, 1974; Richmond et al., 1997). Histones are structurally similar and the nucleosome core consists of an octamer with two copies of each protein (Thomas and Kornberg, 1975; Eickbush and Moudrianakis, 1978). Histone proteins have a core globular domain that is carefully folded, with unstructured tail domains extending out from the histone core (Luger et al., 1997). These tails can be modified at several positions and in numerous combinations, allowing for a complex and fine regulation of the epigenetic state. Histones are modified by an array of enzymes known as ‘writers’ and the epigenetic marks can be removed by ‘erasers’. Histone modifications are generally considered to act through two main mechanisms. The first mechanism is through directly affecting nucleosome charge and thereby nucleosome density, the second is by acting as docking sites for proteins known as ‘readers’ which recognise and bind specific histone and DNA modifications (Fig.1.4).
Fig.1.4: Schematic illustrating writers, readers and erasers. A writer adds a methyl (Me) group to a lysine and a phosphorylation modification (P) to a serine (S). These modifications are recognised by a reader protein and removed by an eraser protein.

The first identified epigenetic modification was histone methylation of lysine residues (Murray, 1964). It is now understood that that histone lysines can be mono- di- or tri- methylated, with different readers and functional outcomes associated with each modification. Lysine acetylation, arginine methylation, lysine monoubiquitination and serine phosphorylation (Allfrey et al., 1938; Phillips 1962) are just a few of the many key post-translational histone modifications identified to date. Interestingly, multiple residues on both the same or different histone protein can be modified simultaneously and the modification of one residue can lead to the modification of another. For example, in yeast, H3K4 and H3K79 methylation depends first on the ubiquitylation of another residue, H2BK123. In contrast, certain modifications are antagonistic, and will elicit distinct outcomes. The recruitment of heterochromatin binding protein 1 (HP1) normally takes place on H3K9me2/3. However, during cell division, the neighbouring
H3S10 is phosphorylated, preventing HP1 binding. The crosstalk between histone modifications is essential for the formation of complex and dynamic chromatin structure (Kouzarides, 2007; Bannister and Kouzarides, 2011).

Histone phosphorylation is very dynamic and adds a negative charge to the DNA, which is thought to change the structure and open up the DNA to chromatin and DNA binding factors. The phosphorylation mark is deposited by specific kinases and removed by phosphatases (Kouzarides, 2007). H3S10 phosphorylation during the cell cycle causes chromatin condensation, required for the correct separation of chromosomes (Wei et al., 1998). The histone variant γH2Ax, is also phosphorylated in response to DNA damage and recruits the DNA damage machinery (Kouzarides, 2007). In post-mitotic hippocampal neurons transient H3S10 phosphorylation is observed in response to neuronal stimuli and corelates with the induction of neuronal activity genes, supporting a role for histone phosphorylation in regulation of gene expression as well as cell cycle control (Crosio et al., 2003).

1.2.1.1 Histone Acetylation

Histone acetylation is often associated with active gene expression, as the modification neutralises the positive charge of the histone tails, weakening the interaction with the DNA, and opening up chromatin for transcription (Allegra et al., 1987; Hansen, 1992; Wang et al., 2001). Studies looking at the location of acetylated histones further demonstrated the link between histone acetylation and transcriptionally active genes, with H3K9 and K14 acetylation detected at the promoters of active genes (Hebbes, Thorne and Crane-Robinson, 1988; Megee et al., 1990; Roh, Cuddapah and Zhao, 2005).
The acetyl mark is deposited on histone lysine residues by histone acetyltransferase (HAT) enzymes which transfer an acetyl group from acetyl-CoA to the target lysines (Allfrey et al., 1964). HATs are extremely diverse in sequence and function, but can be broadly classified into three families, Gcn5-related N-acetyltransferase, the MYST family and the third family of p300/CBP (Sterner and Berger, 2000; Yang, 2003; Neuwald and Landsman, 1997; Carrozza et al., 2003). p300/CBP are key transcriptional activators which interact with a number of co-transcriptional regulators and have a variety of cellular functions, including cell growth suppression and p53 regulation, as well as regulating Wnt signalling (Janknecht and Hunter, 1996; Goodman and Smolik, 2000). p300/CBP were also shown to be histone acetyltransferases, providing a mechanism for their broad activation roles (Ogryzko et al., 1996; Schiltz et al., 1999). A role for HATs in DNA replication was identified through the activity of the acetyltransferase HB01, which is required for initiation of replication through its acetylation of H4 and binding to origins of replication complexes during the cell cycle (Iizuka and Stillman, 1999; Kouzarides, 2007). The acetylation modification itself can be recognised by innumerable proteins or ‘readers’, all of which contain a characteristic bromodomain, which enable the interactions with the acetylated histone (Haynes et al., 1992; Zhou et al., 1999; Owen, 2000). The downstream consequences of histone acetylation can vary depending on the reader, and include gene activation, chromatin remodelling and deacetylation. For example, the bromodomain proteins BRD2/3 recognise hyperacetylated histones within active regions, specifically H4K5, H4K12 and H3K14. The binding of the Brd2/3 proteins enables RNA polymerase II to transcribe through the gene, and is
therefore necessary for active gene expression (LeRoy, Rickards and Flint, 2008). Another reader, the testis specific bromodomain protein BRDT contains double bromodomains and recognises H4K5ac/H4K8ac. The binding of this reader initiates genome wide chromatin remodelling during spermiogenesis, thus regulating male germ cell differentiation (Morinière et al., 2009; Gaucher et al., 2012). The acetylation mark is also recognised and removed by the eraser histone deacetylases (HDACs). HDACs are classified into classes I-III based on the homology to yeast proteins (reviewed in Gray and Ekström, 2001, Taunton et al., 1996). HDACs have variable levels of specificity towards acetylated histone residues. For example Sirt2, a class III HDAC preferentially deacetylates H4K16Ac and is important for deacetylation and subsequent chromosome condensation (Vaquero et al., 2006). Conversely, HDAC1, a class I HDAC recognises a broader spectrum of acetylated histone residues (Taunton et al., 1996). The activity of such HDACs is associated with transcriptional repression, as it reverses an active mark (Kouzarides, 2007).

1.2.1.2 Histone Methylation

Histone methylation is regulated by enzymes with higher specificity towards individual histone marks compared to the HATs and HDACs of acetylation. Both lysine and arginine resides can be methylated and histone methylation is associated with both active and repressive chromatin states (Murray, 1964; Paik and Kim, 1971). Lysine methylation is mediated by methyltransferase enzymes, which are classified into approximately four families, based on the catalytic methyl transferase SET domains and include SET1, SET2, SUV39 and ROZ. Each family contains other specific domains, that in some cases contribute to the
specificity of the methyltransferase activity (Kouzarides, 2002). The precise effect of histone lysine methylation on the local chromatin environment depends both on the histone residues modified and the number of methyl groups added. For instance, in yeast, H3K4 methylation has different effects depending on whether it is di- or tri- methylated. Di-H3K4 methylation is found at both inactive and active genes, whereas tri- methylation is only found at active genes (Santos-Rosa et al., 2002). H3K9 di- and tri- methylation are typically associated with transcriptional repression as they recruit the HP1 protein thereby propagating the spreading of silent heterochromatin chromatin in a process similar to the X-chromosome inactivation (Rea et al., 2000; Heard et al., 2001).

Demethylation of lysine residues is carried out by histone demethylase enzymes. LSD1 (Lysine specific demethylase) was the first specific demethylase discovered and demethylates H3K4. Knockdown of LSD1 leads to an increase in H3K4 methylation and expression of LSD1 target genes that are normally repressed. LSD1 activity can also be modulated by its interaction with other complexes and it is found within a number of co-repressor complexes (Shi et al., 2004). For instance, within the coREST complex LSD1 demethylates mono- and di-methylated H3K4, but when associated with the Estrogen receptor (ER) LSD1 mediates H3K9 demethylation increasing DNA accessibility and promoting ER-mediated transcription (Kozub et al., 2017). A second large family of histone demethylases is characterised by the JmjC domain which specifically demethylates H3K26 methyl groups. The JmiC domain was first identified in the JHDM1 protein, but has been found in numerous other demethylases (Tsukada et al., 2006; Shi and Whetstine, 2007).
1.2.2 Nucleosome Remodelling

Nucleosomes physically reduce the accessibility of DNA to nuclear factors and therefore the positioning of the nucleosomes is a key mechanism of regulating gene expression. Work on mostly synthetic and random DNA substrates has shown that although any DNA sequence can be incorporated into the nucleosome certain sequences have higher or lower affinities, depending on the energy required to assemble the nucleosome (Lowary and Widom, 1997, 1998). AT-rich and GC-rich sequences allow easier DNA bending and as such may play a role in making the wrapping of DNA around the nucleosome more energetically favourable (Segal et al., 2006). Whilst an ‘ideal’ nucleosome position sequence has been generated for use in in vitro assays, this does not take into account the in vivo DNA sequences, nor the local 3D conformation of the DNA and nuclear structure (Lowary and Widom, 1998; Partensky and Narlikar, 2009; Clark, 2010). Studies on nucleosome positioning in yeast and Drosophila have shown that there is some degree of genome-directed positioning (Yuan et al., 2005; Segal et al., 2006). Genes that are highly transcribed, such as tRNA and rRNA genes have a higher percentage of low affinity nucleosome sequences and accordingly, lower nucleosome occupancy. Conversely, repressed centromeric DNA has sequences with higher affinity and therefore higher nucleosome occupancy (Segal et al., 2006; Segal and Widom, 2009). In addition, TATA boxes, located at transcription start sites also show less affinity for nucleosome binding, allowing a more dynamic and fast transcriptional response to stimuli (Ioshikhes et al., 2006; Mavrich et al., 2008). Nucleosome position is changed through the activity of chromatin remodellers, which are multi-subunit
complexes that mediate the dynamic changes of chromatin structure through nucleosome sliding, exchange and eviction (Partensky and Narlikar, 2009) (Fig.1.5).

Fig.1.5: Schematic of principal nucleosome remodelling activities. Diagram demonstrates nucleosome sliding, histone exchange and nucleosome eviction. In nucleosome sliding the nucleosomes are repositioned on the DNA, opening up sections of DNA once blocked (indicated by pink line in diagram) and wrapping around new sections of DNA (indicated by orange). In nucleosome exchange the DNA wrapped around the DNA remains the same but the histones incorporated into the nucleosome core are different (indicated by grey histones). In nucleosome eviction, the nucleosomes are removed from the DNA entirely, exposing a longer stretch of DNA (indicated by pink and orange lines).

1.2.2.1 Nucleosome Sliding and Chromatin Remodelling

Nucleosomes are shifted along the DNA by ATPase remodelling proteins that bind to nucleosomal DNA at a central point of the core particle, disrupting histone-DNA contacts and enabling movement of the DNA along the nucleosome (Saha, Wittmeyer and Cairns, 2005). Following binding of the chromatin remodeller to the nucleosome and the release of energy from ATP hydrolysis, there is a conformational change in the enzyme which causes a stretch of DNA to loop out from the nucleosome core (Strohner et al., 2005; Zofall et al., 2006) (Fig.1.6). New DNA is pulled in to the nucleosome at the entry site and 'old' DNA
is pushed around the nucleosomes toward the exit site by translocase activity, and so the loop is propagated around the nucleosome. The loop of DNA may also create a twist in the DNA that similarly diffuses around the nucleosome to the exit site of the nucleosome or aids its active translocation by the chromatin remodeler. As a consequence of the remodelling activities, nucleosome position is altered (Saha, Wittmeyer and Cairns, 2002, 2005; Whitehouse et al., 2003; Edayathumangalam et al., 2005; Lia et al., 2006).

Fig.1.6. Schematic of loop propagation model of nucleosome sliding. A chromatin remodeler (yellow) binds the nucleosome. Next a conformational change causes a loop to bulge out. This is propagated around the nucleosome (dashed arrows show direction of DNA translocation behind image). As a consequence, DNA is pulled in from the entry site (orange line) and pushed out of exit site (black line) and nucleosome positioning is altered.
It should be noted that the sliding mechanism depends, at least in part, on the family of remodellers and the structure of the nucleosome substrate, such as the length of linker DNA. Moreover, even if they belong to the same family of ATP remodelling enzymes, individual members can display different mechanisms. For instance, within the CHD family, CHD6, 7, and 8 have different remodelling activities. Whilst CHD6 and CHD7 bind nucleosomes with short ~20 bp linker DNA on either side, CHD8 requires longer linker DNA, ~40 bp to bind nucleosomes efficiently. Furthermore CHD7 and CHD8 slide nucleosomes to change positioning, and CHD6 disrupts nucleosome-DNA interactions without sliding the nucleosome (Manning and Yusufzai, 2017). Different families of remodellers can also have distinct remodelling mechanisms. SWI/SNF enzymes can bind nucleosomes without linker or extra-nucleosomal DNA and disrupt the DNA-H2 interactions, causing a loop in the DNA which propagates along the nucleosomal DNA. In contrast the ISWI family requires longer linker DNA to bind nucleosomes and translocates the DNA in small steps, interacting with H4 tails. This translocation creates torsion in the DNA which pulls in new DNA, changing DNA-nucleosome contacts (Zofall et al., 2006; Narlikar, Sundaramoorthy and Owen-Hughes, 2013).

1.2.2.2 Histone Exchange and Histone Eviction

Histone eviction and exchange are often coupled, as eviction of pre-existent histones or nucleosomes can be required for the exchange and insertion of new histones. This occurs during processes such as DNA damage, or at specific stages in the cell cycle, such as during DNA replication (Workman, 2006). The histone H3 variant H3.3 is deposited after DNA replication, replacing the
replication fork H3.1 variant, and is found at transcribed genes with a number of active chromatin marks (Ahmad and Henikoff, 2002; McKittrick et al., 2004). H3.3 and H3.1 are deposited through different chaperone proteins with distinct mechanisms, with the CAF-1 chaperone able to assemble H3.3 nucleosomes without DNA replication, whilst the H3.1 nucleosome assembly is dependent on the HIRA chaperone and DNA replication (Tagami et al., 2004). Additional histone variants include the γ H2A.X protein that is incorporated and phosphorylated during DNA damage and used as a marker for double strand breaks (Rogakou et al., 1998; Fillingham, Keogh and Krogan, 2006) and H2A.Z, which is enriched at TSSs and is required for early mouse embryonic development (Faast et al., 2001; Mavrich et al., 2008). The INO80 family and other members of this remodelling complex are required for H2A.Z exchange. The H2A.Z variant is delivered by a histone chaperone, Nap1/Chz1, which binds to the INO80 complex, bringing the H2A.Z variant close to the DNA. The INO80 remodeller then unwraps the DNA from the current nucleosome leading to octamer disassembly, but subsequent reassembly with the new H2A.Z variant incorporated (Mizuguchi et al., 2004; Luk et al., 2007).

Histone eviction is a key aspect of DNA replication and transcriptional regulation, as removal of nucleosomes from the DNA is necessary for the progression of the transcription and replication machineries along the DNA. Interaction of TFs with nucleosomal DNA has also been shown to promote histone displacement (Lorch, Maier-Davis and Kornberg, 2006; Gutiérrez et al., 2007). Histone eviction is mediated by a number of histone modifying complexes, including the SWI/SNF complex and chaperones which act as histone sinks, to
prevent the reassembly of histones into nucleosomes (Schwabish and Struhl, 2007).

1.2.3 Chromatin Remodelling Complexes

ATP-dependent remodellers use energy from ATP hydrolysis to change nucleosome position and are found within complexes containing a variety of other subunits. Chromatin remodelling complexes are divided into four families and are classified based on the domain structure of the core enzymatic ATPase. (Fig. 1.7)

Fig. 1.7: Schematic of the four main chromatin remodelling enzyme structures. The proteins contain key domains for chromatin binding and ATPase helicase activity. These include the helicase/SANT domain (HSA), the conserved ATPase/Helicase DEDx sequence motif (DEDx), and the HAND domain which recognises and bind histones and DNA. The SANT domain binds unmodified histone tails, the SANT-like but with several insertions (SLIDE) domain binds nucleosomal DNA. The PHD finger domains mediate protein interacts and the Bromodomains (BD) domains recognise acetylated lysines on histones, and the Chromodomains (CHD) domains recognise and interact with chromatin.

Chromatin remodelling complex families include the SWI/SNF (Winston and Carlson, 1992), the ISWI family (Elfring et al., 1994; Corona et al., 1999), the CHD family (Delmas, Stokes and Perry, 1993) and the INO80 family (Shen et al., 2000). Other proteins found in the complexes include scaffolding proteins,
chromatin or DNA binding proteins that ensure target specificity and chromatin modifying enzymes such as HATs or HDACs. Members of the same family can display different remodelling activity depending on cell context, the type of nucleosome substrate, and the presence of other subunits (Becker and Workman, 2013). The consequences of chromatin remodeller activity can also go beyond chromatin changes and transcriptional regulation. For instance, in yeast, double strand breaks (DSB) recruit the nucleosome remodeller Fun30 which helps the access of the repair machinery to the damaged DNA ends. The SWI/SNF remodeler is also important in homologous recombination, alongside the RSC complex, which itself has been implicated in nonhomologous-end-joining repair mechanisms (Chen et al., 2012; Stanley, Moore and Goodarzi, 2013; Seeber and Gasser, 2017). In mammals the INO80 complex is recruited to DSBs to evict histones and allow DNA repair machinery access to the DNA, whereas the BRG1 remodeler complexes help spread the γH2AX deposition around the site of DNA damage (Stanley, Moore and Goodarzi, 2013). Chromatin remodelers also assist during DNA replication, as the ACF remodeler is required for replication through chromatin and the INO80 complex promotes replication fork progression and helps to resolve stalled replication forks (Collins et al., 2002; Poli, Gasser and Papamichos-Chronakis, 2017).

1.2.3.1 Remodelling Complexes in Cortical Development

The combinatorial assembly of different subunits within the chromatin remodelling complexes allows both diversification and specificity of functions. For example, the BAF complex which is a member of the SWI/SNF family, has different assemblies for each neuronal developmental stage. In ESCs, a specific
BAF complex (esBAF) regulates ESC self-renewal and pluripotency by preventing polycomb-mediated repression of the ES cell genes Oct4 and Nanog (Ho et al., 2009, 2011). At later times a different BAF complex, npBAF, is assembled in neural progenitors. npBAF contains some subunits found in the esBAF but crucially also has the specific subunit BAF53a. npBAF activates the Notch pathway and maintains the progenitor pool (Lessard et al., 2007). The final nBAF assembly is specifically detected in post-mitotic neurons and switches BAF53a with BAF53b and includes additional nBAF specific proteins. nBAF is required for dendritic development and ensures the correct connectivity of the cortical neurons (Wu et al., 2007). Inclusion of specific BAF subunits is regulated by the micro-RNA machinery of miR-9* and miR-24 that bind and inhibit the expression of the npBAF subunits. In neural precursors, this mi-RNA mediated repression is prevented by repressive transcription factor REST, that binds to sites within the miR-9* and miR-24 genes and inhibits their expression. Consequently, BAF53a is expressed and incorporated into the npBAF complex. After exiting the cell cycle, REST is repressed thereby allowing miR-9* and miR-24 expression. These miRNAs then bind to BAF53a transcripts preventing BAF53a expression, and as a result, the expressed BAF53b is incorporated into the complex instead, leading to the assembly of the neuronal nBAF complex (Yoo et al., 2009; Ballas et al., 2005; Conaco et al., 2006).

1.2.4 The NuRD Complex

The nucleosome remodelling and deacetylase chromatin remodelling, NuRD complex combines histone deacetylation with ATPase remodelling activity (Wade et al., 1998; Xue et al., 1998) and can act as either a transcriptional
activator or repressor at its target genes (Nitarska et al., 2016). All NuRD complexes contain a catalytic chromodomain helicase DNA binding (CHD) ATPase remodelling protein, a HDAC, a methyl CpG binding domain protein (MBD), a retinoblastoma binding protein (RBBP), a metastasis associated protein (MTA) and a zinc finger protein (GATAD2) (Wade et al., 1998; Wolffe et al., 1999; Zhang et al., 1999) (Fig. 1.8). The majority of the subunits, in particular the RBBP, CHD and HDAC proteins can also be found outside the NuRD complex and associated with other chromatin remodelling complexes. Similar to BAF, each of the six subunits expresses a number of isoforms, that are usually mutually exclusive, leading to different assemblies of the NuRD complex (Hendrich and Bird, 1998; Schultz et al., 2001; Fujita et al., 2003; Le Guezenec et al., 2006).

NuRD plays a key role in a number of biological processes, including DNA damage repair (Larsen et al., 2010; Smeenk et al., 2010) and chromosomal maintenance during the cell cycle (Helbling Chadwick et al., 2009; Sims and Wade, 2011).

**Fig.1.8:** Schematic of principal subunits of the NuRD complex. This illustration is not indicative of stoichiometry or structural interactions. The main subunits are the chromodomain helicase DNA binding (CHD) remodeller, a histone deacetylase (HDAC), a retinoblastoma binding protein (RBBP), a metastasis associated protein (MTA), and a GATA Zinc Finger Domain containing proteins (GATAD2).
Importantly NuRD is required during several stages of cortical development and the specific combination of CHD3/4/5 subunits with MBD3, MTA2, HDAC2 and RBBP7/4 regulates neuronal development (Yamada et al., 2014; Nitarska et al., 2016; Yang et al., 2016).

CHD3, CHD4 and CHD5 are the large ~200kDa catalytic ATPase proteins found associated with the NuRD complex; they belong to the CHD class II ATPase family, CHD1-CHD9. The majority of the CHDs have a broad expression pattern whereas CHD5 is restricted to neural cell types (Woodage et al., 1997; Marfella and Imbalzano, 2007). CHDs interact with histone tails through their PHD domains, and with DNA through the chromodomains, they also contain the SNF2-like ATPase domain, which has helicase activity (Morra et al., 2012) (Fig. 1.7). While the CHDs can bind unmodified nucleosomes, it is likely that different histone modifications regulate the recruitment of CHDs to chromatin, with H3K9me3 favouring CHD4 binding, whereas H3K4 methylation prevents this interaction (Musselman et al., 2009). Assembly of the CHDs into the NuRD complex is thought to happen through smaller sub-complexes, with the CHDs brought in as peripheral subunits. As such it is possible to find the CHDs present outside the NuRD complex (Low et al., 2016; Zhang et al., 2016). Only CHD3, 4 and 5 are associated with the NuRD complex in cortical development, generating complexes with different functions. CHD5 containing NuRD regulates neural radial migration and the expression of neuron-specific genes and transcription factors (Egan et al., 2013, Nitarska et al., 2016). NuRD assemblies with CHD4 are required for neural progenitor maintenance, and for setting the timings during cell fate determination (Reynolds et al., 2012; Sparmann et al., 2013; Nitarska et
NuRD complexes containing CHD4 also play a role in the DNA damage repair pathway and maintaining genome stability (Polo et al., 2010; Gong et al., 2017).

The primary HDACs associated with NuRD are HDAC1 and HDAC2, and they are mutually exclusive within the complex. HDAC1 and HDAC2 are widely expressed in most tissues although HDAC2 is more predominately expressed in neurons. Both HDAC1 and 2 are found in many chromatin remodelling complexes and can often compensate for the loss of the other (Zupkovitz et al., 2006; MacDonald and Roskams, 2008; Montgomery et al., 2009). HDAC1 and 2 are highly important during brain development and double knock out mice of HDAC1 and HDAC2 have severe phenotypes which culminate in embryonic lethality (Hagelkruys et al., 2014).

The MBD family comprises MBD2 and MBD3 which are mutually exclusive within the NuRD complex. MBD2 binds both methylated and hydroxymethylated DNA, whilst MBD3 binds only hydroxymethylated DNA (Hendrich and Bird, 1998). MBD3 is essential for ESC differentiation and embryonic survival whereas MBD2 NuRD complexes are not essential, as knock out of MBD2 does not culminate in lethality (Le Guezenneec et al., 2006; Kaji, Nichols and Hendrich, 2007). MBD3 is also required for the overall assembly of the NuRD complex and therefore has crucial functions in the regulation of neural cell fate determination and terminal differentiation in the cerebral cortex (Zoubovsky et al., 2011; Shimbo et al., 2013; Knock et al., 2015; Hainer et al., 2016).

The MTA proteins MTA 1/2/3 have all been found linked to different NuRD assemblies and are thought to be mutually exclusive subunits (Yao and Yang,
The role of these proteins has been most studied in cancer, where the three MTA proteins are highly upregulated. MTA1 stabilises p53, whilst MTA2 deacetylates p53 to inhibit its downstream targets. MTA1 and MTA2 also support tumour progression, while MTA3 prevents tumour progression (Kumar and Wang, 2016). Structural studies have demonstrated that the MTA proteins contribute to the assembly of the NuRD complex. MTA1 can bind two RBBP subunits, helping to assemble the full NuRD complex and establishing the NuRD stoichiometry (Zhang et al., 1999; Millard et al., 2016; Schmidberger et al., 2016).

RBBP7 and RBBP4 are highly homologous and conserved proteins (Qian and Lee, 1995) that act as histone chaperones. Similar to the HDACs, RBBP7 and RBBP4 are found in several other chromatin remodelling complexes, including the PRC2 complex (Smits et al., 2013; Zhang et al., 2013). Unlike other NuRD subunits, RBBP7/4 can co-exist or compensate for each other within the NuRD complex. Both proteins play a fundamental role in the structural assembly of the complex, as they contain several WD protein binding repeats, which interact with the histones and other proteins within the complex (Alqarni et al., 2014). Structural studies have shown that MTA-RBBP interact with a 1 MTA:2 RBBP stoichiometry and that RBBP can bind to histone H4 at a groove between an extended loop of H4 and blade six of RBBP7. Interestingly it has been suggested that the binding sites of MTA and H4 on the RBBP7/4 proteins are the same, preventing RBBP proteins from interacting with histone H4 when bound to MTA. However, the RBBPs can bind H3 at a separate domain which suggests that the MTA-RBBP interaction may be important for determining the histones targeted. The RBBP-H3 interaction is further influenced by H3 methylation status,
as RBBP4 has higher affinity to unmethylated H3 (Murzina et al., 2008; Alqarni et al., 2014; Millard et al., 2016; Chen et al., 2018).

The highly conserved GATAD2A/B proteins are primarily scaffolding proteins, interacting with the other NuRD subunits, RBBP7/4, MTA2, HDAC1/2 and MBDs. Post-translation modification of GATAD2a by lysine sumoylation enhanced the interaction with HDAC1 and RBBP4 (Brackertz et al., 2002; Gong, Brackertz and Renkawitz, 2006). Heterozygous knockout mice with reduced GATAD2A are viable but the null mice are embryonic lethal, and the phenotype indicates a role for GATAD2 in DNA methylation, potentially mediated by its NuRD interactions (Marino and Nusse, 2007). The interaction of GATAD with the ZMYND8 protein is also required for recruitment of GATAD2A- NuRD to sites of DNA damage, illustrating the importance of GATAD2 as a NuRD scaffold protein (Spruijt et al., 2016).

1.2.4.1 Role of the NuRD Complex during Cortical Development

Work from our lab has demonstrated that, similar to BAF, NuRD complexes are assembled in a stage specific manner, and that the core CHD ATPase remodelers undergo a subunit switch (Nitarska et al., 2016). CHD4 is assembled into the NuRD complex at the earlier stages of development between E12.5 and 15.5, at a time when neuronal progenitor cells proliferate, and this assembly is required for the exiting of the NEC from the cell cycle. From around E15.5 through to E18.5 NuRD complexes containing CHD3 regulate the correct late radial migration of post-mitotic neurons and layer specification. CHD5 is already present at E15.5 and is required for early radial migration. The three CHD containing complexes show target gene specificity during these stages and
activate distinct sets of genes, hence the different functions. Importantly the developmental defects induced by lack of a certain CHD cannot be rescued with another CHD, indicating distinct and non-overlapping functions of the different CHD contain NuRD complexes (Fig.1.9).

**Fig.1.9: CHD developmental stage and target specificity.** At early stages during neuronal progenitor proliferation CHD4 is incorporated into the NuRD complex. During the early neural migration CHD5 is required instead of CHD4, and at the later neuronal migration and deafferentation stages CHD3 is the key CHD subunit found in NuRD complexes (Figure from Nitarska et al., 2016).

The switch of CHD subunits is regulated in part by the expression levels, as CHD4 is detected from E12.5 throughout development, whereas CHD5 and CHD3 expression only increase at E15.5 (Nitarska et al., 2016). However, the
lack of compensatory effect between CHD3, CHD4 and CHD5 at late developmental stages, even when they are all detected, indicates that expression level is not the only factor that determines their incorporation into the NuRD complexes. We hypothesise that post-translational modifications of NuRD subunits may affect protein interactions at various stages of cortical development. Furthermore, post-translational modifications of the NuRD complex containing different CHDs could also regulate the recruitment to specific sets of target genes (Nitarska et al., 2016).

1.3 Nitric Oxide Signalling

Intra and intercellular signalling pathways involve a number of different signal transmitters. The gaseous molecule Nitric oxide (NO) was discovered as a signalling molecule in 1987 (Ignarro et al., 1987; Palmer, Ferrige and Moncada, 1987) and the initial findings were based on the fact that nitroglycerin and exogenous NO caused vasodilation by activating the soluble guanylate cyclase (sCG) monophosphate (cGMP) pathway (Katsuki, Arnold and Murad, 1977; Stinson et al., 1988). Around the same time NO was identified as the endothelial derived relaxation factor (EDRF), known to mediate smooth muscle relaxation.

The first established target of NO signalling was the sGC and cGMP pathway. NO produced by NOS enzymes binds to the heme group of sGC, causing a conformational change in the enzyme and increasing the catalytic activity, leading to an increased synthesis of cGMP from GTP. cGMP regulates a number of downstream signalling cascades, either directly or through the activation of protein kinase G (PKG). PKG is a broad-spectrum serine-threonine kinase and
phosphorylates and activates a number of ion channels, including calcium and calcium activated potassium channels. The influx of Ca\(^{2+}\) and K\(^{+}\) ions then feed into numerous downstream signalling pathways and elicit a variety of responses, including neuronal excitability and vasculature relaxation (Koesling and Fribe, 1999; Ahern, Klyachko and Jackson, 2002; Martínez-Ruiz, Cadenas and Lamas, 2011). However, NO also covalently binds cysteine thiols, in a post-translational modification known as S-nitrosylation (Ignarro et al., 1980; Stamler et al., 1992) which represents an alternative mechanism of NO signalling.

1.3.1 Synthesis of NO

The principle endogenous pool of NO in cells is generated through the activation of nitric oxide synthase (NOS) enzymes. There are three NOS isoforms, neuronal NOS (nNOS, also known as NOS1), which is highly expressed in the brain and in neurons (Bredt and Snyder, 1990, 1992), inducible NOS (iNOS or NOS2) which is expressed in macrophages during the inflammatory response (Xie et al., 1992) and endothelial NOS (eNOS), which is expressed in blood vessels and found in numerous other tissues (Lamas et al., 1992). NO is generated from the conversion of L-arginine and oxygen to L-citrulline and NO. The conversion is mediated by the transfer of electrons, generated from the reduction of NAPDH to NAPD\(^{+}\), to the flavin adenine and the heme domain. This electron transfer is dependent on calmodulin binding (Fig.1.10), which serves as one level of control of NOS enzymatic activity. Whilst calmodulin is constitutively bound to iNOS, the binding of calmodulin to eNOS and nNOS is calcium dependent (Lamas et al., 1992; Lantin-Hermoso et al., 1997; Spratt et al., 2007). eNOS and nNOS activity is triggered by the increase of intracellular calcium.
levels, which often is activated by many signalling pathways. In endothelial cells stimuli such as acetylcholine and histamines increase intracellular calcium levels, activating eNOS. In neurons, early studies demonstrated that glutamergic stimulation and NMDA channel activation increased intracellular Ca\(^{2+}\), which led to Ca\(^{2+}\) dependent calmodulin binding to nNOS and subsequent nNOS activation (Bredt and Snyder, 1992; Zhao, Vanhoutte and Leung, 2015).

**Fig.1.10: Domain structure and enzymatic activity of the nitric oxide synthase enzymes.** nNOS, iNOS and eNOS. Electrons (e-) gained from the reduced form of NADPH are transferred between the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) groups. This is dependent on Zinc dependent dimerisation (not shown) and calmodulin binding to the calmodulin binding domain (CaM). Electrons are passed to the heme iron in the Heme/ oxygenase domain. L-arginine and oxygen (O\(_2\)) is converted to L-citrulline and nitric oxide (NO) at the active site of the oxygenase domain.

NO synthesis also depends on the intracellular levels of NOS enzymes. For instance iNOS is only expressed in response to inflammation whilst the expression of eNOS and nNOS can vary depending on cell context, such as the increase of nNOS expression during embryonic cortical development (Xie et al., 1992; Bredt and Snyder, 1994). Post-translational modification of NOS can also influence activity and NO production. In cortical neurons, nNOS is phosphorylated at Ser\(^{1412}\) by protein kinase B in response to glutamate and this
event increases nNOS activity and NO synthesis. In contrast CAMKII-dependent phosphorylation of nNOS at Ser^{847} inhibits NOS activity (Rameau et al., 2007). The S-sulfhydration of eNOS also promotes phosphorylation, leading to increased eNOS activity (Altaany et al., 2014).

In addition, NO is also produced from other sources, including the intracellular breakdown and reactions of nitrates and nitrites, available due to reactive nitrogen species and exogenous sources such as diet (Hess et al., 2005; (Bredt and Snyder, 1990, 1992),

1.3.2 Protein S-Nitrosylation

Modification of proteins by S-nitrosylation is prevalent in many eukaryotic systems, including yeast, plant and mammalian cells. S-nitrosylation can influence a number of protein functions including enzymatic activity. For example the S-nitrosylation of caspase 3 inhibits its activity, whilst in stroke conditions S-nitrosylation of matrix metalloproteinases activates the extracellular proteases (Mannick et al., 1999; Gu et al., 2002). S-nitrosylation may also alter protein interactions with chromatin, as demonstrated by the S-nitrosylation of HDAC2 which causes its dissociation from chromatin (Nott et al., 2008) and protein localisation, as seen with the S-nitrosylation of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which leads to translocation from cytoplasmic to nuclear localisation (Kornberg et al., 2010).

The addition of the nitrosyl group to a target protein at cysteine thiols (R-SNO) can occur in a number of ways, including through different biochemical pathways (Fig.1.11). NO reacts with oxygen, metals and other nitrogen species present in the cell, however all these alternative pathways result in NO moieties
that can react with the thiol and lead to protein S-nitrosylation (Hess et al., 2005; Martínez-Ruiz et al., 2013; Umbreen et al., 2018) (Fig.1.11).

Fig.1.11. Generation of S-nitrosothiols (R-SNO). Nitric oxide (NO) is produced from NOS enzymes, exogenous donors, and from Nitrate/Nitrates provided by external sources such as diet. NO can then enter the cGMP pathway or lead to S-nitrosylation. NO can react with O$_2^-$ to form peroxynitrite (ONOO$^-$). NO can react with oxygen (O$_2$) to form nitrogen oxides and nitrate species to form N$_2$O$_3$. NO radicals also react with transition metals to form metal–nitrosyl complexes (M-NO). Each pathway leads to S-nitrosylation of cysteine thiols.

NO is also added to target proteins through protein-protein interactions. One mechanism entails the direct interaction between the substrate protein and the NOS enzyme. This was observed for the S-nitrosylation of cyclooxgenase2, which requires a protein-protein interaction with iNOS (Kim, Huri and Snyder, 2005). The interaction of NOSs with target proteins can also be mediated by chaperone proteins, and the NO can even be passed between proteins via a mechanism known as transnitrosylation (Scharfstein et al., 1994; Kornberg et al., 2010; Jia et al., 2014). Transnitrosylation can come from a variety of donor mechanisms, including the best characterised GSNO and Trx systems. GSNO, or S-Nitrosoglutathione, is a small endogenous peptide present in cells and the
GSNO peptide is held in equilibrium with SNO-proteins. GSNO can transnitrosylate a number of targets by transferring its NO moiety to substrate proteins, including haemoglobin (Singh et al., 1996; Liu et al., 2001; Romeo, Capobianco and English, 2003; Hess et al., 2005). The GSNO reductase pathway has particular significance in regulating plant defence mechanisms (Malik et al., 2011). It has also been proposed that S-nitroso-CoA may act as a NO donor and transnitrosylase donor in yeast cells (Anand et al., 2014). A second transnitrosylation system is through the thioredoxin (Trx) enzyme which can be S-nitrosylated, and transnitrosylate targets including caspases (Haendeler et al., 2002; Mitchell et al., 2007; Anand and Stamler, 2012; Stanley, Moore and Goodarzi, 2013). Other proteins have also been demonstrated to have transnitrosylation ability, including GAPDH (Kornberg et al., 2010) and SNO-caspase-3 which can transnitrosylate XIAP (Nakamura and Lipton, 2013). Transnitrosylation is highly important for the S-nitrosylation of nuclear proteins as NOS enzymes are either cytoplasmic or membrane bound (Villanueva and Giulivi, 2010) and have not been detected in the nucleus. Transnitrosylation is one of the mechanisms that have been proposed to explain the S-nitrosylation of nuclear proteins. One important example is provided by GAPDH. This metabolic enzyme is S-nitrosylated at Cys\textsuperscript{150}, both by NO donors and activation of endogenous nNOS (Jaffrey et al., 2001). The SNO modification leads to the binding of SNO-GAPDH to Sia1, which mediates its translocation into the nucleus. Once there, GAPDH transnitrosylates other substrates, including SIRT1, HDAC2, and DNA-activated protein kinase (Hara et al., 2005; Kornberg et al., 2010). The temporal regulation of S-nitrosylation primarily depends on the kinetics of NOS enzymes.
Expression and activation of iNOS for example, takes place up to two hours after exposure to inflammatory stimuli such as lipopolysaccharide, leading to a delayed S-nitrosylation of target proteins (Kelleher et al., 2011). In contrast nNOS responds quickly to extrinsic stimuli that increase intracellular calcium, leading to the S-nitrosylation of proteins that can be detected within 10 minutes of stimulation (Riccio et al., 2006; Nott et al., 2008). Transition metals also accelerate S-nitrosylation, owing to the redox chemistry of the modification (Stubauer, Giuffrè and Sarti, 1999). In haemoglobin the metal groups Fe binds first NO with high efficiency and then transfers the NO to Cys\textsuperscript{93} upon haemoglobin oxygenation. When the S-nitrosohemoglobin becomes fully oxygenated, the subsequent change in structure causes the release of NO (Stamler, Jia, et al., 1997; Gow and Stamler, 1998).

S-nitrosylation is a reversible modification. Denitrosylation can occur as a consequence of transnitrosylation but is also mediated by at least two additional pathways. One mechanism is indirect, through the GSNO/GSNOR system. As the endogenous S-nitrosylated peptide GSNO is held in equilibrium with SNO-proteins, any changes to GSNO levels impact on SNO-protein levels. The denitrosylase enzyme GSNOR reduces the GSNO peptide which leads to the loss of NO from S-nitrosylated proteins and reduces levels of SNO-proteins. If, however, levels of GSNOR are low, the levels of GSNO and SNO-P increase (Liu et al., 2001; Rizza et al., 2018). A second system is direct and is through the thioredoxin (Trx) and thioredoxin reductase (TrxR) system. Trx can reverse S-nitrosylation through transnitrosylation, but also binds directly to the SNO-protein though disulphide bonds, leading to release of reduced NO (HNO) from S-
nitrosylated proteins. TrxR is required to recycle Trx so it can continue to react with target S-nitrosylated proteins. In plants the oxidoreductase Thioredoxin-h5 (TRXh5) selectively reverses SNO modifications, and this de-nitrosylation is required for plant immunity (Kneeshaw et al., 2014). Trx/TrxR has been shown to denitrosylate several S-nitrosylated substrates in mammals, including caspase3, caspase-9 and even eNOS and nNOS, which themselves can be S-nitrosylated (Nikitovic and Holmgren, 1996; Martínez-Ruíz et al., 2013).

1.3.2.1 SNO-Motif

Precise linear sequence motifs that may guide S-nitrosylation specificity and favour this cysteine modification have not been identified to date, however the primary and tertiary structure of the amino acid sequence adjacent to the target cysteine are likely to determine whether or not S-nitrosylation will take place (Stamler et al., 1997; Cho et al., 2009; Marino and Gladyshev, 2010; Jia et al., 2014). Analysis of known S-nitrosylation targets and the potential biochemical mechanisms of NO binding have indicated that hydrophobic residues, and basic or acidic amino acids may enable S-nitrosylation through creating a local environment permissive for the chemical reaction required (Marino and Gladyshev, 2010; Smith et al., 2018). We have recently identified a lysine motif present in a number of potential S-nitrosylation targets, and discovered that nearby lysine residues are required for the S-nitrosylation of MBD3 and HDAC2 (Smith et al., 2018). SNO specificity also depends on the interaction of the NOS enzymes with chaperones or target proteins, which also allows the targeting of specific cysteines depending on the cell type and signalling pathway involved. For example, iNOS-dependent S-nitrosylation of GAPDH
following IFNγ stimulation is mediated by the interaction of iNOS with the transnitrosylase S100A9, which interacts with the chaperone S100A8 to transnitrosylate GAPDH at Cys247. In contrast, nNOS dependent S-nitrosylation of GAPDH at Cys150 mediates the interaction with Siah1, allowing GAPDH translocation to the nucleus (Kornberg et al., 2010; Jia et al., 2014).

1.3.2.2 Identification and Characterisation of SNO-Proteins

Despite the established importance of NO and S-nitrosylation as important mechanisms that regulate intracellular signalling pathways, most known S-nitrosylated proteins have only been identified using candidate-based approaches. This is principally due to technical limitations. Firstly, as yet there is no accurate and reliable pan ‘SNO’ antibody, as a SNO modification can have a different structure depending on the SNO protein, and few reliable SNO-protein antibodies have been generated for use in the mammalian system. Furthermore, SNO-thiols are highly unstable in vitro, as they can be easily degraded by light, reducing agents, and reactive metal species (Forrester et al., 2009). Consequently, an indirect but relatively specific approach to identify S-nitrosylated proteins, termed the biotin switch technique (BST) was developed. This technique replaces the unstable SNO modification with a biotin label, allowing for isolation and identification of the SNO protein using protein specific antibodies (Jaffrey et al., 2001). Furthermore the BST approach can be modified to S-nitrosothiol resin-assisted capture (SNO-RAC) technique which couples the technique with mass spectrometry analysis (Forrester et al., 2009). Several screens were carried out to try to identify potential SNO proteins in a variety of cell types and models, including recent screens to identify S-nitrosylated proteins.
in brains or neurons in response to specific stimuli. These included S-nitrosylation in the retina in response to light stimulation, which was linked to nNOS activity (Tooker and Vigh, 2015), SNO proteins in brains exposed to radiation (Nicolas et al., 2015), or in models of Alzheimer’s disease brains (Zahid et al., 2014; Żaręba-Kozioł et al., 2014; Seneviratne et al., 2016). Despite these, few nuclear targets of S-nitrosylation relevant to fundamental regulation of gene expression have been identified in neurons, partly due to the disease contexts studied, the high abundance of cytoplasmic proteins present when whole lysates are studied, and the methodological sensitivity of the approaches used to isolate SNO-proteins.

We recently performed a comprehensive screen to identify S-nitrosylated proteins in the nucleus of rat cortical neurons (Smith et al., 2018). The exogenous donor, SNO-L-cysteine was used to induce S-nitrosylation of neuronal nuclear extracts and the SNO-RAC technique was carried out to identify potential targets of S-nitrosylation. This screen provided 614 new nuclear neuronal targets of NO, including previously known targets such as HDAC2 and GAPDH. Of the proteins identified, 555 were novel neuronal targets of S-nitrosylation. Importantly several potentially S-nitrosylated proteins were involved in gene regulation, including CREB and members of the NuRD remodelling complex. Furthermore, the analysis of the S-nitrosylated peptides identified putative sites of S-nitrosylation for 360 proteins (Smith et al., 2018).

1.3.3 S-Nitrosylation in Cortical Development

The main source of NO during cortical development is nNOS, and nNOS expression itself increases during cortical development (Bredt and Snyder, 1994).
In rats, nNOS expression is first observed at E15 when it is restricted to the post-mitotic neurons of the cortical plate. nNOS levels then increase until E17 and begin to decline in the postnatal stages. This expression pattern is also seen in the equivalent mouse developmental stages (Fig.1.12). In the adult cortex, high nNOS expression is only seen in GABAergic interneurons, and is greatly reduced in the pyramidal cortical neurons, where it is restricted to dendritic spines (Aoki et al., 1998).

**Fig.1.12**: nNOS expression during rodent cortical development. Top panel: nNOS expression in the developing rat brain, using IF for nNOS and darkfield images. Expression in the cortex (CX) which begins prior at E10.5, and increases as development progresses E15 and E17, peaking at E18-19. nNOs expression then decreases postnatally (P0). Figure taken from Bredt and Snyder, 1994. Bottom panel: nNOS staining in the mouse neocortex, staining using nos1 antibody, expression pattern in ventricular zone (VZ), subventricular zone (SVZ) and cortical plate (CP) which contains the layers 1-VI. Data provided by Dr Nitarska.
The role of nNOS during cortical development was initially investigated using transgenic mice models. The first knockout model, KN1, carried a deletion of the exon encoding the PDZ domain present in two nNOS isoforms, nNOSμ and nNOSα. Although these two isoforms are absent in KN1 mice, a third isoform nNOSβ remains, providing low but significant nNOS residual activity (Huang et al., 1993; Eliasson et al., 1997). KN1 mice have normal brain structure, but impaired cortical development as fewer cortical neurons reach the CP and the layer identity is abnormal (Nott et al., 2013). KN1 mice have behavioural defects, including increased aggression, impaired social interactions, and problems with learning and memory (Huang et al., 1993; Zoubovsky et al., 2011; Walton et al., 2013). These behavioural defects are possibly due to defective glutamate metabolism, since NO is required for the S-nitrosylation of proteins that regulate the metabolic cycle which recycles synaptic glutamate, affecting glutamate transport and impairing neurotransmission (Raju et al., 2015). More recently, a second nNOS knockout mouse, KN2, was generated with the deletion of exon 6, which encodes the oxygenase domain common to all isoforms. These mice are less well characterised than the KN1 but again display increased male aggressive behaviour (Gyurko, Leupen and Huang, 2002). The importance of NO signalling in cortical development was further demonstrated in cultured cells. Long term exposure of the neuroepithelial cell line PC12 to NGF induced nNOS expression and promoted differentiation whilst preventing cell proliferation. In cerebral granular cells, inhibition of nNOS by L-NAME, an arginine analogue that prevents NOS enzymatic activity, also increased proliferation, confirming the role of NOS in cell differentiation (Peunova and Enikolopov, 1995; Ciani et al., 2004).
S-nitrosylation plays a key role during neurogenesis and cortical development, at least in part through the regulation of the CREB pathway. CREB is a transcription factor in neurons that binds to CRE elements present in many neuronal genes, including genes required for dendritogenesis and plasticity (Finkbeiner et al., 1997; Okamoto and Lipton, 2015). The increase of intracellular calcium in response to neuronal depolarisation and neurotrophins leads to CREB activation and expression of target genes (Sheng, McFadden and Greenberg, 1990; Mayr and Montminy, 2001). Transcription depends on CREB phosphorylation, and the recruitment of the HAT p300/CBP (Mayr and Montminy, 2001). S-nitrosylation pathways converge with CREB activation as is seen through the S-nitrosylation and nuclear localisation of GAPDH. Once in the nucleus GAPDH mediates the degradation of a histone methylase, SUV38H1, which normally deposits silencing marks on neuronal genes. In the absence of this mark, CREB binds more efficiently to the DNA, leading to neuronal gene activation (Sen and Snyder, 2011). Furthermore, in cortical neurons stimulated with BDNF, nNOS is activated resulting in increased synthesis of NO and increased binding of CREB to target neuronal genes (Riccio et al., 2006). In addition, the S-nitrosylation dependent dissociation of HDAC2 from chromatin leads to increased histone acetylation and CREB-dependent transcription (Okamoto and Lipton, 2015).

Regulation of HDAC2 by S-nitrosylation has been shown to be important during cortical development and neural radial migration (Nott et al., 2008, 2013). S-nitrosylation of HDAC2 at Cys^{262} and Cys^{274} in response to neurotrophins leads to its dissociation from chromatin and is necessary for dendritic growth. This
demonstrated a role for NO and specific protein S-nitrosylation in the correct transcriptional response of neurons to external cues, activity and depolarisation. Furthermore, in the absence of SNO-HDAC2 neurons fail to reach the CP, and levels of the nBAF complex specific Bhrama subunit are reduced, which impairs radial migration (Nott et al., 2008, 2013). The S-nitrosylation of the nuclear distribution element-like protein 1 (NDEL) at Cys\textsuperscript{203} is also required for dendritogenesis and it has been implicated foetal alcohol syndrome (Saito et al., 2016).

1.3.4 S-Nitrosylation in Disease

Aberrant NO signalling and S-nitrosylation play a key role in several human pathological conditions, including neurodegenerative conditions and behavioural disorders. Dysregulation of NO levels leads to increased levels of reactive oxygen and nitrogen species (ROS/RNS) levels which contributes to oxidative stress (Nakamura et al., 2015). During oxidative stress NO and O\textsubscript{2}\textsuperscript{-} interact, producing the toxic free radical peroxynitrite (ONOO\textsuperscript{-}), which leads to DNA strand breaks and DNA damage. Nitration by peroxynitrite of mitochondrial proteins also causes mitochondrial dysfunction by disrupting the electron transfer chain (Szabó, Ischiropoulos and Radi, 2007). Tyrosine residues can also be modified by NO\textsubscript{2} groups through peroxynitrite. Nitration of Hsp90 leads to activation of the Fas pathway and neuronal cell death (Franco et al., 2013). A decrease of GSNOR levels has been observed during aging and cell senescence, which leads to an increase in S-nitrosylated proteins, including the Drp1 protein. GSNOR is further associated with impaired mitophagy and mitochondrial stress, as cells lacking GSNOR displayed fragmented mitochondria and reduced ATP levels (Rizza et
S-nitrosylation of specific proteins has been widely studied in a number of different disease contexts, including neuronal damage caused by stroke, known as cerebral ischemic injury. In these conditions, overstimulated NMDA leads to a dysregulated and increased calcium influx. Consequently, the RIP3 protein, which senses stress and regulates apoptosis becomes S-nitrosylated at Cys\(^{119}\), leading to its overactivation and leading to increased neuronal cell death (Miao \textit{et al.}, 2015). In neurodegenerative diseases, increased levels of SNO-MEF2 are thought to contribute to the pathological development of Parkinson’s disease. When the transcription factor MEF2 is S-nitrosylated this prevents its DNA binding and therefore inhibits MEF2 target gene transcription, which contributes to the neuronal damage (Okamoto \textit{et al.}, 2014). Furthermore, the S-nitrosylation of Parkin is also implicated in Parkinson’s disease, as SNO-Parkin can no longer ubiquitinate its targets, inhibiting its protective function (Chung \textit{et al.}, 2004). A number of screens carried out using neurodegenerative disease model systems revealed a large number of S-nitrosylated proteins in Alzheimer’s disease (Zahid \textit{et al.}, 2014; Zaręba-Kozioł \textit{et al.}, 2014; Seneviratne \textit{et al.}, 2016). S-nitrosylation of the mitochondrial protein Drp1 is increased in Alzheimer’s disease leading to mitochondrial dysfunction, fragmentation and neurotoxicity. Interestingly, the AB oligomers that are the hallmarks of AD also induce S-nitrosylation of Drp1(Cho \textit{et al.}, 2009). There are also links between the regulation of nNOS and schizophrenia, as the nNOS binding protein NOS1AP, which to reduces NMDA receptor signalling, is mutated in this condition (Carrel \textit{et al.}, 2009; Carrel, Hernandez, Kwon, Mau, Meera P. Trivedi, \textit{et al.}, 2015). Studies have shown the overexpression of this protein reduced neuronal
migration and dendritogenesis, which could contribute to the characteristic reduction of dendrite number seen in schizophrenic brains (Carrel et al., 2009; Carrel, Hernandez, Kwon, Mau, Meera P. Trivedi, et al., 2015). Collectively, these studies show the widespread importance of nNOS and NO signalling in maintaining healthy neuron and brain function.

**Aims**

The aim of my project is to investigate the role of S-nitrosylation in regulating the NuRD complex in neurons. The NuRD complex is a chromatin remodelling complex that plays a key role in modulating gene expression during cortical development. We recently identified many NuRD subunits as potential targets for S-nitrosylation.

The main questions that I addressed during my graduate research are as follows:

1. Do NuRD subunits undergo S-nitrosylation, and what are the sites of this modification?

2. Does S-nitrosylation regulate NuRD complex assembly and chromatin remodelling activity?

3. Does S-nitrosylation of NuRD subunits play a role during neuronal development?
2. Results

2.1 NuRD subunits are S-nitrosylated

To identify novel nuclear neuronal proteins that could be modified by S-nitrosylation Dr Jacob Smith, a former graduate student and post doc in our laboratory performed a screen in E17 rat cortical neurons (Smith et al., 2018). Nuclear extracts were exposed to the exogenous NO donor CysNO and subjected to SNORAC technique (Forrester et al., 2009) and analysed by mass spectrometry to identify S-nitrosylated proteins. The screen identified potential targets of S-nitrosylation, and for a subset of hits, the target cysteine residues (SNO-peptides). A number of chromatin remodelling and gene regulation proteins were identified as potential targets of S-nitrosylation and in particular, the majority of NuRD complex subunits (Table 2.1.).

<table>
<thead>
<tr>
<th>Protein</th>
<th>SNO-Peptide</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD5</td>
<td>LVCLD</td>
<td>Zinc Finger</td>
</tr>
<tr>
<td></td>
<td>ECLAЕ</td>
<td></td>
</tr>
<tr>
<td>CHD4</td>
<td>MVCLD</td>
<td>Zinc Finger</td>
</tr>
<tr>
<td></td>
<td>CCNHP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECLAES</td>
<td></td>
</tr>
<tr>
<td>CHD3</td>
<td>LVCLD</td>
<td>Zinc Finger</td>
</tr>
<tr>
<td></td>
<td>CTCPVL</td>
<td>Chromodomain</td>
</tr>
<tr>
<td></td>
<td>WACLВ</td>
<td>Helicase</td>
</tr>
<tr>
<td></td>
<td>CCNHP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HLCEP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECLAЕ</td>
<td></td>
</tr>
<tr>
<td>MBD3</td>
<td>WЕCPА</td>
<td>Adjacent to Coiled coil</td>
</tr>
<tr>
<td></td>
<td>AQPLCK</td>
<td>Coiled coil</td>
</tr>
<tr>
<td></td>
<td>ACAЕЕ</td>
<td></td>
</tr>
<tr>
<td>RBBP4</td>
<td>GECNP</td>
<td>Adjacent to WD</td>
</tr>
<tr>
<td>RBBP7</td>
<td>GECNP</td>
<td>WD 2</td>
</tr>
<tr>
<td>MTA2</td>
<td>ALDСSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVLСR</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. NuRD subunits identified as S-nitrosylated. Table shows subunits identified as potential SNO targets in neurons for the first time, and the associated cysteine peptide and domain for each subunit. Data from screen performed by Dr. Jacob Smith (Smith et al., 2018).
The NuRD complex plays a key role during cortical development, as shown by our lab and others (Egan et al., 2013; Knock et al., 2015; Nitarska et al., 2016). However, despite these recent findings there is still limited understanding on how the activity of the complex is regulated. Because most NuRD subunits were identified as potentially S-nitrosylated (Table 2.1) I investigated if this modification plays a role in the regulation of NuRD assembly, recruitment to chromatin and function.

2.1.1. Biotin Switch Technique to identify S-nitrosylated proteins

In order to confirm that NuRD subunits are able to be S-nitrosylated I used the Biotin Switch Technique (BST) (Jaffrey and Snyder, 2001; Michael T. Forrester et al., 2009). In this assay the unmodified free cysteines are blocked using the methlayting agent S-methylmethanethiosulfonate (MMTS) or the alkylation agent N-Ethylmaleimide (NEM). Following this step, the S-nitrosylated proteins are reduced by treatment with sodium ascorbate (Asc), making cysteines susceptible to binding by biotin HPDP. Biotinylated proteins are pulled down using streptavidin beads, ensuring that any protein isolated with this technique was originally modified by S-nitrosylation (Fig.2.1). See methods for a more detailed outline of the protocol.

**Fig.2.1: The Biotin Switch Technique.** On S-nitrosylated proteins free cysteine thiols are blocked using MMTS or NEM. The S-nitrosylated residues are then reduced by sodium ascorbate. Samples are then incubated with Biotin HPDP which binds the free cysteines, allowing for pull down of the biotinylated proteins. Samples are then separated on SDS-PAGE and proteins of interest identified by immuno-blotting.
2.1.1.1 Induction of S-nitrosylation

S-nitrosylation is induced by activation of endogenous NOS enzymes or by adding exogenous NO donors. Donors such as CysNO and GSNO are nitrosylated in vitro and added to the cells. The donors are taken up by cells, and then the NO is released to nitrosylate target proteins. It has been reported that some targets of S-nitrosylation are only identified when samples are treated with specific donors or labels (Chung et al., 2015; Seth and Stamler, 2015) and therefore, where possible, both CysNO and GSNO treatment were used to stimulate S-nitrosylation. GSNO is a more physiological donor as it is present in cells and has an established role in S-nitrosylation signalling (Singh et al., 1996; Romeo, Capobianco and English, 2003; Rizza et al., 2018). Negative controls of the unmodified molecules Cys and GSH were used as appropriate.

To induce endogenous synthesis of NO, I depolarised neurons by addition of KCl. KCl is a widely used stimulus which depolarises neurons and stimulates intracellular calcium signalling pathways in a way that mimics the response to neuronal activity, leading to a physiologically relevant in vivo transcriptional response (Malik et al., 2014). It is well known that neuronal activity and KCl stimulation activation of nNOS (Fontana et al., 1997).

2.1.2 Validation of S-nitrosylation sites

I first generated DNA overexpression vectors containing each of the NuRD subunits of interest (Table 2.2). I also generated mutant constructs in which the potential sites of S-nitrosylation identified in the screen were substituted with serine, which is structurally the closest amino acid to cysteine. If the cysteines are targets of S-nitrosylation the mutation would prevent S-nitrosylation and
detection of the protein by BST. For RBBP4, RBBP7 and MBD3, the open reading frames were PCR amplified from rat (RBBP4/7) or mouse (MBD3) cDNA and ligated into the myc-CMV vector. WT constructs for flag tagged mCHD4, mCHD3 and hCHD5 driven by the pCIG overexpression promoter had already been generated in the lab (Nitarska et al., 2016).

<table>
<thead>
<tr>
<th>NuRD Subunit</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD5</td>
<td>pCIG-Flag-hCHD5</td>
</tr>
<tr>
<td>CHD4</td>
<td>pCIG-Flag-mCHD4</td>
</tr>
<tr>
<td>CHD3</td>
<td>pCIG-Flag-hCHD3</td>
</tr>
<tr>
<td>MBD3</td>
<td>pCMV-myc-msMBD3</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{C8S})</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{C215S})</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{C266S})</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{C215S_C266S})</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{C215S_K264A})</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-msMBD3(^{K264A})</td>
</tr>
<tr>
<td>RBBP4</td>
<td>pCMV-myc-rtRBBP4</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-rtRBBP4(^{C167S})</td>
</tr>
<tr>
<td>RBBP7</td>
<td>pCMV-myc-rtRBBP7</td>
</tr>
<tr>
<td></td>
<td>pCMV-myc-rtRBBP7(^{C168S})</td>
</tr>
</tbody>
</table>

Table 2.2. Expression vectors of the NuRD subunit. CHD constructs previously made in the Riccio laboratory by Dr. Nitarska.

In order to determine whether putative cysteines were targeted by S-nitrosylation vectors were transfected into HEK 239T cells. This cell line was used as transfection rates are much higher than for primary neurons, ensuring that sufficient protein was expressed for the technical challenges of BST.
2.1.3 RBBP7 is S-nitrosylated at Cys^{166}.

RBBP7, also known as RbAp46, was first identified through the binding to the tumour suppressor protein Rb (Qian and Lee, 1995) but its role in neurons is only recently being characterised. RBBP7 is present in a number of important chromatin remodelling complexes that regulate neural gene expression. In addition to being in the NuRD complex, RBBP7 is present in the histone methyltransferase Polycomb Repressive Complex 2 (PRC2) which is required for maintaining the balance of cell renewal and neuronal differentiation in cortical progenitors. RBBP7 is also a key component of the mammalian Sin3a complex, which interacts with REST and binds to REST binding elements in neuronal genes to repress neuronal gene expression in non-neuronal cell lineages (Roopra et al., 2000; Pereira et al., 2010). RBBP7 is highly conserved between species, with 99.8% amino acid identity between mice, rats and humans, potentially indicating a common role across species.

In order to confirm that RBBP7 undergoes S-nitrosylation and to identify the cysteine targeted by of S-nitrosylation, I transfected pCMV-myc-rtRBBP7 (RBBP7^{WT}) or pCMV-myc-rtRBBP7^{C166S} (RBBP7^{C166S}) constructs into HEK293T cells. Cys^{166} was chosen as a putative target cysteine of S-nitrosylation as it was in one of the SNO-peptides identified in the screen and had been previously discovered as the site of RBBP7 S-nitrosylation in mouse myocardium (Kohr et al., 2011). 48 hours post transfection, cells were treated with 500 µM CysNO for 20 min, harvested and subjected to BST processing. S-nitrosylation of RBBP7^{WT} was observed when cells were treated with CysNO, whereas this S-nitrosylation
signal was abolished in cells expressing RBBP7\textsuperscript{C166S} (Fig.2.2). This result confirms that RBBP7 can be S-nitrosylated at Cys\textsuperscript{166}.

**Fig.2.2: RBBP7 can be S-nitrosylated at Cys\textsuperscript{166}.** Vectors expressing RBBP7\textsuperscript{WT} or RBBP7\textsuperscript{C166S} were transfected into HEK 293T cells. After 48 h, cells were treated with either Cys or CysNO (500 µM for 20 min) and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE and immunoblotted using an anti-Myc antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to WT + CysNO. n=3 independent experiments. All data shown as mean +/- SEM. One-way ANOVA, compared to ‘WT + CysNO’ column, Fisher’s LSD; *p≤0.05, **p<0.01. (Experiment performed by Sarah Aldous and previously shown in Jacob Smith’s thesis, 2016).

### 2.1.4 RBBP7 is a target of S-nitrosylation in rat cortical neurons

To investigate whether RBBP7 was S-nitrosylated in neurons, rat E17 cortical neurons were treated with the NO donors CysNO or GSNO. Both the NO donors resulted in an increase of endogenous SNO-RBBP7 signal, further validating my findings and illustrating that the S-nitrosylation of RBBP7 can be elicited in neurons and by various sources of NO (Fig.2.3A, B).

In parallel experiments, neurons were treated with 50 mM KCl, an experimental paradigm that induces neuronal depolarisation and stimulates S-nitrosylation \textit{in vitro}. Under these conditions I observed robust S-nitrosylation of
RBBP7 (Fig.2.3C). Importantly this demonstrates that S-nitrosylation of endogenous RBBP7 can occur under physiologically relevant conditions and could therefore be part of an important *in vivo* response.

**Fig.2.3: RBBP7 is S-nitrosylated in rat cortical neurons.** E17 rat cortical neurons were treated with (A) CysNO or Cys (200 μM for 20 min) n=3 and (B) GSH or GSNO (200 μM for 20 min) n=2 (C) Cortical neurons were exposed to 50 mM KCl (n=2 30 min, n=1 KCl 20 min. Data combined for analysis). 30 min experiments performed by Jacob Smith. Cells were then and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using antibodies against RBBP7. Densitometry analysis was performed using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to + CysNO All data are shown as mean +/- SEM (Unpaired t-test, **p=<0.01).
2.1.5 RBBP4 is S-nitrosylated at Cys\(^{167}\).

RBBP4, also known as RbAp48, was also identified as a binding partner of the tumour suppressor Rb protein (Qian and Lee, 1995). A close homologue of RBBP7, RBBP4 is frequently found in the same complexes, including NuRD, PRC2 and Sin3a (Roopra et al., 2000; Pereira et al., 2010). RBBP7 and RBBP4 are 88.3% identical to each other in both rats and mice and have a similar WD propeller structure. The site of RBBP7 S-nitrosylation Cys\(^{166}\), is conserved in RBBP4 and Cys\(^{167}\) was also indicated to be the site of S-nitrosylation for RBBP4 (Fig.2.4, Table 2.1).

![Fig.2.4: RBBP7 and RBBP4 sequence and structural similarity.](image)

Fig.2.4: RBBP7 and RBBP4 sequence and structural similarity. Alignment of RBBP4 and RBBP7 in mice and rat indicates high identity between the proteins and the potential target cysteine, marked in red box, is also conserved. Cys\(^{166}\) and Cys\(^{167}\) are just outside the WD regions for RBBP4 and within WD repeat 2 of RBBP7 respectively (indicated by the arrows on the structural models). Alignment carried out using UNIPROT and CLUSTALO. Structural models from SwizModel ID Q71UF4 for RBBP7 and Q60972 for RBBP4.
In order to investigate whether Cys\textsuperscript{167} of RBBP4 is S-nitrosylated, I transfected HEK 293T cells either with pCMV-myc-rtRBBP4 (RBBP4\textsuperscript{WT}) or pCMV-myc-rtRBBP4\textsuperscript{C167S} (RBBP4\textsuperscript{C167S}). 24 hours after transfection, cells were exposed to the CysNO donor for 20 min and subjected to BST. I observed a strong increase of RBBP4\textsuperscript{WT} S-nitrosylation following treatment with CysNO. Strikingly, the signal was significantly reduced when cells were transfected with the mutant RBBP4\textsuperscript{C167S}, demonstrating that this is the site of S-nitrosylation (Fig.2.5).

**Fig.2.5:** RBBP4 can be S-nitrosylated at Cys\textsuperscript{167}. Vectors expressing RBBP4\textsuperscript{WT} and RBBP4\textsuperscript{C167S} were transfected into HEK 293T cells. After 24 hours, cells were treated with Cys or CysNO (500 \(\mu\)M for 20 min) and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using an anti-Myc antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to WT+CysNO. \(n=6\) independent experiments. All data shown as mean +/- SEM. One-way ANOVA, compared to ‘WT+CysNO’ column, (Fisher’s LSD; *\(p\leq0.05\), **\(p<0.01\)).

**2.1.6 RBBP4 is S-nitrosylated in cortical neurons.**

I next sought to determine if endogenous RBBP4 is S-nitrosylated in cortical neurons, using both CysNO treatment and the more physiological donor GSNO.
I observed that both stimuli induced robust S-nitrosylation of RBBP4 (Fig. 2.6 A, B). Importantly, KCl treatment of E17 cortical neurons also increased S-nitrosylation of RBBP4 (Fig. 2.6 C).

Fig 2.6: RBBP4 is S-nitrosylated in rat cortical neurons. Cortical neurons were treated with (A) CysNO or Cys (200 μM for 20 min) n=3 and (B) GSH or GSNO (200 μM for 20 min) n=2 (C) Neurons were exposed to 50 mM KCl n=3 (n=2 KCl 30’, n=1 KCl 20’ data combined for analysis). Post treatment cells were harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using antibodies against RBBP4. Densitometry analysis was performed using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to + CysNO. All data are shown as mean +/- SEM (Unpaired t-test, **p<=0.01).
The S-nitrosylation of a cysteine in the WD propellers of both RBBP7 and RBBP4 could affect the binding to protein partners, since the WD domains are important structural platforms for regulating protein-protein interactions (Murzina et al., 2008). The binding interactions between RBBP proteins and the other NuRD subunits are known to be important for NuRD assembly in a step wise manner (Low et al., 2016; Millard et al., 2016; Brasen et al., 2017) and therefore it is possible that the assembly of NuRD complexes is influenced by RBBP7/4 S-nitrosylation at these WD propeller domains.

2.1.7. CHD3, CHD4 and CHD5 are targets of S-nitrosylation.

The core chromodomain helicase proteins are the active ATP-dependent nucleosome remodellers of the NuRD complex and are mutually exclusive within the complex. CHD3, CHD4 and CHD5 are found associated with NuRD at different times during cortical development, with distinct and non-overlapping target genes and functions. CHD4 NuRD complexes are required for appropriate neuronal progenitor proliferation, CHD5 complexes for correct radial migration, and CHD3 complexes are involved in the regulation of later migration stages and the terminal differentiation of neurons (Nitarska et al., 2016). It is currently unknown what regulates the differential association of each CHD protein with the NuRD complex, but one possible mechanism entails the post-translational modification of the subunits. In keeping with this hypothesis, CHD3, 4 and 5 were identified as S-nitrosylated in our screen. In order to determine whether the CHD subunits are S-nitrosylated I transfected HEK 293T cells with either pCIG-Flag-
hCHD5, pCIG-Flag-mCHD4 or pCIG-Flag-hCHD3, treated with CysNO and subjected samples to BST (Fig.2.7).

**Fig.2.7:** CHD3, CHD4 and CHD5 can be S-nitrosylated. Vectors expressing flag-tagged hCHD3, mCHD4 and hCHD4 were transfected into HEK 293T cells. After 24 hours, cells were treated with Cys or CysNO (200 µM for 20 min) and subjected to BST. NEM (n=3) and MMTS (n=3) blocking conditions were used and data combined for analysis. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using an anti-Flag antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to +CysNO. n=6 independent experiments. All data are shown as mean +/- SEM ( *p≤0.05**p<0.01, ***p<0.001. Unpaired t-test.)

S-nitrosylation of all three CHDs was observed in response to treatment with CysNO, confirming the CHDs can be S-nitrosylated. Owing to the technical
challenges of the high molecular weight protein (CHDs are ~220kDa) coupled with the limitations of BST, I have been unable to detect S-nitrosylation of endogenous CHDs in neurons, in response to either CysNO donor or KCl. However, the confirmation that these subunits are able to be S-nitrosylated not only confirms the findings of the screen, but also supports the hypothesis that NuRD S-nitrosylation could regulate CHD activity or target specificity.

2.1.8. MBD3 is S-nitrosylated at two target cysteines.

MBD3 is a DNA protein that regulates early embryonic development in mice and is essential for NuRD complex assembly (Kaji, Nichols and Hendrich, 2007). In order to study whether MBD3 was S-nitrosylated I generated a MBD3WT construct, (pCMV-myc-msMBD3WT) and an MBD3 construct with Cys8 mutated (MBD3C8S), the cysteine indicated by the peptide WECPA (Table 1) as potentially S-nitrosylated. In initial experiments HEK293T cells were transfected with msMBD3WT or msMBD3C8S over expression constructs and lysates were treated with CysNO 200 µM for 20 min donor and subjected to BST. Under these conditions I observed a strong S-nitrosylation of msMBD3WT, however mutation of Cys8 to Ser8 did not abolish this signal. These results indicate that MBD3 is S-nitrosylated, but that Cys8, the cysteine indicated by the peptide WECPA (Table 2.1) as potentially S-nitrosylated was not a target of S-nitrosylation in this system or was not the only target (Fig.2.8). Based on SNO peptides indicated from the screen, I also generated further constructs with mutation with either Cys215 (MBD3C215S) and Cys266 (MBD3C266S) or both (MBD3C215S/C266S).

I transfected HEK 293T cells with either MBD3WT, MBD3C215S, MBD3C266S, or a double MBD3C215S/C266S construct and exposed the cells to CysNO 24 hours
after transfection, and then processed the sampled for BST. Under these conditions I again observed strong S-nitrosylation of msMBD3\textsuperscript{WT} and saw that the mutations of Cys\textsuperscript{266} partially abolished the S-nitrosylation signal, whilst mutation of Cys\textsuperscript{215} did not impact on MBD3 S-nitrosylation. Importantly the double MBD3\textsuperscript{C215S/C266S} mutation completely prevented S-nitrosylation of MBD3, indicating that MBD3 is S-nitrosylated at both Cys\textsuperscript{215} and Cys\textsuperscript{266}.

Fig.2.8: MBD3 can be S-nitrosylated at Cys\textsuperscript{215} and Cys\textsuperscript{266}. Vectors expressing MBD3\textsuperscript{WT}, MBD3\textsuperscript{C215S}, MBD3\textsuperscript{C266S}, and MBD3\textsuperscript{C215S/C266S} were transfected into HEK 293T cells. After 24 h, cells were treated with Cys or CysNO (200 µM for 20 min) and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using an anti-Myc antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to WT + CysNO. n=3 independent experiments. All data shown as mean +/- SEM. One-way ANOVA, compared to ‘WT + CysNO’ column. (Fisher’s LSD; *p<0.05, **p<0.01. ****p<0.0001)
Cysteines 215 and 266 are adjacent to and within the coiled coil domain of MBD3, respectively. This coiled coil domain is important for the interaction of MBD3 with the GATAD2 subunits of NuRD (Brackertz et al., 2002; Allen, Wade and Kutateladze, 2013) and S-nitrosylation may stabilise or disrupt this MBD3-GATAD2 interaction. MBD3 is a crucial structural component of the NuRD complex, and it is known that in the absence of MBD3 NuRD cannot properly assemble (Kaji, Nichols and Hendrich, 2007). Thus, the potential modulation of the MBD3-GATAD binding by S-nitrosylation could contribute to the overall complex stability.

2.1.9. Identification of an S-nitrosylation motif

Despite extensive evidence demonstrating the importance of S-nitrosylation in regulating a wide variety of cellular processes, information regarding the amino acid sequence(s) that may mediate the specificity of S-nitrosylation sites is currently lacking. Sequences of nearby acidic/basic residues, or a hydrophobic motif, demonstrated to be present in several S-nitrosylated proteins have been identified as possible facilitators of S-nitrosylation (Stefano M Marino and Gladyshev, 2010; Jia et al., 2014). It is possible that these residues enable S-nitrosylation through providing a local three-dimensional environment permissive for the transfer and binding of NO to target cysteines. However these sequences have only been found in a small subset of proteins, and in some cases, for specific chaperon proteins (Jia et al., 2014). A broader consensus sequence has yet to be identified. Our SNO-RAC screen identified 942 unique SNO-sites that were assigned to 360 proteins (Smith et al., 2018). To search for a potential
motif associated with these SNO-sites the SNO peptides sequences were run through Motif-X (motif-x.med.harvard.edu). SNO-peptides that had been aligned to the corresponding protein were analysed, and this revealed four motifs present in 277 of the 849 (32.6%) mapped SNO-peptides, each containing a lysine residue at a different position relative to the central target cysteine (Fig.2.9).

Fig.2.9: Motif-X analysis of S-nitrosylated cysteine containing peptides. Cysteine-containing peptides associated with nuclear SNO-proteins were subjected to SNO-site analysis using Motif-X and the IPI rat proteome as background. Within the graphical representation of the motifs the size of each letter indicates the probability of occurrence of that amino acid in cysteine containing peptides. Summary table displayed for clarity. Lysine motif number 1 has a lysine reside at -2, motif number 2 at +6, motif 3 at -9 and motif 4 at -6, relative to central nitrosylated cysteines. **Motif-X analysis carried out by Dr Jacob Smith.**

I therefore asked whether any of the NuRD subunits were S-nitrosylated at one of these putative SNO-motifs. MBD3 was found to contain SNO-motif 1,
as the lysine reside at K264 is -2 amino acids away from the S-nitrosylated Cys^{266}. To determine whether K264 is necessary for MBD3 S-nitrosylation at Cys^{266}, I generated mutant MBD3 constructs bearing a mutation of Lys^{264} to alanine. Alanine was chosen as the substitute amino acid so that there could be no compensation in terms of chemical properties. I reasoned that if the Lys^{264} residue was required for S-nitrosylation, then mutating this site would have the same effect as the MBD3^{C266S} mutation and would inhibit S-nitrosylation. Therefore, I generated a single MBD3^{K264A} construct, to compare to the single MBD3^{C266S} mutant. I also generated a double mutant construct, MBD3^{C215S/K264A} to compare to the MBD3^{C215S/C266S} construct previously used and which abolished all MBD3 S-nitrosylation.

The MBD3 constructs were transfected into HEK 293T cells and the impact of the K264A mutation on S-nitrosylation of MBD3 was assessed by BST. Strikingly the single K264A mutation, MBD3^{K264A}, was sufficient to reduce MBD3 S-nitrosylation to a greater extent than the mutation of its nearby Cys^{266} alone, (Fig.2.10) confirming its importance for the modification. In line with this result, mutation of Lys^{264} alongside Cys^{215} Serine mutant, MBD3^{C215S/K264A} also reduced the S-nitrosylation of MBD3 to non-detectable levels. This is comparable to the double cysteine mutant MBD3^{C215S/C266S}, confirming that Lys^{264} is as critical for MBD3 S-nitrosylation as the Cys^{266} that is actually targeted by this modification.
Fig. 2.10: Characterisation of a SNO-Motif in MBD3. (A) MBD3 contains the putative SNO-motif number 1 with a lysine at position 264, marked in purple relative to the S-nitrosylated cysteine 266, marked in red. The other S-nitrosylation site C215 is also marked for clarity. (B) Vectors expressing MBD3WT, MBD3C215S, MBD3C266S, MBD3C215S/C266S, MBD3K264A and MBD3C215S/K264A were transfected into HEK 293T cells. After 24 hours, cells were treated with Cys or CysNO (200 µM for 20 min) and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using an anti-Myc antibody. Densitometry analysis was carried out using ImageJ. SNO-signals were normalised to total inputs and expressed as fold change relative to WT + CysNO. n=3 independent experiments. All data shown as mean +/- SEM. One-way ANOVA, compared to ‘WT + CysNO’ column, Fisher’s LSD; *p<0.05. Non-contiguous lanes from the same experiment and blot are shown side by side, as indicated by the separation evident in the figure. Data are from the same experiment as those shown in Fig. 2.8, with additional lanes and densitometry reanalysed for the complete data set.

Four lysine-containing motifs were identified, and whilst I have characterised one target in MBD3, additional data also shows the importance of lysine motif 2 (+6) in the S-nitrosylation of HDAC2.

Cys262 and Cys274 had been previously identified as the sites for HDAC2 S-nitrosylation (Nott et al., 2008), and Cys274 was found to be part of SNO-motif 2, with Lys280 +6 to the central cysteine. I therefore generated HDAC2 mutation constructs, HDAC2K280A and HDAC2C262A/K280A to use alongside the HDAC2WT
and HDAC2\textsuperscript{C262A/C274A} constructs previously generated in the lab. Constructs were transfected into HEK cells in order to determine the impact of the Lys\textsuperscript{280} mutation, and as before, the mutation of the single lysine dramatically reduced HDAC2 S-nitrosylation, comparable to the levels of the HDAC2\textsuperscript{C262A/C274A} mutation (Fig.2.11).

Fig.2.11: Characterisation of a SNO-Motif in HDAC2. (A) HDAC2 contains the putative SNO-motif number 2 with a lysine at position 280, marked in purple relative to the S-nitrosylated Cys\textsuperscript{272}, marked in red. The other S-nitrosylation site Cys\textsuperscript{264} is also marked for clarity. (B) Vectors expressing HDAC2\textsuperscript{WT}, HDAC2\textsuperscript{C262A/C274A}, HDAC2\textsuperscript{C262A/K280A} or HDAC2\textsuperscript{K280A} were transfected into HEK 293T cells. After 24 hours, cells were treated with Cys or CysNO (200 µM for 20 min) and harvested for BST. Isolated proteins and total inputs were separated by SDS-PAGE then immunoblotted using an anti-Myc antibody. Densitometry analysis was carried out using ImageJ and was performed by Dr J Smith. SNO-signals were normalised to total inputs and expressed as fold change relative to WT + CysNO. n=2 independent experiments. All data shown as mean, with individual experiments also shown.

This data shows for the first time that a single lysine located nearby to a target cysteine is required to promote S-nitrosylation, however the mechanism by which these lysine motifs facilitate modification by S-nitrosylation remains to be further
investigated. These critical lysines may assist in the binding of NO to the cysteine thiol or regulate crosstalk between S-nitrosylation and other post-translational modifications such as lysine methylation, acetylation and ubiquitination. Of note, bioinformatic analysis indicated that cysteines near an ubiquitinated lysine are more likely to be S-nitrosylated (Fowler et al., 2017).

2.2 NO regulates NuRD subunit interactions

Having established that multiple NuRD subunits are able to be S-nitrosylated I wanted to determine the impact of this modification on the structure of the complex as a whole. Given that the CHDs undergo a developmentally regulated subunit switch, and so I investigated whether S-nitrosylation regulates subunit interactions with the other components of the NuRD complex. I performed co-immunoprecipitation (CoIP) of the three CHDs on rat cortical neurons exposed to the CysNO donor for either 5 or 20 minutes. In order to preserve S-nitrosylation modifications, CoIPs were carried out in dark conditions as for BST. I treated the neurons for 20 min as this was the time used to identify the S-nitrosylation of endogenous RBBP7 and 4 in cortical neurons. The 5 min treatment was chosen to see if there were more immediate SNO signalling dynamics involved in NuRD complex regulation, as SNO signalling can have different timescales, depending on source of NO and proteins involved (Nott et al., 2008; Kelleher et al., 2011).
2.2.1. CHD3 interaction with RBBP7 increases with CysNO treatment

Following Cys or CysNO treatment of the neurons I performed a CoIP using a CHD3 antibody. The samples were then processed for western blot analysis and blotted for the key NuRD subunits. Interestingly treatment of neurons with CysNO for 5 min seems to lead to an increase in interactions between RBBP7 and CHD3, although this is not statistically significant (Fig.2.12).

![Western Blot Analysis](image)

**Fig.2.12:** S-nitrosylation regulates the interaction of RBBP7 with CHD3. E17 rat cortical neurons were cultured for 4 days, treated with 200 μM CysNO or Cys for 5 or 20 minutes and subjected to co-immunoprecipitation using an antibody against CHD3. As a control, pooled samples (of Cys and CysNO) were subjected to immunoprecipitation using an IgG antibody. Western blot analysis was carried out on eluates and inputs using indicated antibodies. Densitometry analysis of each timepoint Cys only samples are normalised to CysNO treated samples of corresponding timepoint. (n=3 independent experiments, unpaired t-test.)
Strikingly RBBP7 was the only subunit to change interactions with CHD3 as a consequence of CysNO treatment, as the interactions with other NuRD subunits remained the same across conditions (Fig.2.13).

Fig.2.13: Other NuRD subunits interactions with CHD3 do not change upon CysNO treatment. Full densitometry analysis of co-immunoprecipitated NuRD subunits shown in relation to co-immunoprecipitated CHD3 (n=3 independent experiments, unpaired t-test). Analysis carried out as for RBBP7 graph shown above. Densitometry was carried out by normalising proteins pulldown signal to inputs, then dividing this value by the pulldown signal for CHD3. For each timepoint Cys only samples are normalised to CysNO treated samples of corresponding timepoint.

2.2.2 CHD5 interactions with HDAC2 and RBBP4 change with CysNO treatment

I next investigated the effect of CysNO treatment on the interaction of CHD5 with other NuRD subunits. As before neurons were treated with CysNO or Cys for 5 or 20 min and then the samples were subjected to co immunoprecipitation with an antibody against CHD5.

The interaction between CHD5 and HDAC2 again seems to increase with CysNO treatment of 5 minutes, however this is not yet a significant change. I also
observed a change in the interactions between RBBP4 and CHD5, with a significant decrease in interaction seen after 20 min treatment of the CysNO donor (**Fig.2.14**). Of note, there was no change in RBBP7-CHD5 interaction at either timepoint, or of interaction of CHD5 with the remaining NuRD subunits (**Fig.2.15**). Therefore S-nitrosylation of CHD3 has a different impact on interactions with other NuRD complex members to S-nitrosylation of CHD5.

**Fig.2.14**: S-nitrosylation regulates the interaction of CHD5 with RBBP4 and HDAC2. After 4 days in culture E17 rat cortical neurons were treated with 200 μM CysNO or Cys for 5 or 20 minutes and subjected to co-immunoprecipitation using an antibody against CHD5. IgG control was again taken from pooled samples. Western blot analysis was carried out using indicated antibodies. Cys only samples are normalised to CysNO treated samples of corresponding timepoints for densitometry analysis. (n=3 independent experiments, **p<0.01, unpaired t-test).
Fig.2.15. Other NuRD subunits do not change interaction with CHD5 due to CysNO treatment. Full densitometry analysis of co-immunoprecipitated NuRD subunits shown in relation to co-immunoprecipitated CHD4 (n=3 independent experiments, unpaired t-test). Densitometry was carried out by normalising proteins pulldown signal to inputs, then dividing this value by the pulldown signal for CHD4. For each timepoint Cys only samples are normalised to CysNO treated samples of corresponding timepoint.

2.2.3 CHD4 interaction with RBBP7 increases with CysNO treatment

I also investigated the effect of CysNO treatment on the interaction of CHD4 with other NuRD subunits. As before neurons were treated with CysNO or Cys for 5 or 20 min and then samples were subjected to co immunoprecipitation with a CHD4 antibody. Interestingly the interaction between CHD4 and RBBP7 increased when samples were treated with CysNO for 20 min (Fig.2.16). This is the same change in interactions as seen for CHD3, although RBBP7-CHD3 increased only at the shorter exposure time, whereas CHD4-RBBP7 increased only at the later timepoint.
Fig. 2.16: S-nitrosylation regulates the interaction of RBBP7 with CHD4. After 4 days in culture E17 rat cortical neurons were treated with 200 μM CysNO or Cys for 5 or 20 minutes and subjected to co-immunoprecipitation using an antibody against CHD4. IgG control was again taken from pooled samples. Western blot analysis was carried out using indicated antibodies. Cys only samples are normalised to CysNO treated samples of corresponding timepoints for densitometry analysis. (n=3 independent experiments, **p<0.01, unpaired t-test.)

As for CHD3, none of the other NuRD subunits displayed a change of interactions with CHD4 after CysNO treatment (Fig. 2.17).
Fig. 2.17. Other NuRD subunits do not change interaction with CHD4 with CysNO treatment. Full densitometry analysis of co-immunoprecipitated NuRD subunits shown in relation to co-immunoprecipitated CHD4 (n=3 independent experiments, unpaired t-test). Densitometry was carried out by normalising proteins pulldown signal to inputs, then dividing this value by the pulldown signal for CHD4. For each timepoint Cys only samples are normalised to CysNO treated samples of corresponding timepoint.

2.2.4 S-nitrosylation of RBBP7 at Cys166 regulates interaction with CHD4

I next investigated whether the change in interaction between CHD4 and RBBP7 following CysNO treatment could be dependent on the nitrosylation of RBBP7 at the known S-nitrosylation site of Cys166. I overexpressed pCIG-Flag-mCHD4 together with either HA-RBBP7WT or HA-RBBP7C166S in HEK 293T cells. Cells were treated with CysNO donor for 20 min and a CoIP was carried out using an anti-HA antibody for the RBBP7 constructs, followed by western blotting with an anti-FLAG antibody. As for endogenous proteins in cortical neurons, the interaction between CHD4 and WT RBBP7 increased after 20 min CysNO. Excitingly, this increase was abolished in cells expressing the non-nitrosylatable HA-RBBP7C166S construct (Fig. 2.18). This strongly suggests that S-nitrosylation
of RBBP7 Cys\textsuperscript{166} helps regulate the interaction between RBBP7 and CHD4 within the NuRD complex.

<table>
<thead>
<tr>
<th>HA IP</th>
<th>HA-RBBP7</th>
<th>WT</th>
<th>WT</th>
<th>C166S</th>
<th>C166S</th>
</tr>
</thead>
<tbody>
<tr>
<td>FlagCHD4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CysNO</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig.2.18: S-nitrosylation of RBBP7 at Cys\textsuperscript{166} promotes the interaction with CHD4.** HEK293T cells were co-transfected with Flag-mCHD4 and HA-RBBP7WT or HA-RBBP7C166S, and then 24hr post transfection treated with 200 μM CysNO for 20 mins. IP was carried out on eluates using an antibody against HA. Western blot analysis to probe interactions was carried using indicated antibodies. Densitometry analysis of FLAG-CHD4 displayed in relation to HA-RBBP7, normalised to FLAG-CHD4 inputs (n= 3 independent experiments, **p<0.01, unpaired t-test).

These co-immunoprecipitation data demonstrate that S-nitrosylation of NuRD subunits impacts on NuRD assembly and subunit interaction. It has been shown that the complex is formed in a stepwise manner, with small sub-complexes coming together first, and the CHDs joining as a more peripheral subunit added (Kloet et al., 2015; Low et al., 2016; Millard et al., 2016; Brasen et al., 2017). This is in keeping with our evidence of a subunit switch during development (Nitarska et al., 2016) and now my findings illustrate a role for S-nitrosylation in NuRD complex assembly regulation.
2.3. NO and NuRD recruitment to target genes

In order to determine if CHD or NuRD S-nitrosylation could be involved in targeting NuRD complexes to chromatin, I investigated the recruitment of CHDs to known target genes in WT and nNOS knock out cortices (nNOS-/-). I reasoned that if NO or S-nitrosylation influenced their binding, the recruitment of CHDs to chromatin would be different with and without nNOS. The target genes selected were the neuronal progenitor genes Sox2 and Tbr2, which are known to be bound and activated by CHD4 NuRD in neural progenitors, and then bound and repressed by CHD3 NuRD in post-mitotic neurons (Nitarska et al., 2016). These targets were initially identified in a cell culture system, using neural progenitor cells (NPCs) and post-mitotic neurons (PMNs) differentiated from dissociated E12.5 cortex. The NPCs approximately correspond to cells from E12.5, whilst PMNs are the equivalent of the E18.5 stage of cortical development (Nitarska et al., 2016). I also studied CHD binding at neuronal migration genes ApoER and Dcx, which have been established as CHD5 targets and mediate neuronal migration in post-mitotic neurons. A gene free region of chromosome 19 (NEG) was used as a negative control (Nitarska et al., 2016).

2.3.1. CHD recruitment to target genes in E15.5 cortices

E15.5 WT and nNOS-/- cortices were processed for Chromatin Immunoprecipitation (ChIP) using antibodies against CHD3, CHD4 and CHD5, and the interaction with known targets analysed by RT-qPCR (Fig.2.19).
Fig. 2.19: Analysis of CHD3, 4 and 5 binding to target genes in E15.5 cortex. CHD binding was analysed at the Sox2, Tbr2, ApoER and Dcx gene promoter regions in E15.5 WT and nNOS -/- cortices. The gene free region of chromosome 19 was used as a negative control for background binding. Cortices were lysed and processed for ChIP using antibodies against CHD3, 4 and 5. Binding to gene regions measured by RT-qPCR, and data normalised to input and displayed as fold over IgG, mean ± SEM (n=2). Dashed line indicated average of Neg region binding of WT and nNOS-/- . Two-way ANOVA performed but all values ns.

At E15.5 there appears to be a potential trend whereby binding of CHD3 and CHD4 increases at some target genes in the nNOS-/- cortices. This could suggest that NO is required to prevent or reduce CHD binding at these gene regions. However, these results are preliminary and further experiments will be necessary to confirm these initial findings.

In WT cortices, CHD3 appeared not to bind to the selected progenitor genes, nor the migration genes, which is in line with previous findings (Nitarska et al., 2016). However, in nNOS-/- cortices CHD3 binding at the Sox2, Tbr2 and Dcx targets seems to increase, suggesting that nNOS and CHD3 S-nitrosylation
could prevent incorrect recruitment of CHD3 containing NuRD at this stage, since CHD3 containing NuRD is not bound at these targets in WT cortex.

In WT cortices, I did not detect CHD4 to progenitor or migration target genes. CHD4 binds and activates Sox2 in NPCs, (Nitarska et al., 2016), suggesting it may dissociate upon early differentiation. In nNOS-/− cortex, CHD4 binding at Sox2 seemed to increase, suggesting that NO conditions could be required to remove activated CHD4-NuRD from target genes upon differentiation. CHD4 binding to ApoER and Dcx also increases in nNOS-/− conditions. As these have not been identified as a CHD4 target gene during this stage of development, again it may be possible that NO conditions ensure the specificity of CHD4 binding.

In contrast to CHD3 and CHD4, binding of CHD5 to target genes did not seem to change between WT and nNOS-/− conditions, so it could be possible that NO and S-nitrosylation does not regulate CHD5 targeting to chromatin at this timepoint. More robust binding of CHD5 to ApoER and Dcx might have been expected, as they have been previously identified as CHD5 targets in PMNs. This lack of robust enrichment may indicate technical or antibody problems or be due to the highly heterogenous nature of cell types present in the cortex, in comparison to the homogenous PMN cell culture system.

2.3.2 CHD recruitment to target genes in E18.5 cortices

E18.5 WT and nNOS-/− cortices were processed as for E15.5 and recruitment of CHD3,4 and 5 to the same target genes analysed (Fig.2.20).
Fig. 2.20: Analysis of CHD3, 4 and 5 binding to target genes in E18.5 cortex. CHD binding to Sox2, Tbr2, ApoER and Dcx gene promoter regions was analysed in E18.5 WT and nNOS−/− cortices. The gene free region of chromosome 19 was used as a negative control for background binding. Cortices were lysed and processed for ChIP using antibodies against CHD3, 4 and 5. Binding to gene regions measured by RT-qPCR, and data normalised to input and displayed as fold over IgG, mean ± SEM (n = 3). Dashed line indicated average of Neg region binding of WT and nNOS−/−. Two-way ANOVA performed but all values ns.

In E18.5 cortices, CHD3, but not CHD4 or CHD5, binding to target genes appears to potentially increase between WT and nNOS−/− genotypes. This suggests that NO is involved in regulation of CHD3 binding to chromatin, throughout development.

CHD3 seems to bind to Sox2 and Tbr2 in WT cortex, which is in keeping with CHD3-containing NuRD complexes targeting and silencing these genes in differentiated PMNs (Nitarska et al., 2016). In nNOS−/− cortex, CHD3 may bind to Sox2 and Tbr2 with higher enrichment, but the data is highly variable. This
supports the idea that S-nitrosylation of CHD3 prevents aberrant binding to chromatin.

CHD4 is not robustly enriched at any of the target genes in either WT or nNOS-/− cortex, as expected since CHD4 is not incorporated into NuRD complexes at this stage, and is no longer found at these targets in WT PMNs (Nitarska et al., 2016). Likewise, CHD5 binding of the progenitor and migration genes is low in both WT and nNOS-/− conditions, again consistent with the established findings that CHD5-containing NuRD are no longer the predominant NuRD complex at this late stage of development.

These initial data suggest that at E15.5, NO signalling may be involved in regulating the correct binding of CHD3 and CHD4 to target genes, since enrichment of these remodellers at target genes is enhanced in nNOS-/− cortices (Fig.2.19). Preliminary results also could indicate that NO and S-nitrosylation may have an impact on CHD3 binding later in development at E18.5 (Fig.2.20), when CHD3-containing NuRD is the predominant complex. It should be noted however that these findings are preliminary, and further experiments will be necessary to further investigate the role of NO in regulating CHD targeting to chromatin.

2.4. NO and NuRD chromatin remodelling

I also investigated whether NO and S-nitrosylation could be involved in regulating CHD remodelling activity though DNase hypersensitivity assays, using the WT and nNOS-/− cortices. In this assay, chromatin that is open and accessible is sensitive to digestion by the DNasel enzyme, and therefore these sites are less able to be detected by RT-qPCR. I reasoned that if the remodelling activity of the
CHDs was regulated by NO signalling, then chromatin accessibility, as assessed by DNase digestion would be different in WT and nNOS-/− cortices.

2.4.1. DNaseI hypersensitivity in E15.5 cortices

Cortices were analysed at E15.5, since my previous ChIP data indicated this could be the stage NO signalling had the most impact. DNaseI hypersensitivity was measured by RT-qPCR using primers for the same CHD targets used in the ChIP assay (Fig.2.19, Fig.2.20). As a negative control I used follicle-stimulating hormone (Fsh NEG), a gene that is not expressed in neurons and known to be resistant to DNaseI digestion (Policarpi et al., 2017).

![DNaseI hypersensitivity changes in E15.5 WT and nNOS-/− samples.](image)

Fig.2.21: DNaseI hypersensitivity changes in E15.5 WT and nNOS-/− samples. Cortices were lysed and treated with DNaseI (1 unit for 20 min, 1U) or untreated (0U). Histograms show DNaseI digestion efficiency at target genes and negative regions (FSH NEG) and expressed as fold over undigested. Data are represented as mean ± SEM (n = 3). Two-way ANOVA performed but all values ns.
My results suggest that NO has a potential impact on chromatin remodelling, with genes that are accessible in WT cortices becoming more resistant to DNAseI digestion, and therefore inaccessible, in nNOS-/- cortex.

In E15.5 WT cortex, Sox2 appeared to be inaccessible to digestion. This finding is consistent with my ChIP data (Fig.2.19) and with previous findings in NPCs (Nitarska et al., 2016). Sox2 seems to remain inaccessible in nNOS-/- samples, although the data are highly variable (Fig.2.21). Conversely, Tbr2 appears to be accessible in the WT conditions and inaccessible in nNOS-/- conditions. This inaccessibility could correspond to the increased binding of CHD3 at Tbr2 as seen in nNOS-/- cortex (Fig.2.19), as CHD3 represses Tbr2 expression (Nitarska et al., 2016). It is therefore difficult to separate the effect of NO on the binding of CHD3 versus its remodelling activity, although the repressive remodelling activity of CHD3 appears to be maintained in nNOS-/- cortex.

ApoER and Dcx both appear to show accessibility in WT conditions, in keeping with previous findings that identified these genes as bound and activated by CHD5 in PMNs (Nitarska et al., 2016). Both these targets however could be seen as becoming more resistant to DNAaseI, and inaccessible in nNOS-/- cortex. This inaccessibility could be due to reduced CHD5 binding at these targets in the absence of nNOS, although this was not found in the preliminary ChiP data (Fig.2.19). Therefore, the inaccessibility in nNOS-/- cortex may be due to a requirement for NO signalling to regulate CHD5 remodelling and activation of the target genes.
Together, these initial ChIP and DNase experiments may indicate that during the mid-stage of cortical development (E15.5) NO and S-nitrosylation could possibly be involved in CHD recruitment and remodelling. It is possible that NO signalling may be involved in the regulation of CHD3 and CHD4 recruitment to target genes, but not their remodelling activities. In contrast, NO signalling may not impact CHD5 binding but instead be required for CHD5 remodelling activity.

However, these data are not statistically significant and further repeats of these experiments are required to confirm if these preliminary findings are indeed indicative of a role for NO and CHD S-nitrosylation in regulating chromatin remodelling.

2.5 S-nitrosylation regulates NuRD subunit function

Having identified a role for RBBP7 S-nitrosylation in regulating NuRD interactions, I wanted to see if this modification also had biological significance during neuronal differentiation. I investigated if S-nitrosylation of RBBP7 at Cys\textsuperscript{166} was required for neuronal development and morphogenesis. Dendritogenesis is the process through which neurons respond to extracellular cues and signalling pathways to extend dendrites that will be necessary to establish synaptic contact with other neurons. Dendritogenesis is regulated by a number of signalling pathways, including \textit{Ca}\textsuperscript{2+} signalling and transcriptional regulation (Wu et al., 2007; Jan and Jan, 2010). NO signalling is also known to be important during this process, and the S-nitrosylation of HDAC2 at Cys\textsuperscript{262} and Cys\textsuperscript{274} enhances dendritogenesis (Nott et al., 2008). In this assay neurons are treated with KCl to induce neuronal depolarisation and which increases dendritic growth and
branching. Arbor complexity is quantified through Sholl analysis (imagej.net/Sholl_Analysis) which provides a read out of the number of branch points located at different distances from the soma.

2.5.1 Single knockdown of RBBP7 does not affect dendritogenesis

In order to determine the importance of RBBP7 in *in vitro* dendritogenesis I first validated an siRNA that targets endogenous RBBP7 in Neuro-2a cells (**Fig.2.22**).

**Fig.2.22: RBBP7 knockdown in Neuro-2a cells.** Neuro-2a cells were transfected with a GFP expression vector together with 200nM of either control siRNA (CTL) or siRNA against (siRBBP7). Cells were harvested 48 hr after transfection and RBBP7 levels analysed by western blot using ImageJ. RBBP7 signal was normalised to tubulin and GFP. ****p<0.0001 unpaired t-test. n=3 independent experiments.

I transfected mouse cortical E15.5 neurons with either the control (CTL) or RBBP7 siRNA. Three hours after transfection, neurons were treated with PBS (control) or KCl for 48 hours. Cells were fixed and blinded prior to imaging and Sholl analysis. The Sholl analysis indicated that the knockdown of RBBP7 did not affect dendritogenesis (**Fig.2.23**). Both siCTL and siRBBP7 transfected neurons responded well to the KCl treatment with a comparable increase in neuronal branching and complexity, indicative of normal dendritogenesis.
**Fig.2.23: RBBP7 knockdown and dendritogenesis analysis.** Phenotype of RBBP7 knockdown on activity-induced dendritogenesis in cortical neurons. Control siRNA (CTL) or siRNA against RBBP7 (siRBBP7) was transfected into E15.5 mouse cortical neurons alongside a GFP expression vector and mycEV. Neurons were maintained in normal media (Ctl) or in the presence of 50mM KCl (KCl) for 2 days then immunostained using an anti-GFP antibody. Images were analysed using Fiji Sholl plugin. Maximal projections show representative neurons for each condition. Scale bar 100μM. Summary of Sholl data for each condition is shown. 3 biological replicates were carried out and 10 neurons analysed per experiment (30 neurons in total). Shown are the mean values for number of intersections (y axis) against distance from soma (x axis; μm). Readings were taken every 10μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-way ANOVA with Sidak’s test for multiple comparisons. Red stars indicate KCl value is significantly greater than Ctl. Slides were blinded prior to imaging and Sholl analysis (performed by Dr Smith) and then data de blinded and collated.

**2.5.2 RBBP7 Cys^{166} is required for normal dendritogenesis in vitro.**

We considered the possibility that RBBP4, which is a close homolog of RBBP7 frequently found in the same complexes and is S-nitrosylated at a conserved cysteine (Fig.2.4, 2.5), could be compensating for the lack of RBBP7. Therefore, I carried out a double knock down of both RBBP7 and RBBP4, first in
Neuro-2a cells (Fig.2.24A). I also generated siRNA resistant constructs of both the RBBP7\textsuperscript{WT} and RBBP7\textsuperscript{C166S} constructs (siResRBBP7\textsuperscript{WT} and siResRBBP7\textsuperscript{C166S}), in order to perform rescue experiments, and to see if any potential phenotype could be due to S-nitrosylation defects. First, I confirmed in Neuro-2a cells that these constructs were siRNA resistant and expressed to the same level in Neuro-2a cells (Fig.2.24B).

**Fig.2.24: Confirmation of RBBP4/7 double knockdown.** Neuro-2a cells were co-transfected with GFP expression vector and 400 nM total RNA of either control siRNA (CTL) or siRNA’s against RBBP4 and RBBP7 in a 1:1 ratio (siRBBP4/7). Cells were harvested 48 hr post transfection and analysed by western blot. Western blot of RBBP7, RBBP4 and HSP90 is shown. Densitometry analysis of western blots carried out using ImageJ. RBBP7 and RBBP4 levels were normalised to HSP90. All data are shown as mean +/- SEM. **p<0.01, unpaired t-test (n=3 independent experiments). (B) Expression of siRNA-resistant myc-RBBP7\textsuperscript{WT} RBBP7\textsuperscript{C166S}. Neuro-2a cells were transfected with either myc-EV, siRNA-resistant myc-RBBP7\textsuperscript{WT} or RBBP7\textsuperscript{C166S} and siRNA CTL or the double knockdown siRBBP4/7. 48 hr after transfection cells were harvested and levels of RBBP7, RBBP4, HSP90 analysed by Western blot. RBBP7 levels in RBBP7\textsuperscript{WT} and RBBP7\textsuperscript{C166S} conditions were quantified by densitometry, using HSP90 as a loading control. ns= not significant (unpaired t-test, n=3 independent experiments). Data are shown as mean +/- SEM.
Neurons were transfected with siCTL or siRBBP7/4 to knock down both RBBP7 and RBBP4. The siRNAs were co-transfected alongside either an empty mycEV vector, siResRBBP7\textsuperscript{WT} or siResRBBP7\textsuperscript{C166S} and after 48 hours treatment with KCl, neurons were fixed and dendritogenesis analysed. Strikingly, the double knock down of both RBBP7 and RBBP4 induced a marked phenotype, with neurons having impaired dendritogenesis and failing to respond to the KCl treatment (Fig.2.25). This was confirmed by the branching analysis, as siRBBP7/4-treated neurons showed significantly reduced branching around the soma after KCl treatment, compared to control conditions (Fig.2.26).

Importantly, the dendritogenesis defects observed in siRBBP7/4 conditions were rescued by co-transfection of the siResRBBP7\textsuperscript{WT} construct, whereas siResRBBP7\textsuperscript{C166S} that could not be S-nitrosylated did not rescue this deficit (Fig.2.25-26). These findings indicate that S-nitrosylation of RBBP7 at Cys\textsuperscript{166} is required for normal dendritogenesis \textit{in vitro}, further illustrating the biological significance of NuRD subunit S-nitrosylation in neuronal differentiation and function.
Fig.2.25: S-nitrosylation of RBBP7 promotes dendritogenesis in cortical neurons. Control siRNA (CTL) or siRNAs against RBBP4 and RBBP7 (siRBBP4/7) were transfected into E15.5 mouse cortical neurons alongside a GFP expression vector and EV, siRNA-resistant myc-RBBP7WT or myc-RBBP7C166S as shown. Neurons were maintained in control conditions (Ctl) or exposed to 50 mM KCl (KCl) for 2 days and immunostained for GFP. Maximal projections show representative neurons for each condition. Scale bar: 100 μm.
Fig. 2.2: Sholl analysis for RBBP7/4 knock down and myc-RBBP7WT or myc-RBBP7C166S rescue experiments. Control siRNA (CTL) or siRNAs against RBBP4 and RBBP7 (siRBBP4/7) were transfected into E15.5 mouse cortical neurons alongside a GFP expression vector and EV, siRNA-resistant myc-RBBP7WT or myc-RBBP7C166S. Images were analysed using FIJi Sholl plugin, analysis performed by Dr Smith. 3 biological replicates were carried out in which 10 neurons were analysed per experiment (30 neurons in total). Shown are mean values for number of intersections (intersections) against distance from the soma (μm). Readings were taken every 10 μm. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Red stars indicate KCl value is significantly greater than Ctl, black stars indicate Ctl value is significantly greater than KCL. Means two-way ANOVA with Sidak’s test for multiple comparison.
3. Discussion

Regulation of neuronal and non-neuronal gene expression is essential for the correct development and function of the mammalian cortex. One way to control this process is through the activity of chromatin remodelling complexes such as NuRD, which alter the epigenetic landscape of the gene and affect the accessibility of the DNA to transcription machinery. Importantly, NuRD has mutually exclusive subunits resulting in distinct complexes with specific functions during cortical development (Hendrich and Bird, 1998; Schultz et al., 2001; Fujita et al., 2003; Le Guezennec et al., 2006). Therefore, the regulation of NuRD assembly itself is fundamental to its function and to gene regulation. However, the mechanisms regulating this combinatorial assembly of NuRD are not well studied.

The sequential switch of CHD3, CHD4 and CHD5 in NuRD is regulated at least in part, by the relative expression levels of each CHD. For example, CHD4 expression is detected in cortices at E12.5, when there is no expression of CHD3 or CHD5 (Nitarska et al., 2016). However, this does not explain how at later timepoints, when all three CHDs are expressed (E18.5), only one CHD is incorporated into NuRD. Importantly, each CHD-containing NuRD is recruited to specific genes with different transcriptional outcomes, and therefore there is likely to be an as yet undiscovered mechanism that controls the specificity of NuRD activity. S-nitrosylation is a post-translational modification with established roles during neuronal and cortical development that may control protein-protein interaction, protein-chromatin interactions, and enzymatic activity (Mannick et al., 1999; Gu et al., 2002; Nott et al., 2008; Kornberg et al., 2010). NuRD subunits
were first identified as potential targets of S-nitrosylation in our screen of rat cortical neuronal nuclear extracts (Smith et al., 2018) and my data now indicates that SNO-modification of NuRD could contribute to the regulation of NuRD subunit interactions and function.

**S-nitrosylation regulates NuRD subunit interactions**

My data demonstrated that CHD3, CHD4 and CHD5 are able to be S-nitrosylated (Fig.2.7), supporting the hypothesis that SNO modifications are involved in regulating the CHD subunit switch (Nitarska et al., 2016; Smith et al., 2018). Furthermore, my data show that S-nitrosylation regulates the interactions between the CHDs and other NuRD subunits. I observed changes in CHD5 binding to RBBP4 and possibly also HDAC2 (Fig.2.14), changes in CHD3 and RBBP7 interactions (Fig.2.12) and between CHD4 and RBBP7 (Fig.2.16). Importantly, I have been able to identify Cys\(^{166}\) of RBBP7 as important for mediating the interaction between CHD4 and RBBP7 (Fig.2.18).

What is intriguing is the consequence of this alteration in CHD-subunit interactions, and how it could have a wider impact on NuRD assembly. Not all subunits change interactions with a CHD under S-nitrosylation conditions (Figs.2.13, 2.15, 2.17) and the three CHDs seem to display different dynamics and behaviours as a consequence of S-nitrosylation. This could be due to differences in the levels of available NO, which are known to increase during the different stages of cortical development (Bredt and Snyder, 1994, Nitarska and Riccio observations). Alternatively, these differences could be due to distinct cysteines on each CHD being targeted by the SNO-modification, which could lead to different changes as a consequence of S-nitrosylation.
nNOS is not expressed at the early stages of cortical development, (Fig.1.11) (E12.5) when CHD4 is expressed and incorporated into the NuRD complex (Nitarska et al., 2016), but is expressed during later stages when CHD4 is no longer the main CHD within NuRD complexes. It is possible that a more stable RBBP7-CHD4 subcomplex is generated in response to the S-nitrosylation of one or both factors, and this could lead to the sequestering of CHD4 away from NuRD during the later developmental stages, thus allowing for the subunit switch and sequential addition of the other CHDs. In addition, CHD4-NuRD complexes have been shown to be involved in additional processes such as DSB repair (Larsen et al., 2010; Smeenk et al., 2010) and this NO dependent sub-complex could be recruited to these alternative functions.

CHD5-containing complexes are the predominant NuRD assemblies at E15.5, when nNOS expression has started to increase and NO is accumulating (Bredt and Snyder, 1994, Nitarska and Riccio observations). CHD5 interactions with HDAC2 potentially increase with CysNO, and the interaction between RBBP4 and CHD5 decreased. The decrease in RBBP4-CHD5 interaction could indicate a CHD5-RBBP4 subcomplex disassembly, either through the disassociation of CHD5 or RBBP4. The subcomplex may have been involved in the recruitment of CHD5 to the NuRD complex at this time, but once part of the larger complex, the CHD5-RBBP4 interaction is weakened. The change in CHD5 interaction with HDAC2 could likewise be due to an initial increase in binding between the proteins as CHD5 is incorporated into the NuRD complex.

Although with a different dynamic, S-nitrosylating conditions appear to also affect the interaction of CHD3 and RBBP7. CHD3-containing complexes are the
main NuRD complex present at later developmental stages of neuronal migration and differentiation (E18.5) (Nitarska et al., 2016). nNOS expression and NO production is at its peak during this stage, E18.5 (Bredt and Snyder, 1994, Nitarska and Riccio observations). Here S-nitrosylation of NuRD could be required for incorporation of CHD3 into NuRD, and the quick formation of a stable RBBP7-CHD3 complex could enable the efficient delivery of CHD3 to NuRD at this stage. These possible dynamics could be further validated and investigated through detection of endogenous CHD S-nitrosylation at the E12.5, E15.5 and E18.5 developmental stages.

The identification of Cys^{215} and Cys^{266} as the S-nitrosylation sites for MBD3 could also have important implications for NuRD subunit interactions and complex assembly. Cys^{215} and Cys^{266} are within the binding domain for the GATAD2 subunits, and S-nitrosylation modifications at this site could either prevent or enable interactions between MBD3 and GATAD2A/B. Furthermore, since MBD3 is known to be essential for NuRD assembly and function (Kaji, Nichols and Hendrich, 2007) changes in MBD3-NuRD interactions could affect assembly of the complete complex.

**S-nitrosylation affects NuRD subunit function**

The S-nitrosylation of endogenous RBBP7 by KCl-dependent depolarisation (Fig.2.3, Fig.2.6), suggests that S-nitrosylation of RBBP7 could be part of the neuronal response to activity. I found that RBBP7 regulates dendritogenesis in vitro, and that mutation of Cys^{166} attenuates depolarisation-dependent dendritogenesis (Fig.2.25). Dendritogenesis is an important process during cortical development, and the establishment of arbor complexity is an
essential step in neuronal terminal differentiation and maturation. Once in the correct layers dendritogenesis enables cortical neurons to establish the connections that are required for neuronal complexity and function (Wu et al., 2007; Barnes and Polleux, 2009; Jan and Jan, 2010). Calcium dependent transcription of CREST target genes has also been demonstrated to regulate neuronal morphogenesis, and illustrates how the response to external cues such as depolarisation, is essential for proper, functional, dendritogenesis (Aizawa et al., 2004). This role for SNO-RBBP7 in in vitro dendritogenesis could extend to roles in vivo during cortical development and may even be implicated in behavioural defects. The cortical neurons used in this experiment were taken from E15.5 cortices, and SNO-RBBP7 could also be required for the correct maturation of mid layer neurons, and the migration of later born neurons at E15.5 (Wu et al., 2007; Barnes and Polleux, 2009; Sokpor et al., 2018).

Dysregulation of dendritogenesis during the early embryonic stages can also have long lasting impacts on brain development and function, and has been shown to contribute to behavioural disorders (Broadbelt, Byne and Jones, 2002; Carrel et al., 2009) and impaired fear response pathways in mice (Wu et al., 2007; Jan and Jan, 2010; Lin et al., 2017). Overexpression of a nNOS interacting protein, NOS1AP, during the early stages of cortical development, led to decreased dendrite number and neuronal migration. NOS1AP has also been linked to the development of schizophrenia, which is characterised by a reduction in dendrite number, demonstrating how impaired dendritogenesis early in development could contribute to disorders later in life (Carrel et al., 2009; Carrel, et al., 2015). Furthermore, the intellectual disability condition Rhett syndrome is
characterised by mutations in the MECP2 protein, which leads to impaired neuronal development and defects in dendritogenesis, again illustrating the importance of this process in maintaining proper brain function (Jentarra et al., 2010). It could therefore be possible that impaired S-nitrosylation of RBBP7 could lead to defects in developmental dendritogenesis, which could subsequently be linked to behavioural disorders in adulthood.

It should be noted that the role of RBBP7 and SNO-RBBP7 in this context may not be due solely to its role within the NuRD complex, as RBBP7 can be found in other chromatin binding complexes, including PRC2 and Sin3a (Roopra et al., 2000; Pereira et al., 2010; Smits et al., 2013; Zhang et al., 2013). A role for SNO-RBBP7 in these complexes could be indicative of a more widespread mechanism, whereby S-nitrosylation of histone binding subunits mediates the activity of a number of chromatin remodelling complexes with diverse and severe consequences of dysregulated S-nitrosylation on the development and function of the nervous system.

**S-nitrosylation as a regulator for the recruitment of NuRD to target genes**

Post-translational modifications have been shown to change the interactions of nuclear proteins with chromatin. Poly-ADP-Ribosylation of ISWI, for example, causes its dissociation from chromatin (Sala et al., 2008; Toto, D’Angelo and Corona, 2014). Furthermore, our laboratory has showed that S-nitrosylation of HDAC2 leads to its dissociation from chromatin (Nott et al., 2008). Thus, S-nitrosylation could also alter the interaction of the CHD proteins with chromatin, and so change the recruitment of NuRD to target genes. This S-nitrosylation dependent regulation of CHD targeting or dissociation from
chromatin could be through S-nitrosylation of the CHDs themselves, or through modification of the RBBP7 and RBBP4 proteins.

I have carried out preliminary ChIP experiments to investigate whether the binding of the CHDs changes when NO and S-nitrosylating conditions are absent using nNOS-/- cortices (Fig.2.19, 2.20). My initial findings indicate that NO signalling is possibly involved in preventing aberrant CHD3 and CHD4 binding to chromatin. As enrichment of these CHDs increases at target gene regions in the absence of nNOS, this could suggest that, similar to HDAC2, NO causes dissociation of CHD3 and CHD4 from chromatin. CHD4 is initially bound at target genes at E12.5, when there is little nNOS expression (Bredt and Snyder, 1994, Nitarska and Riccio, observations) but must be removed from these target genes during development for the correct regulation and silencing of the progenitor genes. A possible mechanism could therefore be that CHD4 is bound and un-nitrosylated at specific genes at E12.5, but as nNOS begins to be expressed and NO accumulates at E15.5, CHD4 becomes S-nitrosylated. This S-nitrosylation subsequently leads to the dissociation of CHD4 containing NuRD from the target genes. NO could also regulate CHD3 binding at chromatin during this stage in development, perhaps preventing premature binding of CHD3 to target genes, but allowing the enrichment of CHD3 at these targets later in development at E18.5. The exact nature of this mechanism would need to be further elucidated but could be determined by the exact CHD3 cysteines targeted by S-nitrosylation or be due to SNO modification of other NuRD subunits.

Although the mechanisms by which CHDs are targeted to chromatin remain to be fully elucidated, RBBP7 and RBBP4 are key chromatin binding
proteins for a number of remodelling complexes, including Sin3a and PRC2, and may perform a similar role for NuRD. RBBP7 and RBBP4 interact indirectly with Sin3a, and are thought to be required for the recruitment of the complete complex to nucleosomes and transcriptional silencing (Grzenda et al., 2009; Kadamb et al., 2013). RBBP7 and RBBP4 also play a key role in targeting the PRC2 complex to chromatin through nucleosome binding (Pereira et al., 2010). The interaction of RBBP7 and RBBP4 with histones has been studied by a number of groups. Murzina et al., demonstrated that RBBP7 binds to the C-terminus of H4, at helix 1 of the histone protein (Murzina et al., 2008). This binding is mediated by a hydrophilic face of the helix and causes a conformational change of H4 as the histone partially unfolds. This histone flexibility may aid binding and recognition of other chromatin factors associated with RBBP7. It has also been demonstrated that RBBP4 interacts with H4 in a similar way, and causing a similar conformational change (Murzina et al., 2008; Zhang et al., 2013). Studies have also indicated that whilst isolated RBBP7 and RBBP4 bind H4 (Zhang et al., 2013), when in complex with other NuRD proteins and specifically MTA, they can only interact with H3. These findings suggest that the interaction with MTA can act as a mechanism for determining histone binding and recruitment to chromatin (Millard et al., 2016). Moreover, RBBP4 and RBBP7 targeting to histone H3 can be regulated by histone post translational modifications. H3 phosphorylation at Ser10 for example, prevents RBBP7 binding, as does symmetric dimethylation of H3Arg2 (Migliori et al., 2012; Klingberg et al., 2015). It is possible that modification by S-nitrosylation also directly influences interactions between
RBBP7, RBBP4 and MTA or histone proteins, thereby acting as a regulator for the interaction of RBBP proteins with chromatin.

**S-nitrosylation as a regulator for CHD remodelling activity**

In addition to regulating protein-protein, or protein-chromatin interactions, it is understood that S-nitrosylation can impact the enzymatic activity of target proteins (Cho *et al.*, 2009; Anand and Stamler, 2012; Nakamura *et al.*, 2015; Cui *et al.*, 2018; Mitchell *et al.*, 2018). I hypothesised that S-nitrosylation could potentially impact on the core remodelling activity of the CHDs, if the CHDs were still properly targeted in the absence of NO. In order to begin to investigate this I carried out DNAse hypersensitivity assays to compare the chromatin accessibility of CHD targets in WT and nNOS-/- cortices (Fig.2). I observed that during cortical development at E15.5, the chromatin accessibility of specific progenitor and migration target genes decreases when nNOS is absent. Although these findings are still preliminary, they demonstrate that NO and S-nitrosylation could be involved in maintaining an open chromatin landscape that is necessary for gene expression and NuRD functions at these sites. More sensitive assays, such as ATAC sequencing, which reveals chromatin accessibility genome-wide (Buenrostro *et al.*, 2015), could be employed to further probe the differences in chromatin landscape at NuRD targets between WT and nNOS-/- mice.

It has been proposed that CHDs belonging to the same subfamily (CHD6, CHD7, CHD8 from subfamily III for example) may have distinct remodelling mechanisms, and different linker DNA requirements (Manning and Yusufzai, 2017). Studies performed using *in vitro* assays have indicated that CHD3 and CHD4 have distinct mechanisms. Although both proteins function as remodellers
by sliding nucleosomes, regardless of linker DNA length, the final nucleosome positioning is different between the two, which results in a different chromatin landscape at their respective target genes (Hoffmeister et al., 2017), and thus could result in the different transcriptional outputs. However, very little has been investigated regarding the CHD5 remodelling mechanism, and it would be important to compare this alongside the mechanisms for CHD4 and CHD3.

The consequences of S-nitrosylation on SNO-protein structure

As S-nitrosylation of NuRD can impact on NuRD interactions and possibly even remodelling activity and recruitment, a fundamental question that remains is how the modification is eliciting these changes, and, importantly, how labile the modification is.

The NO-thiol bond is unstable and the longevity of the bond varies, with a wide range of reported SNO-P lifetimes (De Oliveira et al., 2002; Hu and Chou, 2006; Paige et al., 2008). S-nitrosylation is stable and long-lasting in many cases, which may be due to the topology of the amino acids surrounding the modified cysteine providing a protective environment for the SNO modification and preventing further modifications or reduction (Paige et al., 2008). However, in some cases the NO-thiol bond is very labile and easily reduced. This then raises the question of how S-nitrosylation can affect the stable changes required of a post-translational modification, if the SNO-modification is transient? One possibility is that the SNO modification is an intermediate in the process of establishing a disulphide bond (Broniowska and Hogg, 2012). Wolhuter et al., have indicated that when NO donors are used at a low concentration, the generation of disulphide bonds precedes the formation of NO-thiols, and it is only
at higher concentrations that the SNO modifications take place. These data suggest that the consequence of physiological NO levels is the formation of disulphide bonds, and that it is only when the system is overloaded with NO that SNO modifications become more stable and can be detected (Wolhuter et al., 2018).

Another key observation is the high proportion of overlap between sites of S-nitrosylation modifications and disulphide bonds. Cysteines that undergo S-nitrosylation are also identified as sites for functionally relevant disulphide bonds, including GAPDH Cys150 (Wolhuter et al., 2018). Interestingly, Wolhuter et al., demonstrated that Calpain1, a protease inhibited by S-nitrosylation was also inhibited by oxidising agents that reduced disulphide bonds, suggesting that the inhibition of Calpain1 could act through the breakage of disulphide bonds rather than the disruption of S-nitrosylation alone (Wolhuter et al., 2018).

It would be interesting to examine the relevance of disulphide bonds with respect to my findings and to delineate the role of the two modifications. Further investigation could determine if RBBP7 and RBBP4 contain disulphide bonds as a consequence of depolarisation as well as becoming S-nitrosylated, or if the sites identified as S-nitrosylated could be predicted to form disulphide bonds. The changes in CHD interactions observed as a consequence of S-nitrosylation conditions could be probed further to determine if these interactions were in fact dependent on disulphide bonds. Since the presence of disulphide bonds does not exclude the importance of SNO modification my data still illustrates the importance of S-nitrosylation modifications in regulating the NuRD complex,
regardless of whether the end point of NuRD S-nitrosylation is the formation of disulphide bonds.

S-nitrosylation could also alter the conformation of the subunits. In this regard, it has been shown that S-nitrosylation can lead to structural changes of 0.8 to 1.3 Angstroms (Marino and Gladyshev, 2010). The site of RBBP7 and RBBP4 S-nitrosylation is on a WD propeller, and these domains act as protein binding platforms, and thus any conformational shift on these structures could prevent or enable a number of proteins binding to this domain. Any conformational change to RBBP7/4 might also have wider impact on the whole complex assembly, since the assembly of NuRD has been shown to be stepwise through the association of small sub-modules (Low et al., 2016; Millard et al., 2016; Brasen et al., 2017). Likewise, structural changes to the CHD domains could influence the protein and chromatin binding ability, or the catalytic activity. More detailed crystal structure models of the NuRD subunits, both WT and Cys mutants, or predictive modelling will be invaluable in revealing the potential impact of S-nitrosylation on protein conformation, and the subsequent impact on subunit interactions and complex assembly.

**Specificity of the SNO modification**

Another facet to the modification of NuRD by S-nitrosylation is how these modifications are regulated, and the mechanisms underlying the targeting of specific cysteines. Not all cysteines undergo S-nitrosylation, and the exact cysteine(s) S-nitrosylated is often cell type and context specific. The sequences of S-nitrosylated proteins have been investigated to identify motifs that determine whether or not a cysteine can be S-nitrosylated. However, a conserved linear
motif has not been convincingly identified so far for S-nitrosylation in mammals. Initial analysis indicated the presence of acidic or basic residues near the target cysteine, and it is thought that these residues can contribute to the catalysis of the SNO reaction (Stamler, Toone, et al., 1997; Pérez-Mato et al., 1999). The tertiary sequence, and the 3D arrangement of nearby residues were also found to be important in aiding S-nitrosylation. A sequence of acidic and basic residues distal (8 Angstroms) to target cysteines gave a more reliable indication of target cysteine S-nitrosylation, when compared to the linear sequence (Marino and Gladyshev, 2010).

Despite the bioinformatic analysis confirming the relevance of nearby acid/basic residues and lysines in promoting S-nitrosylation, these motifs have rarely been confirmed experimentally. Work in macrophages identified a specific motif involving iNOS induced by the inflammatory stimuli LDLox/IFN-γ. Jia et al showed that an I/L-X-C-X2-D/E motif was necessary and sufficient to guide SNO modification, as mutation of the leucine and glutamine prevented S-nitrosylation of the target protein. However, this motif was demonstrated to be specific to S-nitrosylation mediated by iNOS when in a complex with the chaperone and transnitrosylase S100A8/A9, and is likely to be relevant only for the limited number of proteins targeted by this complex (Jia et al., 2014).

Our screen identified four lysine containing motifs in 33% of our S-nitrosylated proteins with mapped SNO-peptides, with lysines at positions -9, -6, -2 and +6 (Fig.2.9) (Smith et al., 2018). Interestingly lysine residues surrounding SNO-cysteines are over represented in both a WT and an Alzheimer’s model mouse (Seneviratne et al., 2016), and two lysine motifs, with Lys at +4 and +6
were identified in a screen characterising the SNO proteome of heart homogenates (Kohr et al., 2011). Strikingly I demonstrated that one of our motifs, lysine motif 2 (+6) was required for the S-nitrosylation of MBD3 (Fig.2.10). SNO motif 2 is also present in HDAC2, and again the mutation of the lysine residue (+6 to Cys274) prevented HDAC2 S-nitrosylation (Fig.2.11) (Smith et al., 2018). Of note, CHD3 also contains SNO motif 2 for the peptide CTCPVLK. Confirming this site and the requirement of the lysine in CHD3 modification by S-nitrosylation (Cys332 and Lys338 in rat and Cys500 and Lys506 in human) experimentally would provide further evidence for the importance of this motif in mediating S-nitrosylation. Furthermore, it will be interesting to further characterise how precise the lysine positions are, if the lysine must always be at the exact positions found; -9, -6, -2 and +6 (Fig.2.9), or if the lysine merely needs to fall within a certain distance of a cysteine (between -9 to +6, for example). The current motif analysis was carried out on SNO-peptides of 21 amino acids in length (Smith et al., 2018). It might be relevant now to extend this parameter to account for residues located distally in sequence, but proximal in space. Encouragingly, whilst the positions do not fall within a precise motif, all the common CHD putative sites are in relative proximity to lysine residues, and RBBP7 and RBBP4 SNO-peptides contain lysines at -7 (Smith et al., 2018). Structural models and the tertiary structure of the proteins would enable us to establish if these more distal lysines would be spatially proximal to the target cysteine and thus able to influence the S-nitrosylation modification. Moreover, additional lysine containing motifs may be identified by analysing other SNO-P screens performed in different tissues.
How a nearby lysine could guide S-nitrosylation also remains to be investigated. The presence of a nearby lysine does not necessarily render every cysteine S-nitrosylatable but may represent a critical factor that regulates SNO specificity and stability. It is possible that the lysine is involved in crosstalk with other post-translational modifications, and evidence suggests that cysteines near an ubiquitinated lysine are more likely to be S-nitrosylated (Fowler et al., 2017). The lysine may also contribute to the acid-base environment thought to promote the oxidation and radical recombination steps in the NO reaction pathway that leads to the formation of a SNO-thiol (Pérez-Mato et al., 1999; Hess et al., 2005).

The acid-base motif was also suggested to be important as a binding site for protein-protein interactions, and to mediate protein transnitrosylation (Marino and Gladyshev, 2010). Whether dependent on a lysine or not, transnitrosylation may also be important in S-nitrosylation of NuRD. It has been shown that HDAC2 is S-nitrosylated by SNO-GAPDH, once SNO-GAPDH has translocated to the nucleus (Kornberg et al., 2010). SNO-GAPDH may therefore be responsible for the transnitrosylation of the NuRD subunits, or SNO-HDAC2 could perform this role. Experiments using these S-nitrosylated proteins as a source of NO would provide an initial indication of whether NuRD subunits undergo transnitrosylation. Importantly, the accumulation of NO has been observed in neuron nuclei (Nott and Riccio, 2009; Nott, Robinson and Riccio, 2008) and it is possible that NuRD is S-nitrosylated directly by NO.

**Future directions**

Currently, my findings regarding CHD S-nitrosylation have been limited to *in vitro* model systems. In order to further validate the identification of SNO-CHDs,
S-nitrosylation of the endogenous CHDs in response to physiological stimuli should be investigated using more sensitive approaches, such as the organomercury capture approach (Devarie-Baez et al., 2013). In this technique SNO-proteins are isolated through the interaction with phenylmercury bound to a tag or beads and eluted with performic acid. Of note, the use of performic acid leads to the formation of sulfonic acid groups on the cysteines, which could be used for site identification (Pesavento et al., 2007; Doulias et al., 2010).

In order to fully investigate the nature of CHD S-nitrosylation, knowledge of the exact cysteines targeted by the modification will become essential. Our SNO-RAC screen identified a number of potential target cysteines (Table.2.1) and findings from the Co-IP, ChIP and the DNase experiments suggest that S-nitrosylation could have a role regulating all three CHDs. It is possible that different cysteines residues are targeted within the CHDs, allowing for differential regulation of interactions with other subunits or chromatin, or it is possible that they are S-nitrosylated at a conserved cysteine residue. Conserved potential sites were identified from the screen for all three CHDs, with CHD3 and CHD4 also containing extra potential sites (Table.2.1). Evidence from my motif data also indicates that lysines are important in aiding relevant S-nitrosylation and the common SNO-peptides also all contain a nearby lysine (Smith et al., 2018). This further indicates that mutating these common cysteine sites may provide useful information as to the S-nitrosylation of the CHDs. Furthermore, these peptides reside within zinc finger domains, which have been illustrated as targets of S-nitrosylation for a number of proteins in plants, most recently for the SRG1 plant
immunity protein (Cui et al., 2018). The identification of the sites for the CHDs therefore remains an exciting approach to follow up in this project.

Having established that CHD-NuRD interactions change in S-nitrosylating conditions, the role for NO and subunit S-nitrosylation in complex assembly could be further studied. It would be interesting to determine using CoIP experiments if RBBP7 Cys$^{166}$ regulated CHD3 interactions as well as CHD4 interactions, and also if RBBP4 Cys$^{167}$ was involved in mediating the decrease seen in CHD5-RBBP4 interactions. The dependence of these altered CHD interactions on S-nitrosylation and NO signalling could be corroborated by utilising nNOS knock out mice for the CoIP experiments. If the interactions between the CHDs and the other NuRD subunits previously observed to increase with CysNO now decreased in nNOS/- neurons, this would indicate that S-nitrosylation was indeed required for mediating these interactions.

An additional approach to explore how NuRD subunit S-nitrosylation impacts on the incorporation of each CHD into the NuRD complex could be to perform glycerol gradient experiments, which result in proteins within a complex being found in the same sub-fraction (Staahl et al., 2013). In this approach the previously known results from WT samples (Nitarska et al., 2016) would be compared to the co-fractionation results from nNOS/- samples. If the proportion of each individual CHD within the NuRD complex during development changed due to the lack of nNOS, this could again indicate that SNO modifications regulate NuRD assembly during cortical development, thus further confirming this as a viable mechanism for regulating the CHD subunit switch.
Preliminary findings have indicated a possible role for NO in regulating CHD-chromatin interactions and CHD remodelling activity. These findings could be expanded on to further elucidate a role for NO and S-nitrosylation in regulating NuRD function. *In vitro* nucleosome remodelling assays utilising recombinant CHDs and nucleosome substrates could be carried out in WT and NO conditions to determine if S-nitrosylation was required for this core catalytic activity, as well as demonstrating the underlying remodelling mechanism for each CHD. Likewise, these findings could also be carried out with Cys/Ser mutants, once the site of S-nitrosylation is known for the CHDs. A second *in vitro* approach to investigate the enzymatic activity of S-nitrosylated CHDs would be to perform ATPase assays. The ability of each CHD to hydrolyse ATP using either S-nitrosylating conditions or non-nitrosylatable mutants would determine the influence of S-nitrosylation on the essential catalytic function of the CHD proteins.

Moreover, the mechanism of CHD recruitment to target genes and the role of RBBP7/4 in this process could be further investigated, in addition to repeating the preliminary ChIP experiments presented here. It would be interesting to investigate the impact of post-translational modification by S-nitrosylation on the interaction of the RBBP proteins with histones, considering the acetylation of histones is known to modulate RBBP binding (Migliori *et al.*, 2012; Klingberg *et al.*, 2015). Firstly, CoIP experiments could be performed with the RBBP7\(^{WT}\) or RBBP7\(^{C166S}\) constructs and specifically looking at the interactions with H3 and H4. Secondly, ChIP experiments in neurons, again utilising the RBBP7\(^{WT}\) or RBBP7\(^{C166S}\) constructs would reveal if there is any difference in chromatin recruitment to known targets between the two constructs.
A role for NuRD S-nitrosylation in vivo

Further investigation of the role of SNO-NuRD function in vivo remains to be carried out and exciting in vivo experiments to study this could utilise the in-utero electroporation technique. Work from our lab has shown the different phenotypes associated with knock down of each individual CHD (Nitarska et al., 2016). It would be interesting to perform the rescue experiments of each individual CHD knockdown, both with WT CHD and non-nitrosylatable constructs. This would reveal if the Cys/Ser mutation prevented the rescue of the phenotype, and whether any phenotype could be attributed to lack of site-specific CHD S-nitrosylation. As before, many different aspects of corticogenesis could be studied including progenitor proliferation, differentiation, neuron migration, morphology and layer marker expression to reveal the exact role of SNO-CHDs in cortical development.

Further exploration of the function of NuRD subunit S-nitrosylation could also build on the in vitro dendritogenesis data. Analysing the proliferation, migration, differentiation and morphology of neurons in embryos lacking endogenous RBBP7, but expressing either RBBP7WT or RBBP7C166S, would reveal the in vivo importance of this modification for neuronal and cortical development. Additionally, these embryos could be allowed to develop into adulthood in order to investigate any behavioural defects that could occur later in life due to impaired dendritogenesis during development, such as the reduction in fear memory. This approach would draw together all my mechanistic and biochemical findings to conclusively demonstrate the role for NuRD S-nitrosylation during cortical development.
Concluding remarks and perspectives

The main findings of my study are that the NuRD subunits RBBP7, RBBP4, MBD3 and CHD3, CHD4, and CHD5 can be modified by S-nitrosylation. These modifications can result in changes to NuRD subunit interactions and subunit function and lead to changes in neuronal dendritogenesis and chromatin landscapes. This NuRD S-nitrosylation is therefore linked to correct neuronal and cortical development (Fig 3.1).

**Fig.3.1 Schematic model of the SNO NuRD role in cortical neurons.** Both neuronal activity and cortical development lead to increased nNOS activity and NO accumulation. This leads to NuRD S-nitrosylation which in turn could contribute to NuRD regulation, and as such impacts on the correct neuronal and cortical development.
My work has also presented an example of how a target cysteine requires an adjacent lysine in order to be modified by S-nitrosylation. The implications of this motif are not limited to NuRD subunits and it could be involved in regulating the S-nitrosylation of a greater number of proteins than those characterised here. Further work investigating the details of how widespread and precise this motif is, and how the lysine residues promote S-nitrosylation will help address key questions regarding the regulation of S-nitrosylation specificity. It may be possible that this lysine motif is restricted to a subset of SNO-proteins and is important for the S-nitrosylation of specific proteins involved in gene regulation. Other chromatin remodelling complexes and their subunits are also possible targets of nitrosylation, including BRD3, BRD4, SIN3A, and members of the Smarca/b/d family (Smith et al., 2018), and it may be possible that this lysine motif also guides S-nitrosylation of these proteins. Alternatively, this motif could be relevant for proteins that are S-nitrosylated specifically in neurons, and therefore important for the modification of proteins with a wide range of functions. Regulation of the S-nitrosylation of these proteins, through the motif and other mechanisms could have implications for the correct proliferation and differentiation of neuronal progenitors, as well as the correct migration and terminal differentiation of cortical neurons. As such, dysregulation of this process could contribute to developmental disorders and later in life, neurodegenerative and behavioural disorders.

Excitingly, the data presented here supports S-nitrosylation as a viable mechanism to regulate NuRD complex assembly and activity. To date only a few mechanisms have been proposed to regulate the combinatorial assembly of
chromatin remodelling complexes. The best characterised example so far is the microRNA mediated mechanism for BAF complex assembly during neuronal development (Yoo et al., 2009; Ho and Crabtree, 2010; Nitarska et al., 2016). This work joins these studies to elucidate an additional mechanism for the regulation of chromatin remodelling complex assembly, through S-nitrosylation post-translational modification. This mechanism may work alongside others for the regulation of NuRD and indeed other chromatin remodelling complexes. These findings also add to our understanding of how post-translational modifications, including S-nitrosylation, of specific subunits can have wider implications not only on individual protein activity, but on overall complex functions such as nucleosome remodelling (Clapier and Cairns, 2009; Basta and Rauchman, 2015; Sokpor et al., 2018). The coupling of the SNO post-translational modification to nNOS expression and NO levels means that modification by S-nitrosylation can be carefully developmentally regulated. Therefore, it could be an attractive and reliable mechanism for regulating chromatin remodelling complexes throughout cortical development. Future studies investigating the S-nitrosylation of additional chromatin remodellers will indicate if my work in fact presents an example of how subunit S-nitrosylation acts as an important and widespread mechanism for the regulation of chromatin remodelling complexes.
4. Materials and Methods

4.1 Cell Culture

HEK293T cells were obtained from ATCC and cultured according to standard conditions listed on the ATCC website for ATCC® CRL-11268™. Cells were maintained in DMEM supplemented with 10% FBS and 1% Glutamine. Neuro2a cells were from ATCC and cultured according to standard conditions for ATCC® CCL-131™ and transfected as described for HEK293T cells. Cells were not cultured past passage 20. N2A cells were maintained in DMEM supplemented with 10% FBS and 1% Glutamine. All cell lines were kept at 37°C, 10% CO₂. All cell culture reagents purchased from Gibco (Thermo Fisher Scientific).

Cell line transfections

HEK293T cells were transfected at 40-50% confluency using lipofectamine 2000 (Invitrogen) with a DNA/lipofectamine ratio of 1 μg DNA/1.25 μl lipofectamine in optimem. Separate mixes of DNA and optimem and lipofectamine and optimem were prepared and incubated for 10 min before combination and incubation for 20 min. Incubations were at room temperature. The transfection mix was then added to optimem in the well/dish as appropriate for 4 h. Transfection was stopped by replacing optimem with normal media. Cells were then harvested 24 h or 48 h after transfection, depending on experiment.

4.2 Animal Procedures

All animal experiments were approved by the UCL Animal Welfare and Ethical Review Body and carried out in accordance to appropriate UK Home Office licenses. Timed pregnant Sprague Dawley rats and C57BL/6J WT mice were used to prepare WT embryonic cortical neurons. Timed matings were set up using the nNOS knock out line KN2 (Jackson Laboratory B6.129S4-Nos1tm2Phy/J Stock No:008519). Heterozygotes were mated and separated after one night in order to check plugs and identify age of embryos. Pregnant females were culled at E15.5 and E18.5 and embryos processed for cortex dissection, limbs and tails were taken for genotyping.
4.3 Preparation of Cortical Neurons

Media: All cell culture reagents and dishes purchased from Gibco (Thermo Fisher Scientific)

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissection buffer</td>
<td>1x HBSS, 2.5 mM Hepes pH 7.4, 30 mM D-glucose, 1 mM CaCl₂, 1 mM MgSO₄, 4 mM NaHCO₃</td>
</tr>
<tr>
<td>Digestion Buffer</td>
<td>1 mM Hepes pH 7.4, 20 mM glucose, 82 mM Na₂SO₄, 30 mM K₂SO₄, 6 mM MgCl₂, 0.25 mM CaCl₂, 0.001% Phenol Red, 0.126mN NaOH</td>
</tr>
<tr>
<td>Plating Media</td>
<td>MEM with 10% FBS, 5% HS and 1 mM glutamine</td>
</tr>
<tr>
<td>Full Neurobasal</td>
<td>NB with 1x B27, 1 mM glutamine, 1x penicillin-streptomycin and 10 μM FdU</td>
</tr>
<tr>
<td>Starvation Neurobasal</td>
<td>1 mM glutamine, 1x penicillin-streptomycin and 10 μM FdU</td>
</tr>
</tbody>
</table>

Table 4.1 Cortical neuron preparation buffers.

Preparation of cortical neurons

Timed pregnant mice or rats were culled, and embryos removed from the uterus. Embryos are then decapitated, and brains removed from the heads in cold dissection buffer. Hemispheres are separated, and cortex dissected out. Isolated cortices are collected in cold dissection buffer and digested for 25 minutes at 37°C in digestion buffer. The digestion buffer is prepared each time by addition of 200 units papain per 10 ml of digestion buffer and then activated with cysteine-HCl and incubated at 37°C for 20 min before being neutralised to pH 7 with NaOH. 20 ml was used per rat ~ 18 embryos. Cortices were washed 4x in plating media by gentle pipetting and dissociated with 8 stronger stripette motions. Cells were strained through a 40 μm (BD Falcon) counted and plated in plating media on 10 cm Nunc plates coated with 20 μg/ml poly-D-lysine and 2 μg/ml laminin. Cells were plated at a density of 12.5 million per dish and kept at 37°C, 5% CO₂. After 1 day in culture, media was replaced with supplemented ‘full neurobasal media. Prior to stimulation and harvesting on day 5, cells were starved for 16 h by replacing 2/3 of media with starvation neurobasal media, without B27.

Preparation of mouse cortical neurons

Mouse cortices were isolated and digested as for rat cortices but were digested in 10 ml of digestion buffer. For dendritogenesis assays neurons were plated on coverslips in 24 well plates coated with 40 μg/ml poly-D-lysine and 2 μg/ml laminin. Cells were plated at 0.35 million per well and kept at 37°C, 5% CO₂ for 48 h before fixing.
Preparation of cortex tissue
Timed pregnant mice were culled, and embryos removed from the uterus in PBS. Embryos are then decapitated, and brains removed from the heads. Hemispheres are separated, and cortex dissected out. 2 cortices from the same embryo are placed in one eppi for use in experiments. Tails are processed for genotyping and identification of nNOS-/- tissue.

4.4 Biotin Switch Technique

As S-nitrosylation is light sensitive and light can also react with ascorbate to induce artefactual signals, all procedures involving detection or stimulation of S-nitrosylation were carried out in minimal light conditions using brown eppendorf tubes and foil covered polypropylene falcon tubes. No glassware/metalware was used to avoid contaminating metal species that could interfere with the reaction (Forrester et al., 2009). All BST reagents purchased from Sigma unless stated.

Donor Preparation
CysNO was prepared fresh each time before use according to Mallis et al. 2001. Briefly, 25 μL of 4 M HCl is added to 220 μL of 220 mM L-cysteine and 220 mM NaNO₂ and incubated for 10 min at room temperature, in the dark. The pH is then neutralized using 25 μL of 4 M NaOH. This gives a final concentration of ~100 mM CysNO. Exact final concentration calculated from absorbance at 338 nm, of a 1/100 diluted solution, using the formula: 

\[
\text{CysNO concentration (mM)} = \frac{\text{Abs}_{338}}{900} \times 100 \times \text{dilution factor} \times 1000
\]

CysNO is used at a final concentration of 200 μM or 500 μM. Cys only negative controls were prepared by adding 220 μL H₂O, 25 μL of 4 M HCl and 25 μL of 4 M NaOH to 220 μL of L-cysteine at room temperature, in the dark.

GSNO donor was prepared by adding 25 μL of 4 M HCl to 220 μL of 220 mM GSH and 220 μL of 220 mM NaNO₂ and incubated for 10 min at room temperature, in the dark. Then 25 μL of 4 M NaOH is added. This gives a final concentration of ~100 mM GSNO. The final mM concentration is calculated using absorbance at 334 nm, of a 1/100 diluted solution, and using the formula: 

\[
\text{GSNO concentration (mM)} = \frac{\text{Abs}_{334}}{767} \times 100 \times \text{dilution factor} \times 1000
\]

GS negative controls were prepared as for GSNO but replacing the 220 μL of 220 mM NaNO₂ with 220 μL of H₂O.
Buffers: All buffers are made up in BPC grade water (Sigma W3513)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEN</td>
<td>100 mM HepesNaOH, 1 mM EDTA, 0.1 mM neocuproine, pH 8.0</td>
</tr>
<tr>
<td>HEN lysis</td>
<td>HEN+ 0.2% NP40</td>
</tr>
<tr>
<td>HENS</td>
<td>HEN buffer with 1% SDS (w/v)</td>
</tr>
<tr>
<td>Neutralization buffer</td>
<td>25 mM HepesNaOH, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.5</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>Neutralization buffer containing 600 mM NaCl</td>
</tr>
<tr>
<td>2X MMTS blocking buffer</td>
<td>2% MMTS, 5% SDS in HEN buffer</td>
</tr>
<tr>
<td>NEM blocking buffer</td>
<td>NEM (n-ethylmaleimide) 140 mM in HEN buffer with SDS at 2.44%</td>
</tr>
<tr>
<td>2X western loading buffer</td>
<td>2% SDS, 2% βME, 20% glycerol, 100 mM Tris 6.8, approx. 0.010% bromophenol blue</td>
</tr>
</tbody>
</table>

Table.4.2 BST buffers.

Biotin switch on neurons
Three 10cm plates of E17 cortical neurons were used per sample. Plates were treated as appropriate with either Cys or CysNO, then washed twice with ice cold PBS and harvested in 180 μL of cold HEN lysis buffer. Samples were then homogenized 5 times with a 25-gauge needle, centrifuged for 10 min at 10,000 g 4°C and the supernatant collected (~2.5-3 mg of protein per 3 plates). Samples were blocked using an equal volume of 2X MMTS Blocking Buffer (2% MMTS, 5% SDS in HEN buffer). Samples were incubated for 30 min at 50 °C and vortexed every 5 min to ensure efficient blocking of free cysteines. Samples were then combined, and 100% acetone added for precipitation with 4 times the volume of cold (-20°C) acetone. Precipitation took place at -20°C for >1h, then samples were pelleted by centrifugation at 20 min 2,000 g 4°C and washed 3 x with 70% acetone. Common post-blocking protocol was then carried out.

Biotin switch on HEK293T cells
For in-plate HEK experiments, a transfected 6cm plate of HEK293T cells was used per condition. Plates were treated as appropriate, then washed 1x with PBS (4°C). Lysates were harvested in 200 μL HEN lysis buffer, kept on ice, then homogenized 5x with a 25G needle. After centrifugation, CysNO was removed from the supernatant by acetone precipitation in -20°C acetone for a minimum of 1 hour. Post precipitation samples were centrifuged for 10 min at 2000 g, 4°C and washed 2x with -20°C 70% acetone and
resuspended in 200 μL HENS. 650 μg of protein was diluted in 500 μL HEN and samples subjected to common post-blocking protocol as described below.

For treatment of cellular lysates, cells were harvested in 200 μL HEN lysis buffer then homogenised and centrifuged as above. Lysates containing 650 μg protein in 500 μL HEN were treated with CysNO or Cys as necessary for 20 min prior to CysNO removal and resuspension as above. MMTS Blocking Buffer was used for RBBP4 experiments. MBD3 experiments used NEM (n-ethylmaleimide) (140 mM in HEN buffer with SDS at 2.44%) and CHD experiments used either condition as noted. Blocking was carried out for 45 min at 50 °C. Precipitation took place at -20°C for >1 h, then samples were pelleted by centrifugation (20 min 2,000 g 4°C) and washed 3 x with 70% acetone. Common post-blocking protocol was then carried out.

Post-blocking protocol

Post experiment specific blocking pellets were dried to ensure lack of acetone, care is taken not to dry out fully. The pellets were resuspended in 220 μL HENS buffer by 4 rounds of careful pipette to avoid bubbles. Samples were then incubated with sodium ascorbate to final concentration of 50 mM for 10 min at RT with gentle mixing by tapping. Biotin HPDP (Thermo 21341) was then added to 1 mM final concentration and samples rotated for 45 min, followed by acetone precipitation and washes as before. Post resuspension in 220 μL HENS, 400 μL neutralization buffer was added to each sample. Samples were then precleared by incubation with 30 μL protein A beads for 1 h at room temperature. At this point samples were in light conditions. Post preclearing samples were then centrifuged at 500 g for 5 min at RT. Total inputs were taken from supernatant and saved and the remaining supernatant transferred to new clear eppendorfs and incubated with 50 μL bead volume of streptavidin agarose beads overnight, rotating at 4°C. 5x western loading buffer was added to a final 2x to total inputs which were frozen. Next day samples were again centrifuged for 5 min at 500 g, 4°C, and washed 4 x in wash buffer. Beads were dried using a 29G needle and 40 μL of 2x western loading buffer was added. Samples were boiled for 10 min then cooled at RT before a final spin down at 10,000 g for 2 min. The supernatant was collected and the whole amount run on 10% polyacrylamide gels or precast NuPage 4-12% Bis-Tris gels. Samples were then analysed by western blotting for specific proteins of interest. Total inputs were also run, on separate gels but at the same time for further processing together with samples.
4.5 Co-IP Protocol

Co-immunoprecipitation experiments were also performed in low light conditions, to maintain S-nitrosylation modification throughout experiment procedure. All reagents purchased from Sigma unless stated.

CoIP Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP Lysis Buffer</td>
<td>50 mM Tris pH 7.5, 150 mM NaCl, 1% TX100, 1% Sodium Deoxycholate</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>50 mM Tris pH 7.5, 150 mM NaCl, 0.1% TX100, 5% glycerol</td>
</tr>
<tr>
<td>RIPA Buffer</td>
<td>50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Deoxycholate</td>
</tr>
<tr>
<td>2X western loading buffer</td>
<td>2% SDS, 2% βME, 20% glycerol, 100 mM Tris 6.8, approx. 0.010% bromophenal blue</td>
</tr>
</tbody>
</table>

Table.4.3 CoIP buffers.

E17 cortical neurons IP

1x 10cm plate was used per condition. Cells were treated with 200 μM CysNO donor or Cys control for 20 min and then washed with cold PBS and harvested in cold IP Lysis buffer containing protease inhibitor cocktail, phosphatase inhibitors 2 and 3 and PMSF (all at 1:100). Samples were lysed on ice for 30 minutes and homogenized by syringing 5x with 25G needle and then cleared by centrifugation at 1000xg for 10 minutes at 4°C. Lysates were pre-cleared with protein G-Sepharose beads (GE healthcare, 17-0618-01) for 2 h at 4°C. 10% total inputs taken for TCA processing. The remaining sample was incubated overnight in the dark 4°C with specific primary antibodies. Lysates were then incubated with protein G–Sepharose beads for 2 hours at 4°C. Beads were washed 1x with wash buffer with protease and phosphatase inhibitors at 1:1000, 2x with wash buffer without inhibitors and 2 x PBS. Excess PBS was removed, and proteins eluted by boiling the beads with 2x western loading buffer. Total inputs and samples were then run on precast gels and analysed by western blotting.

Antibodies: rabbit anti-CHD4 (Abcam, ab72418), rabbit anti-CHD3 (Bethyl Cambridge Biosciences A301-220A), rabbit anti-CHD5 (Gift from Pazin lab) or rabbit IgG (Dako X0903).
HEK IP

Six well plates of HEK 293T cells were co-transfected with Flag mCHD4 and either HA-RBBP7<sup>WT</sup> or HA-RBBP7<sup>C166S</sup>. HEK 293T cells were treated as for E17 neurons then washed with cold PBS and harvested in cold RIPA buffer containing inhibitors at 1:100. Samples were lysed on ice for 30 minutes and homogenized by syringing 5x with 25G needle and then cleared by centrifugation at 1000xg for 10 minutes at 4°C. Aliquots for BCA were taken and remaining lysates were pre-cleared with protein G beads (GE Healthcare). During pre-clearing protein concentration was determined using BCA Protein Assay; 250-400 μg of protein was used per experiment. Samples were adjusted to correct protein amount in 500 μl RIPA buffer and 10% total inputs taken for TCA processing. The remaining sample was incubated overnight in the dark 4°C with rabbit anti-HA (CST 3724S) or rabbit IgG (Dako X 0903). Lysates were then incubated with protein G–Sepharose beads for 2 hours at 4°C. Beads were then washed and proteins eluted as for E17 samples. Samples were then run on precast NuPage 4-12% Bis-Tris gels and analysed by western blotting.

Total inputs for IP samples were processed by TCA precipitation. 1% volume of 2% NaDeoxycholate was added and samples vortexed before 30 min incubation on ice. Then 10% volume of TCA was added and samples again vortexed before overnight incubation at 4°C. Samples were then again vortexed and spun at 17,000 g for 10 min at 4°C. The protein pellets were washed with ice cold 100% acetone, dried and then resuspended in 2x LB + Tris HCl pH 8.5 before boiling and run on gels. Ti westerns blots were then processed side by side with the sample IP westerns.

4.6 Western Blotting

Western blot was carried out according to standard procedures. Samples treated and run on gels as described for individual techniques. Transfer conditions were 330mA for 1 h 30 min in 20% methanol (for Fig 1-3, S6, S7, S8 and S10) or 100V for 3 h at 4°C in 10% methanol (for Fig 4, S11 and S12). PVDF membranes were blocked in 5% Milk/TBST (50 mM Tris pH7.4, 150 mM NaCl, 0.1%Tween-20) for 1 h room temperature prior to overnight incubation at 4°C in in 5% Milk/TBST with primary antibodies at a 1:1000 dilution. Post 4 (minimum) TBST washes membranes were incubated with the appropriate secondary antibody at a 1:20000 dilution in 5% Milk/TBST for 2 hrs RT. Post further TBST washes signal was detected using ECL or ECL Prime detecting reagents (GE Healthcare Life Sciences) and by exposing the immunoblot to the Amersham
Hyperfilm (GE Healthcare Life Sciences). Western blot signal was quantified using densitometry analysis and ImageJ. Samples were normalized to total input signal.

**Antibodies**

The following primary antibodies were used for western blotting: mouse anti-C-Myc (A7 Santa Cruz Sc-56634), mouse anti-Flag (M2 Sigma F3165), mouse anti-HA (Biolegend 901502), mouse anti-HDAC2 (Merck Millipore 05-814), mouse anti-HSP90 (Abcam Ab13492), rabbit anti-RBBP4 (Abcam 79416), mouse anti-RBBP7 (Santa Cruz Sc377197), goat anti-HSP90 (Santa Cruz Sc-1055), Secondary antibodies: anti-mouse HRP GE Healthcare NA931, anti-goat HRP Sigma A5420, anti-rabbit HRP GE Healthcare NA93AV.

### 4.7 Dendritogenesis Assay

E15.5 mouse neurons were transfected using Lipofectamine 2000 2 h post plating with 220 ng Pbird GFP and then 500 ng of either mycEV, mycRBBP7\textsubscript{WT} siRNA resistant or mycRBBP7\textsubscript{C166S} siRNA resistant plasmids. Cells were co-transfected with 400 nM of each siRNA. Cells were transfected with a DNA/lipofectamine ratio of 1 μg DNA/1.25 μl lipofectamine in optimem. Separate mixes of DNA and optimem, and lipofectamine and optimem were prepared and incubated for 10 min at room temp before combination and incubation for 20 min. The transfection mix was then added to optimem in the well. Transfection was stopped, and media was changed 3 h post transfection to neurobasal with 0.33% B27 (NB with 0.33%B27, 1 mM glutamine, 1x penicillin-streptomycin and 10 μM FdU). Coverslips were fixed in 4% PFA/PBS 48 h post transfection. Coverslips were then washed 1x PBS and permeabilised in 0.3% Triton-X/PBS. Coverslips were washed 3x in RT PBS and then blocked in 5% NGS 5%FBS/ PBS for 1 h at room temp and incubated with chicken anti-GFP (Abcam ab13970) overnight at 4°C. Post 3x PBS washes samples were incubated with Dapi and Alexa Fluor goat anti-chicken 488 (Life Technologies A-11039) at 1:1000 for 1 h RT. Coverslips were washed and mounted. Slides were then blinded prior to imaging and analysis. Images were acquired using SPE or SPE3 confocal microscope (Leica) with LAS AF software and processed using ImageJ/Fiji. Sholl analysis was performed using Fiji sholl analysis plugin and slides then de-blinded for final processing.

### 4.8 ChIP Protocol

WT and knockout cortices dissected as above, and flash frozen in liquid nitrogen prior to ChIP protocol being followed.
Cortices were thawed on ice and then crosslinked in 1% PFA/PBS, rotating for 10 min at room temperature. Crosslinking was quenched by addition of 125 mM glycine, rotating for 5 min at RT. Cortices were then washed 3x in ice cold PBS + inhibitors at 1:1000, centrifuging at 1000 g 4°C between each wash. Cortices were then resuspended in ChIP buffer 1, with 10 pipette strokes followed by 10 min rotating at 4°C. The nuclei were then pelleted by centrifugation at 3000 rpm for 10 min rotating at 4°C and resuspended in ChIP buffer 2. Samples rotated for 10 min at 4°C before re-pelleting and resuspension in ChIP buffer 3. Samples were then split to tubes for sonication, with 300 μl per tube. Samples were sonicated using the Diagenode bioruptor Pico system and were sonicated for 30 min (with cycles of 30 sec pulse and 30 sec pause). Post sonication samples were spun down at max speed to collect all material, and a small 15 μl aliquot removed. The remaining samples was then added to 40 μl of pre-swelled protein A sepharose beads for preclearing (Beads swelled in PBS O/N, washed and then resuspended in TE with 1.4 mg salmon sperm DNA, 6 mg BSA, and 0.05% NaN₃). Samples were rotated for +2 h 4°C. During this stage the 15 μl aliquot was supplemented with 163 mM NaCl and incubated at 95°C for 15 min for a quick reversal of crosslinks. The DNA was eluted using the Qiagen PCR purification kit, and DNA concentration was measured using the Qubit fluorometer. Remaining DNA was run on a gel to assess sonication efficiency. Post
pre-clearing, samples were split and concentrations normalised and then supplemented with 163 mM NaCl, 1% Tx100 and 0.1% NaDeoxycholate. Total inputs were taken and stored at 4°C and antibodies were then added to samples as for incubation overnight, rotating at 4°C. Antibodies used as for CoIP. The next day 60 μl of protein A beads were added and samples were rotated for 2+ h at 4°C. Beads were then spun down at 500 g 4°C for 3 min and then washed 2x in ChIP was buffer low salt, Chip wash buffer high salt and ChIP wash buffer LiCl, sequentially. Washes were each 10 min rotating followed by 3 min centrifugation at 500 g 4°C. Post washes beads were rinsed once in TE and then for 5 min in TE buffer. TE was then removed and 100 μl elution buffer added. Samples were then heated to 65 °C whilst shaking for 5 min, before vortexing for 15 min RT. Samples were then centrifuged at max speed RT, and supernatant transferred to new tubes. 120 μl of EB was added to the beads and the vortexing step repeated. Post final centrifugation the supernatant was pooled, inputs were adjusted to the same (200 μl) volume with EB and then 163 mM NaCl added. All samples were then incubated at 65°C overnight to reverse the crosslinks. The following day samples were cooled to RT before purification by the Qiagen PCR purification kit. Samples were eluted in 40 μl of the kit EB diluted 1:3, before a second addition of 40 μl to the columns, and supernatant was again pooled. Interactions of CHDs at specific genomic regions was then analysed by RTqPCR using the SyberSelect master mix (Thermo) and BioRad CFX Connect Real-time system. See below table for primers used.

4.9 DNAseI Hypersensitivity Assay

3 million cells were used per condition (equates to 1 E15.5 cortex). Cortices were thawed on ice and then lysed in DNase lysis buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 0.1% SDS, 0.5% Triton X) for 30 min. Samples were centrifuged for 10 min 4°C, at 3,000 rpm. Cell pellets were then resuspended in 1X DNase Buffer (Roche) and split between samples. Samples were left untreated or 1U of DNase (Roche) added before all samples were incubated at 25°C for 20 min. The enzyme was heat inactivated at 95°C for 20 min and then Proteinase K added to a final concentration of 200 μg/ml. Samples were incubated overnight at 50°C and then purified by phenol/chloroform extraction the next morning. Sensitivity to DNAseI digestion was assessed at the specific genomic regions by qPCR.
Primers used for DNAse assay and/or ChIP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (F)</th>
<th>Reverse Primer (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox2</td>
<td>GAACCCCAAGATGCACAA</td>
<td>GGGAGCGTGTAATTAC</td>
</tr>
<tr>
<td>Tbr2</td>
<td>CGAGAGGAAATGGCTGGTTG</td>
<td>CCGGTGTAAGTTGTCTCT</td>
</tr>
<tr>
<td>ApoER</td>
<td>GTTACCGAAGCTCTACCCA</td>
<td>CGCCGTGTAATAATAC</td>
</tr>
<tr>
<td>Dcx</td>
<td>CTCTCAGCATCTCACCCA</td>
<td>AGAGGGAACACACAGAGAA</td>
</tr>
<tr>
<td>NEG</td>
<td>CCCAGGAGATCGGAAG</td>
<td>AGTTCTGGTGAGCTATAGACAT</td>
</tr>
<tr>
<td>FSH NEG</td>
<td>GGTGTGCTGCCATATCAATT</td>
<td>GCATCAAGTGCTGCTACCTC</td>
</tr>
</tbody>
</table>

Table 4.5. Target gene primers used in DNase and CHIP assays.

4.10 Cloning

msMBD3, ratRBBP4 and ratRBBP7 were generated from genomic cDNA and subcloned into the CMV-myc expression vector using Sal1 and Not1 restriction enzymes. An empty CMV-myc plasmid was also generated by religating cut CMV-myc, and mutating to restore lost restriction sites. Mutagenesis to create pCMV-myc-rRBBP4<sub>C166S</sub>, pCMV-myc-rRBBP4<sub>C167S</sub> and pCMV-myc-MBD3<sub>C8S</sub>, MBD3<sub>C215S</sub>, MBD3<sub>C266S</sub>, MBD3<sub>C215S/C266S</sub>, MBD3<sub>C215S/K264A</sub>, MBD3<sub>K264A</sub>, and the mycRBBP7<sup>WT</sup> and mycRBBP7<sup>C166S</sup>

siRNA resistant plasmids was carried out using QuikChange Lightning mutagenesis kit (Agilent) as per manufacturer’s protocol. HA-RBBP7 was cloned into Adeasy pshuttle CMV using primers against rat RBBP7, the forward primer encoding a HA tag. HDAC2 constructs used were previously generated in the lab, and where cloned into Adeasy vector, and then mutated (Nott et al., 2008). Flag mCHD4, hCHD5 and hCHD3 were generated as previously described (Nitarska et al., 2016). pCIG-NLSCRE plasmid was a gift from Francois Guillemot. pClneoB-3Flag-hCHD3 plasmid containing full sequence of human CHD3 was provided by Odd Stokke Gabrielsen. pCMV-SPORT6-mCHD4 plasmid containing complete coding sequence of mouse CHD4 was purchased from Open Biosystems. pDEST26-hCHD5 plasmid encoding full sequence of human CHD5 was purchased from Source Bioscience. Complete hCHD3, mCHD4 and hCHD5 coding sequences were sub cloned into pCIG-IRES-GFP vector using Gibson Assembly (NEB) (CHD cloning information provided by Dr Nitarska).
Table 4.6. Primers used to generate over expression constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primers Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBD3 WT F</td>
<td>AATGGTCGACATGGAGCGGAAGAGGTGGG</td>
</tr>
<tr>
<td>MBD3 WT R</td>
<td>AATGCGGCGGCGCTACACTCGCTCTGGCTC</td>
</tr>
<tr>
<td>rtRBBP4 WT F</td>
<td>TACGTCGACCATGGCTGACAAAGGACCGG</td>
</tr>
<tr>
<td>rtRBBP4 WT R</td>
<td>TATGCGGCGGCTAGATGCTTCTTCTGG</td>
</tr>
<tr>
<td>rtRBBP7 WT F</td>
<td>CGCGTCGACCATGGCGAGTAAAG</td>
</tr>
<tr>
<td>rtRBBP7 WT R</td>
<td>GCGCGGCCTAAGAGTCTTGG</td>
</tr>
<tr>
<td>HA RBBP7 WT F</td>
<td>GTCGACACCATGTACCCATACGATGTCCAGATTACG</td>
</tr>
<tr>
<td>HA RBBP7 WT R</td>
<td>GCGGCCGTATCCCTTGCCCCT</td>
</tr>
</tbody>
</table>

### Mutant Constructs

<table>
<thead>
<tr>
<th>Mutagenesis Primers</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGAGGTTGGGAGAGCCCGGCGCTC</td>
<td>MBD3 C8S</td>
</tr>
<tr>
<td>ACTGCACTGCTGACTGAAAGGCTTCTCAGG</td>
<td>MBD3 C215S</td>
</tr>
<tr>
<td>CACTGGACACGGCCCGGCACGG</td>
<td>MBD3 C266S</td>
</tr>
<tr>
<td>AGGCCACCACCTGGAGCGCGCGTGTCCAGAGG</td>
<td>msMBD3 K264A</td>
</tr>
<tr>
<td>CAAGTCTGAGTCTTTCTCCAGAGGCTTCTG</td>
<td>rtRBBP4 C167S</td>
</tr>
<tr>
<td>CAGATCCAAATGGAGAGGTCTAGATCTTTAGTTTCAATCTAACTGTCGCGAGTCAAGGCTAAATGTGTAAGAT</td>
<td>rtRBBP7 C166S</td>
</tr>
<tr>
<td>CTTGGTTTTCATCTAATCTCCGAGGTCTGCTAAGGCTAAATGTGTAAGAT</td>
<td>HDAC2 K280A</td>
</tr>
</tbody>
</table>

SiRNAs were generated by Invitrogen.

Sequences: siRBBP7 (5'-3') CCA CAU AAG AUC AUU CUG GCU U and the reverse complement (5'-3') AAG CCA GAA UAG UUU CAU UAU GUG G. siRBBP4: (5'-3') AAA UCU UUC CCU UCA GGC CUG GUC A and the reverse complement (5'-3') UGA CCA GGC CUG AAG GGA AAG AUC U.

### 4.11 Statistical Analysis

Statistics was performed as indicated in figure legends. All analysis was performed using GraphPad Prism Software.
Reference List


pp. 233–44.


Bielle, F. et al. (2005) ‘Multiple origins of Cajal-Retzius cells at the borders of the developing pallium', Nature Neuroscience, 8(8), pp. 1002–1012.


Brodmann, K. and Garey, L. J. (2006) Brodmann’s localisation in the cerebral cortex:


that contains a chromodomain and an SNF2/SWI2-like helicase domain.


Nitric Oxide Synthase Gene in Mice Results in Hypogonadism and Infertility', *Endocrinology*, 143(7), pp. 2767–2774.


Malik, S. I. et al. (2011) ‘GSNOR-mediated de-nitrosylation in the plant defence


De Oliveira, M. G. et al. (2002) ‘Thermal stability of primary S-nitrosothiols: Roles of


transactions of the Royal Society of London. Series B, Biological sciences, 361(1473), pp. 1545–64. doi:


KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2α subunit of NuRD', *Genes and Development*, 15(4), pp. 428–443.


Sekine, K. *et al.* (2011) ‘The outermost region of the developing cortical plate is crucial for both the switch of the radial migration mode and the Dab1-dependent lamination in the neocortex.’, *The Journal of Neuroscience*, 31(25), pp. 9426–39.


Zhang, W. et al. (2016) ‘The Nucleosome Remodeling and Deacetylase Complex


