Nitric Oxide induces Chromatin Remodelling in the Developing Central Nervous System

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Abstract

The development of the nervous system is a complex task that involves precise connections between billions of neurons. This is achieved, at least in part, by the overproduction of neurons and the survival of a select few that compete for limited survival and growth promoting factors, such as neurotrophic factors. The neurotrophin brain-derived neurotrophin factor (BDNF) has been shown to play an important role in proliferation and differentiation of cortical neuronal precursors (Bartkowska et al 2007). Moreover, BDNF induces binding of the transcription factor CREB to gene promoters in a nitric oxide (NO)-dependent manner (Riccio et al 2006). NO positively regulates a large number of transcription factors and genes in the nervous system (Hemish et al 2003; Dhakshinamoorthy et al 2007). I have demonstrated that NO achieves this broad level of gene regulation by influencing chromatin remodelling. My data also show that NO accumulates within the nucleus of cortical neurons upon BDNF stimulation, thereby inducing *S*-nitrosylation of a wide array of nuclear proteins. *S*-nitrosylation of histone deacetylase 2 (HDAC2) decreases its affinity for chromatin, leading to increased histone acetylation levels. This NO-dependent regulation of HDAC2 promotes changes in endogenous gene expression and affects the dendritic length of cortical neurons.

Acknowledgements

Contents

Abbreviations

AcH4	Acetylated Histone H4
APC	Adenomatosis polyposis coli
ASK1	Apoptosis signal-regulating kinase
BDNF	Brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BMP	Bone morphogenetic protein
CaM	Calmodulin
CaMK	Ca^{2+}/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
CAPON	C-terminal PDZ ligand of nNOS
CBP	CREB binding protein
cGMP	Cyclic guanosine-3',5'-monophosphate
ChIP	Chromatin immunoprecipitation
CK2	C-terminal casein kinase-2
CNS	Central nervous system
CRE	cAMP response element
CREB	cAMP-regulated element binding protein
DAF-FM	4-amino-5-mehtylamino-difluororescein-2'7'-diacetate
DAG	Diacylglycerol
DL-AP5	DL-2-amino-5-phosphonovaleric acid
Dlx	Distal-less homeobox
DNMT	DNA methyl-transferase
DRG	Dorsal root ganglia
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase 1 and 2
FLIP	FLICE inhibitory protein
GABA	γ-aminobutyric acid
GAF	Guanylyl cyclase-activating-factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GSNO	S-nitroso-glutathione
HD	Huntington disease
Hda-1	Histone deacetylase-1 (yeast)
HDAC	Histone deacetylase (mammalian)
HDMT	Histone demethylase
HEK293	Human embryonic kidney-293
HMT	Histone methyltransferase
HTT	Huntington protein
iNOS	inducible nitric oxide synthase
IP_3	Inositol-1,4,5-trisphosphate
IZ	Intermediate zone
LTD	Long-term depression
LTP	Long-term potentiation
MAP	Mitogen-activated protein kinase
Mash1	Mammalian achaete scute homolog
MBD	Methyl-CpG-binding domain
MMP	Matrix metalloproteinase
MMTS	Methyl methanethiosulphonate
NCoR	Nuclear receptor co-repressor
NGF	Nerve growth factor
Ngn	Neurogenin
NMDAR	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric Oxide
NOS	Nitric oxide synthase
NPA	N-O-propyl-L-arginine
NT-3	Neurotrophin factor 3
PI3K	Phosphatidylinositide-3-kinase
PIP_2	Phosphatidylinositol-4,5-bidpohsphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PLC-γ1	Phopholipase C-γ1
PNS	Peripheral nervous system
Pol II	Polymerase II
PSD	Post synaptic density
qPCR	Quantitative polymerase chain reaction
RE1	Repressor element 1
REST	RE1 silencing transcription factor
RMS	Rostral migratory stream
Rpd3	Reduced potassium dependency-3
Rsk	p90 ribosomal S6 kinase
RyR1	Ryanodine Receptor/Ca^{2+}-release channel
sGC	Soluble guanylyl cyclase
SIRT	Sirtuins
SMRT	Silencing mediator of retinoid and thyroid receptor
SNO	S-nitrosothiol
SNOC	S-nitrosocysteine
SVZ	Subventricular zone
Trk	Tropomyosin-related kinase
TRPC	Canonical form of transient receptor potential channel
Trx-TR	Thioredoxin-Trx reductase
TSA	Trichostatin A
VZ	Ventricular zone

<u>Chapter 1</u> Introduction

The mature mammalian brain coordinates an overwhelming diversity of functions, including perception, motor coordination, learning and memory. This is reflected in the complexity of its development, which is incomparable to any other biological organ. The development of the nervous system requires the generation of a large number of neuronal cell types followed by migration of neuronal precursors to target destinations, axonal elongation and the establishment of contacts with specific target cells. The major contribution to the functional complexity exhibited by the mammalian brain is attributed to the recent evolution of the neocortex. The rapid expansion of the neocortex in humans is fundamental for our ability for abstract thought and consciousness. Deciphering the molecular mechanisms that underlie cortical development will provide novel insights into biological processes such as cell survival, proliferation, cell fate determination, cell migration and differentiation.

1.1 Transcriptional signalling during cortical development

The predominant cell type generated during mammalian cortical development is the pyramidal projection neuron, which comprises up to 85% of the total neuronal population (Hevner 2006). Pyramidal neurons make long excitatory connections to both cortical and sub-cortical nuclei and uses glutamate as a neurotransmitter. The remaining cortical neurons are interneurons that release the inhibitory neurotransmitter, γ-aminobutyric acid (GABA). They have a more diverse morphology and make short "local" inhibitory connections (Butt et al 2007). Interneurons are generated within the medial ganglionic eminence of the ventral telencephalon

and migrate tangentially into the cortex. In contrast, pyramidal neurons are generated from radial glial cells located along the lateral ventricular surface of the dorsal telencephalon, called the ventricular zone (VZ) (Gupta et al 2007). Progenitor cells migrate radially from the VZ into the subventricular zone (SVZ) and intermediate zone (IZ) were they form pyramidal precursor neurons. These precursor cells later migrate into the cortical plate where they terminally differentiate and form the six-layered structure of the adult neocortex (Gupta et al 2007). The origin of interneurons and pyramidal neurons within distinct telencephalic regions allows differential exposure to extracellular cues. Spatially segregated differentiation determines distinct molecular, migrational and morphological properties of interneurons and pyramidal neurons, providing cell diversity within the cortex.

Neuronal progenitors within the VZ initially express transcription factors that regulate transcription of genes necessary for establishing regional boundaries between the dorsal telencephalon and sub-cortical regions, such as the ventral telencephalon. Regionalization is maintained in part by the expression of transcription factors belonging to the homeodomain, the paired and homeobox, the nuclear receptor, and the basic helix-loop-helix (bHLH) families (Bertrand et al 2002; Schuurmans and Guillemot 2002). Generation of pyramidal neurons from progenitor cells involves two conflicting processes, proliferation and differentiation, with the bHLH transcription factors being the foremost regulators. For example, the Id and Hes families maintain proliferation while suppressing neuronal development, as opposed to neurogenin (Ngn) 1 and Ngn2, which promote neuronal commitment and exit from the cell cycle (Ross et al 2003).

Migration of pyramidal neurons to distinct layers within the cortex is associated with expression of layer-specific transcription factors and has been comprehensively characterised. For example, the paired and homeobox transcription factor, Pax6, is a specific marker of progenitor cells in the VZ and is absent from early neuronal precursor cells of the SVZ. Upon commitment of progenitor cells to a neuronal cell fate, they begin to express the T-domain transcription factor Tbr2. As these newborn neurons migrate out of the SVZ into the IZ, they downregulate Tbr2 and induce the expression of Tbr1. The expression pattern of transcription factors from Pax6→Tbr2→Tbr1 follows the developmental sequence of radial glial→neuronal precursor→postmitotic pyramidal neuron (Hevner 2006). Stringent temporal and spatial expression of transcription factors is important for coordinating cortical development and provides a useful tool for marking progression of neuronal precursor development.

Similarly, specification of neuronal subtypes is determined by the pattern of transcription factors that are induced during development. For example, differentiation of interneurons is dependent on the expression of the bHLH transcription factor, Mammalian achaete scute homolog 1 and 2 (Mash1), whereas Ngn2 is necessary for the development of pyramidal neurons. Expression of Mash1 induces transcription factors, *Dlx1* and *Dlx2*, which subsequently activate genes associated with interneuron specification and migration (Cobos et al 2005; Kitamura et al 2002). These include expression of glutamic acid decarboxylase (*GAD67*), an enzyme essential for GABA synthesis (Anderson et al 1999).

Expression of genes controlled by specific transcription factors is essential for switching from proliferation of progenitor cells to the terminal differentiation of neuronal and glial

populations and for regulating many other neurodevelopmental processes including the onset of differentiation, cell migration, programmed cell death and cell size. Coordination of the temporal and spatial expression of specific transcription factors during development is regulated by intrinsic and extrinsic factors, including the pro-survival and growth-promoting neurotrophins (Ooi and Wood 2008).

1.2 Role of neurotrophins in neuronal development

Nerve Growth Factor (NGF) is the prototypic neurotrophin and was identified half a century ago by Victor Hamburger and Rita Levi-Montalcini (Levi-Montalcini and Booker 1960). It was originally described as a growth factor that promotes survival and differentiation of sympathetic and sensory neurons. This neurotrophin hypothesis is a widely accepted model for the development of the peripheral nervous system (PNS). Neurons are initially generated in great excess and then reduced to a select few during a critical window in development. This selection process is achieved through competition for prosurvival and growth-promoting factors released from peripheral target tissues, which include neurotrophins (Cowan 2001; Huang and Reichardt 2001).

The initial discovery of NGF was closely followed by the identification of three additional mammalian neurotrophins, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Neurotrophins normally function as homodimers however, BDNF is able to form heteromeric associations with NT-3 and NT-4/5 (Strohmaier et al 1996). All four neurotrophins bind with low-affinity to p75NTR, a distant member of the tumour necrosis factor receptor family. It was

later discovered that neurotrophins bind with much higher affinity to tropomyosin-related kinases (Trk), which belong to the tyrosine kinase receptor family. Each of the neurotrophins exhibits a preferential binding for one of the Trk receptors. NGF binds with high-affinity to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC, although NT3 can also bind TrkA and TrkB receptors with a lower affinity (Fig. 1.1) (Reichardt and Huang 2001).
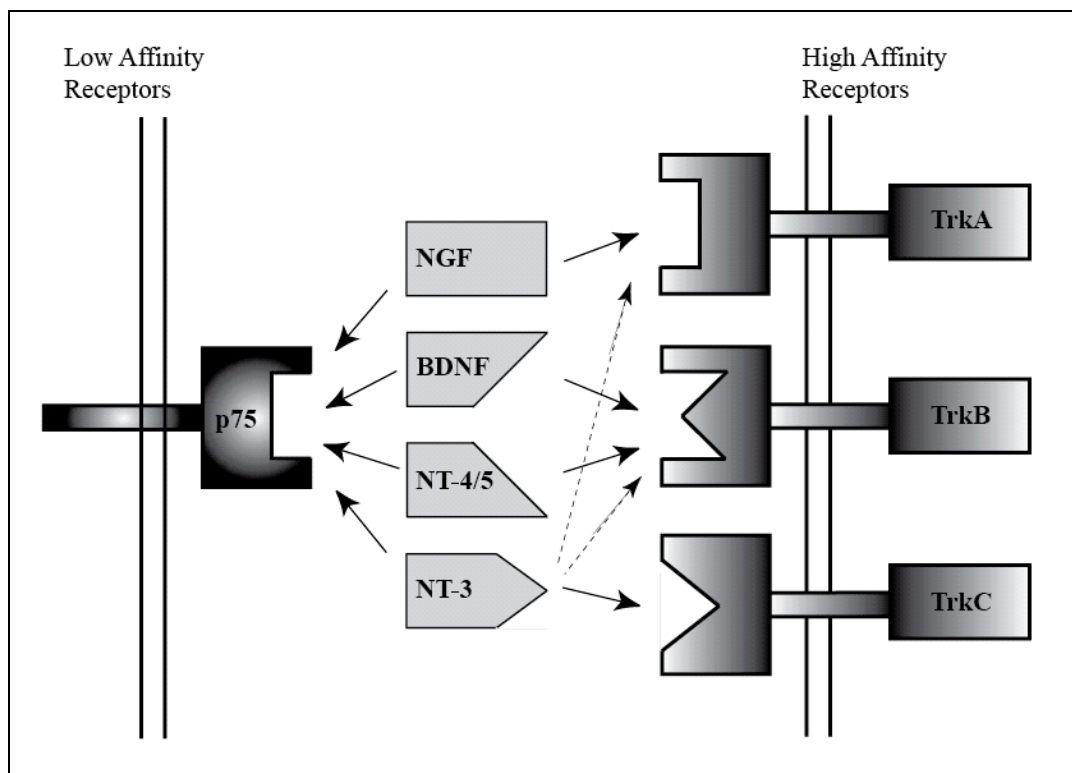
Figure 1.1 *Neurotrophins and their receptors.* All four neurotrophins bind with low-affinity to p75NTR. Each neurotrophin exhibits a preferential binding for one of the Trk receptors. NGF binds with high-affinity to TrkA, BDNF and NT4/5 to TrkB, and NT-3 to TrkC. NT-3 also binds TrkA and TrkB receptors with a lower affinity.

NGF and NT-3 are essential survival factors for peripheral sensory and sympathetic ganglia *in vivo* (Huang and Reichardt 2001; Blum and Konnerth 2005). Different neuronal populations

within the same ganglia can be distinguished by distinct morphological and functional properties, and express receptors for specific neurotrophins. A striking example is demonstrated in dorsal root ganglia (DRG), which contain a mixed neuronal precursor population that can be distinguished according to the Trk receptor expressed. DRG precursors expressing TrkA respond to NGF and form nociceptive neurons, whereas TrkC-positive cells differentiate into proprioceptive neurons in response to NT-3 stimulation (Crowley et al 1994; Smeyne et al 1994; Farinas et al 1996). It is now clear that neurotrophins play additional roles in the development of the PNS besides promoting cell survival, such as axon growth, dendritic development and soma hypertrophy (Purves et al 1988; Snider 1988; Fagan et al 1996; Patel et al 2000; Ye et al 2003).

Neurotrophins also play a key role in the development of the CNS, including cell survival, proliferation, differentiation, synaptic transmission and plasticity (Huang and Reichardt 2003; Blum & Konnerth 2005). During the development of the cortex, precursor cells in the SVZ express the neurotrophins BDNF and NT3, as well as their high affinity receptors TrkB and TrkC (Maisonpierre et al 1990) (Ernfors et al 1992) (Fukumitsu et al 1998). Neurotrophin-mediated TrkB and TrkC signalling is essential for regulating proliferation and differentiation of embryonic cortical precursors. NT3, for example, regulates cell cycle exit and neuronal differentiation of cortical progenitors cells (Ghosh and Greenberg 1995). Cortical precursor cells also synthesize and secrete BDNF that promotes survival and differentiation in an autocrine and paracrine fashion both *in vitro* (Barnabe-Heider and Miller 2003) and *in vivo* (Bartkowska et al 2007). Administration of BDNF into the lateral ventricles of embryonic mice enhances the radial migration of cortical neurons (Ohmiya et al 2002; Fukumitsu et al 2006). BDNF has been shown to facilitate survival and neurogenesis through activation of the phosphatidylinositol-3-kinase

(PI3K) and MEK pathways (Barnabe-Heider and Miller 2003), and to activate pro-neuronal bHLH transcription factors, such as Mash1 and Math1 (Ito et al 2003). Cell-type specific deletion of BDNF high affinity receptor in transgenic mice has shown that deletion of TrkB within cortical pyramidal neurons during development causes altered neuronal arborisation and compression of the cortex (Xu et al 2000). Later phenotypes of TrkB deletion include the loss of suppressed, cAMP-inducible POU (SCIP)-expressing neurons in somatosensory and visual cortices (Xu et al 2000). These data indicates that BDNF/TrkB signalling is necessary for supporting the survival and differentiation of CNS neurons.

1.2.1 Neurotrophin signalling

Tyrosine kinase signalling mediated through activation of Trk receptors promotes developmental processes associated with neurotrophin stimulation, including cell survival and differentiation. Ectopic expression of Trk receptors in embryonic neurons is associated with the acquisition of NGF-mediated survival response (Allsopp et al 1994; Barrett and Bartlett 1994). Neurotrophin-mediated signalling requires Trk receptor dimerization and auto-phosphorylation of three conserved cytoplasmic tyrosine residues. These are located within the autoregulatory loop of the kinase domain and are necessary for tyrosine kinase activity. Seven additional conserved tyrosine residues on Trk receptors can be phosphorylated and act as docking sites for adaptor proteins and kinases containing phosphotyrosine-binding (PTB) or src-homology-2 (SH-2) motifs. Many of these proteins are additional substrates for the Trk receptor tyrosine kinase. Numerous intracellular signalling pathways are initiated upon Trk receptor activation, including

Ras/ERK (extra-cellular signal-regulated kinase) protein kinase pathway, the PI3K/Akt pathway and the phospholipase C (PLC)-γ1 pathway (Fig. 1.2) (Huang and Reichardt 2001).
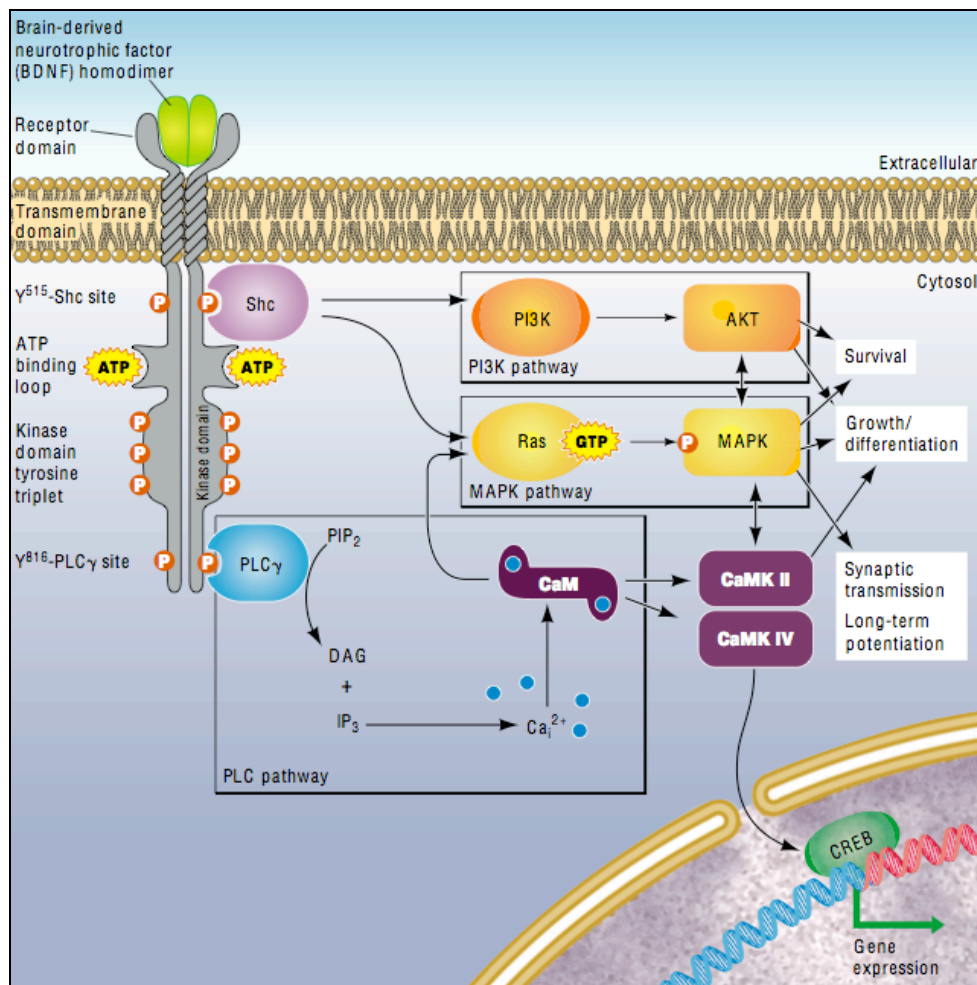
Figure 1.2 *Trk receptor activation and downstream signalling.* Trk receptors form dimers upon neurotrophin binding, which leads to auto-phosphorylation of an intracellular tyrosine kinase domain. Phosphorylated tyrosine residues on Trk receptors act as docking sites for adaptor proteins and kinases, which activate several intracellular signalling pathways including PI3K/Akt, Ras/MAP kinase and PLC-γ1 pathways. Trk-mediated signalling is important for neuronal functions, including survival, growth, differentiation, synaptic transmission and memory. Taken from Blum and Konnerth, 2005.

Ras activation and recruitment to Trk receptors following binding to neurotrophins requires the association of adapter proteins, including Shc and Grb-2 and activation of the Ras exchange factor, SOS. Downstream Ras signalling mediates transient activation of several kinase cascades, including the Extracellular signal-Regulated Kinase (ERK), Mitogen-Activated Protein Kinase (MAPK) and PI3K (Huang and Reichardt 2001). Activated MAPK leads to phosphorylation of p90 ribosomal S6 kinase (Rsk) and activation of pro-survival pathways (Bonni, 1999). This includes sequestering of proapoptotic BAD and activation of the prosurvival transcription factor, cAMP-Regulated Element Binding protein (CREB) (Bonni et al 1999). Importantly, activation of ERK kinase signalling upon neurotrophin stimulation leads to transformation of a neural-derived cell line, PC12, into a differentiation-like phenotype (Tanaka et al 1993; Matsuda et al 1994; Hempstead et al 1994). These examples demonstrate that Ras-activated kinases are important for promoting neuronal survival and differentiation.

PI3K is predominantly activated by Ras; however, PI3K function can also be activated by Shc and Grb-2 independently of Ras (Holgado-Madruga et al 1997)(Kaplan and Miller 2000). PI3K leads to activation of Akt/protein kinase B, and mediates survival of many neuronal populations including cerebellar granule cells and sympathetic neurons (Bonni et al 1999; Vaillant et al 1999). Activated Akt controls various proteins involved in cell death, such as BAD, $I_K B$, human caspase 9 and glycogen synthase kinase 3-β (Datta et al 1999). For example, phosphorylation of $I_K B$ targets it for degradation, which prevents $I_K B$ from binding and sequestering the transcription factor, $NF_K B$. Degradation of $I_K B$ allows nuclear translocation of $NF_K B$ and subsequent activation of pro-survival genes (Middleton et al 2000). Pharmacological

studies have demonstrated that the PI3K pathway is also capable of activating CREB, through mechanisms that remain elusive (Lin et al 2000; Perkinton et al 2002).

Many developmental functions regulated by neurotrophins require the activation of Ca^{2+}-dependent pathways. The major route for Trk-dependent elevation of intracellular Ca^{2+} is mediated by the PLC-γ1 pathway. PLC-γ1 catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to generate inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ induces Ca^{2+} release from intracellular stores, leading to activation of Ca^{2+}-regulated isoforms of protein kinase C (PKC) (Corbit et al 1999). PLC-γ1 signalling further modulates intracellular Ca^{2+} concentrations through the gating of a Ca^{2+}-permeable plasma-membrane channel. The canonical form of transient receptor potential (TRPC) channel is activated upon BDNF-induced synthesis of IP$_3$ and DAG (Li et al 1999; Clapham 2003; Amaral and Pozzo-Miller 2007). BDNF-dependent gating of TRPC channels through PLC-γ1 activation has been associated with growth cone development (Li et al 1999; Li et al 2005).

In addition to PKC, elevated intracellular Ca^{2+} following neurotrophin stimulation activates many different classes of protein kinases. Interaction of Ca^{2+} with its binding protein, calmodulin (CaM), forms a complex that activates several CaM-regulated kinases and phosphatases, including the CREB kinases, CaMKI, CaMKII and CaMKIV (Dash et al 1991; Sheng et al 1991; West et al 2001). Certain subtypes of adenylyl cylases are also regulated by Ca^{2+}, resulting in elevated levels of cyclic adenosine monophosphate (cAMP) and subsequent activation of an additional CREB kinase, cAMP-dependent protein kinase (PKA) (Gonzalez et al 1989). In

summary, neurotrophin-induced activation of Trk signalling leads to activation of several pathways that converge to regulate pro-survival transcription factors, such as NF_KB and CREB.

1.2.2 CREB – a transcriptional target of neurotrophin signalling

One important nuclear target of neurotrophin signalling is the transcription factor CREB (Ginty et al 1994; Bonni et al 1995). NGF stimulation of PC12 cells and sympathetic neurons, and BDNF stimulation of cortical and cerebellar granule cells has been shown to induce phosphorylation and activation of CREB (Ginty et al 1994; Riccio et al 1997; Bonni et al 1999). CREB-mediated gene expression is necessary and sufficient for the survival and differentiation of sympathetic and sensory neurons *in vitro* and *in vivo* (Lonze et al 2002; Mantamadiotis et al 2002; Riccio et al 1999). In addition to promoting neuronal survival, CREB has been implicated in a diverse range of neuronal processes from axonal (Rudolph et al 1998) and dendritic growth (Redmond et al 2002) to regulation of circadian rhythms, learning and memory (Lonze and Ginty 2002).

CREB is a bZIP transcription factor containing a C-terminal basic domain that mediates DNA binding to *c*AMP *R*esponse *E*lements (CREs). CREB activation is mediated by phosphorylation of Ser133 located within its kinase inducible domain. Phosphorylation of Ser133 results in binding of CREB to the transcriptional co-activator, *C*REB *B*inding *P*rotein (CBP). CBP has an endogenous histone acetyltransferase activity and catalyses acetylation of histone proteins. Stimulus-induced CREB phosphorylation recruits CBP to CRE-containing gene

promoters, resulting in hyperacetylation of histones and local chromatin unravelling. A relaxation of chromatin structure permits binding of transcription machinery and gene activation.

CREB regulates hundreds of genes that are diverse in function including neurotransmission, cellular metabolism, transcription and signal transduction (Mayr and Montminy 2001). Despite the fact that all CREB-regulated genes share a similar CRE element, their expression profile varies widely, depending on the cell type and the extracellular stimuli. This is suggestive that CREB regulation requires a more complex mechanism than a simple on-off switch, as described above. The Chromatin ImmunoPrecipitation (ChIP) technique is a highly sensitive assay for detecting DNA-protein complexes and it has been used to demonstrate that CREB is not constitutively bound to many transcriptionally inactive gene promoters (Riccio et al 2006). Neurotrophin-dependent gene expression in cortical neurons requires both CREB phosphorylation and CREB binding to CRE-containing gene promoters. Notably, these two processes occur independently through distinct signalling pathways and are both necessary for CREB-regulated gene activation (Riccio et al 2006). Regulation of CREB binding to promoters of specific genes provides a mechanism for differential activation of CREB-regulated genes in specific cell-types. It is well established that transcription factor binding to gene promoters is dependent on local changes of chromatin structure. Epigenetic modifications, such as histone acetylation, allow stable remodelling of chromatin that favours gene activation or repression. Maintenance of specific genes as either repressive or poised for activation may provide an intrinsic mechanism that dictates which CREB-regulated genes are activated in response to extrinsic stimuli.

1.3 Epigenetic events and neuronal development

During cortical development, neurons are the first cell type generated and are produced in a regulated sequential pattern that results in an inside out layering of the cortex. This initial peak of neurogenesis is followed by the generation of astrocytes and later oligodendrocytes. These diverse cortical cell types arise from the same population of radial glial progenitor cells, which is determined by the temporal expression of specific transcription factors. Many of the transcription factors required for neuronal differentiation have been identified and characterised. However, the binding of transcription factors to target genes is dependent on additional factors other than the DNA primary sequence. Epigenetic modification of DNA and histone proteins changes the overall structure of chromatin, which regulates the access of transcription factors to gene promoters and drives transcription.

Adrian Bird has recently proposed a revised definition of epigenetics as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird 2007). Epigenetic modification of chromatin is an intrinsic mechanism that allows regulation of gene transcription, DNA repair, replication, and chromosome condensation (Kouzarides 2007). DNA is packaged into chromatin through association with protein complexes called nucleosomes. A nucleosomal unit comprises 146 base pairs of DNA wrapped around an octameric histone complex composed of one H3/H4 tetramer and two H2A/H2B dimers (Luger et al 1997). Nucleosomes are connected by 20-60 base pairs of DNA that associate with the linker histone H1. Chromatin exists either as highly condensed heterochromatin or less compact euchromatin. Heterochromatin is resistant to endonuclease

digestion, is late-replicating, rich in repetitive sequences, and transcriptionally silent. Euchromatin organisation is more dynamic and can change transiently, allowing for gene activation and repression in response to extracellular cues. Epigenetic regulation of chromatin involves covalent modification of both DNA and the N-terminal tail of histone proteins (Kouzarides 2007).

1.3.1 DNA modifications

DNA methylation is associated with gene silencing and plays a major role in modulating differentiation of neuronal progenitor cells (Allen 2008). Methylation of CpG nucleotide islands is catalysed by two classes of DNA methyl-transferases (DNMT): the ubiquitously expressed DNMT1 uses hemi-methylated DNA as a template ensuring inheritance of established methylation patterns from parental to daughter DNA strands, whereas DNMT3a and DNMT3b catalyse *de novo* DNA methylation and exhibit a greater degree of differential expression during the development of the CNS (Robertson and Wolffe 2000; Goll and Bester 2004). *Dnmt3b* for example, is expressed earlier during development (E10-14) in progenitor cells of the VZ, coinciding with the major period of cortical neurogenesis (Feng et al 2005). In contrast, *Dnmt3a* expression persists postnatally, indicating a role in gene silencing necessary for neuronal maturation (Feng et al 2005). Recruitment of DNMTs for *de novo* methylation is dependent on ATP-dependent remodelling complexes. DNA methylation can repress gene expression through either direct or in-direct mechanisms. DNA methyl groups situated within the major groove of double helix DNA can directly disrupt the binding ability of transcription factors (Watt and Molloy 1988; Takizawa et al 2001). Alternatively, DNA methylation sites recruit methyl-CpG-

binding domain (MBD)-containing proteins, such as MeCP2, MBD1 and MBD2. Methylation-dependent repression can occur through interaction of MBD proteins with the transcriptional machinery or by modifying chromatin structure (Takizawa et al 2001). For example, MeCP2 has a transcriptional repression domain (TRD) that binds the transcriptional corepressor Sin3A, which recruits HDAC activity to nucleosomes (Matijevic et al 2008).

MeCP2-mediated repression is important for postnatal neuronal maturation and synaptogenesis (Smrt et al 2007). Disruption of MeCP2 expression results in Rett syndrome, a neurodegenerative disorder characterised by a regression of language and motor skills, followed by mental retardation the first year after birth (Matijevic et al 2008). Over-expression of *Bdnf* in MeCP2 mutant mice rescues the locomotor deficit, demonstrating a functional interaction between MeCP2 and BNDF *in vivo* (Chang et al 2006). MeCP2 regulates the expression of *Bdnf* by selectively binding and repressing the BDNF promoter (Chen et al 2003; Martinowich et al 2003). Neuronal activity causes Ca^{2+} influx and MeCP2 phosphorylation, which is released from the BDNF promoter. BDNF expression is associated with neuronal plasticity, learning and memory, which may account for certain Rett syndrome phenotypes. MBD proteins, such as MeCP2, are all components of chromatin-remodelling complexes and closely couple gene repression with histone methylation and acetylation (Fuks 2005).

1.3.2 Histone modifications

Histones can undergo a large number of posttranslational covalent modifications at their N-terminal tails, including acetylation, methylation, phosphorylation, ADP-ribosylation,

sumoylation and ubiquitination (Fig. 1.3) (Kouzarides 2007). Histone acetylation and methylation are currently the most extensively studied epigenetic modifications and will be the focus of my literature review.
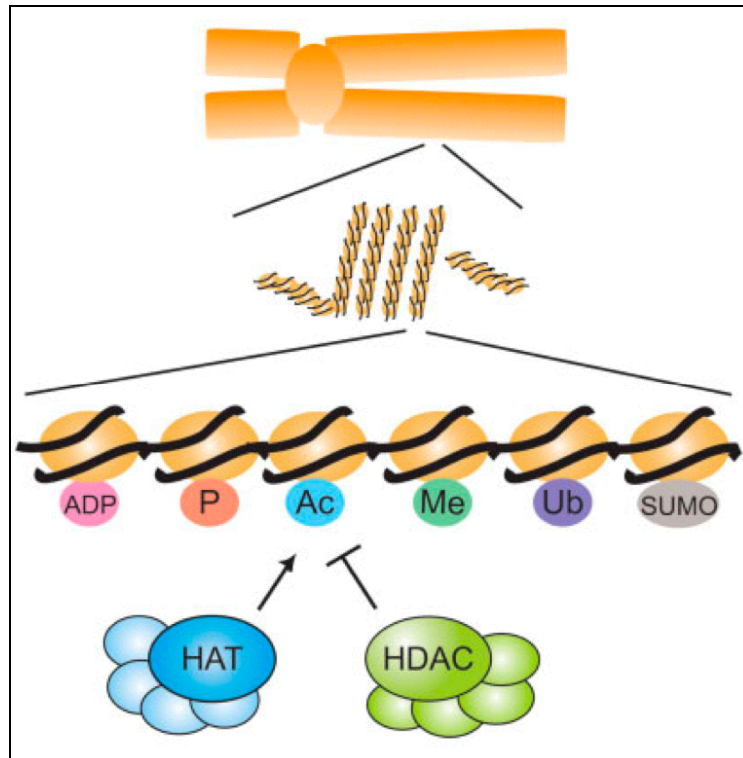
Figure 1.3 *Schematic representation of chromatin (top), chromatin fibre (middle) and nucleosome structure (lower)*. DNA is depicted in black and nucleosome histone cores as yellow spheres. Histone posttranslational modifications have been depicted as circles: ADP-ribosylation (ADP, pink), phosphorylation (P, orange), acetylation (Ac, blue), methylation (Me, green), ubiquitination (Ub, purple) and sumoylation (SUMO, grey). Histones are acetylated by Histone Acetyltransferases (HAT, blue) and acetyl groups are removed by Histone Deacetylases (HDAC, green). Taken from Kimura et al, 2005.

1.3.3 Histone methylation

Methyl groups are added to histone lysine and arginine residues by histone methyltransferases (HMT). In humans and mice, fourteen different HMTs have been identified that generally exhibit high specificity, often methylating a single residue on specific histone proteins (Bannister and Kouzarides 2005). Lysine residues can be mono-, di- or tri-methylated and arginines can be either mono- or di-methylated (symmetrically or asymmetrically), adding another level of complexity (Berger 2007). The addition of small methyl groups is unlikely to change charges on lysine and arginine residues, which suggests that chromatin structure will remain unaltered. Epigenetic regulation of transcription following histone methylation is likely to be dependent on recruitment of nuclear proteins via chromo-like binding domains. Histone lysine demethylases (HDMT) have recently been discovered and display the ability to specifically interact with mono-, di-, or tri-methylated lysine residues (Shi et al 2004; Metzger et al 2005; Tsukada et al 2006). So far, no ariginine demethylases have been identified.

During early stages of development, global chromatin environments are established, with large regions that remain silent (heterochromatin) whereas other regions become transcriptionally active (euchromatin). Methylation of H3K4 and H3K9 is present at the boundary of euchromatin with heterochromatin and is important for dictating and preserving the two types of chromatin domains (Kouzarides 2007). Heterochromatin regions of DNA are associated with high levels of methylation at specific histone lysine residues, H3K9, H3K27 and H4K20 (Barski et al 2007). Euchromatin has a dynamic biological output compared to heterochromatin and therefore a more flexible modification pattern to reflect this. Transcriptionally active euchromatin is associated with trimethylated histone H3K4 and H3K36, conversely methylation of these residues is low in silent regions of euchromatin (Barski et al 2007). The chromo-domain proteins that recognise

methylated histone lysines dictate the mechanistic and functional consequence of these modifications. H3K9 methylation recruits the repressive protein Heterochromatin Protein 1 (HP1), which provides a platform for the binding of DNA methyltranserases (Lehnertz et al 2003; Fuks et al 2003). This event results in DNA methylation near chromatin regions containing H3K9 methylation and the subsequent recruitment of MBD proteins associated with gene repression (Fuks 2005). In contrast, the transcriptional activator Chromo-ATPase/Helicase-DNA binding Domain 1 (CHD1), binds to methylated H3K4, which recruits chromatin remodelling complexes associated with gene activation (Pray-Grant et al 2005). These two examples demonstrate that the function of methylated histone lysines is dependent on recruitment of specific activating or repressing methyl-binding proteins.

1.3.4 Histone acetylation

Histone acetylation, the most widely studied epigenetic modification, involves the cleavage of acetyl groups from acetyl-coenzyme A and its addition to the ε-NH^+ of lysine residues. Acetylation of histones is catalysed by histone acetyl-transferases (HATs), which are divided into three main families, CBP/p300, MYST and GNAT and exhibit virtually no preference for specific lysine residues (Roth et al 2001; Carrozza et al 2003). The majority of acetylated lysine residues are located within the more accessible N-terminal tail of histones. Acetylation of histones has a direct influence on chromatin structure as it reduces the positive charge on lysine residues, which decreases the affinity of histones for negatively charged DNA. This allows a localised 'unravelling' of chromatin, making it more accessible for binding of further co-activators and basal transcription machinery. Histone acetylation also provides a platform for

chromatin remodelling proteins that contain bromo-domains, such as the SWI/SNF complex (Kouzarides 2007). The assembly of these large multiprotein complexes is important for initiation of transcription, DNA replication and repair and further histone modifications.

Acetylation of histone H3 at lysine residues 9 and 14 (AcH3K9/K14) and histone H4 at lysine residues 5, 8, 12, and 16 (AcH4) has been mapped on transcriptional initiation sites of active genes (Pokholok et al 2005). Hyperacetylation of histone H3 and H4 correlate positively with DNA binding of various HATs and DNA polymerase II (Pol II). It should be considered that different histone modifications are not mutually exclusive, for example tri-methylation of histone H3K4 exhibits a similar genomic profile as described for AcH3K9/K14 (Guenther et al 2007). However, tri-methylation of histone H3K36 is associated with transcriptional elongation, is enriched within the coding regions of active genes and is almost absent at the transcriptional initiation site of active genes (Guenther et al 2007).

The removal of acetyl groups is catalysed by histone deacetylase proteins (HDACs) and is associated with chromatin condensing and transcriptional repression. There are four classes of HDACs, class I (HDACs 1-3 and 8), class II (HDACs 4-7, 9 and 10) and class IV (HDAC 11) that are all zinc-dependent, whereas class III (SIRT 1-7) require the cofactor, NAD^+ (Thiagalingam et al 2003; Yang and Gregoire 2005). Class I HDACs show high homology to yeast Reduced potassium dependency-3 (Rpd3) and with the exception of HDAC3, are ubiquitously expressed and principally localised in the nucleus. Class II HDACs are homologous to yeast Histone deacetylase-1 (Hda-1). They exhibit a tissue-specific expression and shuttle between the nucleus and cytoplasm (as does HDAC3). HDAC11, the sole member of class IV

HDACs, shows an intermediate homology to both Rpd3 and Hda1. Sirtuins (class III HDACs), are homologous to silent information regulator-2 (sir2) and are evolutionarily distinct from classes I, II and IV HDACs.

1.3.5 Epigenetic control of neurogenesis

The expression of neural genes during development requires the turnover of large transcriptional complexes that often contain HATs, HDACs, HMTs and HDMTs (Allen 2008). During the early stages of neuronal development, progenitor cells are maintained in a proliferative state through expression of Notch effectors. These include inhibitory bHLH transcription factors, such as Hes1, that act in concert with co-repressive complexes to inhibit neurogenic bHLH genes, such as *Ascl1* (Cau et al 2000). The repressive activity of Hes1 is mediated through the recruitment of Sin3A/HDAC complexes that results in localised histone hypoacetylation and gene silencing (Nuthall et al 2004). Exposure of progenitor cells to neurogenic stimuli leads to ADP-ribosylation of the Hes1 complex, which results in dissociation of Sin3A/HDAC, recruitment of HATs and hyperacetylation of local histone proteins (Ju et al, 2004). The switch of Hes1 from a transcriptional repressor to an activator is dependent on the presence of HDACs and HATs within the complex. Thus, chromatin remodelling is essential for transcriptional regulation of neural genes.

Differentiation of progenitor cells into a neuronal phenotype is further regulated by the nuclear receptor co-repressor (N-CoR). Repressive complexes containing N-CoR silence the gene promoters of down-stream effectors of Notch signalling through the recruitment of HDAC3

(Kao et al 1998). The absence of N-CoR in cortical progenitors prevents FGF-dependent self-renewal of neuronal progenitors and promotes their differentiation into astrocytes (Hermanson et al 2002). Ciliary neurotrophic factor (CNTF) triggers glial differentiation and leads to Akt1 kinase-dependent phosphorylation of N-CoR and subsequent relocalization to the cytoplasm (Hermanson et al 2002). These data imply that N-CoR/HDAC3 complexes play important functions in regulating the differentiation of early-stage progenitor cells specifically by repressing gliogenic over neurogenic genes.

Many neuronal genes have a conserved motif called the Repressor Element 1 (RE1), that upon methylation binds the zinc-finger transcriptional repressor RE1 silencing transcription factor (REST) (Chong et al 1995; Schoenherr and Anderson 1995). REST recruits Sin3A/HDAC, as well as repressive cofactors CoREST and MeCP2 to sites of gene repression. CoREST is a co-repressor that mediates long-term gene silencing by recruitment of HDACs, H3K9 histone methyltransferases and the H3K4 demethylase LSD1. REST silences many neuron-specific genes in non-neuronal cells, including those encoding ion channels, neurotransmitter receptors, synaptic vesicle proteins and adhesion proteins (Johnson et al 2007). Regulation of REST allows a tight regulation of neuronal development, and later, the plasticity of mature neurons (Ballas and Mandel 2005). A high level of REST in neuronal embryonic stem cells prevents their exit from the cell cycle and blocks premature neuronal differentiation. Posttranslational downregulation of REST protein allows the activation of selected genes, which leads to the developmental progression of progenitor cells to neuronal precursors. As neuronal development proceeds, the *Rest* gene itself is transcriptional repressed to allow precursor cells to differentiate into mature neurons. The absence of the REST/HDAC/Sin3A/CoREST repressive

complex from gene promoters allows the expression of neuronal terminal differentiation genes (Schoenherr et al 1996). However, the transcription of certain genes, including *Bdnf* and calbindin, remains low due to the binding of CoREST and MeCP2 complexes at additional methylation sites (Ballas et al 2005). Neuronal depolarisation results in phosphorylation of MeCP2 and initiation of *Bdnf* transcription. Regulation of MeCP2 repressive complexes binding to neural gene promoters in response to neuronal activity provides a platform for synaptic plasticity in mature neurons (Ballas and Mandel 2005).

1.3.6 Regulation of HDAC functions

The function of HDAC proteins can be classified into two areas, the enzymatic deacetylase activity of the protein and the functional repressive activity of the protein. HDACs exist as part of large repressive complexes where they play a key role for the recruitment of other corepressor proteins. Binding of these repressors to chromatin is often sufficient for transcriptional inhibition in the absence of deacetylase activity (Yang and Seto 2008). Moreover, HDAC functions are also regulated by post-translational modifications (phosphorylation, ubiquitination and sumoylation), subcellular localisation, intracellular protein levels and proteolytic cleavage.

1.3.7 HDAC co-repressors

With the exception of yeast HOS3 and mammalian HDAC8, purified HDACs are enzymatically inactive, as they require assembly into large multi-subunit complexes in order to function (Carmen et al 1999; Hu et al 2000; Lee et al 2004). Additionally, HDAC proteins do not

exhibit target selectivity, as they deacetylate both histone and non-histone substrates and do not directly bind DNA (Sengupta and Seto 2004). Therefore, HDAC repressor function depends on interaction with specific corepressors that target them to specific chromosomal loci and regulate their enzymatic activity. HDACs are regulated by multiple signalling pathways and can associate with numerous different repressive complexes.

HDAC1 and HDAC2 exist in three distinct native complexes, the Sin3A, NuRD and CoREST complexes. These HDAC-containing complexes comprise similar core proteins, notably HDAC1, HDAC2, RbAp46 ad RbAp48, with the addition of unique polypeptides that make up the native holo-complex (Sengupta and Seto 2004). Purified HDAC core complexes exhibit limited deacetylase activity that can be restored through the addition of specific corepressors, such as MTA2 (Zhang et al 1999). The best example of an HDAC activity regulated by protein-protein interaction is provided by the association of HDAC3 with Silencing Mediator of Retinoid and Thyroid receptor (SMRT) and N-CoR (Wen et al 2000; Guenther et al 2001; Zhang et al 2002). The deacetylase activity of HDAC3 is significantly enhanced following SMRT/NCoR binding and is therefore regulated by SMRT/NCoR nuclear levels.

The majority of corepressors that enhance class I HDAC enzymatic activity contain a SANT domain (Sengupta and Seto 2004). These motifs are putative DNA binding domains and target HDAC complexes to chromatin. Interestingly, deletion of SANT domains demonstrated that they are also important regulators of HDAC activity (Yu et al 2003). These data demonstrate a key role of protein-protein interaction in regulating HDAC repressive function by modulating both its localisation and enzymatic activity.

1.3.8 HDAC posttranslational modification

In addition to binding with specific corepressors, HDACs are regulated by posttranslational modifications such as phosphorylation. HDAC1 and HDAC2 contain two C-terminal casein kinase-2 (CK2) phosphorylation consensus domains, whereas HDAC3 has one. Serine phosphorylation of HDAC1-3 increases deacetylase activity, possibly through changing the protein conformation to an enzymatically active form (Pflum et al 2001; Tsai and Seto et al 2002). Importantly, HDAC mutants that can no longer be phosphorylated exhibit impaired association with the corepressors RbAp48, MTA-2, Sin3A and CoREST (Pflum et al 2001; Tsai and Seto et al 2002). HDAC phosphorylation may increase the affinity of HDAC binding with corepressors, which subsequently enhances its deacetylase activity. In contrast, HDAC8 does not have a CK2 consensus domain but is phosphorylated by PKA *in vitro* and *in vivo* (Lee et al 2004). HDAC8 is phosphorylated at a non-conserved serine residue, which decreases its deacetylase activity (Lee et al 2004). Class II HDACs may also be phosphorylated however, there are conflicting evidences supporting either calcium/calmodulin-dependent kinase (CaMK) or *E*xtracellular signal-*R*egulated *K*inases 1 and 2 (ERK1/2) as potential HDAC kinases (McKinsey et al 2000; Zhou et al 2000). Interestingly, phosphorylation of class II HDACs does not affect their catalytic activity, instead it affects their subcellular localisation.

HDACs are also regulated by a post-translational modification called sumoylation. The addition of small ubiquitin-related modifier 1 (SUMO-1) to HDAC1 increases its deacetylase activity independently of corepressor binding (David et al 2002). Sumoylation of class II

HDACs, including HDAC4 and HDAC6 is dependent on interactions with nuclear pore complex proteins, such as RanBP2, and is therefore associated with HDAC nuclear localisation and increased transcriptional repression (Kirsh et al 2002). In summary, post-translational modification of class I HDACs predominantly affects deacetylase activity, whereas modification of class II HDACs affects mostly their subcellular localisation.

1.3.9 HDAC subcellular localisation

Class I HDACs, with the exception of HDAC3, are nuclear proteins and therefore their localisation is not regulated. Class II HDACs shuttle in and out of the nucleus and are sequestered within the cytoplasm upon binding to 14-3-3. Association of HDACs with 14-3-3 is dependent on HDAC phosphorylation, which leads to HDAC localization in the cytoplasm. Binding of 14-3-3 to class II HDACs occludes the nuclear localisation signal and disrupts their association with importin-α. The change in subcellular localisation of class II HDACs is often regulated by extracellular stimuli. In the hippocampus, for example, nuclear export of HDAC4 is induced by spontaneous electrical activity, whereas HDAC5 export is induced by Ca^{2+} influx following activation of *N*-methyl-D-aspartate receptors (NMDARs).

1.3.10 HDAC protein levels

Targeting components of HDAC complexes to proteosomal degradation provides a mechanism to alleviate HDAC repression and allow gene activation. HDAC1/Sin3A is targeted for proteosome-mediated degradation during preadipocyte differentiation (Wiper-Bergeron et al

2003). N-Cor, a key component of HDAC3 repressive complexes, is targeted for degradation by Siah1 in a cell-type specific manner (Zhang et al 1998). Since N-CoR greatly enhances HDAC3 activity, reducing the availability of N-CoR will inhibit HDAC3. HDAC levels may also be regulated at the transcriptional level. Interestingly, HDACs can regulate their own transcription. Disruption of HDAC1 in embryonic stem cells, for instance, increases the expression of many other class I HDACs (Lagger et al 2002).

Although HDACs have been established as key regulators of transcription, the regulation of HDACs themselves has not been fully explored. It is likely that in addition to the mechanisms described here, additional modifications may also influence HDAC activity. Nuclear shuttling of class II HDAC4 and HDAC5, for example, is regulated by Nitric Oxide (NO) signalling (Illi et al 2008). Similarly, a recent study indicated that binding of class I HDAC2 to gene promoters is regulated by NO-dependent signalling (Riccio et al 2006). However, the mechanisms by which NO influences HDAC functions remain to be elucidated.

1.4 Nitric oxide (NO)

A decade ago, Robert Furchgott, Ferid Murad, and Louis Ignarro were awarded the Nobel Prize for demonstrating that a gas is capable of acting as a signalling molecule. This gas, called nitric oxide (NO), is now known to regulate many important physiological functions in the cardio-vascular, immunological and nervous systems.

1.4.1 NO synthases

NO is synthesised by the enzyme NO synthase (NOS) through oxidation of the terminal guanidine of L-arginine in the presence of oxygen (Fig. 1.4) (Palmer et al 1988). There are three mammalian NOS enzymes, the Ca^{2+}-dependent neuronal NOS (nNOS) and endothelial NOS (eNOS), and the Ca^{2+}-independent inducible NOS (iNOS) (Bredt and Snyder 1994). All three isoforms exist as homodimers, with subunits ranging in size from 130 to 160 KDa (Schmidt et al 1991). They require a large heterogeneous group of cofactors for their activity, including FAD, FMN, heme groups, tetrahydrobiopterin and calmodulin, and two co-substrates NADPH and O_2 (Alderton et al 2001). The NOS enzymes have a C-terminal domain homologous with cytochrome P450 reductase (CPR) that contains binding sites for NADPH, FAD and FMN (Fig. 1.4) (Schmidt et al 1993).

The synthesis of NO involves the transfer of five electrons to arginine guanidine nitrogen and is catalysed over two independent steps (Fig. 1.4). First, a two-electron oxidation reaction of L-arginine produces a tightly bound N^{ω}-hydroxyarginine (NHA) intermediate utilizing 1 eq of NADPH and 1 eq of O_2. This reaction requires tetrahydrobiopterin and Ca^{2+}/calmodulin as co-activators. The second step involves the production of NO and L-citrulline, the mechanism of which is less clear. Enzymatically, both steps are dependent on all six co-factors and oxygen however, only 0.5 eq of NADPH are consumed in the second step, with an additional electron possibly provided by NHA (Stuehr and Ikeda 1992).

Phase 1

O_2

Fe^{2+} → L-arginine

1 mol NADPH → FAD/FMN → (P_{450})

Fe^{3+} → H_2O

N^{ω}-OH-L-arginine

Phase 2

NBT O_2

0.5 mol NADPH → FAD/FMN → BH_4 → $O_2^{\cdot-}$ → H_2O / L-citrulline

·NO

0.5 mol NADPH → FAD/FMN → q-BH_2

NBT formazan H_2O_2

NADPH diaphorase
NADPH oxidase
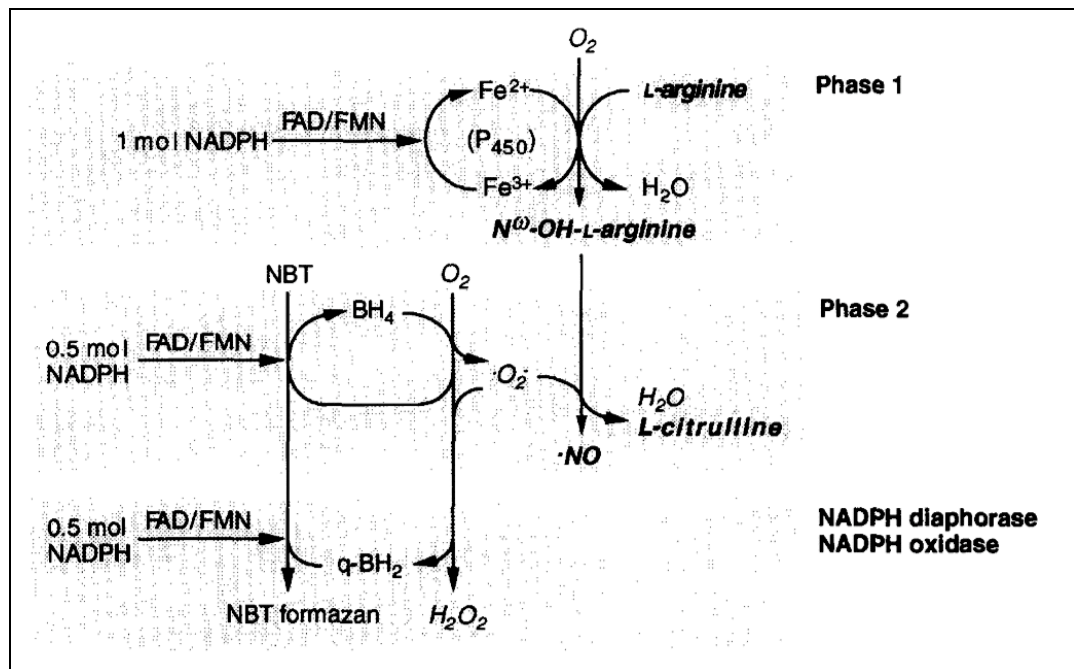
Figure 1.4 *Two-phase reaction mechanisms for NO synthesis.* Electron transfer from NADPH to O_2 are necessary for both phases and require the flavin cofactors, FAD and FMN, and the cytochrome P_{450} domain. Phase 1, L-arginine is hydroxylated to N^{ω}-hydroyarginine, which requires 1 mol NADPH. Phase 2, N^{ω}-hydroxyarginine is oxidated to form L-citrulline and NO. This step consumes 0.5 mol NADPH and is dependent on tetrahydrobiopterin (BH_4). NOS enzymes are capable of oxidase activity in the absence of L-arginine (NADPH oxidase activity) generating hydrogen peroxide (H_2O_2). Taken from Schmidt et al, 1993.

The co-factor tetrahydrobiopterin substantially increases NO synthesis through a structural mechanism that stabilises NOS dimerisation, a prerequisite for its enzymatic activity (Stuehr et al 1991). Ca^{2+}/calmodulin binding to NOS is necessary for electron transfer and oxygen activation, but is not required for arginine binding (Matsuda and Iyanagi 1999). Calcium signalling allows a very rapid activation of NOS and induction of NO-dependent events, including neurotransmitter release upon glutamate stimulation of NMDA receptors and vasodilation upon activation of acetylcholine muscarinic receptors in blood vessel endothelial cells (Kuriyama and Ohkuma 1995; Elhusseiny and Hamel 2000). iNOS co-purifies with calmodulin in the absence of Ca^{2+},

suggesting that it exists in a native heterotetrameric complex. iNOS activity is transcriptionally regulated and its expression is induced by extracellular stimuli such as interferon-γ and lipopolysaccharide signalling (Hecker et al 1999).

Studies in cardiovascular, nervous and musculoskeletal systems have demonstrated that phosphorylation of both eNOS and nNOS play a key role in regulating NO production (Mount et al 2006). The two NOS isoforms can be phosphorylated at multiple serine/threonine residues, which in the case of eNOS can either inhibit or enhance NO synthesis (Fulton et al 2001). Biochemical studies have demonstrated a negative regulation of nNOS following phosphorylation by a number of different kinases, including PKC, CaMK and cGMP-dependent protein kinase (PKG) (Dinerman et al 1994a; Bredt et al 1992; Hayashi et al 1999). Ca^{2+} signalling regulates NOS activity at two levels, initially by activating NOS through Ca^{2+}/calmodulin binding and later by inhibiting NOS through activation of CaMKs. Additionally, NO can activate soluble guanylyl cyclase (sGC) by binding to the sGC heme group. This event increases the levels of cyclic guanosine 3',5'-monophosphate (cGMP) and activates PKG. Interestingly, NO can directly inhibit NOS activity by binding the N-terminal heme co-factor, thereby functioning as a potential negative feedback inhibitory mechanism (Rogers and Ignarro 1992; Rengasamy and Johns 1993; Assreuy et al 1993).

In addition to regulating its enzymatic activity, phosphorylation of eNOS also determines its cellular localisation. Under basal conditions, eNOS is localised at the plasma membrane of endothelial cells. Upon treatment with bradykinin, it becomes rapidly phosphorylated and translocates to soluble fractions (Michel et al 1993). NOS localisation is important for spatially

restricting NO signalling by targeting NOS enzymes to subcellular compartments, thereby allowing NOS interaction with specific binding partners. A striking example is provided by the localization of NOS isoforms in cardiomyocytes, where eNOS and iNOS are targeted to the plasma membrane and sarcoplasmic reticulum respectively, resulting in distinct isoform-specific inotropic responses (Petroff et al 2001; Ashley et al 2002; Sears et al 2003). In neurons, nNOS localisation is achieved through interaction with scaffold proteins, including synaptic density proteins (PSD93/95) and carboxy-terminal PDZ ligand of nNOS (CAPON) by interaction with PDZ domains (Brenman et al 1996; Jaffrey et al 1998). PSD93/95 interacts with NMDARs, which upon glutamate binding allows Ca^{2+} influx, leading to nNOS activation and NO synthesis (Kornau et al 1995). NO directly modifies NMDAR subunits, inhibiting its activity, and thus acting as an inhibitory feedback loop (Lipton et al 2002). Conversely, the small G-protein Dexras 1 is activated upon NMDAR-induced NO signalling and interacts with nNOS via the soluble scaffold protein, CAPON (Fang et al 2000). Competitive binding of nNOS with CAPON and PSD95 is a possible mechanism that regulates nNOS shuttling between synaptic and non-synaptic sites. Interaction with CAPON sequesters nNOS from NMDA receptor-mediated Ca^{2+} influx, reducing the physiologic and pathologic affects of synaptic-induced NO signalling. Localisation of nNOS to specific subcellular compartments will determine its exposure to local increases of Ca^{2+} and will influence the level of NO signalling.

1.4.2 NO signalling

NO has rapidly emerged as a dynamic regulator of a vast number of proteins. NO was initially demonstrated to bind and regulate the activity of transition metal-containing proteins. In

addition to this, it is now well established that NO can directly modify target proteins by nitrosylation of cysteine (S-nitrosylation) or tyrosine residues (Tyr-nitration). NO binding to the transition metal centre of proteins will either stimulate or inhibit their intrinsic catalytic activity. The prototypic example of a transition metal-containing protein regulated by NO is sGC (Murad et al 1978; Bohme et al 1978). Upon binding of NO to the heme moiety of sGC, the heme-iron is dislocated, causing a conformational change that results in enzymatic activation. Activated sGC catalyses the synthesis of cGMP, which leads to the activation of several downstream cGMP-dependent effectors (Schmidt et al 1993). cGMP activates cGMP-gated channels, phospodiesterases and PKG. These mediators are important for a number of processes, including retinal rod response, olfactory reception, steroidogenesis, renal and intestinal ion transport, platelet aggregation, and cardiac and smooth muscle contractility (Schmidt et al 1993). It should be noted that sGC is also activated independently of NO signalling through other pathways, such as CO, •OH and guanylyl cyclase-activating factors (GAFs). In the nervous sytem, sGC exhibits better co-localisation with the GAF protein, heme oxygenase, than with nNOS (Snyder 1992; Schmidt et al 1992). Expression of sGC and nNOS within the cerebellum, for example, is mutually exclusive, with nNOS restricted to granule and basket cells and sGC restricted to Purkinje cells (Bredt et al 1990; Nakane et al 1983). This indicates that NO may not be the primary activator of sGC in the nervous system. However, NO is capable of freely diffusing between cells that are in close proximity. For example, stimulation of parallel fibres leads to NO production and diffusion to postsynaptic Purkinje cells where it subsequently activates sGC (Lev-Ram et al 1995).

NO signalling mediates many physiologic and pathologic affects through post-translational modification of proteins. Covalent addition of a NO moiety occurs through direct interaction of higher order NO oxide (NO_x) species with target tyrosine and cysteine residues, independent of additional enzymatic catalysis. Tyr-nitration is responsible for many of the pathologic effects associated with excessive NO signalling in cardiovascular and neuronal diseases or following inflammatory insults (Ohmori and Kanayama 2005; Peluffo and Radi 2007; Torreilles et al 1999). Tyrosine nitration involves the oxidation of a Tyr phenolic ring (Tyr•) followed by a radical-radical termination reaction with $•NO_2$, forming 3-nitrotyrosine (3-NT) (Souza et al 2008). The production of the Tyr• radical is accomplished by the one-electron oxidants, $CO_3•^-$, $•OH$, and $•NO_2$ or by numerous peroxidases. The generation of 3-NT is ultimately dependent on NO which upon reaction with superoxide, forms a powerful intermediate nitrating agent called peroxynitrite (Souza et al 2008). Peroxynitrite can homolyze to form the various oxidants mentioned for 3-NT formation. Protein tyrosine nitration is a selective process, occurring on specific residues within loop and irregular folded regions exposed to solvent or near the metal centre of metalloproteins (Souza et al 1999). Only a few proteins are capable of undergoing tyrosine nitration and the level of cellular 3-NT detected under basal conditions is low. However, significant increases in 3-NT can be detected after nitrosative stress associated with cardiovascular disorders and during inflammation (Leeuwenburgh et al 1997; Shishehbor et al 2003). Proteins that change biological function upon nitration, such as α–synuclein and tau, are often associated with pathological conditions. Nitrated α–synuclein and tau are thought to initiate aggregate formation in neurodegenerative diseases, such as Parkinson's and Alzheimer's (Souza et al 2000; Hodara et al 2004; Reynolds et al 2006). It should also be considered that

certain nitrated proteins exhibit no change in biological function, the scale of which is probably masked by difficulties in publishing negative data (Gole et al 2000).

After discovering NO as a physiological signalling molecule, it became apparent that cysteine thiol groups could couple NO through *S*-nitrosylation, a modification akin to phosphorylation. Due to the labile nature of this modification, early studies of *S*-nitrosylated proteins were performed *in vitro* using large quantities of exogenously supplied NO. Within the last decade a new methodology that replaces the nitrosyl group with a more stable biotin-moiety called the biotin-switch assay, has allowed the analysis of endogenously nitrosylated proteins (Jaffrey and Snyder 2001). Within a short time period, more than 100 proteins bearing cysteines that form *S*-nitrosothiols (SNO) have been identified (Jaffrey et al 2001). The major contributor of this modification is NO generated by NOS, as well as nitrites and other higher order NO species. Proteins can also be nitrosylated by the exchange of nitrosyl groups from other cellular SNOs in a process called transnitrosylation (Pawloski et al 2001). The low-mass SNO *S*-nitroso-glutathione (GSNO) is metabolized by GSNO-reductase and is the major source of cellular transnitrosylating SNO.

Despite the expression of at least one NOS isoform in most cell types and that the majority of proteins contain a cysteine residue, *S*-nitrosylation appears to be a highly specific modification. Many proteins that are *S*-nitrosylated often contain multiple cysteines but only one or very few are targeted by NO *in vivo*. The specificity of cysteines targeted by NO is dependent, at least in part, on the presence of an acid-base consensus motif (Stamler et al 1997). Deprotonation of the thiol group to form a more reactive thiolate anion (RS⁻) is suppressed and

enhanced by the presence of proximal acidic and basic groups, respectively. This can be enhanced by the presence of Zn^{2+} that reduces the pKa of neighbouring cysteine thiols, as observed for metalloproteins (Dudev and Lim 2002). The presence of an acid-base motif may be determined by the tertiary or quaternary structure of a protein, such as for caspase-3 and haemoglobin therefore, prediction of *S*-nitrosylation sites from the primary sequence is not always possible (Hess et al 2001). An additional mechanism involves the concentration of NO and O_2 within hydrophobic pockets, resulting in the formation of NO species that readily nitrosylate nearby cysteines. These hydrophobic pockets can reside within the protein tertiary structures or within a juxtamembrane position, as with the Ryanodine Receptor/Ca^{2+}-release channel (RyR1) and the NMDAR, respectively (Choi et al 2000; Nedospasov et al 2000; Sun et al 2001; Hess et al 2005). Metal-centres of proteins can also catalyse nitrosylation of intrinsic cysteine residues (eg haemoglobin) or cysteines on differing protein substrates (eg superoxide dismutase). As discussed earlier, coupling of nitrosyl groups to cysteine residues can also occur by transnitrosylation. This direct transfer of a nitrosyl group from other SNOs present in the cell is specified by protein-protein interactions.

S-nitrosylation has been reported to regulate the activity of a number of proteins, affecting catalytic activity, protein-protein interaction and/or subcellular localisation (Hess et al 2005). The enzymatic activity of all caspases, and certain protein tyrosine phosphatases (PTPs) is inhibited by nitrosylation of critical cysteine residues within the active site (Mannick et al 2001). MAP kinases, including ASK1 (apoptosis signal-regulating kinase-1) and JNK (Jun N-terminal kinase) are also negatively regulated by *S*-nitrosylation however, other kinases such as Src (non-receptor protein tyrosine kinase p60[Src]) have enhanced activity upon nitrosylation (Akhand et al

1999; Park et al 2000; Park et al 2004). Similarly, *S*-nitrosylation of ion channels can either inhibit ion influx, such as the NMDAR, or enhance it, as observed with RyR1 (Choi et al 2000; Eu et al 2000). Nitrosylation can also enhance GDP–GTP exchange of certain monomeric GTPases, including activation of p21^{Ras} and Dexras in neurons (Yun et al 1998; Jaffrey et al 2002). These few examples illustrate the variety of effects that NO can have on protein functions, demonstrating its ability to act as a very flexible signalling molecule.

1.4.3 *S*-nitrosylation: a reversible modification

S-Nitrosylation of proteins is dependent on cellular levels of NO and its higher oxide derivatives and on intracellular SNO content that is dictated largely by the tight regulation of NOS activity, expression and localisation. However, removal of nitrosyl groups (denitrosylation) is also essential. The finding that constitutively *S*-nitrosylated proteins, such as mitochondrial caspase-3 and eNOS, become activated upon stimulus-coupled denitrosylation support this mechanism (Mannick et al 1999; Ravi et al 2004).

In vitro studies have identified the redox proteins glutathione-dependent formaldehyde dehydrogenase (GS-FDH), protein disulphide isomerase (PDI) and the thioredoxin–thioredoxin reductase (Trx-TR) system as denitrosylases that reduce SNO levels (Nikitovic et al 1996; Liu et al 2001; Sliskovic et al 2005). Both GS-FDH and PDI are capable of reducing GSNO to produce GSH and NO, thus providing an additional source of cellular NO (Liu et al 2001; Sliskovic et al 2005). Additionally, PDI is capable of forming a fairly stable intermediate (PDI-SNO) and is

continuously secreted from NO-like endothelial cells, possibly providing a mechanism for intra- and intercellular transport of NO (Sliskovic et al 2005).

The Trx-TR system reduces low molecular mass and protein *S*-nitrosothiols. Unlike other redox proteins associated with denitrosylation, both Trx and TR are ubiquitously and constitutively expressed in most cells types (Nikitovic and Holmgren 1996; Ravi et al 2004; Sengupta et al 2007). Nitrosylated proteins that are reduced by Trx include glutathione, caspase-3, albumin, metallothionenin and eNOS. Trx-dependent denitrosylation has been observed in many species from yeast to humans, and more recently in *Arabidopsis thaliana* (Nikitovic et al 1996; Ravi et al 2004; Stoyanovsky et al 2005; Tada et al 2008). Trx contains a vicinal dithiol (Cys-X-X-Cys) which reduces the disulphide bridges of substrate proteins through a mixed disulphide intermediate, and then forms a disulphide ring closure (Trx(SH)$_2$) upon release of the reduced substrate. Reduction of Trx(SH)$_2$ back to Trx requires the NADPH-dependent enzyme, TR (Arner and Holmgren 2000). The same two Trx cysteine residues are necessary for the reduction of *S*-nitrosolyated proteins with a mechanism that remains elusive (Fig 1.5) (Benhar et al 2008). Trx reduction of *S*-nitrosylated proteins also exhibits some isoform specificity. Notably, Trx-2 reduces the constitutively *S*-nitrosylated mitochondrial caspase-3 upon exposure of cells to pro-apoptotic stimuli, whereas Trx-1 maintains a low basal steady-state level of cytosolic SNO, including cytosolic caspase-3 (Benhar et al 2008).
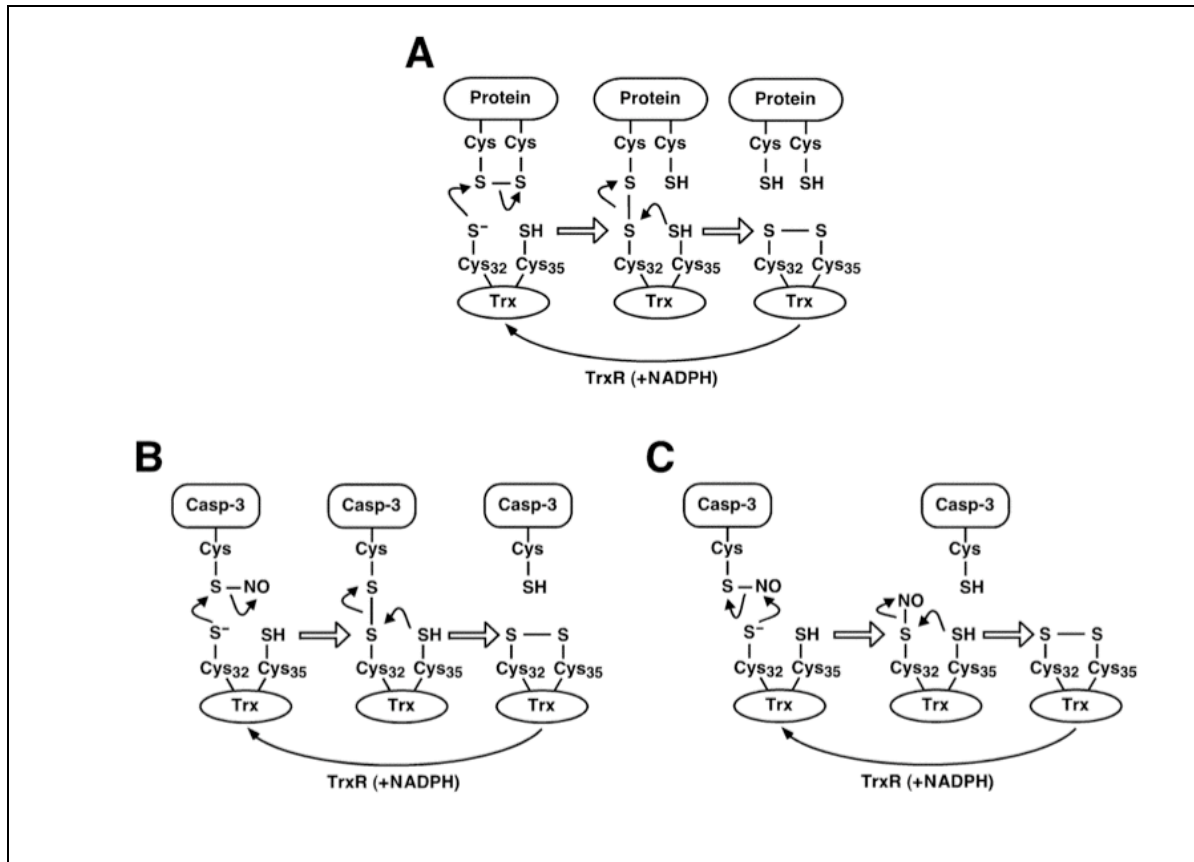
Figure 1.5 *Mechanisms for reduction of SNO-proteins by Trx.* **A**. Initially, the N-terminal cysteine thiolate within a vicinal dithiol (Cys-X-X-Cys) attacks the substrate disulphide bond and produces a mixed disulphide intermediate. Attack by the C-terminal cysteine thiolate transfers the disulphide link to Trx, (Trx-SH$_2$) which results in reduction and release of the substrate. Trx-SH$_2$ is reduced back to Trx by NADPh-dependent TrxR. **B**. A possible mechanism for denitrosylation of SNO-protein by Trx, as proposed by Benhar et al, 2008. **C**. An alternative mechanism for denitrosylation of SNO-protein based on transnitrosylation. Taken from Benhar et al, 2008

1.4.4 NO is a retrograde signal that influences learning and memory

Learning results from changes in the efficacy of chemical synaptic transmission, and can be either facilitative (long-term potentiation; LTP) or depressive (long-term depression; LTD). Short-term memory normally involves covalent post-translational modifications without *de novo* protein synthesis. Long-term memory, lasting from days to years, requires initiation of gene

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transcription and presumably involves translocation of a signal from the synapse to the nucleus. Synaptic plasticity is predominantly localised to the presynaptic neuron leading to the popular theory that LTP requires a retrograde signal from the post-synaptic site back to the presynaptic neuron (Arancio et al 1996; Emptage et al 2003). NO is both water and lipid soluble and presumably capable of free diffusion between cells, making it a good candidate for a retrograde signal (Schuman and Madison 1991; Zhuo et al 1993).

LTP is mostly studied in the CA1 pyramidal neurons of the hippocampus following glutamate stimulation of Schaffer collaterals. Glutamate binding to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors (AMPARs) on pyramidal neurons activate voltage-dependent NMDARs and induces Ca^{2+} influx. An increase in intracellular Ca^{2+} results in the activation of secondary messenger cascades, including Ca^{2+}/calmodulin binding and activation of nNOS. The CA1 pyramidal neurons release postsynaptic NO, which can then signal back to the original presynaptic sites or possibly to other adjacent synapses (Susswein et al 2004). This hypothesis is supported by the ability of NO to induce LTP when paired with a weak tetanus not normally sufficient to cause LTP (Zhuo et al 1993). Injection of membrane-impermeant NO scavengers into the presynapse are also sufficient to block LTP (Arancio et al 1996). Analysis of NOS transgenic mice indicates that the absence of both eNOS and nNOS is associated with the disruption of LTP in the hippocampal CA1 region (Son et al 1996). This implies that non-neuronal cells that express eNOS may also be required for the establishment of LTP. However, in discrete areas of the CA1 region, LTP remains unaffected by nNOS and eNOS disruption (Son et al 1996). Therefore, additional NO-independent mechanisms must exist. It should be considered that the role of NO in mediating LTP and the establishment of memories remains

controversial as several studies failed to find NO-dependent effects on LTP (Bannerman et al 1994a; Cummings et al 1994; Murphy et al 1994), or memory in the hippocampus (Bannerman et al 1994b; Tobin et al 1995).

The molecular mechanism that underlies NO-mediated LTP in the hippocampus is still a matter of debate. Pharmacological studies have demonstrated a requirement of activated sGC and PKG as secondary messengers of NO signalling for hippocampal LTP (Zhuo et al 1994; Son et al 1998). One group observed that PKG activation is necessary within the presynatic terminus, further implicating NO as a retrograde messenger from the postsynaptic to the presynaptic neuron (Arancio et al 2001). However, additional studies on hippocampal LTP favour NO induced ADP-ribosylation of proteins, with no effect observed upon blocking of the cGMP pathway (Schuman et al 1994; Kleppisch et al 1999). NO can also suppress LTP in post-synaptic neurons by reducing the efficacy of NMDA receptors (Murphy and Bliss 1999). The discrepancies on NO and its involvement in hippocampal LTP may be due to differences in experimental conditions and protocols, as well as the use of different animal models at differing stages of development.

The molecular mechanisms underlying learning and memory have also been extensively studied in the cerebellum, where LTD can be observed in Purkinje cells following activation of presynaptic parallel fibres. Purkinje cells are inhibitory neurons therefore, a reduction of Purkinje activity following LTD will lead to increased activity of target neurons. As observed with hippocampal LTP, increased NO or Ca^{2+} levels within postsynaptic Purkinje neurons are sufficient for the establishment of LTD through an sGC-dependent mechanism (Lev-Ram et al

1997). LTP in the cerebellum is also NO dependent however, it does not require cGMP or cAMP signalling (Lev-Ram et al 2002). NO produced in Purkinje cells can act as a retrograde signal to induce LTP in presynaptic parallel fibres (Jacoby et al 2001). This is analogous to NO produced in CA1 neurons, which serves as a retrograde signal that activates sGC in presynaptic parallel fibres. The majority of NO studies in the cerebellum often rely heavily on NO donors and manipulation of the cGMP pathway, with little or no use of NOS inhibitors (Lev-Ram et al 1997; Smith et al 2003). Therefore caution should be taken when considering the effects of NO on LTD and learning in the cerebellum.

1.4.5 NO and cell survival – a Janus molecule

NO is infamously regarded as a Janus molecule, as it has been implicated in both pro- and anti-apoptotic signalling responses. Cells maintained in basal conditions generate a low level of NO that activates anti-apoptotic pathways necessary for cell survival. An increased level of NO following exposure to apoptotic stimuli initiates both pro- and anti-apoptotic mechanisms and under these conditions cell survival will depend on the level of NOS activation, with a higher concentration of cellular NO favouring a pathologic outcome.

It is well established that a number of apoptotic stimuli induces nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to its nuclear translocation and cell death (Sawa et al 1997). Nuclear translocation of nitrosylated GAPDH is dependent on its interaction with an E3 ubiquitin ligase, Siah1. GAPDH increases Siah1 stability and nuclear accumulation, facilitating Siah1-dependent degradation of nuclear proteins and apoptosis (Hara et

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al 2005). NO-mediated apoptosis is often studied in models of stroke and neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Matrix metalloproteinase-9 (MMP-9) and parkin are examples of pro-apoptotic proteins positively regulated by NO signalling (Gu et al 2002; Chung et al 2004). MMP9 activity requires S-nitrosylation of a specific cysteine residue that disrupts its coordination with Zn^{2+} allowing the entry of the obligate catalytic ligand H_2O (Gu et al 2002). Further cysteine oxidation to sulphinic or sulphonic acid irreversibly activates MMP9 resulting in extracellular proteolytic cascades and apoptosis (Gu et al 2002). Parkin, an E3 ubiquitin ligase, is inactivated upon S-nitrosylation and is thought to be responsible for the intracellular accumulation of misfolded proteins and apoptotic death of dopaminergic neurons in patients affected by Parkinson's disease (Chung et al 2004).

Interestingly, apoptosis mediated by caspase activation requires a mechanism that involves denitrosylation. Caspases belong to a family of cysteine proteases associated with initiation and execution of apoptotic pathways. They are constitutively expressed as inactive caspase zymogens, which in response to pro-apoptotic signals are cleaved to form active caspase enzymes. Under basal conditions, caspases are inhibited by nitrosylation of a critical active site cysteine residue (Dimmeler et al 1997; Kim et al 1997; Li et al 1997; Mannick et al 1999). S-nitrosylation of caspase-3 occurs predominantly within a subpopulation associated with mitochondria and is essential for inhibiting apoptosis (Mannick et al 1999). Caspase-3 is denitrosylated following Fas stimulation, which results in caspase activation and initiation of apoptosis (Mannick et al 1999; Matsumoto et al 2003).

Conversely, pro-apoptotic stimuli can inhibit pro-apoptotic proteins through NO signalling, thus acting as a negative feedback mechanism. Increased NO synthesis following interferon-γ stimulation induces nitrosylation of ASK1, which prevents ASK1 from binding to substrate kinases and blocks apoptosis (Park et al 2004). Stimulation of glutamate-dependent NMDAR induces Ca^{2+} influx, which leads to activation of nNOS and NO synthesis (Garthwaite et al 1988). Subsequent S-nitrosylation of NMDARs inhibits further Ca^{2+} influx, thereby preventing excitotoxicity and activation of pro-apoptotic pathways (Lipton et al 1993; Choi et al 2000). Interestingly, NMDAR activation inhibits expression of the Trx inhibitor, *Txnip*, thus enhancing an antioxidant system known to reduce intracellular SNO (Papadia et al 2008). Similarly, Fas stimulation leads to S-nitrosylation of FLICE inhibitory protein (FLIP) preventing its ubiquitination and proteasomal degradation (Chanvorachote et al 2005). FLIP interferes with caspase-8 binding to the Fas receptor complex, which inhibits caspase-8 activation and apoptosis (Chanvorachote et al 2005). Other stimuli, including neurotrophin signalling in the nervous system, induces NOS activation and promotes cell survival.

Opposite responses to NO signalling may be due to differences of subcellular NO concentrations and compartmentalisation of NOS activity. Basal S-nitrosylation of proteins when intracellular NO levels are low may be facilitated by co-localisation of substrates with NOS. For instance, caspase-3 directly associates with all three isoforms of NOS. S-nitrosylated proteins also bind adaptor proteins or are located within microdomains containing NOS, such as the intermembrane space in mitochondria. Interestingly, Fas stimulation increases nitrosylation of cytosolic FLIP, while inducing denitrosylation of mitochondrial caspase-3. The ability of Fas signalling to induce both addition and removal of the same modification may be achieved by

segregating these opposite mechanisms to subcellular compartments, such as nitrosylation within the cytoplasm and denitrosylation in mitochondria.

1.4.6 NO and cell proliferation

nNOS is the primary isoform expressed in neurons however, specific populations within the CNS express also eNOS (astrocytes and vascular endothelial cells) and iNOS (microglia and astrocytes) (Dinerman et al 1994b; Heneka and Feinstein 2001). Neuronal NO has been implicated in a number of neuronal processes, including proliferation, survival, differentiation, ischemic cell death and neurodegeneration. Neurotrophins are important mediators of neuronal proliferation and differentiation. Exposure of neuronal cultures and neuronal-derived cell lines to either NGF or BDNF induces expression of NOS and stimulates NO synthesis (Peunova and Enikolopov 1995; Cheng et al 2003; Riccio et al 2006). NO has anti-proliferative effects in cerebellar granule cells, as well as in several neuronal-derived cell lines without affecting cell viability or apoptosis (Peunova and Enikolopov 1995; Murillo-Carretero et al 2002; Ciani et al 2004). Inhibition of NOS activity has also been shown to delay differentiation of PC12 cells and neural progenitor cultures (Rialas et al 2000; Murillo-Carretero et al 2002; Cheng et al 2003).

Neurogenesis persists in two regions of the adult brain, the SVZ and the subgranular zone of the dentate gyrus in the hippocampus. Within these regions discrete populations of neurons express nNOS, which are in very close proximity to neuronal precursor cells. Neuroblasts from the SVZ migrate tangentially along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate to form mature interneurons. NOS positive cells are found along the

RMS, with abundant processes that intermingle with the migrating precursor cells (Moreno-Lopez et al 2000). Interestingly, progenitors in the adult SVZ that do not initially express NOS become NOS positive as they migrate to the olfactory bulb, suggesting that NO may play a role in neuronal differentiation. Both genetic ablation of nNOS and systemic or intraventricular infusion of NOS inhibitors augmented the number of cells generated within the SVZ and dentate gyrus of adult mice (Moreno-Lopez et al 2004; Matarredona et al 2004; Packer et al 2003). Moreover, NO inhibits proliferation of neuronal cultures obtained from the SVZ of postnatal mice without affecting cell survival (Matarredona et al 2004; Torroglosa et al 2007).

1.4.7 Role of NO in the development of the nervous system

In the developing nervous system nNOS is transiently expressed in restricted regions of the brain, including the embryonic cortical plate and the olfactory epithelium (Bredt and Snyder 1994). Within these populations, nNOS is not detectable during the early phases of neurogenesis when neural stem cells are actively proliferating. nNOS mRNA is first detected in neural precursor cells at E10, with levels progressively increasing as precursors differentiate to form post-mitotic mature neurons (Bredt and Snyder 1994; Ogura et al 1996; Cheng et al 2003). Neurotrophin-dependent precursors in sensory and sympathetic ganglia also express high levels of nNOS when they exit the cell cycle and differentiate (Bredt and Snyder 1994; Thippeswamy and McKay 2005). nNOS expression sharply decreases postnatally once the axons have established connections with their peripheral targets (Thippeswamy and McKay 2005).

The observation that NO triggers growth arrest in NGF-induced differentiating PC12 cells provided the initial indication that NO is an important regulator of neuronal development (Peunova and Enikolopov 1995; Phung et al 1999). Similarly, reduced proliferation and increased differentiation was observed in neuroblastoma cell lines NB69 and Sk-N-BE upon exposure to endogenous and exogenous NO (Murillo-Carretero et al 2002; Ciani et al 2004). Exogenous application of NO donors and NOS inhibitors to primary cultures of cerebellar and cortical neurons also demonstrated that NO inhibits proliferation and promotes differentiation (Cheng et al 2003; Ciani et al 2004). Stimulation of mouse cortical neuroepithelial cluster cultures with either NO donors or BDNF decreases cell proliferation and increases neuronal differentiation (Cheng et al 2003). Interestingly, BDNF application increases expression of nNOS indicating that it may act as a positive feedback loop for NO signalling (Cheng et al 2003). Application of NO inhibitors to embryonic brain slice cultures inhibits granule cell migration and increases proliferation in the cerebellar cortex (Tanaka et al 1994). Similarly, treatment of rat pups (postnatal day 3) with NOS inhibitors increases proliferation of cerebellar precursor cells (Ciani et al 2006).

These data strongly implicates a role of NO in the development of the CNS however, apart from early work performed by Tanaka et al., studies *in vivo* have primarily focused on the role of NO in adult neurogenesis (Tanaka et al 1994). Developmental processes require long-lasting, tightly regulated changes in gene transcription. It is therefore, not surprising that NO signalling is capable of affecting gene expression.

1.4.8 Transcriptional targets of NO

Recent studies have suggested that NO signalling plays an important role in regulating the expression of multiple genes by activating promoter cis-elements (Hemish et al 2003; Dhakshinamoorthy et al 2007). NO modulates gene expression through activation of multiple transcription factors including CREB, N-Myc, NF-κB, p53 and in bacteria, OxyR. A role of NO in regulating gene expression was first demonstrated for CREB. Ca^{2+}-dependent NO signalling induced the expression of the CREB-regulated gene, *Fos* (Peunova and Enikolopov 1993). NO-dependent activation of CREB regulates various neuronal processes, including neuronal survival, differentiation and LTP (Lu et al 1999; Ciani et al 2002; Puzzo et al 2005). Several groups have demonstrated that NO signalling and activation of the sGC/PKG pathway results in CREB phosphorylation on Serine 133 (Ciani et al 2002; Nagai-Kusuhara et al 2007). Phosphorylation of CREB is essential for its activation and is necessary and sufficient for promoting neuronal survival (Ciani et al 2002; Nagai-Kusuhara et al 2007). Similarly, LTP in the CA1 region of the hippocampus is dependent on activation of NO and sGC/PKG pathways, followed by phosphorylation of CREB (Lu and Hawkins 2002). NO can also regulate CREB activity through a mechanism independent of CREB phosphorylation. Upon exposure of cortical neurons to BDNF, NO mediates the binding of CREB to target gene promoters (Riccio et al 2006). These conflicting mechanisms of NO-mediated CREB activation may reflect differences in neuronal cell type, developmental stage and experimental conditions.

N-Myc is an oncogene and a transcription factor that is essential for the expansion of progenitor cell populations during normal brain development (Contestabile 2008). NO and

cGMP/PKG signalling have both been demonstrated to inhibit N-Myc expression, as well as proliferation rates of neonatal precursor granule neurons (Ciani et al 2004; Ciani et al 2006). Similarly, neuroblastoma cell line SK-N-BE and embryonic carcinoma stem cells exhibited reduced transcription of genes associated with tumour progression following a NO-mediated suppression of N-Myc (Park et al 2002; Ciani et al 2004; Contestabile 2008). NO-based treatments are therefore, considered as potential novel therapeutic tools for neural-derived tumours. Despite evidence that NO regulates both CREB and N-Myc activity, *S*-nitrosylation of either transcription factor has not been observed. Interestingly, the transcription factor AP1 binds to DNA in a redox-sensitive manner. NO inhibits AP1 binding to DNA *in vitro* through nitrosylation of two cysteine residues within the DNA binding domain of its components, c-jun and c-fos (Nikitovic et al 1998). Moreover, NO can negatively regulate AP1 binding through addition of glutathione (*S*-glutathionylation) to a specific cysteine within the active site of c-jun (Klatt et al. 1999). Interestingly, upregulation of c-jun expression following neurotrophin deprivation of sympathetic neurons has been linked to cell death (Ham et al 1995); therefore NO inhibition of c-jun may be beneficial for cell survival. NO induces expression of the c-fos-regulated gene tyrosine hydroxylase, as well as induction of *Fos* itself, in a number of neuronal systems (Wu et al 2000; Najimi et al 2002; Chan et al 2004; Riccio et al 2006). This may serve as a negative feedback mechanism, with NO initially inducing expression of *Fos* and later inhibiting AP1 activity through *S*-nitrosylation of c-fos and c-jun.

Nitric oxide regulates transcription factors containing a zinc-finger domain, including Egr-1 and NF-κB, by disrupting the zinc-sulphur clusters essential for zinc binding and subsequent targeting to DNA. However, in living cells there is no direct evidence that zinc mobilization

occurs upon NO synthesis. The expression and activity of Egr-1 has been shown to increase in neuronal-derived PC12 and SH-SY5Y cells following exposure to NO donors, although the mechanism by which NO mediates this transcriptional activation remains unclear (Thiriet et al 1997; Cibelli et al 2002). In mammals, NF-κB is constitutively expressed as a p50/p65 heterodimer and is retained within the cytoplasm by its inhibitor IκB. Upon IκB phosphorylation and subsequent proteolysis, NF-κB translocates into the nucleus and binds to target gene promoters. In immune cells, *S*-nitrosylation of p50 and tyrosine nitration of p65 negatively regulates NF-κB binding to DNA (Marshall and Stamler 2001; Park et al 2005). NO signalling further inhibits NF-κB function by reducing proteasomal degradation of its inhibitory component, IκB (Marshall and Stamler 2002; Reynaert et al 2004). Conversely, striatal and spinal cord neurons show an increase of NF-κB binding to DNA upon NO signalling (Simpson and Morris 1999; Miscusi et al 2006).

Conflicting data on the regulation of AP1 and NF-kB suggest that NO may regulate gene expression independent of transcription factor *S*-nitrosylation. Two recent studies have demonstrated that NO elicits a very broad level of control on transcriptional regulation (Hemish et al 2003; Dhakshinamoorthy et al 2007). NO is capable of modulating important cellular processes, including cell cycle exit and differentiation, which require tightly regulated and large-scale changes in gene expression. However, the molecular target of NO that would enable such a global change in transcription has yet to be elucidated.

1.5 Aims and Questions

A major aim of my thesis was to test whether neurotrophin activates NO signalling in nuclei of developing neurons. I utilised a NO-sensitive fluorescent dye, 4-amino-5-methylamino-difluororescein-2'7'-diacetate (DAF-FM DA), to visualise NO synthesis in neurons. I demonstrated that BDNF treatment of mouse embryonic cortical neurons induces accumulation of NO within both nuclear and cytoplasmic compartments. Neurotrophins are responsible for stable long-term changes in gene expression, which requires chromatin remodelling. This begged the question, does neurotrophin-induced NO signalling modulate gene transcription during development? Analysis of proteins modified by NO was performed using a complex biochemical assay, the biotin-switch assay, which replaces labile cysteine nitrosyl groups with a stable biotin-moiety. By employing this technique, I demonstrated that exposure of cortical neurons to BDNF induces *S*-nitrosylation of HDAC2 at two specific cysteine residues. ChIP is a procedure that determines whether a given protein is associated with a specific region of DNA. ChIP experiments were performed to test whether HDAC2 or acetylated histones were detectable at CREB regulated gene promoters. ChIP experiments revealed that BDNF stimulation induced a substantial loss of nitrosylated HDAC2, hyperacetylation of histones H3 and H4 and increased expression of endogenous genes. Finally, I demonstrated that nitrosylation of HDAC2 is necessary for normal dendritic growth *in vitro* and radial migration of cortical progenitor cells *in vivo*.

Chapter 2 BDNF induces nuclear NO accumulation in cortical neurons

Neurotrophin stimulation and NO signalling are important modulators of cortical neurogenesis. Both BDNF and NO signalling inhibit proliferation and promote neuronal differentiation (Barnabe-Heider and Miller 2003; Cheng et al 2003; Packer et al 2003; Moreno-Lopez et al 2004; Bartkowska et al 2007). Importantly, expression of BDNF and NOS is spatially restricted within identical regions of the cortex during development (Bredt and Snyder 2004; Cheng et al 2003). The stimulatory effect of BDNF on differentiation of neuronal precursor cells can be blocked using NOS inhibitors, thereby implicating a direct role of NO on neurotrophin-induced cortical development (Cheng et al 2003). BDNF induces nNOS expression and NO synthesis in neurons (Cheng et al 2003; Nott et al 2008), however, there are no direct evidence of BDNF-dependent accumulation of NO in neurons.

NO synthesis was directly visualise in cortical neurons using the fluorescent probe DAF-FM DA, and was performed in collaboration with Mr James Robinson (Fig. 2.1). Embryonic cortical neurons were incubated with the membrane-permeant, DAF-FM DA, which is deacetylated in the cytoplasm to form the cell-impermeable non-fluorescent DAF-FM (Fig. 2.1). Any excess probe was removed by washing the cultures with fresh medium prior to treatment with BDNF (Fig. 2.1). The fluorescence quantum yield of DAF-FM is low (~0.005) but increases about 160-fold after reacting with NO. DAF-FM fluorescence is detected within a spectrum similar to flourescein at emission/excitation maxima of 495/515nm (Kojima et al 1998). E17 rat cortical neurons were either stimulated with BDNF (75ng/ml) for 10 minutes or left

untreated and subjected to DAF-FM staining (Fig. 2.1). Neurons exposed to BDNF exhibited a two-fold increase in the number of DAF-FM positive cells when compared to cultures treated with vehicle. This corroborates earlier studies that showed that BDNF induces NO synthesis in neurons (Cheng et al 2003; Riccio et al 2006). Confocal imaging revealed that NO could be detected in both cytoplasmic and nuclear compartments of cortical neurons. This is the first evidence that NO accumulates within the nucleus and has important implications for NO modulation of nuclear proteins. In unstimulated neurons, a basal level of NO was detected, which may be a consequence of NMDA receptor activation induced by washing the cells during the DAF-FM protocol (Fig. 2.2, 2.3). To reduce the background fluorescence, neurons were cultured in a reduced-serum medium containing the NMDA antagonist, DL-2-amino-5-phosphonovaleric acid (DL-AP5) for 16 hours prior to stimulation. Treatment of neuronal cultures with DL-AP5 greatly reduced basal DAF-FM fluorescence however, a residual amount of fluorescence persisted in 20% of unstimulated neurons. Detection of residual NO under basal culture conditions may be a result of endogenously synthesised BDNF that may stimulate neurons in a paracrine and autocrine manner (Maisonpierre et al 1990; Ernfors et al 1992; Fukumitsu et al 1998). It is important to note that the percentage of NO-positive cells never exceeds 50%, even in cultures treated with exogenous BDNF. This result reflects previous observations that during development, cells positive for nNOS are observed in the intermediate zone and cortical plate, but are absent in progenitor cells of the VZ and SVZ (Cheng et al 2003).

NO accumulation in cortical neurons following treatment with BDNF is extremely rapid and is detected within minutes of stimulation (Fig. 2.2, 2.3). The fast rate of accumulation of NO in neurons may reflect the requirement of Ca^{2+} for nNOS activation and rapid kinetics of Ca^{2+}

signalling. To test whether BDNF-dependent NO accumulation in neurons is generated by nNOS activation, cultures were pre-treated with the specific nNOS inhibitor N-O-propyl-L-arginine (NPA). The number of DAF-FM positive cells in cultures pre-treated with NPA and subsequently exposed to BDNF was dramatically lower, when compared to cultures treated with BDNF (Fig. 2.2, 2.3). This result demonstrates that specific inhibition of nNOS blocks BDNF-dependent NO synthesis in neurons (Fig. 2.2, 2.3), thereby supporting a model by which neurotrophin stimulation of embryonic cortical neurons leads to nNOS activation and increased intracellular NO.

Since the discovery of NO as a physiological signal, a multitude of proteins regulated by this small gaseous molecule have been identified (Hess et al 2001). The prevalent mechanism of NO-dependent modulation of protein functions is through *S*-nitrosylation, a covalent modification of cysteine thiol groups. A major obstacle to the detection of endogenously *S*-nitrosylated proteins is the labile nature of this posttranslational modification, as nitrosothiol groups are easily lost upon exposure to UV light and reducing agents such as glutathione, ascorbic acid and reducing metal ions. An additional technical obstacle in detecting endogenous nitrosylated proteins is the lack of reliable anti-nitrosocysteine antibodies. The development of the biotin-switch assay by Jaffrey and Snyder in 2001 was a seminal step that allowed the detection of *S*-nitrosylated proteins by substituting the labile nitrosothiol group with the more stable biotin-moiety (Fig. 2.4) (Jaffrey and Snyder 2001). This complex biochemical assay requires the neutralization of free cysteine thiol groups with the thiol-specific methylthiolating agent, methyl methanethiosulphonate (MMTS). Treatment with ascorbic acid specifically reduces nitrosylated cysteines to free thiol groups, while cysteines in alternative oxidative states remain unaffected

(Forrester et al 2007). Finally, the newly-formed free thiols are labelled using the sulphidyl-specific biotinylating agent, *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP) (Jaffrey and Snyder 2001). As a result, proteins that were originally *S*-nitrosylated are labelled with a stable biotin-moiety, allowing separation on an acrylamide gel and western blot analysis. Alternatively, biotinylated proteins can be precipitated using streptavidin conjugated to agarose beads, as streptavidin has a high affinity for biotin. The biotinylated protein is eluted from the beads and candidate proteins are individually detected by western blotting.

To investigate whether BDNF-induced NO accumulation results in *S*-nitrosylation of endogenous proteins, cortical neurons were exposed to BDNF (75ng/ml) for 10 and 30 minutes, respectively and cell lysates were subjected to the biotin-switch assay followed by biotin western blotting. I observed that BDNF stimulation of embryonic cortical neurons induced nitrosylation of many endogenous proteins. Exposure of neurons to BDNF for 10 minutes resulted in the appearance of nitrosylated proteins of 110, 90 and 75 KDa, whereas after 30 minutes of BDNF treatment prominent bands were observed at 35 and 17 KDa (see red arrows, Fig. 2.5). This demonstrates that BDNF-dependent NO synthesis results in *S*-nitrosylation of many proteins in a time dependent manner. A possible explanation is that nitrosylation of certain proteins may require higher cellular concentrations of NO. These cysteine residues may be buried within hydrophobic pockets and are less exposed to intracellular NO however, once nitrosylated, they may form more stable modifications that persist for longer. The accumulation of NO as a result of endogenous nNOS activation requires elevated levels of intracellular Ca^{2+}. To test whether increased intracellular Ca^{2+} concentration induces protein *S*-nitrosylation in neurons, cortical

cultures were exposed to the Ca^{2+} ionophore A23187. Stimulation of cortical neurons with A23187 (50nM) increased protein S-nitrosylation similarly to BDNF treatment (Fig. 2.5). This result suggests that BDNF-dependent S-nitrosylation in neurons requires nNOS activation as a source of NO.

BDNF stimulation of cortical neurons induces NO accumulation within both the cytoplasm and the nucleus, resulting in nitrosylation of many endogenous proteins. Interestingly, *in situ* nitrosocysteine immunostaining of lung epithelial cells revealed significantly higher levels of nitrosylated proteins within the nucleus, when compared to the cytoplasmic compartment (Ckless et al 2004). However, nitrosylation of nuclear proteins has not been firmly established. To determine whether nitrosylation of nuclear proteins occurs in cortical neurons, nuclear and cytoplasmic extracts were exposed to GSNO for 30 minutes and subjected to the biotin-switch assay followed by anti-biotin western blot. Treatment of neuronal nuclear fractions with NO donors induced S-nitrosylation of multiple proteins, with prominent bands observed at 90, 75, 38, and 25kDa (Fig. 2.6). Importantly, the S-nitrosylated proteins observed in the nuclear fraction were distinct from those found in the cytoplasm (Fig. 2.6). Nuclear translocation of proteins induced by cytoplasmic NO signalling has been demonstrated in a number of cases either through direct nitrosylation, such as GAPDH (Hara et al 2005), or through nitrosylation of regulatory factors, such as HDAC4/5 (Sengupta et al 2004). In contrast, nitrosylation of proteins that reside predominantly within the nucleus, such as the class I HDACs, has never been observed. The finding that NO accumulates within the nucleus opens the possibility that S-nitrosylation may occur while proteins are localised in the nucleus. The ability of NO to modify nuclear proteins *in*

situ implies that NO participates directly to various nuclear functions, including gene transcription, DNA replication and repair.

Figure 2.1 *Schematic of DAF-FM-DA protocol.* **Step 1.** Cells were loaded with membrane permeable DAF-FM-DA (orange cricles) for 30 mins. **Step 2.** The plates were washed to remove excess DAF-FM-DA and incubated for 15 minutes to allow the production of membrane impermeable DAF-FM (purple cricles). **Step 3.** Cells were treated with BDNF (green circles), which induced nNOS activation and NO synthesis (red circles) **Step 4.** NO reacts with DAF-FM to produce a fluorescent compound (yellow stars). Neurons were fixed for 15 mins with 4% formaldehyde and images were captured within 24 hours using confocal microscopy.

Figure 2.2 *BDNF stimulates nuclear accumulation of nitric oxide production in cortical neurons.* Cortical neurons were loaded with DAF-FM (10μM, 30 min) and exposed to either (i) BDNF (100ng/μL, 10 min), (ii) NPA (300μM, 30 min), (iii) NPA followed by BDNF, or (iv) vehicle (Ctrl) (lower panels). Cells were stained using CellTracker and Hoescht to visualise cell morphology and nucleus, respectively (upper panels). Nitric oxide is detected upon reaction with DAF-FM at emission/excitation maxima of 495/515 nm. Images were captured on a Leica DM2500 confocal microscope using an ACS APO 40x/1.15 oil objective lens. Scale bars, 25 μm (top panels) and 75 μm (lower panels).

Figure 2.3 *Quantitative analysis of DAF-FM experiment (Fig 2.1).* Analysis performed on images captured with an Axioplan 2 fluorescence microscope using an 'EC Plan Neofluar' 10x/0.3 air objective lens and FS10 (GFP) filter. Data represents the number of DAF positive cells normalised to the total number of cells, +/- SEM; n=3.

Figure 2.4 *Schematic of biotin-switch assay.* **Step 1.** Reduced thiols were blocked by using the thiol-specific methylthiolating agent, MMTS. Excess blocking agent was removed by acetone precipitation. **Step 2.** Nitrosylated cysteine residues were reduced to free thiol groups using ascorbic acid. **Step 3.** Reduced cysteines were labelled using the thiol-specific biotinylating agent, biotin-HPDP. Proteins that were initially *S*-nitrosylated are now labelled with a biotin-moiety.

Figure 2.5 *Protein* S-*nitrosylation is induced in embryonic cortical neurons upon neurotrophin stimulation.* Neurons were exposed to BDNF (75ng/ml for the indicated times), A23817 (50ng/ml, 20 mins), GSNO (100μM, 30 mins) or vehicle (Ctrl) and processed using the biotin-switch protocol. Positive control substrate obtained from the NitroGlo Nitrosylation Detection Kit (PerkinElmer Life Science, USA) was subjected to GSNO (100μM, Ctrl+) or vehicle (Ctrl-) and processed using the biotin-switch protocol. Samples were subjected to anti-biotin western blot analysis. Anti-HDAC2 western blot indicates equal loading (bottom panel). Red arrows indicate stimulus-induced *S*-nitrosylation of cortical proteins; n=3.

Figure 2.6 *Nuclear proteins are* S-*nitrosylated in cortical neurons upon exposure to NO donors.* Nuclear and cytoplasmic extracts of cortical neurons were exposed to GSNO (100μM, 30 mins) *in vitro* and subjected to the biotin-switch assay. Extracts were subjected to anti-biotin western blot analysis, and the purity of fractions was assessed using anti-histone H4 western blot (bottom panel). Red arrows indicate proteins that are nitrosylated after BDNF stimulation; n=3. NE, nuclear extract; WCL, whole cell lysate; CE, cytoplasmic extract.

Fig. 2.1

Fig. 2.2

Fig. 2.3

Fig. 2.4

MMTS
Step 1

S
S
S-NO
S-H

→

S
S
S-NO
S-S-CH₃

Ascorbic acid   Step 2

S-S-biotin
N
Biotin-HPDP

S
S
S-S-Biotin
S-S-CH₃

←
Step 3

S
S
S-H
S-S-CH₃

Fig. 2.5

BDNF
0'   10'   30'   GSNO   A23187   no biotin   Ctrl +   Ctrl -

97—
64—
51—
39—
28—
19—
14—

anti-biotin

anti-HDAC2

Fig. 2.6

NE   WCL
GSNO   -   +   -   +

188 —
98 —
62 —
49 —
38 —
28 —
17 —
14 —
6 —

14 —

CE   WCL
GSNO   -   +   -   +

188 —
98 —
62 —
49 —
38 —
28 —
17 —
14 —
6 —

anti-biotin

14 —

anti-histone H4
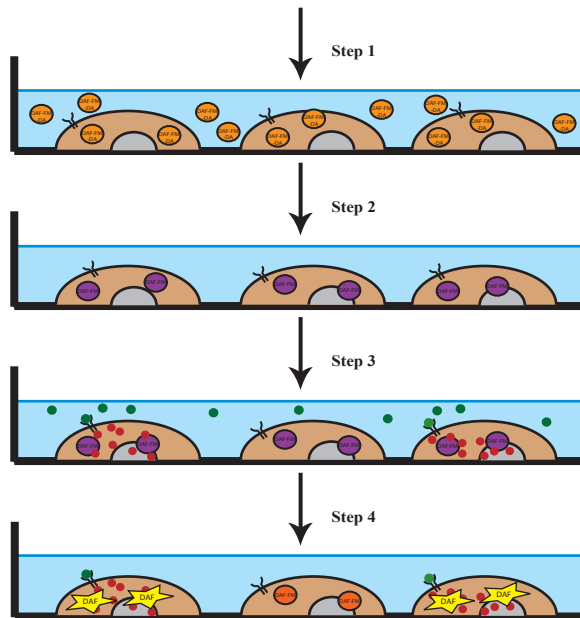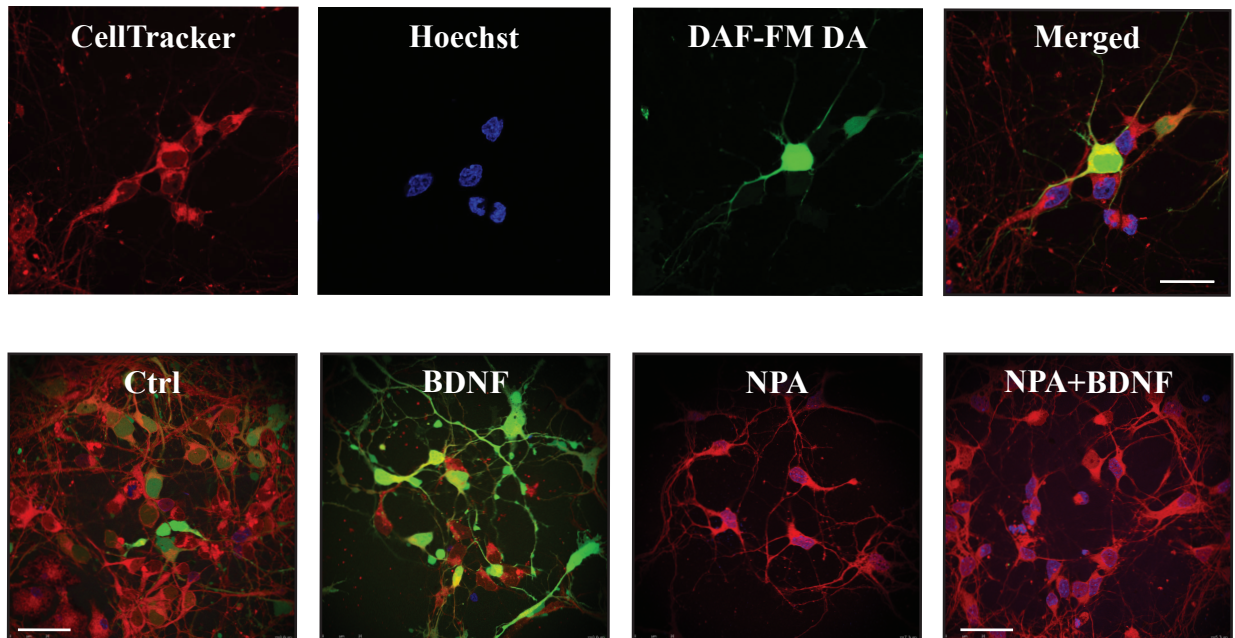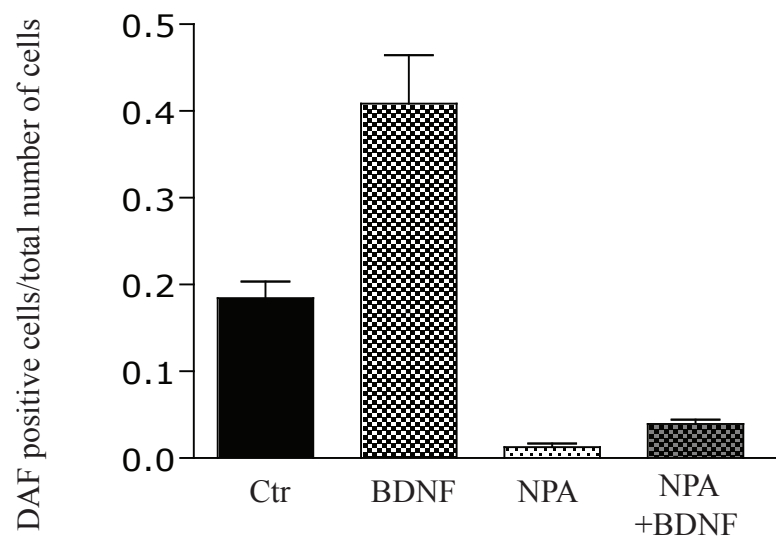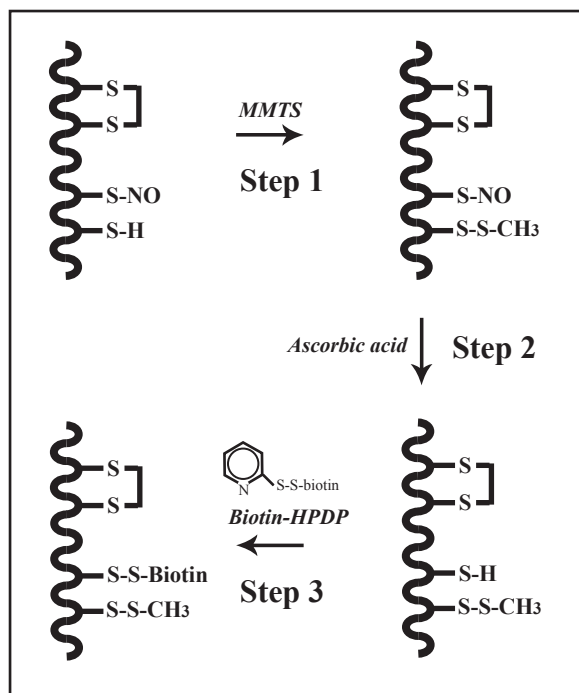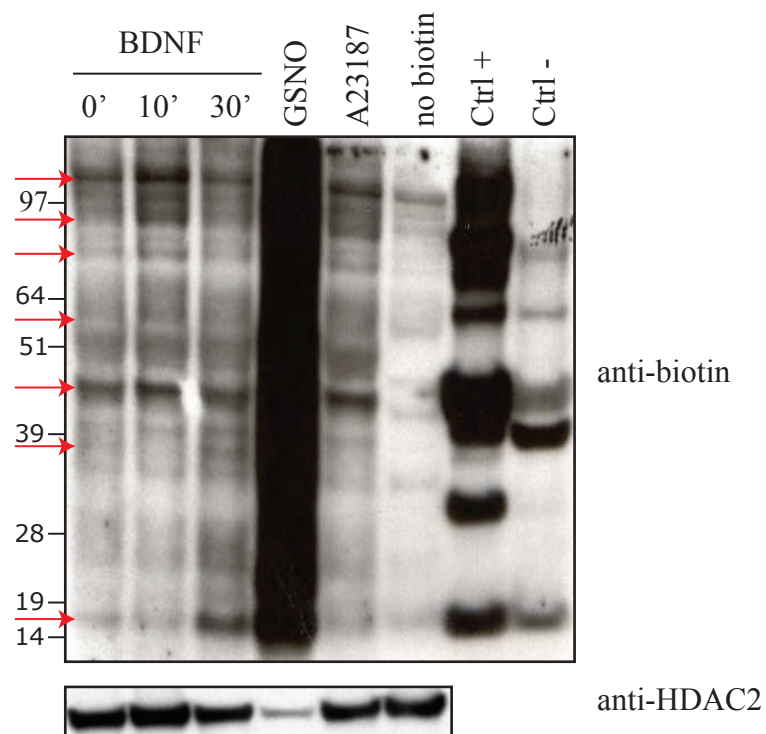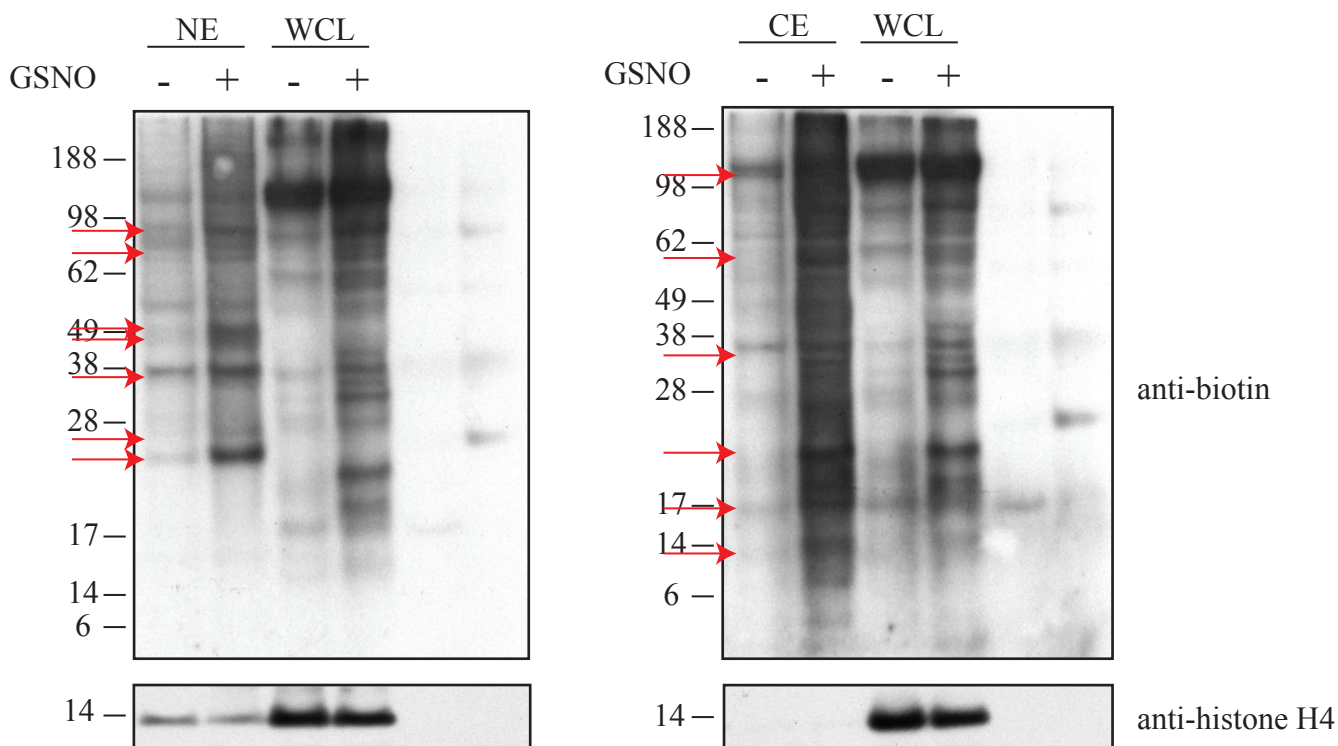
Chapter 3 Histone deacetylase 2 is a nuclear target of NO

A common factor to physiological processes regulated by NO during the development of the nervous system is the requirement for profound and long-lasting changes in gene transcription. Exposure of cells to NO donors induces the transcription of a large number of genes through the binding of transcription factors to various cis-activating elements, including CRE, AP1, AP2, BRN-3, EGR, E2F1 and Sp1 (Hemish et al 2003; Dhakshinamoorthy et al 2007). Genes activated by NO regulate a broad range of genes involved in cell signalling, cell cycle, transcription, stress response, protein degradation, oxidative stress and apoptosis (Hemish et al 2003). The large number of transcription factors activated and the broad range of genes induced suggest that NO may signal at an epigenetic level, reflecting the broad impact that NO has on neuronal physiology. Binding of the transcription factors CREB and N-Myc to DNA is regulated by NO and is not dependent on *S*-nitrosylation of these transcription factors (Riccio et al 2006; Contestable et al 2008). Epigenetic modifications of histone tails, such as lysine acetylation, result in chromatin remodelling that increases accessibility of transcription factors and gene activation. Conversely, repressive complexes induce gene repression by removing these epigenetic modifications. Transcription factor binding and activation during cortical differentiation requires inactivation of various repressive complexes. Activation of neural genes involves coordinated dissociation of repressive complexes containing deacetylase proteins, including HDAC1 and HDAC2 (Ballas and Mandel 2005; Hermanson et al 2002; Ju et al, 2004). A recent study has indicated that NO modulates nuclear shuttling of class II HDACs (Illi et al 2008), which poses the question of whether nuclear class I HDACs are also regulated by NO.

During the development of the CNS class I HDACs are expressed in differentiating progenitor cells (Ajamian et al 2003; Shaked et al 2008). Notably, HDAC2 expression is maintained in both proliferating progenitor cells and differentiating neuronal precursors of the neocortex (Shaked et al 2008; Riccio et al 2006). To determine whether HDAC2 is *S*-nitrosylated in embryonic cortical neurons upon neurotrophin stimulation, E17 rat cortical cultures were exposed to BDNF (75ng/ml) for 5, 15 and 60 minutes. Cell lysates were processed using the biotin-switch assay, followed by precipitation with streptavidin and HDAC2 western blotting. I observed that stimulation of cortical neurons with BDNF induced a robust *S*-nitrosylation of endogenous HDAC2. *S*-nitrosylation of HDAC2 was detected within 5 minutes and accumulated progressively with increased BDNF exposure up to 1 hour (Fig. 3.1, 3.2). This is the first example of a *S*-nitrosylated nuclear protein and provides strong evidence that NO accumulates within the nucleus.

To test whether Ca^{2+} activation of nNOS is required for HDAC2 *S*-nitrosylation, human embryonic kidney 293 (HEK293) cells stably expressing nNOS were treated with the calcium ionophore A23187 (50nM). Cell lysates were subjected to the biotin-switch assay, followed by precipitation with streptavidin and HDAC2 western blotting. I observed that *S*-nitrosylation of HDAC2 was induced by calcium signalling, suggesting that calcium-dependent activation of nNOS provides an endogenous source of NO (Fig. 3.1). As a negative control, *S*-nitrosylation of HDAC2 was analysed in E14.5 cortical cultures from nNOS null mice (Huang et al 1993). Embryonic cortical cultures obtained from both nNOS null and wild-type littermates were exposed to BDNF (75ng/ml) and A23187 (50nM). Cell lysates were subjected to the biotin-switch assay, followed by precipitation with streptavidin and detection by HDAC2 western

blotting. While both BDNF and A23187 induced *S*-nitrosylation of HDAC2 in wild-type cultures, HDAC2 *S*-nitrosylation was completely absent in nNOS null cultures exposed to the same stimuli (Fig. 3.2). This data indicates that HDAC2 nitrosylation requires both BDNF and calcium signalling, suggesting that nNOS activation is the major source of NO synthesis under these conditions. HDAC2 has recently been implicated in cell fate determination of progenitor cells (Shaked et al 2008) and NO has been shown to modulate the ability of cortical precursors to differentiate into mature neurons however, the molecular targets of NO have yet to be elucidated (Packer et al 2003; Matarredona et al 2004; Moreno-Lopez et al 2004). Nitrosylation of a chromatin-remodelling protein provides a direct mechanism whereby NO regulates the transcriptional profile of progenitor cells to define their cell fate.

HDAC2 is a 55KDa protein containing ten cysteine residues that are conserved between human, rat and mouse species (Fig. 3.3). The primary sequence of HDAC2 does not contain any obvious acid-base consensus motifs associated with cysteine nitrosylation. Hydrophobicity plots show the distribution of polar and non-polar residues along the protein sequence and are used to predict hydrophobic regions. Although the hydrophobicity plot of HDAC2 did not reveal cysteine residues within hydrophobic regions, *S*-nitrosylation consensus motifs or hydrophobic pockets might be hidden in the tertiary structure of HDAC2, making them difficult to predict. Unfortunately, the tertiary structure of HDAC2, and its closest relative HDAC1, remains to be determined.

Cysteine residue 152 (Cys152) is highly conserved between class I HDACs, and has previously been implicated in the synthesis of acetate, the by-product of lysine deacetylation

(Vannini et al 2004) (Fig. 3.3). Nitrosylation of Cys152 could result in conformational changes that prevent acetate formation, thus inhibiting the deacetylase activity of HDAC2. To test whether Cys152 was S-nitrosylated, I mutated this cysteine residue to an alanine (HDAC2^{C152A}). Alanine residues do not possess the thiol group targeted by nitrosylation, therefore if Cys152 was the residue targeted by NO, exposure of HDAC2^{C152A} to NO donors should not result in HDAC2 nitrosylation. HDAC2^{C152A} was expressed in HEK293 cells and cell lysates were exposed to the NO donor S-nitrosocysteine (SNOC; 200μM for 30 minutes). Lysates containing HDAC2^{C152A} were subjected to the biotin-switch assay followed by precipitation with streptavidin and HDAC2 western blotting. I observed that exposure to SNOC induced HDAC2^{C152A} nitrosylation, indicating that NO must target a different cysteine residue (data not shown). I generated expression vectors containing HDAC2 bearing a single mutation of each cysteine residue and subjected them to the biotin-switch assay. I found that all single mutations of HDAC2 were nitrosylated upon exposure to NO donors (Fig 3.3, data not shown), suggesting that HDAC2 was nitrosylated on multiple cysteine residues. S-nitrosylation of proteins on multiple cysteine residues is not uncommon and has been observed for a number of proteins, including GAPDH, NMDAR and eNOS (Molina et al 1992; Takahashi et al 2007; Tummala et al 2008).

I next performed further structure to function analysis to assess which cysteine residues were targeted by NO. I was able to exclude 7 cysteine residues as potential targets of NO by exposing truncated forms of HDAC2 to NO donors and testing for nitrosylated cysteines (Fig. 3.4). Six constructs containing N-terminal and C-terminal truncated forms of Myc-tagged HDAC2 were generated. The constructs were expressed in HEK293T cells and lysates were exposed to SNOC (200μM for 30 minutes) and subjected to the biotin-switch assay followed by

streptavidin precipitation and Myc western blotting. I observed that HDAC2 constructs containing amino acids 1-261 and 286-488 were consistently not nitrosylated (Fig 3.4, 3.5), indicating that Cys262, Cys274 and Cys285 were possible targets of NO. I next generated three constructs containing HDAC2 with only two of these cysteine residues mutated to alanine, HDAC2$^{C262A/C274A}$, HDAC2$^{C262A/C285A}$ and HDAC2$^{C274A/C285A}$. The constructs were expressed in HEK293T cells and lysates were exposed to SNOC and subjected to the biotin-switch assay followed by streptavidin precipitation and HDAC2 western blotting. I observed that constructs containing HDAC2 with single mutations of Cys262 or Cys274 were nitrosylated following exposure to NO donors while HDAC2$^{C262A/C274A}$ was not nitrosylated (Fig. 3.6). These results demonstrate that Cys262 and Cys274 of HDAC2 are the residues targeted by NO.

To test whether Cys262 and Cys274 were *S*-nitrosylated in neurotrophin-stimulated neurons, embryonic cortical cultures were transfected with vectors encoding either HDAC2^{WT} or HDAC2$^{C262A/C274A}$ and exposed to BDNF (75ng/ml) for 30 minutes. Cell lysates were subjected to the biotin-switch assay followed by streptavidin precipitation and HDAC2 western blotting. Although the transfection efficiency of cortical neurons was less than 10%, the biotin-switch assay was sensitive enough to allow the detection of nitrosylated HDAC2^{WT} (Fig. 3.7). I was able to detect intracellular nitrosylation of HDAC2^{WT} expressed in embryonic cortical neurons. Importantly, I did not detect *S*-nitrosylation of HDAC2$^{C262A/C274A}$ in cortical neurons exposed to the same stimulus. These results indicate that Cys262 and Cys274 are the cysteine residues nitrosylated in neurons in response to neurotrophin stimulation.

In summary, BDNF stimulation of cortical neurons induces nNOS activation and accumulation of NO within the nucleus. Nuclear NO induces nitrosylation of various nuclear proteins, including the chromatin remodelling protein HDAC2. Previous studies have demonstrated that BDNF and NO are important regulators of proliferation and differentiation of neuronal progenitor cells (Barnabe-Heider and Miller 2003; Cheng et al 2003; Packer et al 2003; Moreno-Lopez et al 2004; Bartkowska et al 2007) and are capable of generating profound changes in gene expression (Hemish et al 2003; Dhakshinamoorthy et al 2007). Modulation of HDAC2 function through *S*-nitrosylation may provide an intriguing link between BDNF and NO-dependent regulation of gene expression in neurons.

Figure 3.1 *Endogenous HDAC2 is S-nitrosylated in embryonic cortical neurons upon neurotrophin stimulation.* Cultured cortical neurons were treated with BDNF (75ng/ml for the indicated times) and GSNO (100μM, 30 min). HEK 293 cells expressing nNOS were treated with A23187 (50μM, 20 min) and GSNO (10μM, 30 min). Cultures were subject to the biotin-switch assay followed by streptavidin precipitation and HDAC2 western blotting. Equal loading prior to streptavidin precipitation was assessed by HDAC2 western blotting (lower panel); n=3.

Figure 3.2 *BDNF-dependent HDAC2 S-nitrosylation is absent in cortical neurons obtained from nNOS null mice.* Embryonic cortical cultures from wild-type and nNOS null mice were treated with BDNF (75ng/ml, 30 mins), A23187 (50μM, 20 min) or vehicle (Ctrl) and subjected to the biotin-switch assay, followed by streptavidin precipitation and HDAC2 western blotting. Equal loading prior to streptavidin precipitation was assessed by HDAC2 western blotting (lower panel); n=3.

Figure 3.3 *HDAC2 is highly conserved between mammalian species.* An amino acid sequence alignment of mouse, rat and human HDAC2 using ClustalW2 (Larkin et al 2007). Blue text indicates Asp178, His180 and Asp267 that are predicted to coordinate with the catalytic zinc ion. Blue arrows indicate additional catalytic residues. Red text indicates conserved HDAC2 cysteine residues and nitrosylated Cys262 and Cys274 are marked by red boxes.

Figure 3.4 *Schematic representations of truncated forms of HDAC2.* A series of N-terminal and C-terminal truncations of HDAC2 were generated (labelled D1-D6). Nitrosylation was detected when the HDAC2 truncations, D1, D2, D5 and D6 (yellow), were exposed to NO

donors. The two HDAC2 truncations, D3 and D4 (red), were not nitrosylated in the presence of NO donors.

Figure 3.5 *Cys262 and Cys274 of HDAC2 are S-nitrosylated.* Myc-tagged N-terminal and C-terminal truncations (D1, D3 & D5) and full-length HDAC2 (WT) were expressed in HEK293T cells and exposed to SNOC (200μM, 30 min) or vehicle (Ctrl). Lysates were subject to the biotin-switch assay, followed by streptavidin precipitation and Myc western blotting (top panel). Equal loading prior to streptavidin precipitation was assessed by Myc western blotting (lower panel); n=3.

Figure 3.6 *Cys262 and Cys274 of HDAC2 are S-nitrosylated.* Flag-tagged mutants, HDAC2^{C262A}, HDAC2^{C274A}, HDAC2$^{C262A/C274A}$, wild-type HDAC2^{WT} and empty vector (EV) were expressed in HEK293T cells. Lysates were exposed to SNOC (200μM, 30 min) or vehicle and subject to the biotin-switch assay, followed by streptavidin precipitation and HDAC2 western blotting (top panel). Equal loading prior to precipitation with streptavidin was assessed by HDAC2 and Flag western blots (lower panels); n=3.

Figure 3.7 *Neurotrophin stimulation of cortical neurons induces nitrosylation of Cys262 and Cys274 of HDAC2.* Embryonic cortical neurons expressing Flag-tagged HDAC2$^{C262A/C274A}$, HDAC2^{WT} and EV were exposed to SNOC (200μM, 30 min) and subject to biotin-switch assay, followed by streptavidin precipitation and HDAC2 western blotting (top panel). Equal loading prior to precipitation with streptavidin was assessed by HDAC2 and Flag western blotting (lower panels); n=3, Flag-HDAC2, Flag-tagged HDAC2; End-HDAC2, Endogenous HDAC2.

Fig. 3.1

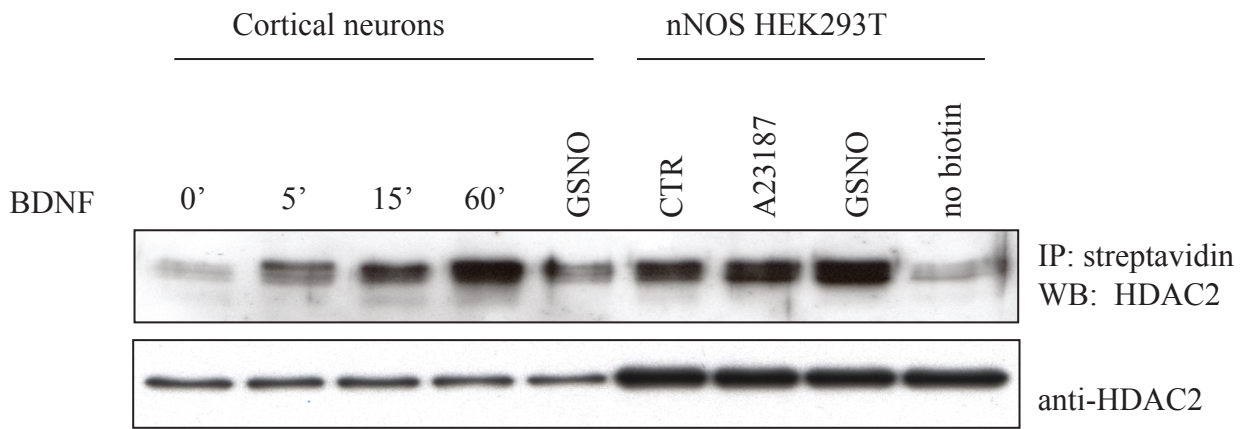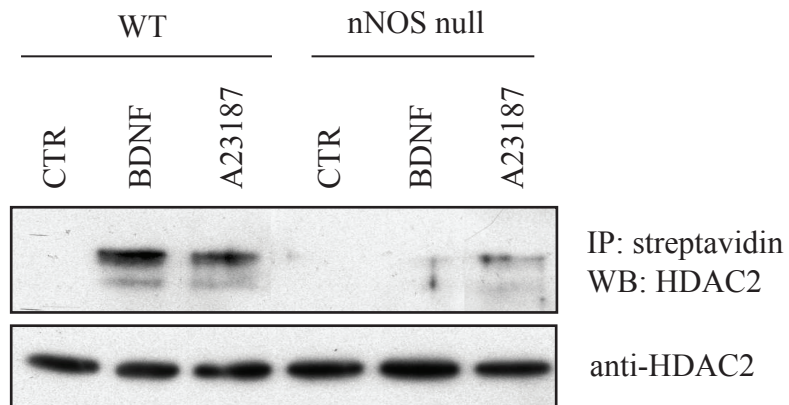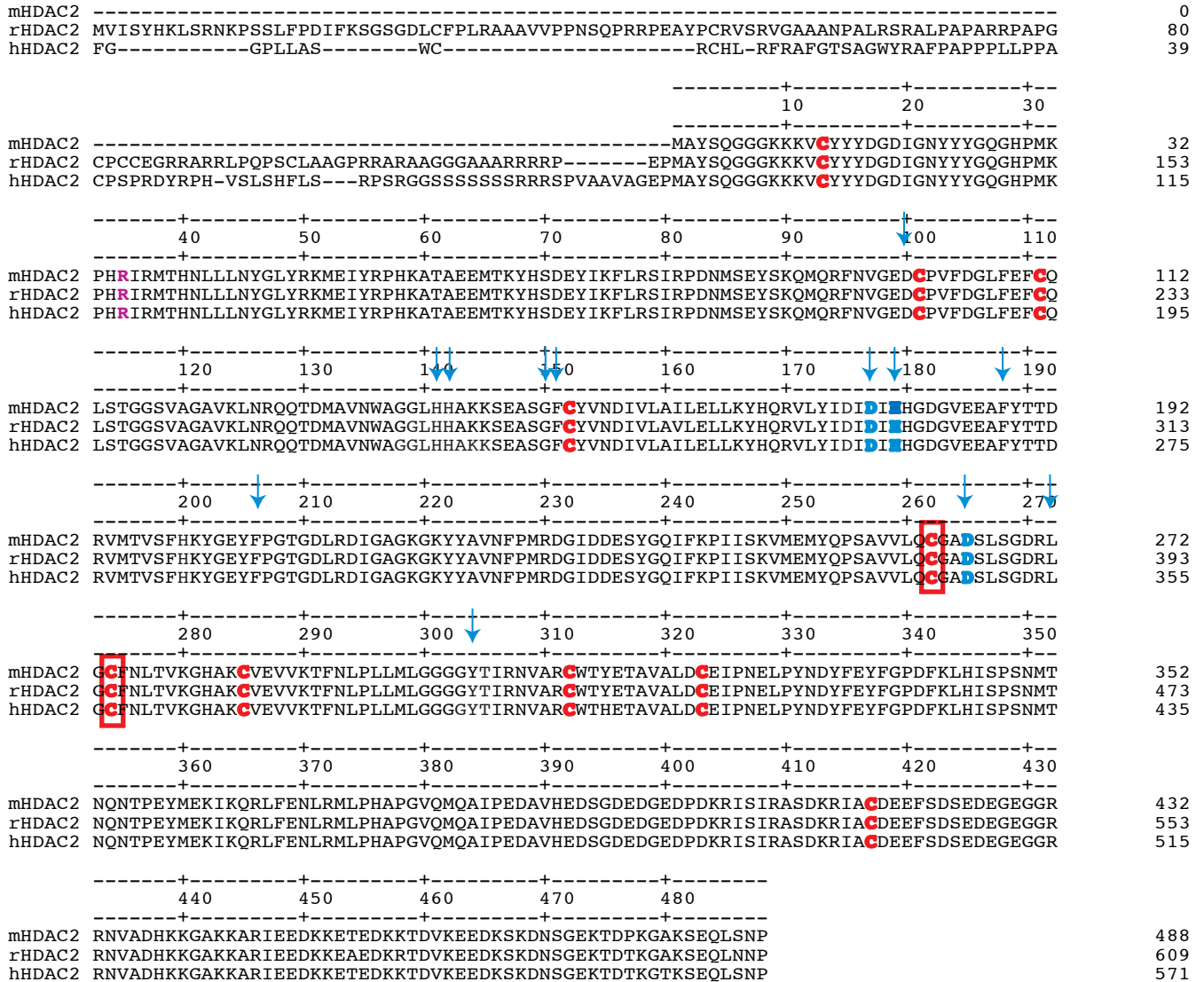Cortical neurons   nNOS HEK293T

BDNF   0'   5'   15'   60'   GSNO   CTR   A23187   GSNO   no biotin

IP: streptavidin
WB: HDAC2

anti-HDAC2

Fig. 3.2

WT   nNOS null

CTR   BDNF   A23187   CTR   BDNF   A23187

IP: streptavidin
WB: HDAC2

anti-HDAC2

Fig. 3.3

```
mHDAC2  --------------------------------------------------------------------------------   0
rHDAC2  MVISYHKLSRNKPSSLFPDIFKSGSGDLCFPLRAAAVVPPNSQPRRPEAYPCRVSRVGAAANPALRSRALPAPARRPAPG  80
hHDAC2  FG-----------GPLLAS-------WC--------------------RCHL-RFRAFGTSAGWYRAFPAPPPLLPPA  39

                                                  ---------+---------+---------+--
                                                          10        20        30
                                                  ---------+---------+---------+--
mHDAC2  -------------------------------------------------------MAYSQGGGKKKVCYYYDGDIGNYYYGQGHPMK  32
rHDAC2  CPCCEGRRARRLPQPSCLAAGPRRARAAGGGAAARRRRP-------EPMAYSQGGGKKKVCYYYDGDIGNYYYGQGHPMK  153
hHDAC2  CPSPRDYRPH-VSLSHFLS---RPSRGGSSSSSSSRRRSPVAAVAGEPMAYSQGGGKKKVCYYYDGDIGNYYYGQGHPMK  115

        -------+---------+---------+---------+---------+---------+---------+--↓------+--
              40        50        60        70        80        90       100       110
        -------+---------+---------+---------+---------+---------+---------+---------+--
mHDAC2  PHRIRMTHNLLLNYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQ  112
rHDAC2  PHRIRMTHNLLLNYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQ  233
hHDAC2  PHRIRMTHNLLLNYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMQRFNVGEDCPVFDGLFEFCQ  195

        -------+---------+---------+----↓↓---+-↓↓-------+---------+------↓+--↓-----+-↓--
              120       130       140       150       160       170       180       190
        -------+---------+---------+---------+---------+---------+---------+---------+--
mHDAC2  LSTGGSVAGAVKLNRQQTDMAVNWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHGDGVEEAFYTTD  192
rHDAC2  LSTGGSVAGAVKLNRQQTDMAVNWAGGLHHAKKSEASGFCYVNDIVLAVLELLKYHQRVLYIDIDIHGDGVEEAFYTTD  313
hHDAC2  LSTGGSVAGAVKLNRQQTDMAVNWAGGLHHAKKSEASGFCYVNDIVLAILELLKYHQRVLYIDIDIHGDGVEEAFYTTD  275

        -------+------↓--+---------+---------+---------+---------+-------↓-+--↓------+-↓
              200       210       220       230       240       250       260       270
        -------+---------+---------+---------+---------+---------+---------+---------+--
mHDAC2  RVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNFPMRDGIDDESYGQIFKPIISKVMEMYQPSAVVLQCGADSLSGDRL  272
rHDAC2  RVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNFPMRDGIDDESYGQIFKPIISKVMEMYQPSAVVLQCGADSLSGDRL  393
hHDAC2  RVMTVSFHKYGEYFPGTGDLRDIGAGKGKYYAVNFPMRDGIDDESYGQIFKPIISKVMEMYQPSAVVLQCGADSLSGDRL  355

        -------+---------+---------+---↓-----+---------+---------+---------+---------+--
              280       290       300       310       320       330       340       350
        -------+---------+---------+---------+---------+---------+---------+---------+--
mHDAC2  GCFNLTVKGHAKCVEVVKTFNLPLLMLGGGGYTIRNVARCWTYETAVALDCEIPNELPYNDYFEYFGPDFKLHISPSNMT  352
rHDAC2  GCFNLTVKGHAKCVEVVKTFNLPLLMLGGGGYTIRNVARCWTYETAVALDCEIPNELPYNDYFEYFGPDFKLHISPSNMT  473
hHDAC2  GCFNLTVKGHAKCVEVVKTFNLPLLMLGGGGYTIRNVARCWTHETAVALDCEIPNELPYNDYFEYFGPDFKLHISPSNMT  435

        -------+---------+---------+---------+---------+---------+---------+---------+--
              360       370       380       390       400       410       420       430
        -------+---------+---------+---------+---------+---------+---------+---------+--
mHDAC2  NQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHEDSGDEDGEDPDKRISIRASDKRIACDEEFSDSEDEGEGGR  432
rHDAC2  NQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHEDSGDEDGEDPDKRISIRASDKRIACDEEFSDSEDEGEGGR  553
hHDAC2  NQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHEDSGDEDGEDPDKRISIRASDKRIACDEEFSDSEDEGEGGR  515

        -------+---------+---------+---------+---------+--------
              440       450       460       470       480
        -------+---------+---------+---------+---------+--------
mHDAC2  RNVADHKKGAKKARIEEDKKETEDKKTDVKEEDKSKDNSGEKTDPKGAKSEQLSNP  488
rHDAC2  RNVADHKKGAKKARIEEDKKEAEDKRTDVKEEDKSKDNSGEKTDTKGAKSEQLNNP  609
hHDAC2  RNVADHKKGAKKARIEEDKKETEDKKTDVKEEDKSKDNSGEKTDTKGTKSEQLSNP  571
```

Fig. 3.4

Fig. 3.5

Fig. 3.6

	C262A		C274A		C262/274A		WT		EV	WT no biotin
SNOC	−	+	−	+	−	+	−	+	+	+

IP: streptavidin
WB: HDAC2

anti-HDAC2

anti-FLAG

Fig. 3.7

	EV		WT		C262/274A	
BDNF	−	+	−	+	−	+

flag-HDAC2 →
end-HDAC2 →

IP: streptavidin
WB: HDAC2

flag-HDAC2 →
end-HDAC2 →

anti-HDAC2

anti-FLAG

Chapter 4 HDAC2^{C262A/C274A} strongly associates with chromatin

S-nitrosylation of HDAC2 may affect its function by either modulating its catalytic activity or by affecting its localisation. Crystal structures have been determined for human HDAC8 and *Aquifex aeolicus* HDAC-like protein (HDLP), which share 41% and 35% sequence homology with HDAC2, respectively (Finnin et al 1999; Vannini et al 2004; Somoza et al 2004). The deacetylase core consists of a narrow 12Å-deep pocket lined with hydrophobic residues conserved between all class I HDACs. The bottom of the pocket contains a Zn^{2+} ion essential for the deacetylase activity, which is coordinated by Asp178, His180 and Asp267 (Fig. 4.1). The crystal structure of HDAC8 reveals that two conserved residues, Arg-37 and Cys153, are necessary for the synthesis of acetate, the by-product of lysine deacetylation (Fig. 4.1) (Vannini et al 2004). The hydroxamic acid inhibitor used to generate HDAC8 crystals was bound to His142, His143 and Tyr306 (Vannini et al 2004). These residues are highly conserved between class I HDACs and are likely to be involved in HDAC catalysis. A predicted tertiary structure of HDAC2 based on crystallography data of HDAC8 revealed that nitrosylated Cys262 and Cys274 should not interfere stochiometrically with amino acid residues essential for Zn^{2+} coordination and deacetylase activity (Fig. 4.1) (Vannini et al 2004; Somoza et al 2004). Cys262 is buried within a hydrophobic tunnel, whereas Cys274 is located at the protein surface (Fig. 4.1). The location of Cys262 and Cys274 away from the active site residues suggests that *S*-nitrosylation of HDAC2 may have little effect on its deacetylase activity. Furthermore, NO has been previously demonstrated to regulate HDAC4 and HDAC5 independent of their catalytic activity through modulation of subcellular localisation (Illi et al 2008). NO may regulate HDAC2 function by modulating its affinity for certain repressive complexes, thereby determining its association with

specific gene promoters. However, the possibility remains that a post-translational modification such as *S*-nitrosylation may cause dramatic conformational changes that could possibly impair or enhance HDAC2 deacetylase activity.

To determine whether BDNF-dependent nitrosylation of HDAC2 influences its activity, a fluorometric HDAC activity assay was employed in collaboration with Dr P. Marc Watson. The HDAC activity assay is dependent on a non-fluorogenic peptide BOC-(acetyl)lys-AMC, which upon deacetylation becomes susceptible to trypsin digestion, generating a fluorogenic moiety detected at excitation/emission maxima of 360/460nm. Experiments were performed by immunoprecipitating HDAC2 followed by incubation with BOC-(acetyl)lys-AMC for 1 hour at 37°C, during which deacetylation occurs. The reaction was then quenched using the potent HDAC inhibitor trichostatin A (TSA), followed by a short incubation with trypsin. To test whether either neurotrophin or NO signalling affected the catalytic activity of HDAC2, embryonic cortical cultures were treated with BNDF (75ng/ml) or SNOC (200μM). Cell lysates were immunoprecipitated using a HDAC2 antibody and analysed by performing the HDAC activity assay. We observed that neither BDNF nor NO stimulation of cortical neurons influenced the catalytic activity of endogenous HDAC2 (Fig. 4.2). These data indicate that NO regulates HDAC2 function independently of its deacetylase activity. However, *S*-nitrosylation of HDAC2 may cause conformational changes that prevents or enhances its association within activating or repressive complexes.

To investigate further whether NO signalling regulates HDAC2 function I employed Flag-tagged HDAC2^{WT} and HDAC2$^{C262A/C274A}$ constructs. Functional analysis requires that Flag-

tagged HDAC2 constructs retain their endogenous deacetylase activity. PC12 cells expressing either HDAC2^{WT} or HDAC2$^{C262A/C274A}$ were exposed to NGF (100ng/ml), SNOC (200M) or vehicle. Cell lysates were immunoprecipitated using a Flag antibody, and subjected to HDAC activity assay. As expected, NGF and NO donors had no affect on HDAC2^{WT} and HDAC2$^{C262A/C274A}$ enzymatic activity, when compared to unstimulated cultures (Fig. 4.3). However, we observed that the deacetylase activity of HDAC2$^{C262A/C274A}$ was somewhat lower than HDAC2^{WT} (Fig. 4.3). This may be due to the fact that cysteine residues frequently form disulphide bridges, which is a covalent bond that occurs when two cysteine residues oxidise together. Formation of disulphide bonds is often associated with the stabilization of protein tertiary structure. The absence of two cysteine residues in HDAC2$^{C262A/C274A}$ may make it more susceptible to protein unfolding and protease-mediated degradation. The HDAC activity assay is based on a protocol that includes a 1 hour incubation step at 37°C. This may increase the susceptibility of HDAC2$^{C262A/C274A}$ to degradation by proteases released during cell lysis. Protein degradation was minimised by decreasing the duration of immunoprecipitation from overnight to 2 hours, which resulted in reduced degradation of HDAC2$^{C262A/C274A}$. Moreover, when protease inhibitors were included during the 37°C incubation, degradation of HDAC2$^{C262A/C274A}$ was further reduced. In summary, I observed that neurotrophin-dependent S-nitrosylation of HDAC2 does not influence its enzymatic activity. *S*-nitrosylation has been demonstrated to regulate protein functions by modulating protein-protein interactions. For example, *S*-nitrosylation of *N*-ethylmaleimide sensitive factor (NSF) enhances its binding to GluR2 receptors, which increases cell surface localisation of AMPA receptors (Huang et al 2005). The finding that HDAC2 activity is unaffected by NO signalling led us to investigate whether *S*-nitrosylation of HDAC2

may affect its ability to bind specific corepressors, thereby dictating its association with gene promoters.

To test whether *S*-nitrosylation of HDAC2 influences its localisation at specific gene promoters, the Chromatin ImmunoPrecipitation (ChIP) technique was employed in collaboration with Dr Antonella Riccio. This is a powerful technique that allows the detection of proteins bound to DNA, such as histones, or proteins associated with DNA, such as repressive/activating complexes and proteins within the transcriptional machinery. Proteins are cross-linked to DNA using formaldehyde, followed by cell lysis and sonication to shear the genomic DNA. Proteins cross-linked to DNA are immunoprecipitated using an appropriate antibody. Cross-linking of immunoprecipitated DNA-protein complexes is reversed and the DNA is purified. The presence of immunoprecipitated proteins on specific promoters can be detected by quantitative Polymerase Chain Reaction (qPCR) analysis. Previous ChIP experiments have demonstrated that endogenous HDAC2 is bound to the *Fos* promoter in unstimulated embryonic cortical cultures (Riccio et al 2006). Treatment of cortical cultures with BDNF leads to the release of HDAC2 from the *Fos* promoter (Riccio et al 2006). BDNF-stimulated release of HDAC2 from the *Fos* promoter can be blocked using NOS inhibitors (Riccio et al 2006) suggesting that neurotrophins regulate HDAC2 DNA binding through a NO-dependent mechanism.

To study whether nitrosylation of HDAC2 influences its association with DNA, PC12 cells were transfected with either Flag-tagged HDAC2^{WT} or HDAC2$^{C262A/C274A}$ constructs and exposed to NGF (50ng/ml), SNOC (200μM) or vehicle. ChIP assays were performed by immunoprecipitating cell lysates with a Flag antibody followed by qPCR of gene promoters. I

choose to analyse CREB-regulated gene promoters that are activated following neurotrophin stimulation and NO signalling in cortical neurons (Riccio et al 2006). Treatment with either NGF or SNOC led to a 5-fold reduction in HDAC2^{WT} occupancy at the *Fos* promoter when compared to unstimulated cells (Fig. 4.3, 4.4), confirming that neurotrophin–dependent NO signalling modulates the association of HDAC2 with DNA. In contrast both NGF and SNOC did not affect the association of HDAC2$^{C262A/C274A}$ with the *Fos* promoter (Fig. 4.4, 4.5) demonstrating that nitrosylation of HDAC2 on Cys262 and Cys274 is necessary for the release of HDAC2 from DNA.

To test whether NO regulates HDAC2 binding at multiple gene promoters, we performed ChIP analysis of *Egr1*, *Vgf* and *Nos1* promoters. Neurotrophin and NO treatment strongly reduced the binding of HDAC2^{WT} to *Egr1*, *Vgf* and *Nos1* promoters, while HDAC2$^{C262A/C274A}$ binding to the same promoters was not affected by either NGF or NO stimulation (Fig. 4.6). These data demonstrates that nitrosylation of HDAC2 following neurotrophin stimulation influences HDAC2 binding to multiple gene promoters.

Many neuronal genes contain a conserved RE1 motif that binds to the transcriptional repressor, REST. Neuronal genes of progenitor cells are silenced by the recruitment of Sin3A/HDAC complexes following REST binding. An intriguing possibility is that nitrosylation of HDAC2 disrupts its interaction with the REST complex. This would result in a loss of HDAC2 from neuronal gene promoters, followed by increased histone acetylation and gene activation.

Figure 4.1 *Predicted tertiary model of HDAC2.* A predicted tertiary structure of HDAC2 based on crystallography data of HDAC8 using Swiss – PdbViewer software. Helical structures are highlighted in green and β-strands in blue. Cys262 and Cys274 are labelled in red. Asp177, His179 and Asp265, which are known to coordinate with Zn^{2+}, are labelled in yellow.

Figure 4.2 *Endogenous HDAC2 deacetylase activity is unaffected by neurotrophin or NO stimulation of cortical neurons.* Embryonic cortical neurons were exposed to BDNF (75ng/ml, 20 min), SNOC (200μM, 20 min), TSA (1 μM, 20 min) or vehicle (Ctrl), and subjected to HDAC2 immunoprecipitation followed by HDAC activity fluorometric assay. As a negative control, the HDAC activity assay was performed on lysates immunoprecipitated with normal IgG. Data is presented as average fold change of fluorescence over IgG control; n=3 (upper panel). Equal loading was assessed by HDAC2 western blotting of lysates before (immunoprecipitation) or after (input) immunoprecipitation.

Figure 4.3 *NGF and NO stimulation do not change deacetylase activity of $HDAC2^{C262A/C274A}$ and $HDAC2^{WT}$.* PC12 cells expressing N-terminal Flag-tagged $HDAC2^{WT}$, $HDAC2^{C262A/C274A}$ or an EV control were treated with NGF (100ng/ml, 20 min), SNOC (200μM, 20 min) or vehicle (Ctrl), and subjected to Flag immunoprecipitation followed by HDAC activity fluorometric assay. Data is presented as an average of arbitrary units +/- SEM; n=3. Equal loading was assessed by Flag western blot (lower panel).

Figure 4.4 *$HDAC2^{C262A/C274A}$ is strongly associated with the Fos promoter.* PC12 cells expressing $HDAC2^{WT}$, $HDAC2^{C262A/C274A}$ or EV control were treated with NGF (100ng/ml, 20

min), SNOC (200μM, 20 min) or vehicle (Ctrl) and subject to ChIP assay. Lysates were immunoprecipitated with Flag antibody and analysed by qPCR for the *Fos* promoter; n=3. PI, immunoprecipitation with pre-immune serum.

Figure 4.5 *HDAC2$^{C262A/C274A}$ is strongly associated with multiple CREB-regulated gene promoters.* PC12 cells expressing HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or EV control were treated with NGF (100ng/ml, 20 min), SNOC (200 μM, 20 min) or vehicle (Ctrl). Lysates were subjected to ChIP using Flag immunoprecipitation followed by qPCR for the indicated gene promoters. Data is normalised to total input and represented as average fold change over vehicle control (Ctrl) +/- SEM; *P<0.05; **P<0.005; n=3.
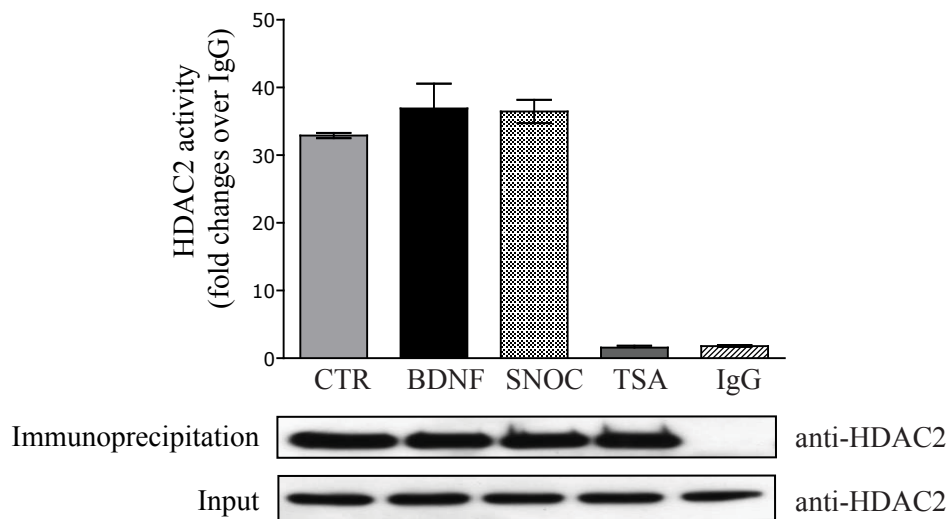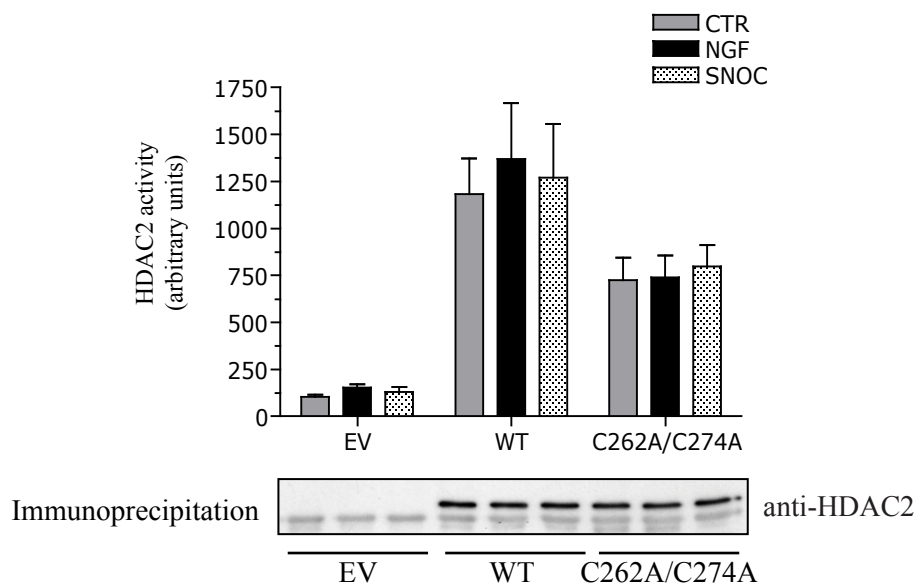
Fig. 4.1

Fig. 4.2

Fig. 4.3

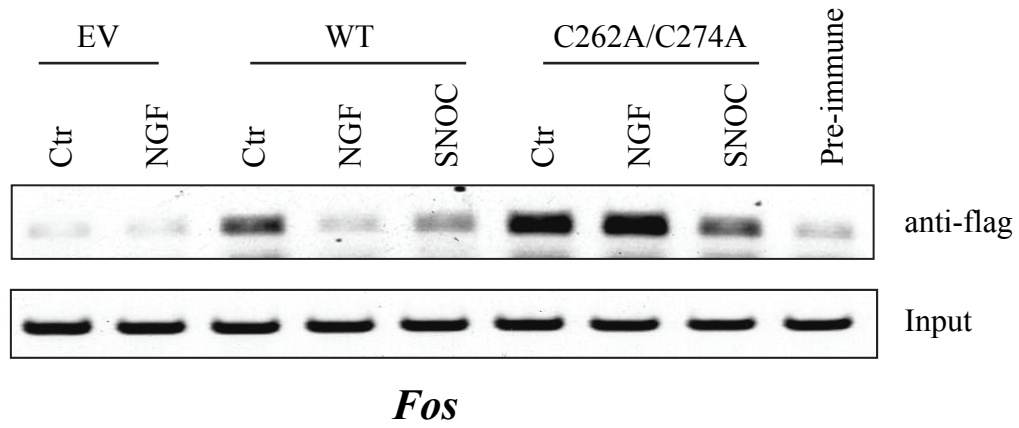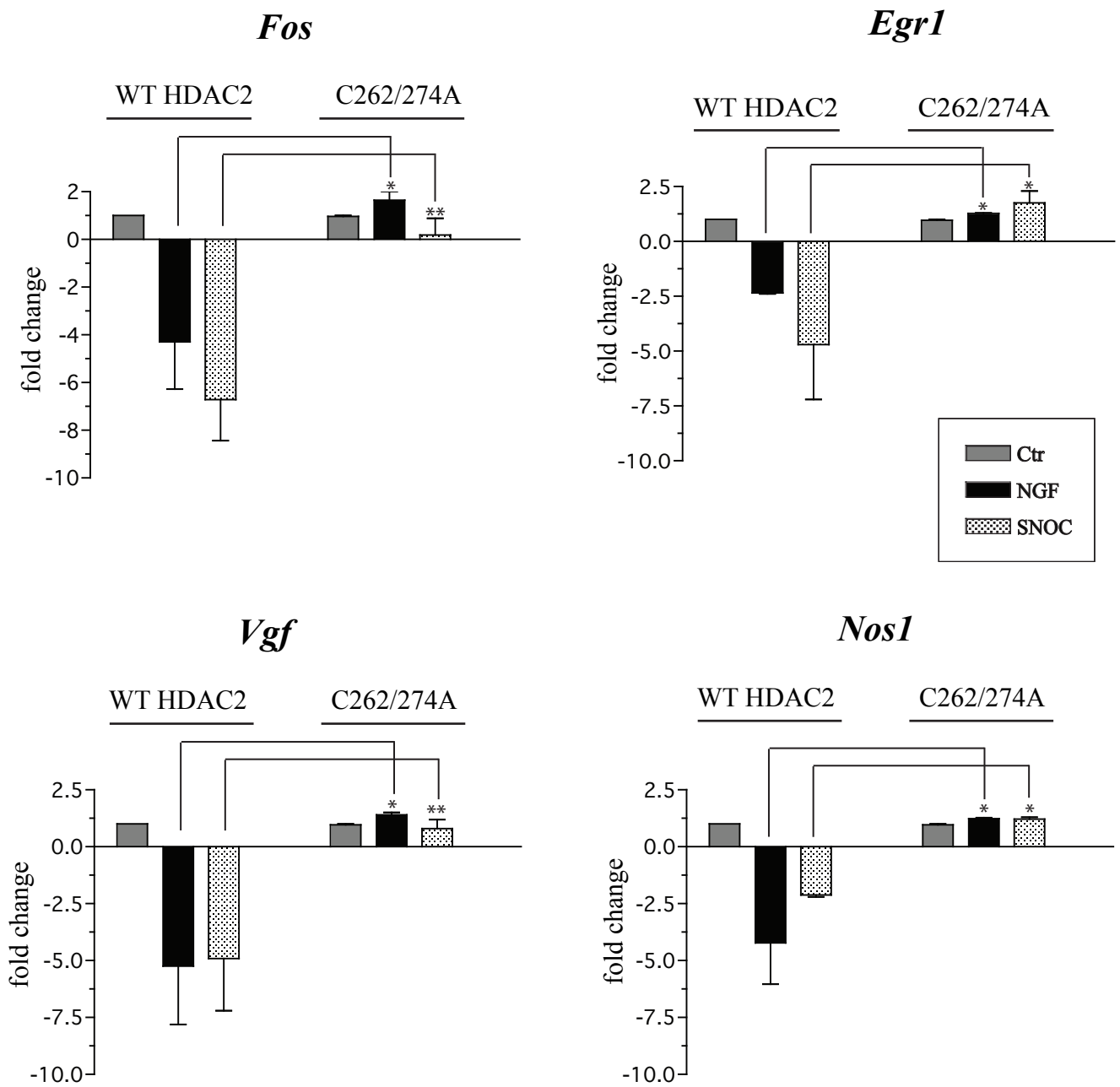**Fig. 4.4**

Fos

Fig. 4.5

Fos

Egr1

Vgf

Nos1

Chapter 5 HDAC2$^{C262A/C274A}$ is a powerful transcriptional repressor

HDAC2 was initially characterised for its ability to remove acetyl groups from histone proteins (Rundlett et al 1996; Yang et al 1996). Acetylation of histone H3 Lys9 and Lys14 (H3K9/K14) is associated with gene activation in human embryonic stem cells (Pokholok et al 2005; Guenther et al 2007). Acetylation of histone H3K9/K14, together with the recruitment of RNA polymerase II is necessary for initiation of transcription and it is now widely recognized as a marker of transcriptionally active genes (Pokholok et al 2005; Guenther et al 2007). To study whether *S*-nitrosylation of HDAC2 influenced histone acetylation, the ChIP assay was employed in collaboration with Dr Antonella Riccio. PC12 cells expressing either HDAC2^{WT} or HDAC2$^{C262A/C274A}$ or empty vector (EV) were exposed to NGF, SNOC or vehicle and immunoprecipitated with pan-acetylated histone H4 (AcH4) or acetylated histone H3 K9/K14 (AcH3K9/K14) antibodies. DNA was purified from immunoprecipitated DNA-histone complexes and analysed by qPCR for various gene promoters. Treatment of PC12 cells expressing HDAC2^{WT} with NGF and SNOC induced a robust increase in acetylation of histone H4 and H3 at the *Fos*, *Egr1*, *Vgf and Nos1* promoters (Fig. 5.1). In stark contrast, histone acetylation of these promoters remained unchanged in PC12 cells expressing HDAC2$^{C262A/C274A}$ (Fig. 5.1). These data indicate that nitrosylation of HDAC2 following neurotrophin and NO signalling increases acetylation of histones H3 and H4 at multiple gene promoters.

Acetylation levels however, varied greatly between gene promoters. For example, acetylation of histone H3 and H4 of *Egr1* and *Vgf*, was induced 8-fold following NGF and SNOC

treatment, compared to a 4-fold induction observed at the *Fos* promoter (Fig. 5.1). In contrast, NGF and SNOC treatment resulted in little or no induction of histone H4 acetylation of the *Nos1* promoter (Fig. 5.1). Acetylation of lysine residues on histone N-terminal tails is thought to reduce positive charges and decrease their association with negatively charged DNA (McGhee and Felsenfeld 1980). Histone acetylation is associated with conformational changes in chromatin structure that allows the binding of transcription machinery and gene activation. Differences of histone acetylation at specific residues between gene promoters may reflect different modes of activation in response to neurotrophins. An increase of AcH3K9/K14 has been demonstrated to strongly correlate with actively transcribed genes (Pokholok et al 2005; Guenther et al 2007). Therefore, a rapid induction of H3 acetylation on K9/K14 may indicate rapid activation of genes that were previously silent. Exposure of neurons to neurotrophins rapidly induces the transcription of immediate early genes (Callela et al 2007). This may explain the 8-fold induction of AcH3K9/K14 observed at the *Egr1* promoter. Transcriptional responses following acetylation of histone H4 are less well characterised, but may reflect activation of gene promoters that already exhibit a basal level of transcription, which may explain the 10-fold induction observed at the *Vgf* promoter.

Alternatively, differences of histone acetylation may be a consequence of the primers used for the qPCR analysis. Promoters are generally considered as a region of non-coding DNA situated 5' of the initiation site that contains enhancer elements responsible for initiation of transcription. Gene promoters often span several kilobases, and may contain multiple enhancer elements that are differentially regulated. Enhancer elements have also been located outside of promoter regions, including intronic sequences. Acetylation of histones within gene promoters

allows the binding of transcriptional activators to their respective enhancer elements. However, different enhancers within the same promoter can be differentially regulated, which may reflect local variations of histone acetylation at gene promoters. The ChIP assay requires that the genomic DNA is sheared into ~500 bp fragments, which results in immunoprecipitated protein-DNA complexes that contain short DNA fragments. qPCR analysis of DNA, purified from immunoprecipitated complexes, is performed using primers designed to amplify 300 base pair fragments or smaller. Therefore, primers designed to loci slightly upstream or downstream of enhancer elements responsive to neurotrophin signalling may show lower acetylation levels.

I next tested whether histone acetylation was induced in PC12 cells expressing HDAC2$^{C262A/C274A}$ following neurotrophin and NO stimulation. PC12 cells expressing HDAC2$^{C262A/C274A}$ were stimulated with NGF (50ng/ml) and SNOC (200μM) and cell lysates were subjected to ChIP assay followed by qPCR analysis (Fig. 5.1). I found that acetylation levels of *Fos*, *Egr1*, *Vgf* and *Nos1* promoters remained low when PC12 cells, expressing HDAC2$^{C262A/C274A}$, were treated with NGF and SNOC (Fig. 5.1). Thus, nitrosylation of HDAC2 is necessary for neurotrophin-dependent histone acetylation at multiple gene promoters. It is conceivable that HDAC2$^{C262A/C274A}$ is unable to dissociate from the chromatin and remains strongly bound to gene promoters regardless of stimulation by neurotrophins. My findings indicate that neurotrophin signalling induces nitrosylation of HDAC2 and subsequent dissociation of HDAC2 from gene promoters, which increases acetylation of histones.

I next addressed whether neurotrophin-dependent gene transcription is mediated by HDAC2 nitrosylation by employing the dual luciferase reporter assay. Embryonic cortical

neurons were co-transfected with a firefly-luciferase construct containing the *Fos* promoter (-41 to -498) together with various HDAC2 constructs or empty Flag vector. Activation of the *Fos* promoter results in transcription of a firefly-luciferase protein, which can be measured using a luminometer. As control, neurons were co-transfected with a *Renilla*-luciferase construct driven by the thymidine-kinase promoter (TK-luciferase). The level of transcription mediated by the thymidine-kinase promoter is independent of neurotrophin stimulation, thereby serving as an internal control for transfection efficiency. Cortical neurons expressing empty vector were exposed to BDNF (75ng/ml, 6 hours) and subjected to the dual luciferase assay. BDNF stimulation of cortical neurons resulted in a 2-fold induction of *Fos* promoter activity. Pre-treatment of cultures with the nNOS inhibitor, NPA (300μM) completely inhibited BDNF-dependent activation of *Fos*. This finding supports previous evidences that neurotrophin-dependent gene expression in cortical neurons requires nNOS activity (Riccio et al 2006).

To test whether activation of the *Fos* promoter requires nitrosylation of HDAC2, cortical neurons were transfected with HDAC2^{WT} or HDAC2$^{C262A/C274A}$ followed by BDNF stimulation and processed using the dual luciferase assay. As expected, treatment with BDNF of cortical neurons expressing HDAC2^{WT} induced activation of the *Fos* promoter (Fig. 5.2; P>0.001). Treatment of cortical cultures with NPA completely abolished BDNF-dependent activation of *Fos* (Fig. 5.2). To investigate whether BDNF-dependent nitrosylation of HDAC2 mediated *Fos* expression, cortical neurons expressing HDAC2^{C262A}, HDAC2^{C274A} or HDAC2$^{C262A/C274A}$ were stimulated with BDNF (75ng/ml) and cell lysates were subjected to the dual luciferase assay. Activation of the *Fos* promoter was induced following stimulation with BNDF in neurons expressing HDAC2^{C262A} and HDAC2^{C274A} however, expression of HDAC2$^{C262A/C274A}$ blocked

BDNF-induced activation of the *Fos* promoter. Thus, *S*-nitrosylation of HDAC2 at both Cys262 and Cys274 is necessary for activation of the *Fos* gene promoter. These data imply that BDNF-induced activation of the *Fos* promoter requires that nitrosylated HDAC2 dissociates from gene promoters, thereby facilitating transcriptional initiation.

I next tested whether HDAC2 nitrosylation is necessary to induce the expression of neurotrophin-dependent endogenous genes by performing northern blot analysis of *Fos*, *Egr1* and *Vgf*. PC12 cells were transfected with HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or empty vector and exposed to NGF for 30, 60 and 180 minutes. Total cellular mRNA was run on a denaturing agarose gel and transferred onto a nylon membrane. The presence of RNA for specific genes was detected using a ^{32}P labelled complementary DNA probe. An increase of RNA upon treatment with NGF indicates an initiation in gene transcription that is dependent on neurotrophin stimulation. I observed that endogenous expression of *Fos*, *Egr1* and *Vgf* was induced upon NGF treatment of PC12 cells expressing either en empty vector or a vector containing HDAC2^{WT} (Fig. 5.3). In contrast, the expression of all three genes following NGF stimulation was reduced in PC12 cells expressing HDAC2$^{C262A/C274A}$ (Fig. 5.3). These data indicate that HDAC2 is a key target of NO signalling following neurotrophin stimulation and confirms that HDAC2$^{C262A/C274A}$ is a strong transcriptional repressor.

The development of the cortex depends on progenitor cells switching from a highly proliferative state into mature post-mitotic neurons. This dramatic change in cellular identity requires long-lasting changes in expression of multiple genes. I have demonstrated that neurotrophin-dependent *S*-nitrosylation of HDAC2 induces histone acetylation in embryonic

cortical neurons and increases the expression of multiple genes. Neurotrophin modulation of HDAC2 through NO signalling may provide an epigenetic mechanism by which the temporal activation of multiple neural genes can be tightly regulated during development.

Figure 5.1 *Nitrosylation of HDAC2 modulates histone acetylation.* PC12 cells expressing HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or EV control were treated with NGF (100ng/ml, 20 min), SNOC (200 μM, 20 min) or vehicle (Ctrl). Lysates were subjected to ChIP analysis using either pan-acetylated histone H4 (AcH4) or K9/K14-acetylated histone H3 (AcH3K9/K14). Immunoprecipitation was followed by qPCR analysis of promoter regions of *Fos*, *Egr1*, *Vgf* and *Nos1*. Data is normalised to total input and represented as average fold change over vehicle control (Ctrl) +/- SEM; *P<0.005; n=3.

Figure 5.2 *Nitrosylation of HDAC2 regulates Fos expression in cortical neurons.* Embryonic cortical neurons co-expressing HDAC2 vectors (as indicated), Fos-luciferase vector (firefly) and TK-luciferase vector (Renilla) were treated with either BDNF, (75ng/ml), SNOC (200μM), NPA (300 μM), BDNF plus NPA, or vehicle (Ctrl) for 6 hours and subjected to dual luciferase reporter assay (Promega). Raw values were normalised by TK-luciferase expression. Data is presented as fold induction over control Fos-luciferase expression, +/- SEM; *P<0.001; n=4. EV is empty vector control.

Figure 5.3 *Nitrosylation of HDAC2 regulates the expression of multiple endogenous genes.* PC12 cells expressing HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or EV control were treated with NGF (100ng/ml) for the indicated times and subjected to Northern blot analysis for *Egr1*, *Vgf*, *Fos* and *Gapdh* mRNA; n=3.
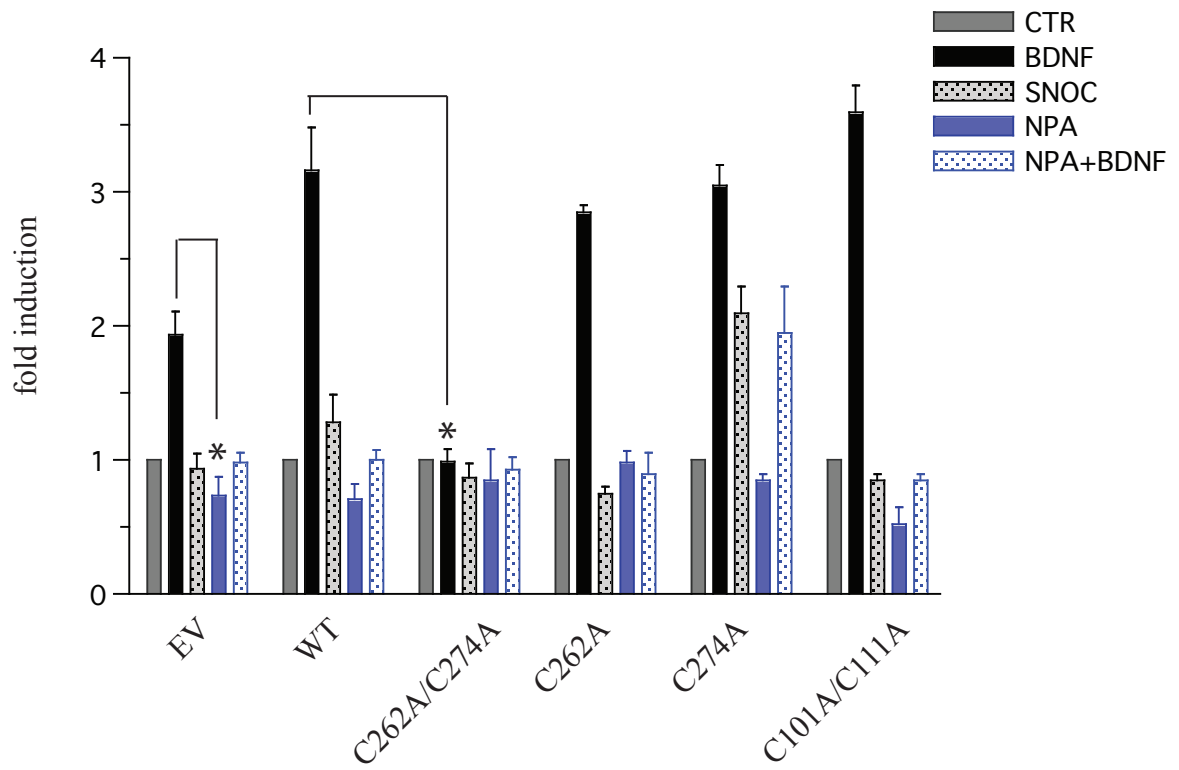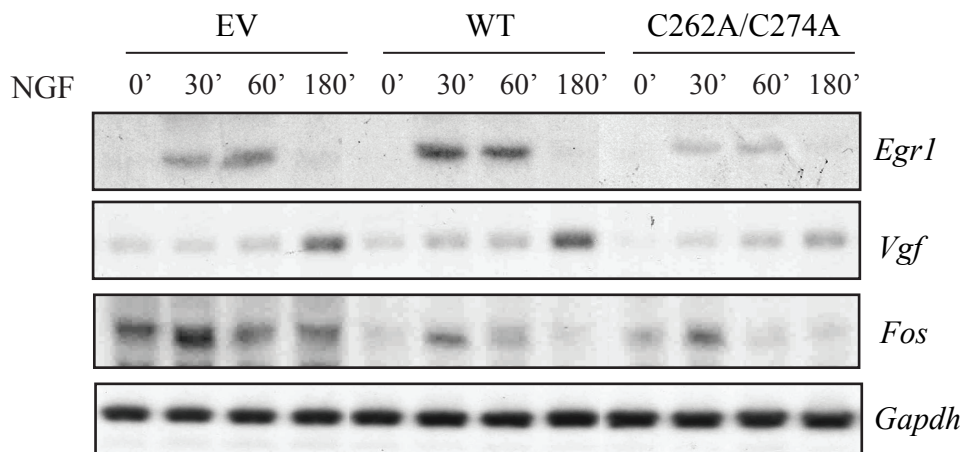
Fig. 5.1

Fos

Egr1

Vgf

Nos1

Fig. 5.2

Fig. 5.3

Chapter 6   Implications of HDAC2 *S*-nitrosylation for the development of cortical neurons *in vivo* and *in vitro*

The development of the cortex is associated with progenitor cell differentiation and their migration from the VZ to distinctive layers within the cortical plate (Fig. 6.1).   Cortical neurogenesis is characterised by a tight regulation of cell proliferation and differentiation which is mediated by extrinsic signals, including Notch, ephrins and neurotrophins, and intrinsic effectors, such as chromatin remodelling factors (Barnabe-Heider and Miller 2003; Ito et al 2003; Cheng et al 2003; Ciani et al 2004). *In vivo* neurogenesis of cells within the SVZ is enhanced in adult nNOS null mice (Packer et al 2003) whereas neurogenesis is decreased in mice over-expressing nNOS within the cortex, hippocampus and striatum (Packer et al 2005).   Notably, TrkB deletion in pyramidal cells during development leads to the compression of cortical layers II/III and V, suggesting that BDNF signalling is critical for neocortical development (Xu et al 2000). We have shown that neurotrophin stimulation of cortical neurons induces *S*-nitrosylation of the nuclear protein HDAC2, which results in transcriptional activation of multiple genes. This provides a mechanism whereby neurotrophin-induced NO signalling influences cell differentiation and migration during cortical development.

To test whether activity-dependent *S*-nitrosylation of HDAC2 affects neuronal development *in vitro*, embryonic cortical neurons were co-transfected with green fluorescent protein (GFP) and either HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or empty vector. I observed that average dendritic lengths of neurons transfected with HDAC2$^{C262A/C274A}$ were significantly shorter when compared to neurons expressing HDAC2^{WT} or empty vector (Fig. 6.2, 6.3). Stimulation of cortical neurons

with KCl leads to neuronal depolarisation and increases intracellular calcium. It has been previously demonstrated that exposure of cortical neurons to KCl (50mM) greatly enhances dendritic growth (Redmond et al 2000; Redmond et al 2002). Neurons transfected with either HDAC2^{WT} or empty vector and exposed to KCl for 48 hours showed increased average dendritic length of almost 50 μm (Fig. 6.2, 6.3). Importantly, expression of HDAC2$^{C262A/C274A}$ completely blocked an activity-dependent increase in dendritic length (Fig. 6.2, 6.3). These findings strongly support the hypothesis that HDAC2 nitrosylation mediates the expression of genes necessary for dendritic projection during development.

To ascertain whether NO inhibition of HDAC2 influences cortical development *in vivo,* I employed an *ex vivo* electroporation technique (Fig. 6.4). Development of the neocortex requires the generation of a diverse range of neuronal subtypes characterized by the expression of distinct genes. Migration of neuronal subtypes is restricted to specific cortical layers where precise connections with a subset of target cells are established. The generation of neuronal subtypes and subsequent migration to specific layers follows a tightly regulated temporal pattern (Fig. 6.1). The first post-mitotic neurons migrate radially from the VZ at E11, forming the pre-plate at the pial surface of the brain. The preplate is split into a marginal zone and a deeper subplate by a second wave of postmitotic neurons migrating from the VZ at E13. Postmitotic neurons generated between E14 and E18, leave the VZ at distinct phases and migrate radially to the marginal zone, passing previously established cortical layers to form an 'inside out' patterning of the cortical plate. Once the layering of the cortex has been established, the subplate degenerates, establishing the six-layered neocortex found in the adult brain.

Cortical pyramidal neurons are generated from asymmetric division of radial glial cells, with the generation of an additional self-renewal progenitor cell. Radial glial cells produce intermediate neuronal precursors that retain a limited proliferative capability. Neuronal precursor cells undergo two further cell divisions within the SVZ and IZ to produce four multi-polar early neurons that migrate radially into the cortical plate. Generation of intermediate neuronal precursors allows a rapid increase of pyramidal neurons from a small number of radial glial cells and is thought to be essential for the expansion of the cortical plate during development.

The *ex vivo* electroporation technique involves the microinjection of DNA within the lateral ventricles of the whole brain, followed by electroporation of progenitor cells along the VZ (Fig. 6.4). Mouse brains were electroporated with a modified pCIG2 construct containing an HDAC2-IRES-eGFP expression cassette under the control of a cytomegalovirus (CMV) enhancer and a chicken ß-actin promoter (Hand et al 2005). This promoter cassette allows the expression of HDAC2 and eGFP within undifferentiated cortical progenitor cells. Coronal sections of electroporated E14.5 mouse brains were cultured on semi-permeable organotypic membranes for 5 days (Fig. 6.4). Immuno-staining of eGFP allowed the identification of HDAC2 transfected cells and the analysis of their migration path from the VZ. Cortical morphology was visualised by immuno-staining for nestin-positive radial glial cells. Preliminary data suggests that E14.5 cortical progenitors expressing $HDAC2^{C262A/C274A}$ exhibit impaired radial migration into the cortical plate, when compared to cells transfected with either $HDAC2^{WT}$ or empty vector (Fig. 6.5). This implies that gene activation following nitrosylation of HDAC2 is necessary for normal radial migration of cortical precursor cells and corroborates an early study that observed impaired migration of granule cells in brain slices cultured in the presence of NOS inhibitors (Tanaka et al

1994). Similarly, radial migration of cortical progenitors was impaired following *in utero* electroporation of a dominant-negative TrkB construct or *Sir*TrkB oligonucleotides (Bartkowska et al 2007). I have demonstrated that BDNF induces *S*-nitrosylation of HDAC2 and dissociation of HDAC2 from specific gene promoters. Disruption of HDAC2 *S*-nitrosylation through site-directed mutagenesis of two specific cysteine residues impairs the radial migration of cortical precursors. Possible implications of the *ex vivo* data is that *S*-nitrosylation of HDAC2 following stimulation with BDNF is necessary for the normal layering of the cortex.

The lack of cells expressing HDAC2$^{C262A/C274A}$ within the cortical plate may be a consequence of either increased apoptosis or impaired neurogenesis and migration. To determine whether HDAC2$^{C262A/C274A}$ influences apoptosis, transfected cortical slices will be immuno-stained using an antibody raised against cleaved caspase-3. Cleavage of immature pro-caspases leads to activation of apoptotic pathways and subsequent DNA fragmentation and nuclear condensation therefore, staining of cleaved-caspases and condensed nuclei are good indications of apoptosis. To test whether HDAC2$^{C262A/C274A}$ impairs proliferation of progenitor cells, electroporated slices will be stained for the proliferation marker Ki-67. Ki-67 is expressed during the active phases of the cell cycle, but absent in resting cells therefore, impaired proliferation will result in reduced Ki-67 staining (Scholzen and Gerdes 2000). To further differentiate between defects in proliferation and radial migration, slices will be immuno-stained for the early neuronal markers HuD and βIII-tubulin. Defective radial migration will result in an increased number of neuronal marker positive cells within the SVZ but not in the cortical plate. However, if I observe that staining of HuD and βIII-tubulin is restricted to the cortical plate, I will conclude that radial migration is normal and that the phenotype is due to a defect in either proliferation or survival, or

both. Reduction of cells within the cortical plate following disruption of TrkB signalling is due to impaired proliferation, whereas radial migration remained normal (Bartkowska et al 2007). Since nitrosylation of HDAC2 is induced following TrkB signalling, a reduction in precursor cells expressing HDAC2$^{C262A/C274A}$ within the cortical plate will probably be due to impaired proliferation.

In summary, stimulation of cortical progenitor cells with BDNF increases intracellular Ca^{2+} and leads to the activation of nNOS and nuclear accumulation of NO (Fig. 6.6). A key nuclear target of NO is the chromatin-modifying enzyme, HDAC2 (Nott et al 2008). *S*-nitrosylation of HDAC2 results in its dissociation from repressive complexes at gene promoters regulated by CREB (Fig. 6.6). This leads to an increase of histone acetylation at specific promoter regions and initiation of gene transcription (Fig. 6.6) (Nott et al 2008). Importantly, I have demonstrated that NO modulation of HDAC2 is necessary for dendritic growth *in vitro* (Nott et al 2008). My preliminary data obtained by employing an *ex vivo* technique suggest that nitrosylation of HDAC2 may be important for radial migration of cortical precursors *in vivo*. Two potential targets of NO-dependent inhibition of HDAC2 include the CREB-regulated genes *Bdnf* and *Nos1*. The expression pattern of both *Bdnf* and *Nos1* in the developing cortex is very similar, with expression levels high in the IZ and cortical plate but absent from progenitor cells in the VZ. Furthermore, stimulation with BDNF induces transcription of *Nos1* and *Bdnf* itself (Xiong et al 1999; Cheng et al 2003). A further potential candidate that may be regulated by nitrosylated HDAC2 is the pro-survival factor, *Bcl2*, which has been demonstrated to be induced upon NO-dependent activation of CREB (Riccio et al 1999; Dhakshinamoorthy et al 2007). Nitrosylation

of HDAC2 during cortical development is likely to regulate a broad array of genes, which may

provide further incite into the ability of cells to switch from proliferative to differentiated states.

Figure 6.1 *Formation of the neocortex into distinct neuronal layers.* At E11, the preplate (PP) is established by a postmitotic wave of neurons that has migrated along radial glia (vertical bars) from the ventricular zone (VZ) to the pial surface (PS). A second wave of postmitotic neurons migrates through the intermediate zone (IZ) at E13, splitting apart the PP into the marginal zone (MZ) and the subplate (SP), thus creating the cortical plate (CP). Subsequent waves of neurons exit the VZ through E14-E18, each wave passing previously established layers, forming the 'inside-out' six-layered neocortex. In adulthood the SP degenerates. (Taken from Gupta et al 2002).

Figure 6.2 *S-nitrosylation of HDAC2 regulates cortical dendritic growth.* Examples of cortical neurons transfected with HDAC2^{WT}, HDAC2$^{C262A/C274A}$, or EV control. Cortical cultures were treated with KCl (50mM) or vehicle (Ctrl) for 48 hours before fixation. Images were taken on an Axioplan 2 fluorescence microscope with a Plan Neofluar 25x/0.8 Corr objective lens and FS10 (GFP) filter set. Scale bar, 40 μm.

Figure 6.3 *Quantitative analysis of cortical neuron dendritic growth (Fig. 6.1).* Average dendritic length of cortical neurons transfected with HDAC2^{WT}, HDAC2$^{C262A/C274A}$ or EV control. Neuronal cultures were treated with KCl (50mM) or left untreated (Ctrl). Each condition represents the analysis of 30 neurons per experiment (n=3); +/- SEM, *P<0.005.

Figure 6.4 *Ex vivo electroporation of cortical progenitors and organotypic slice culture allows the study of radial migration.* **a.** pCIG-green construct mixed with 0.5% Fast Green dye is injected into the lateral ventricles of an intact E14.5 mouse embryo. **b.** The intact head is

immediately electroporated and the brain removed. **c.** The brains are embedded in Low Melting Point (LMP) agarose and 250µm sections are sliced using a vibratome. Slices are cultured on semi-permeable organotypic membranes for up to 10 days (Hand et al 2005).

Figure 6.5 *S-nitrosylation of HDAC2 influences radial migration of cortical progenitors.* *Ex vivo* electroporation of mouse E14.5 embryonic cortical progenitors with either pCIG2:EGFP-HDAC2^{WT}, pCIG2:EGFP-HDAC2$^{C262A/C274A}$ or pCIG2:EGFP (empty vector), followed by organotypic slice culture for 5 days *in vitro*. Slices were immuno-labelled for GFP (green) and images captured using a Leica DMI6000 confocal microscope with HC PL APO 20x/0.7 Corr objective lens.

Figure 6.5 *HDAC2 nitrosylation mediates neurotrophin induced gene transcription.* In resting neurons repressive complexes recruit HDAC2 to gene promoters, which result in deacetylation of histones and gene repression. Neurotrophin stimulation of cortical neurons results in nuclear accumulation of NO and nitrosylation of nuclear proteins. Nitrosylation of HDAC2 results in its dissociation from gene promoters, which correlates with an increase in histone acetylation and activation of gene transcription.
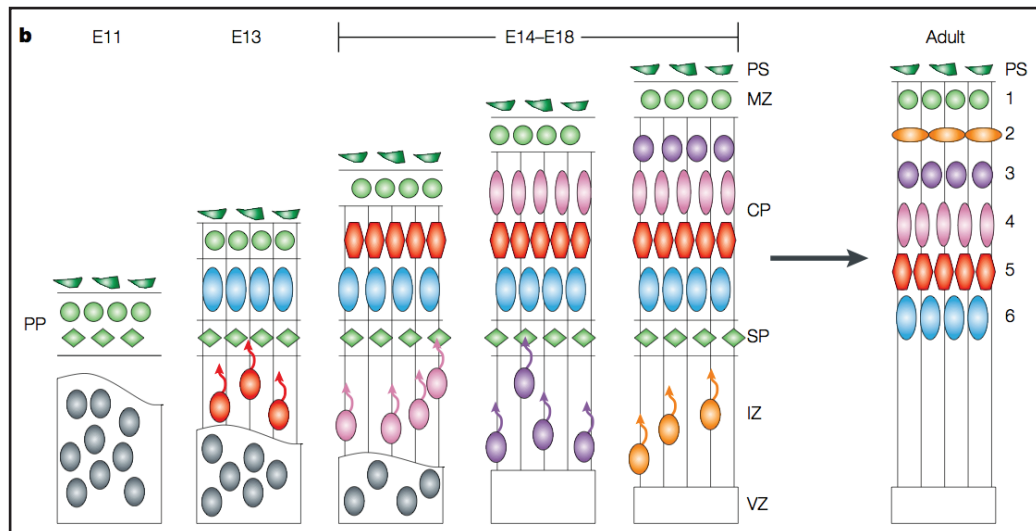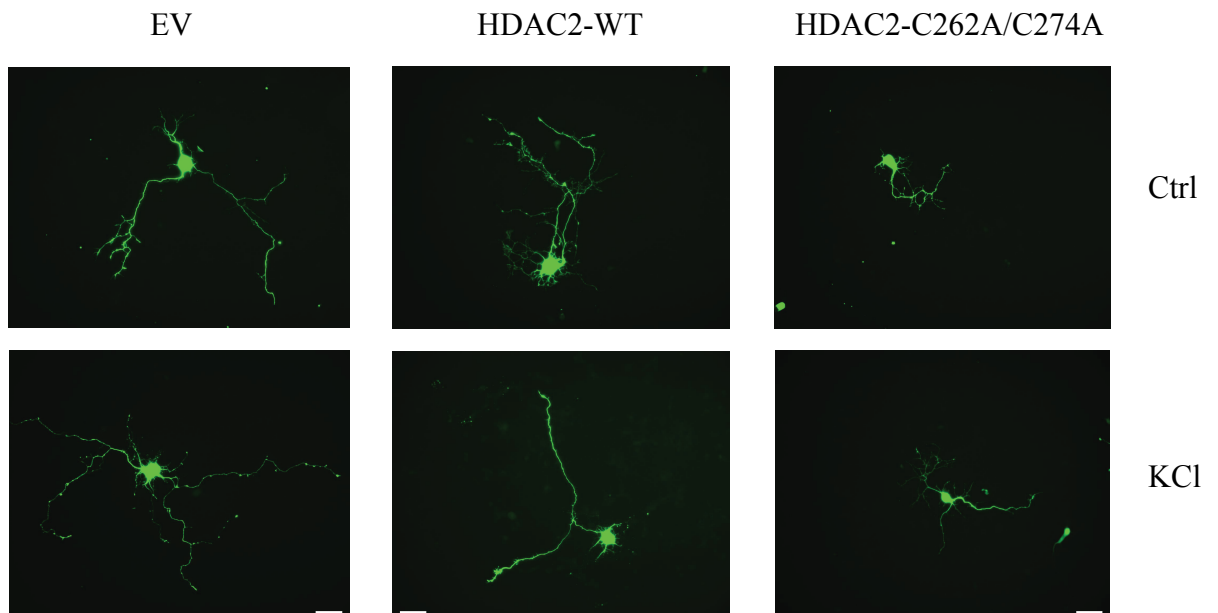
Fig. 6.1

Fig. 6.2
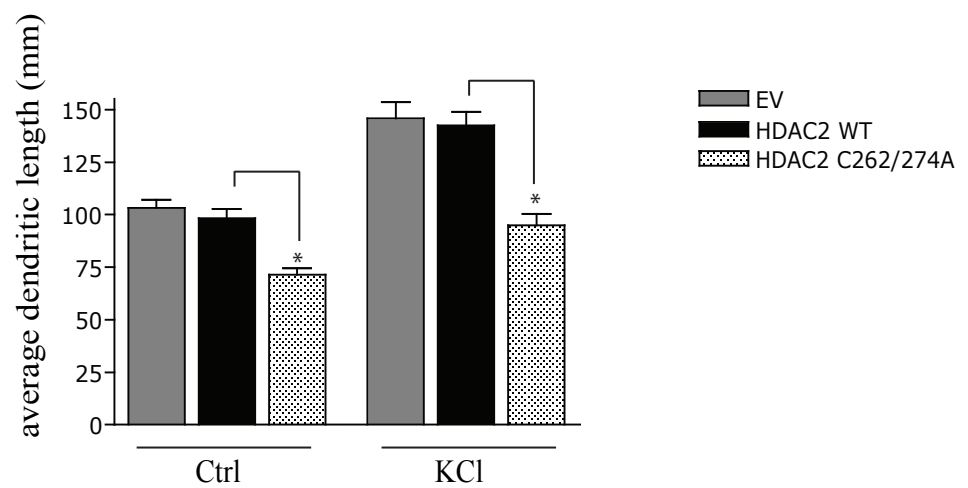

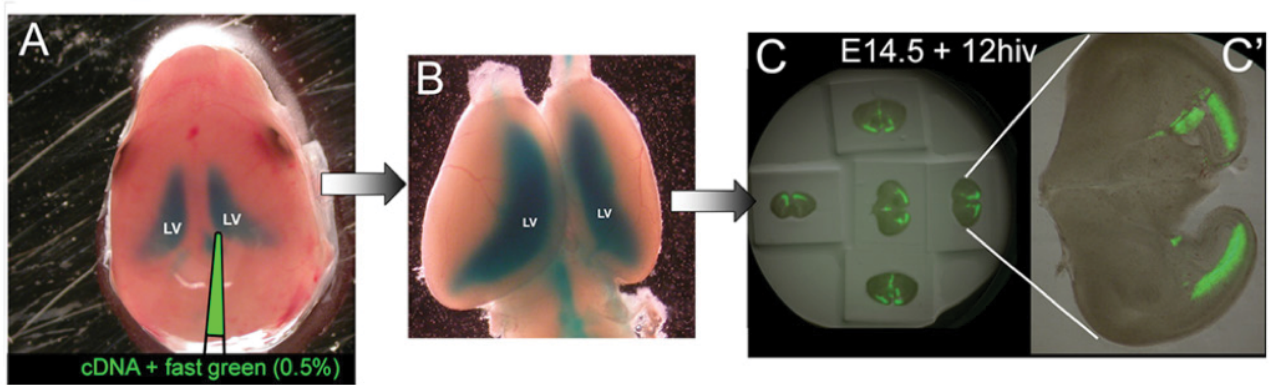| EV | HDAC2-WT | HDAC2-C262A/C274A | |

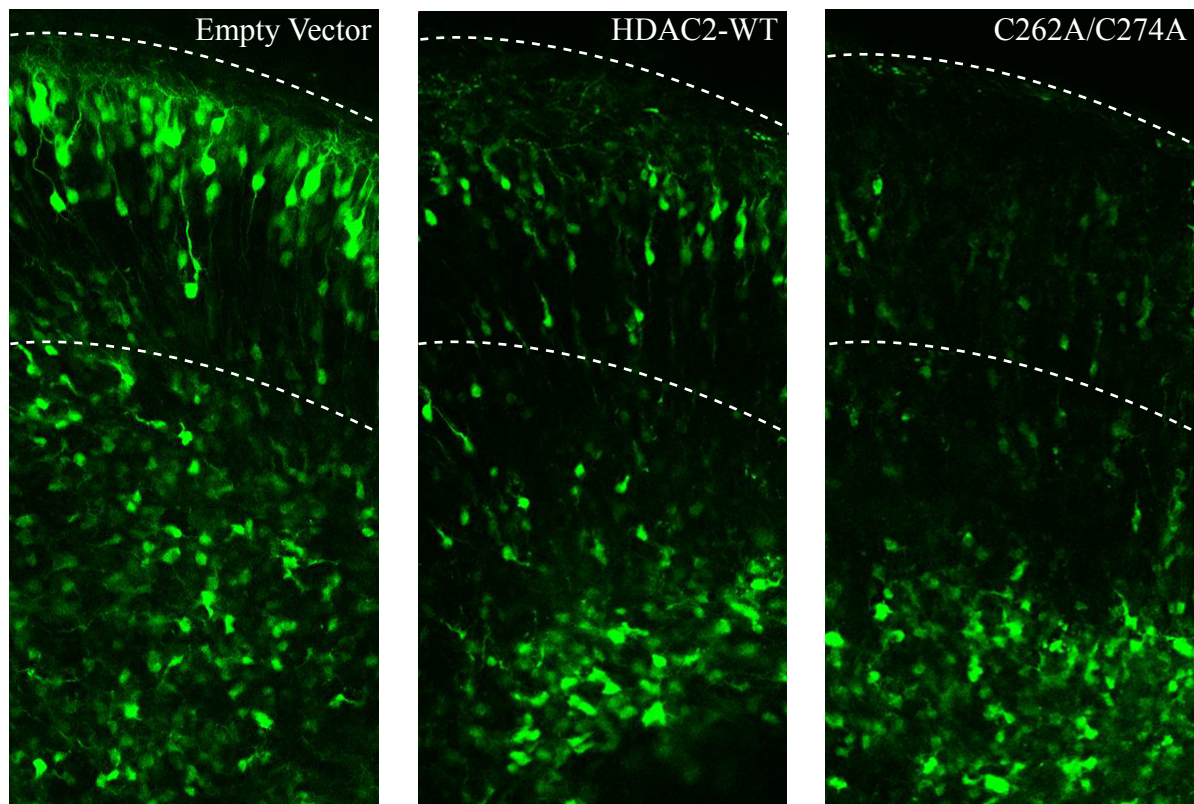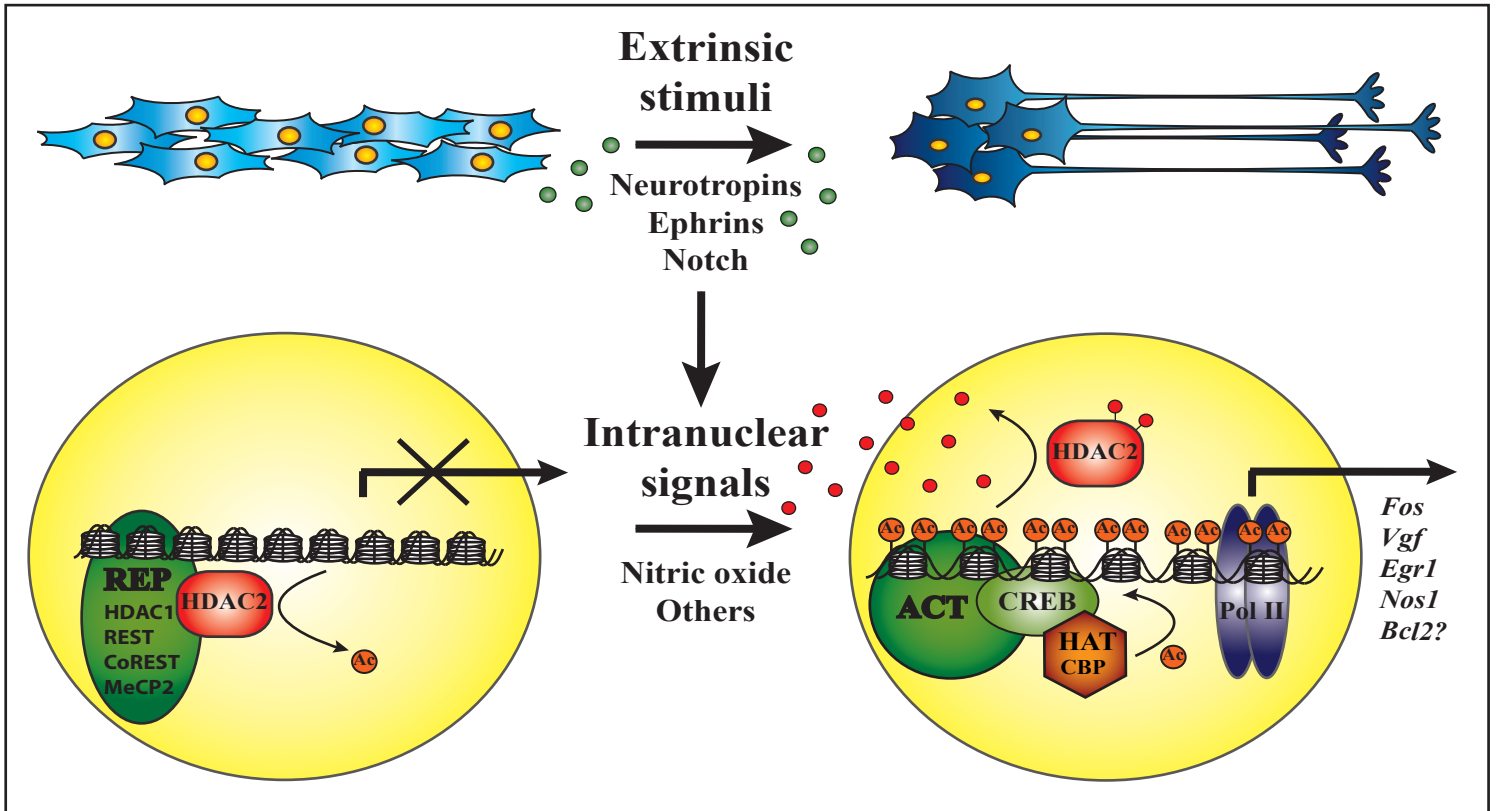**Fig. 6.3**

Fig. 6.4

Fig. 6.5

Fig. 6.6

<u>Chapter 7</u> Discussion

The development of the nervous system is a complex process that involves the generation of a vast number of neuronal and glial cell types from a single uniform population of embryonic stem cells. The birth of distinct neuronal populations is both spatially and temporally regulated. The initial cell migration process precisely defines the final destination of neurons and is followed by the establishment of synaptic contacts within specific populations of target cells. Developmental processes are heavily reliant on the availability of extracellular cues and the responsiveness of progenitor cells to these cues. We have demonstrated a novel mechanism whereby BDNF induces neuronal precursor cell differentiation and migration by regulating NO-dependent HDAC2 function and gene activation (Nott et al 2008).

I demonstrated that BDNF-stimulation of embryonic cortical neurons induces NO accumulation within the nucleus (Nott et al 2008). This accumulation of NO is completely blocked by pre-treatment of cultures with the nNOS specific inhibitor NPA. This finding indicates that the primary source of NO in cortical neurons following BDNF stimulation is endogenous nNOS. Exposure of cortical cultures to BDNF increases intracellular accumulation of NO and *S*-nitrosylation of nuclear and cytoplasmic proteins within minutes. The rapid detection of NO and the requirement of Ca^{2+} as a co-activator of nNOS suggest that a BDNF-dependent elevation in intracellular Ca^{2+} is required. TrkB receptor is expressed in cortical progenitors throughout neuronal development and is maintained in fully differentiated pyramidal neurons. TrkB signalling leads to activation of PLC-γ1, which catalyses the hydrolysis of PIP_2 to produce the two intracellular messenger molecules IP_3 and DAG. Binding of IP_3 to IP_3 receptors

located on the endoplasmic reticulum leads to a rapid influx of intracellular Ca^{2+}. This is probably the primary source of Ca^{2+} in neurons following BDNF stimulation. Activated IP_3 receptors on the endoplasmic reticulum near the nuclear envelope will produce a localised elevation of Ca^{2+} in close proximity to the nucleus. Perinuclear nNOS will subsequently be activated and contribute to nuclear accumulation of NO.

An alternative source of intracellular Ca^{2+} following neurotrophin stimulation is the TRPC ion channel. These plasma-membrane store-operated channels are responsible for capacitance, a process that allows replenishment of depleted intracellular Ca^{2+} stores following activation of IP_3 receptors. TRPC capacitance significantly contributes to BDNF-induced increases in intracellular Ca^{2+} levels. An established mechanism for TRPC ion channel gating in neurons is through BDNF-induced activation of PLC-γ1 (Li et al 1999; Clapham 2003; Amaral and Pozzo-Miller 2007). However, the contributing role of either IP_3 receptors, DAG or Ca^{2+} released from intracellular stores is still controversial (Kiselyov et al 1998; Hofmann et al 1999; Clapham et al 2001). Ca^{2+} influx through activated TRPC channels may further contribute to elevated Ca^{2+} levels following PLC-γ1 signalling. Localisation of TRPC channels to the plasma membrane and IP_3 receptors on the endoplasmic reticulum results in Ca^{2+} influx from two spatially distinct sources. This may allow the activation of specific subsets of NOS enzymes that are differentially localised within the cell, which may in turn initiate distinct signalling pathways.

During the development of the nervous system, BDNF-induced activation of TRPC channels influences growth cone development in the mammalian brain (Li et al 1999; Li et al 2005). Interestingly, a recent report has linked BDNF-dependent neuronal survival with TRPC-

induced activation of CREB (Jia et al 2007). An attractive hypothesis is that an increase of intracellular Ca^{2+} upon PLC-γ1 activation leads to nNOS activation and nuclear accumulation of NO. It has previously been shown that NO-dependent CREB binding in cortical neurons occurs upon BDNF stimulation in a Ca^{2+}-dependent manner and is independent of both the Ras/ERK and PI-3 Kinase pathways (Riccio et al 2006). Further pharmacological analysis will elucidate the role of PLC-γ1-induced activation of IP_3R and TRPC channels for nNOS activation.

I demonstrated that HDAC2 is nitrosylated on two specific cysteine residues (Nott et al 2008). However, thiol groups of cysteine residues can exist in a number of oxidative states, from *S*-nitrosylation (S-NO), sulphenic acid (S-OH), disulphide bond (S-S), sulphinic acid (SO_2^-) to the irreversible sulphonic acid (SO^{3-}). The ability of thiol groups to exist in successive oxidative states may add a further level of complexity to NO modulation of protein functions (Fig. 7.1). The oxidative state of cysteine residues can be viewed as a continuum that depends on the amount, origin, spatiotemporal distribution and order of the NO oxide species (NO/higher nitric oxides (NO_x)/SNO). Progression of protein modifications through oxidative states may allow a transition in cellular response from adaptive to oxidative stress and finally toxicity. A striking example is provided by OxyR, a bacterial transcription factor with a cysteine residue (Cys199) that can stably exist in several oxidative states, either *S*-nitrosylated, *S*-hydroxylated (S-OH) or *S*-glutathionylated (S-SG; mixed disulphide with glutathione) (Kim et al 2002). These different modifications activate OxyR to various degrees, changing its affinity for DNA and its ability to bind in a cooperative or non-cooperative manner. A similar mechanism may exist for HDAC2, whereby different oxidative states could change its tertiary and quaternary structure, influencing its affinity for specific repressive complexes.

My findings indicate that physiological levels of NO following neurotrophin signalling, induces *S*-nitrosylation of HDAC2 and initiates transcription of genes necessary for neuronal development (Nott et al 2008). It could be hypothesised that further increases in NO levels possibly associated with nitrosative stress, may result in sulphenic or sulphinic acid modifications of HDAC2 allowing the expression of genes that reduce intracellular reactive oxygen species (ROS). Likewise, toxic levels of NO may generate irreversible sulphonic acid modifications of HDAC2 and subsequently activate genes associated with apoptosis. The presence of these modifications in a physiological intracellular environment will require the exposure of cells to various oxidative conditions followed by mass spectrometry analysis of HDAC2. Interestingly, a 3D predicted model of HDAC2 based on the crystal structure of HDAC8 estimates that the distance between Cys262 and Cys274 is 15.6Å and that one residue is buried within a hydrophobic tunnel (Fig. 4.1) (Vannini et al 2004; Somoza et al 2004). Similarly, in the reduced form of OxyR, Cys199 and Cys208 are 17Å apart, which is too far for disulphide bond formation. A sulphenic acid intermediate forces Cys199 out of a hydrophobic pocket, permitting bond formation with Cys208 (Kim et al 2002). This allows a more stable conformational change in OxyR and prolonged activation of the transcription factor. A similar mechanism may apply to HDAC2, with the formation of a disulphide bond affecting its ability to oligomerise with additional HDACs or with components of repressive complexes. It should be noted however, that a stable *S*-nitrosylated form of HDAC2 must exist, because the use of ascorbic acid in the biotin-switch assay selectively reduces nitrosothiols only, while cysteine residues in other oxidative states remain unaffected (Forrester et al 2007).

I demonstrated that NO induces expression of multiple genes through *S*-nitrosylation of HDAC2 (Nott et al 2008). These findings may provide a possible explanation for conflicting results of NO-dependent regulation of transcription factors. For example, NO has opposing affects on NF-κB binding to DNA. *S*-nitrosylation of NF-κB subunits reduces its affinity for DNA and stabilises its inhibitory cofactor IκB. However, other studies have suggested that NO signalling induces the expression of genes regulated by NF-κB. Similarly, NO negatively affects the binding of the AP1 transcription factor to DNA, although it also induces the expression of AP-1-regulated genes, including *Fos* itself. It is possible that NO initially increases gene transcription by modulating the acetylation state of histones through *S*-nitrosylation of HDAC2. This event is followed by an inhibitory-feedback loop, mediated by direct nitrosylation of transcription factors. A further level of complexity is provided by the compartmentalisation of NO signalling. For example, NF-κB is nitrosylated within the cytoplasm, whereas HDAC2 is modified within the nucleus. Compartmentalisation of NO synthesis may be achieved in part by local increases of Ca^{2+} and nNOS activation. As previously discussed, activation of PLC-γ1 may lead to Ca^{2+} influx, either through depletion of intracellular stores or activation of TRPC channels at the plasma membrane, providing a possible mechanism for segregated Ca^{2+} entry and signalling.

Compartmentalised NO signalling has been demonstrated in cardiomyocytes though subcellular segregation of two NOS isoforms, which results in discernable isoform-specific cellular properties (Petroff et al 2001; Ashley et al 2002; Sears et al 2003). Cortical neurons were thought to express a single nNOS isoform however, it has become apparent that nNOS exist as several splice variants. There are three major splice variants of nNOS, the predominant wild-type

variant nNOSα and two terminal truncated variants nNOSβ and nNOSγ. The two truncated splice variants are missing an N-terminal PDZ domain that is present in nNOSα. The PDZ domain is responsible for targeting nNOSα to the postsynaptic density proteins PSD93 and PSD95 therefore, nNOSβ and nNOSγ are presumed to be soluble. nNOSγ is missing a further three structural motifs important for its dimerization, resulting in a catalytic activity lower than the other two variants. Differences in expression, subcellular localisation and activity of nNOS N-terminal splice variants would allow the compartmentalisation of NO signalling between the cytoplasm and the nucleus. This may result in NO-dependent regulation of transcription factors, such as NF-κB, which normally reside in the cytoplasm and then shuttle into the nucleus upon activation.

Preliminary *ex vivo* data suggest that *S*-nitrosylation of HDAC2 is necessary for the proper radial migration of cortical progenitors. Previous data have implicated both BDNF and NO signalling in regulating the differentiation and migration of neuronal precursors (Barnabe-Heider and Miller 2003; Ito et al 2003; Cheng et al 2003; Ciani et al 2004). The transition of progenitor cells to pyramidal neurons is determined by the outcome of two conflicting processes, proliferation and differentiation. bHLH transcription factors are the foremost regulators of both these processes: Id and Hes families maintain proliferation, whereas the neurogenin family promotes neuronal differentiation and exit from the cell cycle. Ngn2, a pro-neural bHLH factor expressed in the VZ, is a key regulator of cell cycle arrest and neurogenesis during cortical development (Hevner 2006). The temporal and spatial expression of Ngn2 during cortical development makes it a potential target of NO-dependent HDAC2 regulation (Fode et al 2000; Murciano et al 2002; Miyata et al 2004; Schuurmans et al 2004). Transient expression of Ngn2

triggers a second wave of sustained bHLH expression, including NeuroD, NeuroD2 and Nex. These factors alone are sufficient for promoting cell cycle arrest and neuronal differentiation. Therefore, my preliminary observations that electroporated cortical slices have reduced HDAC2$^{C262A/C274A}$ positive neurons within the cortical plate may reflect a disruption in neurogenesis. This finding is supported by a recent study that shows impaired neurogenesis of cortical progenitors upon inhibition of BDNF signalling, using an *in utero* electroporation system (Bratkowska et al 2007).

The NeuroD/Nex factors are both necessary and sufficient for cell cycle arrest and neuronal differentiation of cortical progenitors. Interestingly, the NeuroD, NeuroD2 and Nex transcription factors are highly expressed in the IZ and cortical plate, which mirrors cortical expression of nNOS (Bredt and Snyder 1994; Lee et al 2000). Since these transcription factors also play a prominent role in cell cycle arrest and differentiation, *S*-nitrosylation of HDAC2 may first regulate expression of NeuroD/Nex factors through inhibiting Ngn2 binding to DNA, and later also regulate DNA-binding of NeuroD/Nex factors. Recent studies have suggested that Ngn2 may modulate additional aspects of cortical development distinct from its proneural activity. Ngn2 null mice exhibit defects in cortical radial migration, directly implicating Ngn2 activation in pyramidal migration and unipolar dendritic morphology (Hand et al 2005; Heng et al 2008). The small GTP-binding protein Rnd2 is induced in progenitor cells by Ngn2 and its expression is sufficient to rescue migration defects in Ngn2-mutant cells (Heng et al 2008). Neurons expressing HDAC2$^{C262A/C274A}$ may have reduced Rnd2 expression, which subsequently impairs cortical radial migration. Therefore, HDAC2$^{C262A/C274A}$–mediated repression of bHLH target genes may have a combined affect on neurogenesis and radial migration.

Cortical development requires the coupling of multiple cellular pathways, many of which appear to be influenced by NO signalling. The cyclin-dependent kinase inhibitor, $p27^{Kip1}$, is capable of binding cyclin/cyclin-dependent kinase complexes to initiate cell cycle withdrawal. $p27^{Kip1}$ is highly expressed in the IZ and cortical plate, as cortical precursors stop proliferating and differentiate to form pyramidal neurons. An exciting study has recently demonstrated that $p27^{Kip1}$ is a modular protein capable of independently regulating multiple aspects of neurogenesis (Nguyen 2006). The N-terminal region of $p27^{Kip1}$ stabilises Ngn2 thus promoting neuronal differentiation whereas the C-terminal region of $p27^{Kip1}$ inhibits RhoA activity thus promoting radial migration of cortical neurons (Nguyen 2006). In fibroblasts, levels of $p27^{kip1}$ are significantly increased upon HDAC inhibition, and this event plays a critical role in determining cell cycle arrest (Chen and Faller 2005). The spatial expression of $p27^{Kip1}$ and its ability to modulate multiple aspects of neurogenesis makes it a potential target of *S*-nitrosylated HDAC2 during cortical development.

HDACs themselves have recently been implicated as possible regulators of corticogenesis (Humphrey et al 2008; Shaked et al 2008). Overexpression of a dominant-negative (dn) HDAC2 construct in neural stem cells led to a two-fold increase of the astrocyte marker glial fibrillary acidic protein (GFAP), with no discernable difference of other neuronal markers (Humphrey et al 2008). GFAP is expressed in radial glial cells and astrocytes, leading the authors to suggest that HDAC2 may repress a transition from an 'early' to 'late' stem cell state, or possibly repress mitotic arrest of newly formed radial glial cells (Humphrey et al 2008). An advantage of expressing dnHDAC2 over a knockdown approach is that the protein is still present and capable

of forming repressive complexes. Therefore, any phenotype observed is likely to be dependent on HDAC activity. It has previously been demonstrated that the corepressor complex, HDAC2/CoREST, is required for REST repression in neurons (Ballas et al 2001). An intriguing possibility is that a loss of HDAC2 activity is required for the generation of astrocytes, while the presence of an inactive form of HDAC2 is still capable of recruiting additional co-repressors to the REST complex. In this model, HDAC2 function is dependent on its ability to act as a scaffold protein for the recruitment of co-repressors to gene promoters and is not dependent on its deacetylase activity.

HDAC inhibition promotes neurogenesis in the cerebral cortex in a Bone Morphogenetic Protein-2 (BMP2)-dependent manner (Shaked et al 2008). TSA inhibition of HDACs induces BMP2 expression with a corresponding increase of cells that exit the ventricular zone (Shaked et al 2008). BMP expression is first detected in the telencephalon at E10.5, and is maintained throughout the period of cortical neurogenesis (Dewulf et al 1995). Application of BMP2 to radial glial cultures increases the number of cells expressing neuronal-specific markers (Li et al 2007), while retroviral transfection of radial-glia with a dominant-negative BMP type I receptor blocks neurite elaboration and cell migration out of the VZ (Li et al 1998). BMP signalling is further complicated by the observation that different concentrations of BMP2 have opposite effects on radial glial responses (Mabie et al 1999). Low BMP2 levels (1-10ng/ml) promote neuronal differentiation, whereas higher levels (100ng/ml) inhibit proliferation and promote apoptosis (Mabie et al 1999). BMP2 gradients appear to have an important function in cortical development. BMP signalling is dependent on expression of the BMP ligand, receptors, as well as BMP inhibitors such as noggin, any of which may be induced within the developing cortex

following nitrosylation of HDAC2. According with these observations, I found that *ex vivo* electroporation of HDAC2$^{C262A/C274A}$ into progenitor cells of the VZ impairs radial migration of neuronal precursors during development. This demonstrates that nitrosylation of HDAC2 has a dramatic affect on processes crucial for cortical development.

Previous observations indicate that histone acetylation plays an important role in the pathogenesis of several neurodegenerative disorders, including Huntington's, Parkinson's and Alzheimer's disease. The majority of studies that investigate HDACs as a therapeutic tool for neurodegenerative disorders have been focused on the treatment of polyglutamine disorders (Butler and Bates 2006). Disruption of neuronal function in polyglutamine disorders, such as Huntington's disease (HD), is associated with the formation of expanded-polyglutamine aggregates that sequester intracellular proteins important for multiple signalling pathways. It has now become apparent that transcriptional cofactors are included within these aggregates, perturbing histone modification and gene expression (Hughes 2002). Transcription factors and co-activators, including p53, CBP, P/CAF and TATA-binding proteins are sequestered within mutant Huntingtin (HTT) aggregates, resulting in global hypoacetylation of chromatin (McCampbell et al 2000; Steffan et al 2000; Nucifora et al 2001; Dunah et al 2002; van Roon-Mom et al 2002). Additionally, HDAC2 association to neuronal gene promoters further exacerbates the transcriptional dysregulation observed in HD (Zuccato et al 2001) (Zuccato et al 2003). In non-neuronal cells, REST associates with co-repressors Sin3A, HDAC1 and HDAC2 and is recruited to neuron restrictive silencing elements (NRSE), resulting in hypoacetylation and silencing of neuronal genes. In healthy neurons, wild-type HTT binds REST within the cytoplasm, preventing its association and targeting of repressive complexes to neuronal genes

(Zuccato et al 2003). However, mutant HTT is not capable of binding REST, resulting in downregulation of neuronal genes, such as BDNF (Zuccato et al 2001). HDAC inhibitors are therefore being considered as novel and promising therapeutic agents for HD.

The deacetylase activity of HDACs involves the removal of an acetyl group through a charge-relay system that consists of a histidine residue and two aspartate residues. The charge-relay system relies on a Zn^{2+} ion bound to the bottom of the active site pocket (Finnin et al 1999; Vannini et al 2004; Somoza et al 2004). The majority of HDAC inhibitors function by binding to the zinc ion and disrupting the charge-relay system. Residues essential for zinc binding and the charge relay system are highly conserved between class I and II HDACs, which greatly hinders the generation of HDAC-specific inhibitors. Another consideration in using HDAC inhibitors as a therapeutic strategy is that a significant number of HDAC substrates are non-histone proteins, whose regulation by HDACs may still be beneficial.

S-nitrosylation regulates HDAC2 localisation at gene promoters independent of its deacetylase activity and provides a novel approach for the design of HDAC inhibitors (Nott et al 2008). The nitrosylated cysteines of HDAC2 are conserved only among class I HDACs, which allows for the design of class-specific drugs. Furthermore, the location of both cysteine residues outside the highly conserved active site will permit the development of individual HDAC-specific inhibitors. A major advantage of targeting nitrosylated residues outside the active site is that HDAC function at specific gene promoters will be affected, without inhibiting global HDAC activity. Hypoacetylation observed in neurodegenerative diseases is due to a loss of HAT activity, while the total HDAC protein level remains comparably similar to healthy neurons (Saha

and Pahan 2006). Therefore, inhibition of HDAC localisation at gene promoters will reduce the acetylation level of chromatin-associated substrates, while non-histone targets will remain unaffected.

HDACs play an important role in regulating cell proliferation, differentiation and apoptosis. Therefore, it is not surprising that they have been a major focus for cancer therapeutics for the past 10 years (Ginsburg et al 1973; Altenburg et al 1976; Boffa et al 1978). Many cancer types exhibit enhanced expression of HDACs, with a corresponding decrease of antiproliferative genes, such as the cyclin-dependent kinase inhibitor p21 (Lagger et al 2002; Glozak and Seto 2007). HDAC2 is highly expressed in gastric carcinomas, colorectal carcinomas, cervical dysplasias and endometrial stromal sarcomas, along with reduced p21 expression (Huang et al 2005; Song et al 2005; Hrzenjak et al 2006). HDAC2 expression in intestinal mucosas is elevated by the loss of function of the tumour suppressor adenomatosis polyposis coli (APC) (Zhu et al 2004). Expression levels of p21 and other proapoptotic genes, such as *Bax*, can be restored in APC null mice by treatment with HDAC inhibitors (Myzak et al 2006). This correlates with increased acetylation of gene promoters and increased apoptosis. However, certain sporadic colorectal carcinomas express a mutant truncated form of HDAC2 that is resistant to current inhibitor treatments (Ropero et al 2006). Designing drugs that disrupt DNA HDAC2 interaction instead of targeting the active site may provide an alternative treatment strategy against mutant variants of HDAC2 that no longer respond to current inhibitors.

Our findings have implications that reach beyond the regulation of cortical development, with possible therapeutic implications for both neurodegenerative disorders and cancer. NO and

chromatin remodelling have been associated with various learning and memory paradigms (Susswein et al 2004). Exposure of mice to a novel enriched environment induces transcription of genes that are impaired in nNOS null mice (Riccio et al 2006). Notably, inhibition of HDAC activity in a neurodegenerative mouse model profoundly improves learning capabilities and retrieval of previously lost memory (Fischer et al 2007). HDAC inhibitors are already considered as a possible therapeutic strategy for improving memory loss associated with neurodegenerative disorders. The regulation of HDACs through NO signalling has unveiled an alternative and exciting avenue for the design of alternative therapeutic approaches.

Chapter 8 Experimental Procedure

8.1 Reagents

Unless otherwise stated, all biochemical reagents described were purchased from Sigma (UK) and all tissue culture reagents from Gibco (Invitrogen, UK). SNOC was always prepared fresh and used immediately by combining 220µl 220mM cysteine, 220µl 220mM sodium nitrate and 25µl 4M HCl (Mallis et al 2001). SNOC was incubate in the dark at room temperature for 10 minutes and then the reaction was stopped with 25µl 4M NaOH. SNOC has an extinction coefficient of $900M^{-1}A.cm^{-1}$ and absorption at 338nm.

8.2 Cell Cultures

Primary cultures were performed on E17.5 rat and E16.5 mouse embryos. Dissociated cultures were plated on Nunc plastic (Invitrogen, UK) coated with 1mg/ml PDL (Sigma, UK) and 1mg/ml Laminin (BD Bioscience, NJ) diluted 1:50 and 1:500 respectively. Brains were removed from the head, the meninges were separated and the cortices were isolated and stored in ice-cold HBSS medium (supplemented with 2.5mM Hepes pH 7.4, 35mM glucose, 1mM $CaCl_2$, 1mM $MgSO_4$, 4mM $NaHCO_3$). After completion of the dissection the HBSS medium was removed and an enzyme solution was added which contained 400U papain (Worthington, NJ) diluted in dissociation medium (81.75mM Na_2SO_4, 30mM K_2SO_4, 5.8mM $MgCl_2$, 250nM $CaCl_2$, 1mM Hepes pH 7.4, 20mM glucose, 0.001% phenol red). The cortices were digested at $37^{o}C$ for 25 minutes, followed by four washes with dissociation medium to remove the papain. The cortices

were pipette dissociated in MEM medium supplemented with 10% foetal bovine serum (FBS), 5% horse serum (HS) and 1mM glutamine and the cells were passed through a strainer. Mouse cultures were dissociated in Neurobasal medium supplemented with 1X B27 and 1mM glutamine. Cultures were plated at 15M cells per 10cm^2 plate for biochemistry, 2.5M per 6-well for the dual luciferase assay, 0.2M per 4-well for DAF imaging, or 0.3M per 4-well for the morphology experiments. Plates were incubated at 37°C, 5% CO_2 and starved overnight prior to experiments in 3% FBS serum and 50μM AP5.

HEK239T cells and PC12 cells were maintained in DMEM supplemented with 10% FBS, and 10% FBS plus 5% HS, respectively. Before experiments, PC12 cells were starved in medium containing 0.5% HS overnight.

8.3 nNOS mouse line

nNOS null mice (Huang et al 1993) or wild-type controls (C57 Black/6) were interbred to produce nNOS null and wild-type litters, respectively. Embryos were taken at E16.5 for dissociated cortical culture.

8.4 Constructs and Transfections

The pME18S-HDAC2 (FLAG-tagged) construct was kindly provided by Dr E Seto (Laherty et al 1997). Cysteine mutants were obtained using the Quickchange® II site-directed mutagenesis kit (Stratagene, UK) according to the manufacturers instructions. HDAC2

truncations were cloned into a pCMV-Myc plasmid (Clontech) using primers that contained 5'SalI and 3'NotI restriction sites. HDAC2 was cloned into a modified pCIG2-IRES-eGFP construct containing an expression cassette under the control of a CMV enhancer and a chicken β-actin promoter using primers that contained 5'EcoRI and 3'XmaI restriction sites (Hand et al 2005).

All transfections were performed using Lipofectamine® 2000 (Invitrogen, UK) as follows, unless otherwise stated. The plating medium was removed and replaced by OptiMEM that contained Lipofectamine 2000 (diluted 1:65) plus 1.5μg/ml of DNA. Medium containing the transfection reagent and DNA was incubated at room temperature for 20 minutes before applying to the cells. Transfections were left at 37°C, 10% for 4 hours before replacing the transfection medium by the original serum-containing medium.

8.5 DAF Imaging

Imaging was performed on E17 rat cortical neurons at 4 days *in vitro*. Neurons were starved for 16 hours in medium that contained 3% serum and AP5 (50μM) prior to experiments. Neurons were loaded with DAF-FM DA dye by replacing medium with DMEM (containing no phenol) plus 1mM glutamine, 50μM APV and 10μM DAF at 37°C, 5% CO_2 for 30 minutes. Cells were washed three times in DMEM medium and incubated at 37°C, 5% CO_2 for 15 minutes. This allowed deacetylation of DAF-FM DA, which produced the cell impermeable and weakly fluorescent DAF-FM. Neurons were treated with BDNF (100ng/ml), NPA (300μM), NPA plus BDNF or vehicle (Ctrl) for 30 minutes. NO released within neurons reacted with

DAF-FM to produce a fluorescent benzotriazole derivative, with excitation/emission maxima of 495/515nm. After treatment, neurons were left in medium which contained 2.67ng/ml Cell Tracker Red CMTPX (Invitrogen, UK) for 30 minutes to allow visualisation of cell morphology. Cells were washed in PBS and fixed in 4% PFA for 15 minutes before mounting. Imaging was performed on a Leica DM 2500 confocal microscope using an ACS APO 40x/1.15 oil objective lens within 48 hours of fixation.

8.6 Biotin-switch Assay

The following is based on a previously described protocol with minor modifications (Jaffrey and Snyder 2001; Forrester et al 2007). All the following steps were performed in the dark. Cells were washed in ice cold PBS and lysed in HEN buffer [250mM HEPES, 1mM EDTA, and 100mM neocuproine). Nuclei were disrupted using a hand-held homogeniser for 15 seconds. Cell debris was pelleted at 750xg for 10 minutes and the resultant lysate adjusted to 0.4% CHAPS. Reduced cysteine thiol groups were blocked using 3 volumes of blocking buffer (HEN buffer plus 2.5% SDS and 200mM Methyl methanethiosulphonate) at 50°C for 1 hour. The blocking buffer was removed by acetone precipitation. Two volumes of -20°C acetone was added to the samples and left at -20°C for 20 minutes to precipitate the protein. The samples were spun at 2000xg for 10 minutes, washed with 1ml -20°C acetone and spun for a second time. Precipitated protein was resuspended in 100μl HENS buffer (HEN buffer plus 1% SDS). Nitrosylated cysteine residues were reduced to free thiol groups using fresh 100mM ascorbic acid. The newly exposed cysteine thiol groups were labelled using 1mM biotin-HPDP at room temperature for 1 hour. Proteins were then separated by SDS-PAGE electrophoresis and detected

by western blotting using a streptavidin-HRP antibody. Alternatively, biotinylated protein were first isolated by immunoprecipitation. Excess biotin-HPDP was removed by a second acetone precipitation and proteins were resuspended in 100μl HENS buffer. Two volumes of neutralization buffer (20mM HEPES, 100mM NaCl, 1mM EDTA, 100mM β–mercaptoethanol [β–ME]) were added with 50μl streptavidin-agarose beads and rotated at room temperature for 1 hour. Beads were washed five times with 1ml high salt neutralisation buffer (neutralisation buffer plus 600mM NaCl). Biotinylated proteins were eluted using 50μl Elution buffer (20mM HEPES, 100mM NaCl, 1mM EDTA, 100mM β–ME) and boiled in SDS-loading buffer for 10 minutes at 96°C.

8.7 Histone deacetylase Activity Assay

The HDAC activity assay required the presence of HDACs to deacetylate the non-fluorogenic peptide, BOC-(acetyl)lys-AMC (Bachem, UK), which could subsequently be digested by trypsin to produce a fluorescent moiety (Wegener et al 2003). Cells (60mm plates) were washed with PBS and lysed in 500μl Low Salt IP Buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 5mM EDTA, 0.5% NP40). Cells were homogenised for 10 seconds and debris spun down at 20,000xg for 15 seconds. HDAC2 was immunoprecipitated using 5μg antibody (anti-FLAG-M2 mouse monoclonal, Sigma; anti-HDAC2 rabbit polyclonal, Santa Cruz Biotechnology) at 4°C for 2 hours. Protein A sepharose beads (50μl) were added and rotated for 1 hour at 4°C. The beads were washed and centrifuged three times using Low Salt IP Buffer at 6100xg. The beads were washed twice in HDAC buffer (25mM Tris-HCl pH8.0, 137mM NaCl, 2.7mM KCl, 1mM MgCl$_2$) to remove NP40 detergent. After the final wash, 60ul HDAC buffer containing 100μM

HDAC peptide was added and agitated vigorously at 37$^\text{o}$C for 1 hour. The HDAC assay was stopped using 1μM TSA (Tocris Bioscience, UK) and the peptide digested using 5mg/ml trypsin at room temperature for 15 minutes. The excitation/emission maxima was measured at 355/460nm on a SpectraMax Gemini plate reader (Molecular Devices, USA).

8.8 Chromatin ImmunoPrecipitation

Approximately 3.5x10^6 cells were used per ChIP. Cells were treated and then the medium was replaced with PBS containing 1% formaldehyde. Cells were rinsed twice in PBS, and harvested in collection buffer (100mM Tris-HCl pH9.4 and 1mM dithiothreitol). Cells were pelleted, washed in PBS and the pellet resuspended in lysis buffer (0.1% SDS, 0.5% Triton X-100, 20mM Tris-HCl, pH8.1 and 150mM NaCl). Samples were sonicated on ice with a 20 sec pulse followed by a 10 sec interpulse interval, repeated six times. Cell debris was removed by centrifugation and the supernatant pre-cleared using Protein A Sepharose beads (Amersham Biosciences) for 1 h at 4$^\text{o}$C. Beads were pelleted by centrifugation and the supernatant was retained for immunoprecipitation. A fraction of the supernatant was collected for pre-immunoprecipitation total input. The volume of each sample was adjusted to 500μl using lysis buffer, and immunoprecipitation was performed overnight at 4$^\text{o}$C using 5-10μg of rabbit polyclonal antibody. The following antibodies were used: anti-HDAC2 (Santa Cruz), anti-acetyl Histone H3 K9/K14 (Upstate), anti-acetyl Histone H4 (Upstate), control IgG (Santa Cruz) and anti-Flag M2 monoclonal antibody (Sigma). Immune complexes were precipitated by incubating with Protein A Sepharose beads for 1 h at 4$^\text{o}$C. Beads were collected and subjected to a series of seven sequential washes at 4$^\text{o}$C as follows: 1x lysis buffer 10 min, 1x lysis buffer 5 min, 1x wash

buffer (0.1% SDS, 0.5% Triton X-100, 2mM EDTA pH 8.0, 20mM Tris-HCl pH8.1, 150mM NaCl) 10 min, 1x wash buffer 5 min, 1x LiCl buffer (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA pH 8.0, 10mM Tris-HCl pH 8.1) 5 min, 1x TE buffer (10mM Tris-base pH 8.1, 1mM EDTA pH8.0) 30 min, 1x TE buffer 5 min. Immune complexes were eluted from beads by vortexing in elution buffer (1% SDS and 0.1M NaHCO$_3$ pH 8.0). DNA-protein cross-linking was reversed by adding NaCl at a final concentration of 0.33M and incubated at 65°C overnight. DNA fragments were purified using the QIAquick PCR purification kit (Qiagen). For PCR, specific sets of primers were designed that flank the CRE element within the upstream regulatory regions of *Fos*, *Egr1*, *Vgf* and *Nos1*. PCR conditions and cycle numbers were determined empirically for the different templates and primer sets. Primers amplified fragments ranging in size from 200-400bp.

8.9 Quantitative real time PCR

PCR reactions (25μl) contained 12.5μl of PCR Sybr Green mix (NEB) and 0.3μM primers. All reactions were performed in duplicate with an Opticon 2 system (MJ Research) and each experiment included a standard curve, a preimmune control and NO template control. Standard templates consisted of gel-purified PCR products of the *Fos*, *Egr1*, *Vgf* and *Nos1* amplicons of known concentration, and each standard curve consisted of seven serial dilutions of the template. At the end of the 46 cycles of amplification, a dissociation curve was performed in which Sybr Green was measured at 1°C intervals between 50°C and 100°C. Melting templates for *Fos*, *Egr1*, *Vgf*, and *Nos1* were 83°C, 88°C, 85°C, respectively. Results were normalised using total input DNA and expressed as fold changes over the unstimulated control.

8.10 Dual Luciferase Reporter Assay

E17 rat cortical neurons were cultured on 6-well plates at 250,000 cells/well and transfected after four days *in vitro*. Neurons were transfected with 2μg HDAC2 vector, 2μg c-fos-luciferase (firefly) and 1μg TK-luciferase (*Renilla*) in 1ml media containing 10μl lipofectamine (Invitrogen, UK). Forty-eight hours after transfection, cells were treated with BDNF (75ng/μl), SNOC (200μM), NPA (300μM) or NPA plus BDNF for 6 hours. Plates were then washed twice with ice cold PBS and processed using the Dual Luciferase® Reporter Assay System (Promega, UK) according to the manufacturers recommended protocol.

8.11 Dendritic Growth Analysis

E17 rat cortical cultures were plated on 4-well plates containing No.1.5 13mm coverslips at 300,000 cells/well. Transfection was performed 5 hours post-plating, allowing time for neurons to attach but before the majority had established projections. Plates were left in the transfection medium for 4 hours before performing a complete medium change. Neurons were either treated with KCl_2 (50mM, 30 mins) or left unstimulated. Coverslips were fixed for 48 hours post-transfection using 4% PFA. Cells were visualised using an Axioplan2 microscope (Zeiss, Germany) with a 'Plan-Neofluar' 25x/0,80|mm Corr. DIC Objective (Zeiss) and FS10 (GFP) filter set (Zeiss). Dendritic growth was analysesd using OpenLab 5.5 (Improvision.com) as previously described (Redmond et al 2000; Redmond et al 2002).

8.12 *Ex Vivo* Electroporation

The following *ex vivo* protocol was developed by Dr Franck Polleux (Polleux and Ghosh 2002). Electroporation was performed using E14.5 mouse embryos. pCIG2-HDAC2-IRES-eGFP constructs (1μg/ml) were microinjected into the lateral ventricles in 0.05% Fast Green Dye using a PLI-100 Pico-injector (Harvard Apparatus, MA) set at pulse length 2ms, P_{INJECT} 0.3, $P_{BALANCE}$ 0.1. Mouse brains were electroporated using ECM 830 Electro-Square-Porator (Harvard Apparatus, MA) with gold plated Genepaddles (Fisher scientific, UK) using three unipolar pulses set to 30V (100ms interval and pulse length). Electroporated brains were immediately transferred to ice cold cHBSS medium (1x HBSS, 2.5M Hepes pH7.4, 30mM D-glucose, 1mM $CaCl_2$, 1mM $MgSO_4$ and 4mM $NaHCO_3$). Electroporated brains were embedded in 7% LMP agarose dissolved in cHBSS medium using Tissue Embedding Molds (Polysciences, PA). Brain slices were obtained using a Vibratome 1000 Plus (Harvard Apparatus, MA) set to 250μM. Slices were cultured on semi-permeable organotypic membranes in slice culture medium (70% MEM, 30% cHBSS, 20mM D-glucose, 1mM L-glutamine, 100units/ml penicillin and 0.1mg/ml streptomycin) at 37°C, 5% CO_2 for up to 5 days *in vitro*. Slices were fixed in 4% PFA overnight, and immuno-stained using Nestin (Santa Cruz) and GFP (Abcam) antibodies. Slices were visualised on a Leica DMI 6000 confocal microscope using a HC PL APO 20x/0.7 Corr. objective lens.

8.13 Statistical Analysis

Statistical analysis was performed using Prism 4 for Mac software. Data for the DAF experiment and HDAC2 Activity Assay (Fig 4.1) were analysed using one-way ANOVA followed by comparison using unpaired T-test. All other quantitative data was analysed using two-way ANOVA.

Chapter 9 References

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