Expression and function of *Npas4* during early development

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Declaration

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Abbreviations

°C	degrees Celsius
µg	microgram
µL	microlitre
6-OHDA	6-hydroxydopamine
ACTH	adrenocorticotropic hormone
ADHD	attention deficit hyperactivity disorder
aha-1	aryl hydrocarbon receptor associated protein
Ahr	aryl hydrocarbon receptor
Akt	protein kinase B
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA receptor
ANOVA	analysis of variance
Arc	activity-regulated cytoskeleton-associated protein
Arnt	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
βIII tubulin	Class III β-tubulin
Bax	BCL-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bdnf	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
Bmal	brain and muscle ARNT-like
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CA	*cornu Ammonis*
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
CAG	CMV/chicken β-actin hybrid
CaMKIV	Ca^{2+}/calmodulin-dependent protein kinase type IV
cAMP	cyclic AMP
$CaPO_4$	calcium phosphate
Cbp	Creb binding protein
CCG	Clock controlled gene
cDNA	complementary DNA
Chem-LTD	chemically induced LTD
Chem-LTP	chemically induced LTP
ChIP	chromatin immunoprecipitation
ChIP-Seq	ChIP followed by sequencing
Clock	circadian locomoter output kaput
cm^2	centimetres squared
CME	central midline element

CMV	cytomegalovirus
CNS	central nervous system
cNXFL	*C. elegans* NXF-like-factor
CO_2	carbon dioxide
COS7	monkey kidney cell line
CRE	cAMP response element
Creb	cAMP response element‑binding protein
CRF	corticotropin-releasing factor
CRY	cryptochrome
CSD	cortical spreading depression
CSF	cerebrospinal fluid
C-terminal	carboxy-terminal
C-terminus	carboxy-terminus
D. Melanogaster	*Drosophila melanogaster*
DAPI	4,6-Diamidino-2-phenylindole dihydrochloride
DG	dentate gyrus
DIG	digoxigenin
DIG-11-UTP	DIG-11-uridine-5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dNXFL	*Drosophila* NXF-like-factor
DRE	downstream regulatory element
DREAM	DRE-antagonist modulator
Drebrin	developmentally regulated brain protein
DSCR	Down's syndrome critical region
DTT	dithiothreitol
dUTP	deoxyuracil triphosphate
Dys	dysfusion
E	embryonic day
E. coli	*Escherichia coli*
EAA	Excitatory amino acid
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor-1 alpha
eGFP	enhanced green fluorescent protein
Egr1	early growth response protein 1
EPL	early primitive ectoderm-like
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Ern1	ER-nucleus signalling 1 protein
eRNA	enhancer RNA
ES cell	embryonic stem cell
ETS	E-twenty six
eYFP	enhanced yellow fluorescent protein
FBS	foetal bovine serum

FGF	fibroblast growth factor
Fox-3	feminizing locus on X 3
FTD	frontotemporal dementia
FTDP-17	FTD and parkinsonism linked to chromosome 17
g	the earth's gravitational acceleration
GABA	γ-aminobutyric acid
$GABA_A$-γ2	$GABA_A$-receptor γ2 subunit
GABAR	GABA receptor
GAD65	glutamic acid decarboxylase
GCL	granule cell layer
gDNA	genomic DNA
Gfap	glial fibrillary acidic protein
GFP	green fluorescent protein
GluR	glutamate receptor
Gpx2	glutathione peroxidase 2
Grp78	glucose-regulated protein 78
H^+	hydrogen
HeBS	HEPES-buffered saline
HEK	human embryonic kidney cell line
HeLa	human cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hES cell	human embryonic stem cell
Hif	hypoxia inducible factor
HMG	high mobility group
HPA	hypothalamic-pituitary-adrenocortical
hPGK	human phosphoglycerate kinase
hr	hour
HRE	hypoxic response element
HSP	heat-shock protein
IEG	immediate-early gene
iGluR	ionotropic glutamate receptor
IP_3	inositol 1,4,5-trisphosphate
IRES	internal ribosome entry site
ISH	*in situ* hybridisation
K^+	potassium
KAR	kainate receptor
kb	kilobase
KCl	potassium chloride
KCN	potassium cyanide
KD	knock-down
kDa	kilodalton
LB	lysogeny broth
LE-PAS	limbic-enriched PAS
LIF	leukaemia inhibitory factor
LTD	long-term depression

LTP	long-term potentiation
LTR	long terminal repeat
M	molar
MAPK	mitogen-activated protein kinase
MAPT	microtubule-associated protein tau
MEK1	MAPK kinase 1
mEPSC	miniature excitatory postsynaptic current
mES cell	mouse embryonic stem cell
mGluR	metabotropic glutamate receptor
min	minutes
mIPSC	miniature inhibitory postsynaptic current
miRNA	microRNA
mL	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
MOP4	member of PAS superfamily 4
mRNA	messenger RNA
MW	molecular weight
MyoD1	myogenic differentiation 1
n	number of independent experiments
Na^+	sodium
NADE	neuronal activity-dependent enhancer
NeuroD1	neurogenic differentiation 1
NFT	neurofibrillary tangle
ng	nanograms
Ngf	nerve growth factor
NHE9	N^+/H^+ Exchanger 9
NLS	nuclear localisation sequence
nM	nanomolar
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
Npas	neuronal PAS domain protein
NPC	neural progenitor cell
NT3	neurotrophin-3
NT4	neurotrophin-4
N-terminal	amino-terminal
N-terminus	amino-terminus
$O2^-$	superoxide
Oct-4	octamer-binding transcription factor 4
$p75^{NTR}$	p75 neurotrophin receptor
Pac	puromycin N-acetyl-transferase
PAN	primitive anterior neuroectoderm
PAS	Per-ARNT-Sim
Pax6	paired box gene 6

PBS	phosphate buffered saline
PBST	PBS Tween solution
PC12	rat pheochromocytoma cell line
PCR	polymerase chain reaction
PD	Parkinson's disease
Per	period
PFA	paraformaldehyde
pg	picograms
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
pmol	picomoles
PTZ	pentylenetetrazol
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative RT-PCR
REST	RE1- silencing transcription factor
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	revolutions per minute
RRE	Rev response element
RT	reverse transcription
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR
s	seconds
SCN	suprachiasmatic nuclei
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SGZ	subgranular zone
shRNA	small hairpin RNA
Sim	single-minded
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
Sox	SRY-related high mobility group box
SSC	saline sodium citrate
SVZ	subventricular zone
TAD	transactivation domain
TEMED	N,N,N,N-tetramethylethylenediamine
Tgo	Tango
$TgTau^{P301L}$	Tau transgenic mice
Trh	trachealess
Trk	tropomysin-related kinase
TS	Tourette syndrome

TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
U	unit
UTR	untranslated region
UV	ultra violet
V	volts
(v/v)	volume/volume
VZ	ventricular zone
(w/v)	weight/volume
XBP-1	X-box binding protein 1
XRE	xenobiotic response element

Notes

1. The literature review is current as of October 2011.
2. The gene nomenclature used throughout this thesis is as follows;

   Human: human gene symbols, transcripts and coding sequences are represented by uppercase italicised text (i.e. *NPAS4*) while proteins are represented by uppercase non-italicised text (i.e. NPAS4).

   Rodent: rodent gene symbols, transcripts and coding sequences are represented by an initial uppercase letter and are italicised (i.e. *Npas4*) while proteins are not italicised (i.e. Npas4).

   Drosophila: *Drosophila* gene symbols, transcripts and coding sequences are represented by all lowercase italicised text (i.e. *dys*) while proteins are not italicised and use an initial capital letter (i.e. Dys).

   Nematode: nematode gene symbols, transcripts and coding sequences are represented by all lowercase italicised letters (i.e. *aha-1*) while proteins are not italicised (i.e. aha-1).

Abstract

Npas4 is an activity-dependent bHLH PAS transcription factor expressed within neurons of the mammalian central nervous system where it regulates the expression of several genes that are important for neuronal survival and synaptic plasticity. In the adult brain, Npas4 plays an important role in several key aspects of neurobiology including inhibitory synapse formation, neuroprotection and memory formation. Consequently, abnormal *Npas4* expression has been implicated in a number of neurological disorders such as autism, Down's syndrome, epilepsy, cerebral ischaemia and Alzheimer's disease. While the expression and function of *Npas4* are beginning to be elucidated in the adult brain, to date little is known regarding the role of *Npas4* during neuro-development. The aim of this study was to investigate the expression and function of *Npas4* during early development.

The expression of *Npas4* during early neuro-development was investigated using several different developmental model systems. *Npas4* was found to be transiently up-regulated during neural differentiation of both mouse and human embryonic stem (ES) cells at a stage of differentiation that is marked by an increase in neural progenitor cell (NPC) proliferation. This was corroborated by analysis of *Npas4* expression in the developing mouse embryo where *Npas4* mRNA was found to be expressed between embryonic day 7.5 and 9.5. The function of *Npas4* in the context of development was investigated by using RNA interference to decrease endogenous Npas4 expression in mouse ES cells undergoing neural differentiation. *Npas4*-specific small hairpin RNA expression constructs were delivered to mouse ES cells using lentiviral transduction to create two independent Npas4 knock-down mouse ES cell lines. An Empty vector control line was also generated by transducing mouse ES cells with a construct that does not produce small hairpin RNAs. When the cell lines were assessed for their ability to undergo neural differentiation, it was found that aspects of NPC identity and neuronal maturation were affected by a reduction in Npas4 expression. The percentage of cells expressing the neuroectoderm marker Sox1 was significantly diminished in Npas4 knock-down cultures while expression of Nestin was not affected. In addition, neurite sprouting defects were also observed at a later stage of differentiation in cultures having a more severe reduction in Npas4 expression. These data suggest that *Npas4* acts upstream of *Sox1* during neural differentiation and is involved in aspects of NPC maintenance and neuritogenesis. When taken together, the data presented in this thesis provide the first evidence that *Npas4* is expressed developmentally by a population of early NPCs and that it may have a developmental role that is unrelated to its function in the adult brain.

1 - Introduction

1.1 Overview

Npas4 is an activity-dependent transcription factor expressed within neurons of the mammalian central nervous system (CNS) where it regulates the expression of several genes that are important for neuronal survival and synaptic plasticity. Npas4 plays an important role in several key aspects of neurobiology including inhibitory synapse formation, neuroprotection and memory formation, and, consequently, abnormal expression of *Npas4* has been implicated in a number of neurological disorders such as autism, Down's syndrome, epilepsy, cerebral ischaemia and Alzheimer's disease. While the expression and function of *Npas4* have, to some extent, been characterised in the adult brain, to date very little research been has focussed on the role of *Npas4* during development. The aim of this thesis is to investigate the expression and function of *Npas4* during mammalian development.

This opening chapter will begin with a brief introduction to Npas4 and the family of related transcription factors to which it belongs. This will be followed by a review of what is known about the expression of *Npas4* in both the adult organism and the developing embryo. Next, the regulation of *Npas4* expression in response to various stimuli will be explored in detail. Following this, the function of Npas4 in the mature brain will be reviewed together with an exploration of how perturbation of this function may relate to various neurological disorders. To gain an evolutionary perspective, attention will then turn to homologs of Npas4 in other species after which the other *Npas* genes will also be briefly discussed. Finally, the aims of this thesis will be outlined including a summary of the rationale behind the project.

1.2 An introduction to *Npas4*

1.2.1 Npas4 is a member of the bHLH PAS transcription factor family

1.2.1.1 Transcription factors

Transcription factors are sequence-specific deoxyribonucleic acid (DNA) binding proteins that, either alone or in conjunction with other proteins, regulate the expression of specific genes by controlling the amount of messenger ribonucleic acid (mRNA) that is transcribed from those loci. Transcription factors can either positively or negatively regulate expression of target genes; transcription factors

that promote transcription are called activators, while transcription factors that inhibit mRNA synthesis are called repressors. Transcription factors are often classified based on their amino acid sequence similarity and hence the tertiary structure of their DNA-binding domains. For example, one superfamily of transcription factors are the basic helix-loop-helix (bHLH) proteins which are characterised by a DNA-binding motif comprised of two α-helices connected by a loop. Proteins of this family are involved in fundamental processes such as cell growth and division, differentiation and development of body systems in the embryo (Dambly-Chaudiere and Vervoort 1998). Well known members of this super family include Myc, myogenic differentiation 1 (MyoD1) and neurogenic differentiation 1 (NeuroD1).

1.2.1.2 The bHLH PAS transcription factor family

A subset of bHLH proteins also contain a dimerisation motif known as a PAS homology domain, named after the first three proteins in which this domain was characterised; period (Per), aryl hydrocarbon receptor nuclear translocator 1 (Arnt) and single-minded (Sim). Members of this subfamily are termed bHLH PAS transcription factors and these proteins regulate processes such as circadian rhythm, embryonic development and cellular response to environmental stresses. A summary of known mouse bHLH PAS transcription factors and their functions is listed in Table 1.1. As these proteins perform functions that are often crucial to the survival of the organism, they are generally well conserved throughout evolution and can be found from nematodes to mammals. Included among the bHLH PAS proteins are; the hypoxia inducible factors (Hifs), which promote angiogenesis and stimulate red blood cell production in response to hypoxia, the circadian locomotor output cycles kaput (Clock) proteins, which regulate circadian rhythm, and the aryl hydrocarbon receptor (Ahr), which is activated by environmental pollutants and is responsible for initiating metabolism of xenobiotic compounds (Kewley *et al.* 2004). A phylogenetic tree showing the evolutionary relationship between the murine bHLH PAS factors is shown in Figure 1.1.

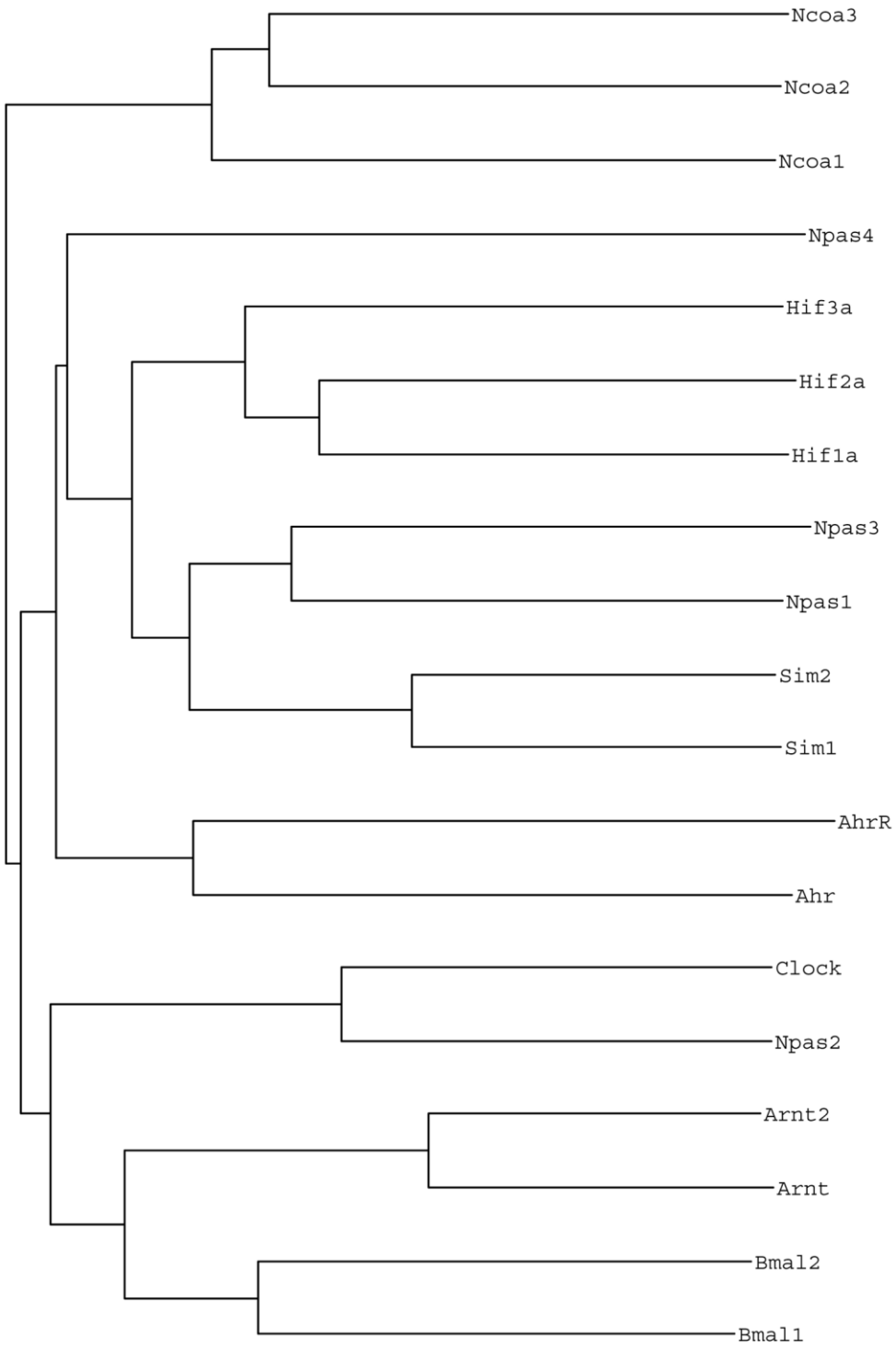


Figure 1.1 – Phylogenetic tree of mouse bHLH PAS transcription factors
The phylogenetic relationships among the mouse bHLH PAS transcription factors were calculated using the Clustal Omega algorithm (http://www.clustal.org/omega/) and a phylogenetic tree constructed using the neighbour joining method. Amino acid sequences can be found in Appendix 1. For a description of each protein, see Table 1.1.

Gene	Synonyms	Class	Development			Adult	
			Expression	Null mutant phenotype	Heterozygote phenotype	Expression	Function
Ahr	Dioxin receptor	I	Neuroepithelium, branchial arches, thymus, heart, lung, somites, kidney, liver,retina, adrenal, ectoderm, bladder, bone and muscle	Decreased body weight Small liver Cardiac hypertrophy Decreased resistance to bacterial infection	Indistinguishable from wildtype	Thymus, lung, brain, liver, kidney (heart and spleen)	Xenobiotic metabolism
Ahrr	Kiaa1234	I	-	Delayed response to skin carcinogenesis	Indistinguishable from wildtype	Ubiquitous Brain, heart, kidney, testis	Xenobiotic metabolism (supresses Ahr function)
Arnt	Hif1β	II	Widely expressed (E9+)	Embryonic lethal at E10.5 (defective vascularisation, placental, hematopoiesis, neural tube defects)	-	Ubiquitous	General partner factor
Arnt2	-	II	Widely expressed (E9+)	Perinatal death (hypothalamic hypoplasia)	Indistinguishable from wildtype	CNS, kidney	General partner factor
Bmal1	Arnt3, Arntl, MOP3	II	Yes (E10+)	Delayed embryonic/reproductive development Decreased body mass Infertility in females Premature ageing Ankylosis Loss of circadian rhythmicity in darkness	Increased postnatal mortality	Brain, skeletal muscle	Partner factor Circadian rhythm
Bmal2	Arntl2, MOP9	II	-	-	-	Brain	Partner factor Circadian rhythm
Clock	-	I	Yes (E10+)	Lengthened circadian period in constant darkness Altered circadian response to light Disrupted eating rhythm - obesity	Lengthened circadian period in constant darkness	Brain, liver, kidney	Circadian rhythm
Hif1α	MOP1	I	Brain, heart, kidney, primitive gut, retina, bladder, testis	Embryonic lethal at E10 (cardiovascular malformations, impaired vascularisation, neural tube defects)	Impaired response to chronic hypoxia Kidney more susceptible to ischaemic injury	Ubiquitous	Hypoxic response
Hif2α	EPAS1, MOP2	I	Blood vessels, bronchial epithelium, choroid plexus, kidney, lung, urogenital ridge, brown fat, lens, retina	Mid-gestational death at E12.5-16.5 (bradycardia)	Kidney more susceptible to ischaemic injury	Lung, heart, kidney, liver, brain, skeletal muscle, testis, spleen	Hypoxic response
Hif3α	MOP7, NEPAS[#], IPAS[#]	I	Heart, lung, brain, liver, kidney	Impaired heart and lung development	-	Thymus, lung, brain, heart, kidney, liver	Hypoxic response (supresses Hif1α function)

Gene	Synonyms	Class	Development			Adult	
			Expression	Null mutant phenotype	Heterozygote phenotype	Expression	Function
Ncoa1	SRC-1, Nrc1	I	Neuroepithelium, cartilage	Partial hormone resistance Impaired development of reproductive organs Prone to obesity Osteopenia	Indistinguishable from wildtype	Brain, adipose tissue, lung, heart, liver, testis	Modulates energy metabolism
Ncoa2	SRC-2, Nrc2, GRIP1, TIF2	I	-	Partial hormone resistance Reduced fertility Protected from obesity	Short stature, developmentally delayed	Adipose tissue, lung, heart, liver, testis	Modulates energy metabolism Fertility
Ncoa3	SRC-3, Nrc3, AIB1, TRAM-1, RAC3, p/CIP, ACTR	I	-	Dwarfism Delayed puberty Reduced female reproductive function Impaired mammary gland development	Slightly shorter stature (approximately 10%)	Hippocampus, oocytes, mammary glands, hepatocytes, smooth muscle, vaginal epithelium, lung(?), heart, liver(?), testis, placenta, pancreas	Involved in growth hormone regulatory pathway
Npas1	MOP5	I	CNS	-	-	Brain, spinal cord	Lung development
Npas2	Clock2, MOP4	I	Not detected	Shortened circadian period in constant darkness Altered circadian response to light Disrupted sleeping rhythm Impaired long-term memory	Indistinguishable from wildtype	Brain, liver, heart, stomach, spleen, kideny, testis	Circadian rhythm - controls food entrainable oscillator
Npas3	MOP6	I	CNS, lung, neuroepithelium, neural tube, developing cardiac valves, limbs, kidneys	Post-natal death (lung failure) Growth retarded Memory deficits Impaired hippocampal neurogenesis	Emphysema Airway hyperactivity	Brain	Lung development Regulates FGF signalling
Npas4	Nxf, LE-PAS, PASD10, MOP22	I	?	Shortened lifespan Anxious, hyperactive Prone to seizure	Mild reduction in lifespan	Brain, testis	Inhibitory synapse formation Neuroprotection Fear memory
Sim1	-	I	Mesencephalon, neural tube, kidney, foregut, muscle, somites	Perinatal death (hypothalamic hypoplasia)	Early onset obesity	Kidney, skeletal muscle	Hypothalamus development Weight regulation
Sim2	-	I	Diencephalon, branchial arches, rib primordia, kidney, muscle, lung, vertebrate	Perinatal death (lung failure) Scoliosis, rib protrusions Craniofacial abnormalities	Scoliosis, rib protrusions	Kidney, skeletal muscle, lung, brain	Up-regulated in aggressive cancers (prostate, colon, pancreas, glioblastoma)

Table 1.1 – The mouse bHLH PAS transcription factor family

The expression and function of all known mouse bHLH PAS transcription factors is summarised. #Names of splice variants.

The bHLH PAS factors are not active individually but function as dimers and thus they can be grouped according to their partnering behaviour (see Table 1.1). Class I proteins do not heterodimerise with each other, nor do they form homodimers, and they include the Ahr, the Hifs and the Sim proteins (Kewley *et al.* 2004). Class II proteins, which are known as the general partner factors, promiscuously form heterodimers with Class I factors and are also able to form homodimers. The most common Class II partner is Arnt which is ubiquitously expressed, though other Class II proteins include the tissue-restricted Arnt2 and the circadian rhythm proteins brain and muscle Arnt-like 1 (Bmal1) and Bmal2. In general, a functional bHLH PAS transcriptional complex is formed when a Class I factor dimerises with its respective partner protein from Class II.

By definition, members of the bHLH PAS family contain several highly conserved domains and thus they also share a common domain organisation. The amino-terminal (N-terminal) half of these proteins contains a bHLH domain which is used in dimerisation with other bHLH PAS proteins and also forms part of the DNA-binding interface of the resulting dimer (Fairman *et al.* 1997). Adjacent to the bHLH domain is the PAS homology domain consisting of two repeats, called PAS A and PAS B, which are separated by a spacer region of variable length. The multifunctional PAS domains are known as 'sensing domains' and are found in many proteins where they respond to various stimuli such as changes in light, oxygen tension or redox state (Taylor and Zhulin 1999). In bHLH PAS factors, PAS domains also regulate partner protein choice by conferring binding specificity for particular proteins, though in some bHLH PAS factors they also contribute to DNA binding (Chapman-Smith and Whitelaw 2006).

1.2.1.3 The newest member of the bHLH PAS family: Npas4

Neuronal PAS domain protein 4, or Npas4, is a member of the bHLH PAS family of regulatory proteins (Flood *et al.* 2004; Moser *et al.* 2004; Ooe *et al.* 2004). It is so called because of its expression pattern, which is restricted primarily to neurons of the brain, and because it contains a conserved PAS domain, however it also has a number of other synonyms including Nxf, Limbic-enriched PAS (LE-PAS) and PASD10. The overall amino acid sequence of the mouse Npas4 protein has a low degree of homology to other proteins, even within the bHLH PAS family. Though Npas4 is the fourth neuronal PAS protein to be discovered, it should be noted that this nomenclature was established solely based on initial observations of the expression pattern of these proteins rather than any functional or evolutionary relatedness (see Section 1.9). Indeed, the murine protein having the highest degree of homology to

mouse Npas4 is Sim2, with which it shares 24% overall identity, though most of this homology is found within the highly conserved bHLH and PAS domains (Ooe *et al.* 2004).

1.2.2 Genomic organisation of the mouse *Npas4* gene

In the mouse genome, the *Npas4* locus is located on the reverse strand of chromosome 19A and spans a region of approximately 5.6 kilobases (kb). The human *NPAS4* gene is located on chromosome 11q13 (Ooe *et al.* 2004). The total length of the primary mouse *Npas4* transcript is approximately 3,274 base pairs (bp) and it consists of a 2,406bp coding region which is flanked by untranslated regions (UTRs) of 155bp at the 5' end and 713bp at the 3' end (Figure 1.2A). The open reading frame is divided into eight exons and encodes a protein of 802 amino acids. The predicted molecular mass of the mouse Npas4 protein is 83 kilodalton (kDa), though *in vitro* transcription and translation of a complementary DNA (cDNA) sequence coding for the full-length mouse Npas4 protein produced a protein of approximately 110kDa (Moser *et al.* 2004).

1.2.3 Domain structure and sequence conservation within the Npas4 protein

Like other bHLH PAS transcription factors, Npas4 is separated into two functional units; the N-terminal half which controls dimerisation and DNA binding, and the carboxy-terminal (C-terminal) half which controls transcriptional regulation (Figure 1.2B). In the mouse Npas4 protein, the bHLH domain is located between amino acids 10-52 and is highly conserved (79% identity) to the consensus domain sequence (Flood *et al.* 2004). Likewise, both of the PAS repeats, PAS A at residues 72-135 and PAS B at residues 216-273, are also highly conserved (93% and 87% identity, respectively) to the consensus sequence (Moser *et al.* 2004). In addition to these characteristic bHLH PAS domains, the C-terminal half of the Npas4 protein contains a region that is used to regulate transcription of target genes. It has been demonstrated in reporter gene assays that Npas4 can act as an activator via a transactivation domain (TAD) that is located between amino acids 597-802 (Ooe *et al.* 2004), though it is not known whether Npas4 also acts as a repressor in other systems. The Npas4 TAD has a rather divergent sequence suggesting that the Npas4 protein may have a function which is distinct to that of other bHLH PAS factors. While the TAD is essential for transcriptional activity, the PAS B repeat also provides a minor contribution as deletion of the PAS B region resulted in a slight decrease in activity in a yeast one-hybrid reporter assay (Hester *et al.* 2007). Currently, it is not known whether the Npas4 PAS domain is involved in sensory functions or regulation of Npas4 activity.

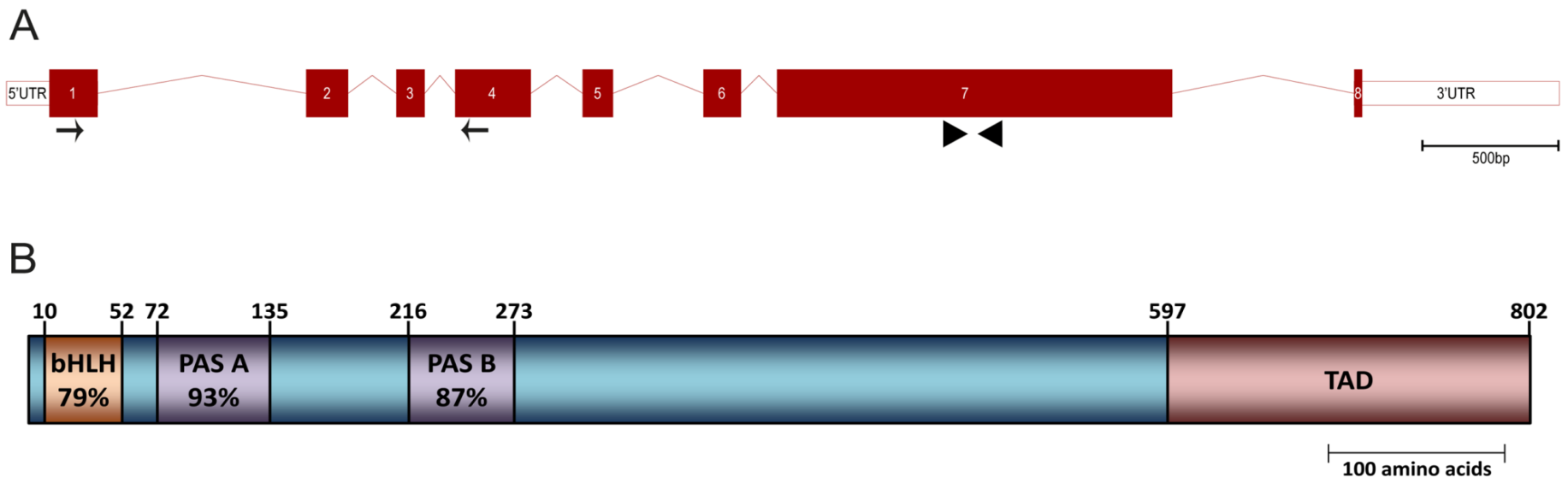


Figure 1.2 – The mouse *Npas4* gene and protein
(A) The genomic structure of the mouse *Npas4* gene. Filled boxes represent exons, empty boxes represent UTRs and lines between filled boxes represent introns. For primers used in this study, the approximate position of primer binding sites is indicated: arrows - primers used for RT-PCR; arrowheads - primers used for qRT-PCR (see Table 2.2). **(B)** The domain structure of the mouse Npas4 protein showing the location of the basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) domains as well as the transactivation domain (TAD). Conservation of each domain to the domain consensus sequence is also indicated (percentage amino acid identity).

1.2.4 Interaction with other bHLH PAS factors

NPAS4 is a Class I bHLH PAS factor and can interact with (in decreasing order of preference) ARNT2, ARNT and BMAL1; the ARNT proteins seem to be the preferred binding partners and both ARNT and ARNT2 co-immunoprecipitate with NPAS4 *in vivo* (Ooe *et al.* 2009c), though a slight interaction was also found with BMAL1 in yeast two-hybrid experiments (Ooe *et al.* 2004). No interaction was observed with CLOCK or SIM2 and, like other Class I factors, NPAS4 is unable to form homodimers (Ooe *et al.* 2004). Although NPAS4 has a greater affinity for ARNT2, the NPAS4/ARNT complex is equal to the NPAS4/ARNT2 complex in transcriptional activity (Ooe *et al.* 2009c). While Arnt2 may be the favoured binding partner of Npas4 *in vivo*, due to its restricted expression pattern there are some regions of the brain, such as the striatum, where Npas4 is expressed but Arnt2 is not (Ooe *et al.* 2009c). It is likely that in these regions Npas4 is partnered by the ubiquitous Arnt. Some Class I bHLH PAS factors must be stabilised or pre-activated before they can dimerise with their partner proteins. This is the case for the Hif proteins which are rapidly degraded under normoxia but become stabilised and transcriptionally active under hypoxic conditions (Fedele *et al.* 2002). Similarly, the Ahr is a ligand-activated factor which is dormant in the cytoplasm in the unbound state but becomes activated upon ligand binding and translocates to the nucleus (Fujii-Kuriyama and Mimura 2005). Unlike these factors, it appears as though the Npas4 protein does not require pre-activation to be functionally active and is able to bind its partner factors in the native state.

1.2.5 DNA binding specificity of Npas4 transcriptional complexes

bHLH PAS dimers bind DNA at specific sites that are recognised by the DNA-binding interface of the protein complex. Therefore, the sequence that is bound is determined by the combination of the two proteins that comprise the dimer. For example, the Sim2/Arnt2 complex recognises a DNA motif called the central midline element (CME) which contains the sequence ACGTG (Wharton *et al.* 1994). Part of the core sequence that is conserved among bHLH PAS binding elements is the GTG sequence which is the part that is recognised by the Class II partner proteins (Swanson *et al.* 1995). Unlike other bHLH PAS dimers, which generally have a single preferred binding sequence, dimers containing the Npas4 protein are promiscuous in their DNA binding. For the Npas4/Arnt2 complex, the critical sequence was found to be NCGTG with varying levels of affinity depending on which nucleotide occupies the first position (Ooe *et al.* 2004). Though results from reporter gene assays have varied between laboratories, the consensus appears to be that the preferred sequence of the Npas4/Arnt dimer is TCGTG, followed by GCGTG though the other variants ACGTG and CCGTG are also tolerated (Moser *et al.* 2004; Ooe *et al.* 2004; Jiang and Crews 2007). This unusual promiscuous binding is

conserved in the *Drosophila melanogaster* (*D. melanogaster*) homolog of Npas4, Dysfusion (Dys), which suggests that it may be biologically important for Npas4 function (Jiang and Crews 2007). Interestingly, the human NPAS4 protein is able to parter with Tango (Tgo), the *D. melanogaster* ortholog of Arnt, to drive gene expression from a reporter construct though it seems that this inter-species heterodimer is more selective in its DNA binding preference; when a reporter construct containing four copies of the TCGTG sequence was used, reporter gene expression levels were comparable between the NPAS4/Tgo and NPAS4/ARNT dimers, however when reporter constructs using other variants were tested, the NPAS4/Tgo dimer was less effective (Jiang and Crews 2007).

Due to its promiscuous binding, the Npas4/Arnt2 dimer is capable of binding DNA sequences that are recognised by other bHLH PAS factors such as the xenobiotic response element (XRE) recognised by the Ahr/Arnt dimer (TNGCGTG), the hypoxic response element (HRE) recognised by the Hif/Arnt dimer (TACGTG) and the CME. Thus, it is possible that Npas4 may compete with other bHLH PAS factors in two ways; in cells where multiple Class I bHLH PAS factors are expressed, there may be competition for Class II partner proteins and/or competition for similar DNA sequences. It is noteworthy that in some cases Npas4 may antagonise the actions of other bHLH PAS factors. For instance, although Npas4 and Sim2 may compete for the same binding sites, Npas4 is a transcriptional activator while Sim2 is a repressor which raises the possibility they may exert opposing regulatory effects on common target genes (see Section 1.7.4).

1.3 Expression of *Npas4*

1.3.1 Tissue distribution of the *Npas4* transcript in the adult organism

Expression of bHLH PAS factors varies from those that are ubiquitously expressed, such as Arnt, to those whose expression is spatially restricted to specific tissues (see Table 1.1). Like the related bHLH PAS factor Sim2, Npas4 has a restricted expression pattern and is found in only a very small number of tissues in the adult organism. Northern blot analysis of various rodent tissues revealed that *Npas4* is expressed primarily in the brain where a major transcript of approximately 3.4kb was detected, though low expression of additional transcripts (ranging from 4-6kb) was also seen in the testis (Moser *et al.* 2004; Shamloo *et al.* 2006; Hester *et al.* 2007). The size of the transcript detected in brain samples corresponds to the expected size of the mature *Npas4* mRNA (see Section 1.2.2), though it is unclear whether the larger transcripts present in the testis are true isoforms of *Npas4*. It is known that the transcripts of some bHLH PAS transcription factors, such as Hif1α (Wenger *et al.* 1998), undergo alternative splicing in different organs, though whether this also occurs in the case of

Npas4 requires further investigation. In humans, the *NPAS4* transcript is also expressed predominantly in brain tissue, though, unlike in rodents, no expression was observed in the testis using Northern blotting (Ooe *et al.* 2004). *NPAS4* mRNA has also been detected in human blood samples (Liao *et al.* 2010).

1.3.2 *Npas4* expression in the adult brain

In a normal brain, *Npas4* is generally expressed at a low level throughout the grey matter, which consists mainly of neuronal and glial cell bodies as well as unmyelinated axons, while being absent from the myelinated white matter (Shamloo *et al.* 2006). Though it is expressed throughout the whole brain at a low level, certain regions, such as the limbic system, are enriched in their expression of *Npas4*. The limbic system is loosely defined, in both an anatomical and functional sense, as there is still some controversy regarding precisely which brain structures comprise the limbic system and hence which aspects of neurophysiology it regulates (Isaacson 1992). Most commonly, the limbic system refers to a collection of subcortical structures including the hippocampus, amygdala, fornix and cingulate gyrus and it is considered to be involved in learning and memory, regulation of emotions and emotional behaviour, sexual function and olfaction (Kotter and Meyer 1992).

In the limbic system, the most prominent site of *Npas4* expression is the hippocampus (Flood *et al.* 2004; Moser *et al.* 2004; Ooe *et al.* 2004; Shamloo *et al.* 2006), though expression of both *Npas4* mRNA and protein has also been detected in the lateral nucleus of the amygdala (Ploski *et al.* 2011). The hippocampus is the part of the brain responsible for the formation of new, and consolidation of old, memories, particularly with respect to spatial (Maguire *et al.* 2000) or declarative memory (Squire 1992), while the amygdala plays a central role in the processing and memory of emotional reactions (Roozendaal *et al.* 2009). Both *Npas4* mRNA and protein are highly expressed in the hippocampus; high levels of expression are present throughout the *cornu Ammonis* (CA) in regions CA1, CA2 and CA3, and also in the dentate gyrus (DG) where staining can be seen in the granule cell layer (GCL) and, to a lesser extent, the hilus (Flood *et al.* 2004; Moser *et al.* 2004; Ooe *et al.* 2004; Shamloo *et al.* 2006). The DG is, along with the subventricular zone (SVZ), one of the few regions of the mammalian brain which retains the capacity for neurogenesis after birth (Gage 2000; Doetsch 2003). The subgranular zone (SGZ), located just beneath the GCL, provides a continual source of self-renewing neural progenitor cells (NPCs) that are able to give rise to new neurons through asymmetric divisions (Cameron and McKay 2001; Seri *et al.* 2001). This area is often referred to as a

'neurogenic niche' as it contains the appropriate microenvironment for the birth, survival and maturation of new neurons in the mature brain (Basak and Taylor 2009).

Brain structures outside of the limbic system that have lower levels of *Npas4* expression include the cortex, striatum, olfactory bulb, cerebellum and the hypothalamus (Moser *et al.* 2004; Ooe *et al.* 2004; Unfried *et al.* 2010; Prentice *et al.* 2011). In the cortex, expression is highest in layers III and V and is present in the frontal, parietal and entorhinal cortices (Moser *et al.* 2004; Ooe *et al.* 2004; Shamloo *et al.* 2006). Some species-specific differences in *Npas4* expression have been observed in the cerebellum; in the rat cerebellum, *Npas4* expression is confined to the Purkinje cell layer, while in the mouse cerebellum *Npas4* is expressed in both the GCL and the Purkinje cell layer (Moser *et al.* 2004; Ooe *et al.* 2004).

1.3.3 Cellular distribution of Npas4 expression

It has been stated that Npas4 is expressed predominantly in excitatory neurons (Lin *et al.* 2008), though there is also evidence that it may be expressed by some immature neurons and NPCs. In the GCL of the DG, Npas4 immunoreactivity was observed in most of the cells expressing the mature neuronal markers Feminizing locus on X 3 (Fox-3) or calbindin (Yun *et al.* 2010). However, in the SGZ, a small number of Npas4-expressing cells were found to also express Sry related high mobility group box 2 (Sox2), a marker of neural progenitor cells (Brazel *et al.* 2005), or doublecortin, which is expressed by immature neurons and migrating neuroblasts (Yun *et al.* 2010).

1.3.4 Subcellular localisation

As is expected of a transcription factor, bioinformatic analysis of the Npas4 amino acid sequence predicted a nuclear localisation for the Npas4 protein and, indeed, when transgenically expressed in the COS-7 monkey kidney cell line, the protein was found to be localised to the nucleus (Moser *et al.* 2004). One group has claimed that, in addition to the nucleus, the Npas4 protein may also be found in other parts of the cell; in rat primary cortical neuron cultures (12 days *in vitro*), endogenous Npas4 protein was detected by immunofluorescence in the soma, neurites and also at synapses where it co-localised with the synaptic marker synaptophysin (Shamloo *et al.* 2006). The same group also reported expression of the Npas4 protein in the microsome and synaptosome, as determined by immunoblotting of rat brain lysates processed for subcellular fractionation (Shamloo *et al.* 2006). Nevertheless, the validity of these results is questionable due to the poor quality of the anti-Npas4 antibody used. Furthermore, these observations do not agree with other reports in which expression

of the endogenous Npas4 protein was found to be largely nuclear (Lin *et al.* 2008). Thus, without convincing evidence to the contrary, the consensus seems to be that Npas4 is a predominantly nuclear protein.

1.3.5 *Npas4* expression during development

Little has been published about the expression of *Npas4* during embryonic development, and what information exists is conflicting and inconsistent. Despite cloning the *NPAS4* cDNA from a human foetal brain library, Ooe *et al.* were unable to detect *Npas4* mRNA expression in the developing mouse embryo using *in situ* hybridisation (ISH) (Ooe *et al.* 2004). It was, however, detected late in development at embryonic day 17 (E17) by the more sensitive method of reverse transcription polymerase chain reaction (RT-PCR) albeit at the cost of forfeiting information about its spatial expression at this stage (Ooe *et al.* 2004). At an even earlier stage, Hester *et al.* found Npas4 protein to be expressed at E10.5 in the dorsal root ganglion of the mouse embryo (Hester *et al.* 2007). These inconsistencies regarding *Npas4* expression during development may be due to differences in the sensitivity of the techniques used, or perhaps differences in the relative amounts of protein and mRNA being expressed in the embryo at different stages. More research is needed to shed light on the expression of *Npas4* during development.

1.4 Regulation of *Npas4* expression

1.4.1 Npas4 is a stimulus-response transcription factor

Many bHLH PAS transcription factors are continually expressed in the cell but only become functional in response to a specific signal. For example, the Hif proteins are rapidly degraded in normoxic conditions but are stabilised and become transcriptionally active during hypoxia (Fedele *et al.* 2002). Similarly, the Ahr is normally sequestered in the cytoplasm in an inactive state by chaperone molecules, but, upon binding of a xenobiotic ligand, it translocates to the nucleus where it dissociates from the chaperone complex and dimerises with Arnt to form a transcriptionally active unit (Kewley *et al.* 2004). Npas4 differs to these proteins in that it is regulated primarily at the transcriptional level and, furthermore, there is no evidence that the Npas4 protein must be pre-activated to be functional (though several post-translational modifications that modulate its activity have been identified; see Section 1.4.4).

In unstimulated neurons, *Npas4* mRNA is expressed at very low levels, if at all, but is rapidly and robustly up-regulated in response to various stimuli. The types of stimuli that can induce up-regulation of *Npas4* are many and varied (discussed below; summarised in Figure 1.7), though the primary signal for induction of *Npas4* expression is an increase in the concentration of nuclear calcium (Ca^{2+}) which, in neurons, is largely modulated by excitatory neural activity. Other conditions which induce up-regulation of *Npas4* mRNA include signalling through neurotrophin receptors, various drugs of abuse and some forms of cellular stress. In each case, *Npas4* induction is extremely rapid with both mRNA and protein expression reaching a maximum within 30-90 minutes (min) of stimulation. However, this up-regulation is generally transient and, once peak expression has been reached, both mRNA and protein levels decline sharply such that *Npas4* expression returns to baseline levels within a few hours. Such a precisely controlled temporal expression profile suggests that down-regulation of *Npas4* may also be tightly regulated by a variety of mechanisms to ensure that its expression is quickly repressed once the stimulatory signal has passed (potential post-transcriptional regulation of *Npas4* expression is discussed in Section 1.4.9). This type of 'rapid-response' expression pattern is characteristic of a group of genes known as 'immediate-early genes' (IEGs) which suggests that Npas4 expression may be regulated in a similar manner to IEGs.

1.4.1.1 Immediate-early genes

The IEGs are a diverse family of stimulus-response genes whose expression is generally low in unstimulated cells but is rapidly and transiently induced in response to extracellular signals such as peptide growth factors, drugs of abuse, cellular stress and small signalling molecules such as forskolin. Several well-known examples of IEGs are *Myc*, c-*jun* and early growth response protein 1 (*Egr1*). Typically, the up-regulation of IEGs is immediate (in some cases detectable within minutes) and is not dependent on *de novo* protein synthesis which is reflective of their origin as homologues of retroviral proto-oncogenes. Many IEGs are derived from viral sequences encoding transcription factors that are expressed before replication of the viral genome (hence the term 'early gene'). Therefore, IEGs are postulated to represent the first genetic signal in response to cellular stimulation as they provide a molecular link between extracellular signals and changes in gene expression via stimulus-transcription coupling. Through control of target gene expression, IEGs are involved in a diverse range of cellular processes including brain development, learning, memory, response to drugs of abuse and synaptic plasticity (Sheng and Greenberg 1990; Perez-Cadahia *et al.* 2011). Many IEGs, for example *c-fos* and activity-regulated cytoskeleton-associated protein (*Arc*), are also up-regulated by neurotransmitter release making them useful as markers of neural activity in the CNS (Kovacs 2008).

1.4.2 Regulation of *Npas4* expression by neural activity

1.4.2.1 Neural activity and NMDAR-mediated Ca^{2+} signalling

1.4.2.1.1 Neural activity

Neural activity can be defined as the propagation of a signal, in the form of a chemical messenger, from one cell to another in a neuronal circuit. This relaying of information between neurons underpins many important physiological processes in the CNS such as synaptic plasticity (Butz *et al.* 2009), memory formation (Kang *et al.* 2001; Jutras and Buffalo 2010), and neuronal survival (Mennerick and Zorumski 2000). In neural networks, information is encoded as discrete membrane depolarisation events called 'action potentials' which travel along the length of a neuron's axon and typically culminate in the fusion of vesicles at the axon terminal to the plasma membrane causing release of neurotransmitters into the synaptic cleft (Figure 1.3). Neurotransmitters are small chemical messenger molecules that bind to receptors present on the plasma membrane of the post-synaptic cell and thereby transmit signals from one cell to another. In this way, neurotransmitters and their receptors are the primary mediators of neural activity in the CNS.

1.4.2.1.2 Neurotransmitters and their receptors

Following neurotransmitter release at the synapse, the incoming signal is processed by post-synaptic transmembrane receptors which undergo conformational changes in response to ligand binding. The nature of these changes depends on the type of receptor that is activated. Two types of neurotransmitter receptors exist:

- Ionotropic receptors – these receptors form ligand-gated ion channels that, when bound by the appropriate neurotransmitter, open to allow passage of ions through the plasma membrane. These receptors mediate fast synaptic responses.
- Metabotropic receptors – these receptors do not form an ion channel pore themselves but instead may activate ion channels indirectly through various signalling mechanisms. These receptors often contain intracellular guanine nucleotide-binding protein domains and generally mediate slower, but more prolonged, synaptic responses.

Regardless of the type of receptor, binding of a neurotransmitter to its cognate receptor triggers a cascade of events within the cytoplasm of the post-synaptic cell that can ultimately lead to intracellular outcomes such as changes in gene expression or the generation of an action potential. The outcome of a synaptic event depends on many factors including the neurotransmitter that is released, the type of receptor that is activated and the number of receptors that are activated. Furthermore, the frequency of synaptic transmission between neurons can also convey information,

such as the intensity of a particular stimulus. Nevertheless, although neurotransmitters can elicit a wide range of diverse effects on their targets, they can be broadly classified as being either 'excitatory' or 'inhibitory'; excitatory neurotransmitters, such as glutamate, are those that generally increase the probability that the target neuron will fire an action potential, while inhibitory neurotransmitters, such as γ-aminobutyric acid (GABA), have the opposite effect.

1.4.2.1.3 Excitatory synaptic transmission: glutamate and glutamate receptors

Glutamate is the most abundant excitatory neurotransmitter in the nervous system and its action is modulated by a large family of glutamate receptors (GluRs) that are collectively termed 'excitatory amino acid (EAA) receptors' (Watkins and Jane 2006). In addition to glutamate, there are a number of small molecules structurally similar to glutamate that are also capable of binding to, and activating, GluRs including N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate. The various analogues of glutamate differ in their affinity for particular receptor subtypes and hence GluRs can be classified according to their binding preferences (Figure 1.4). Ionotropic GluRs (iGluRs), for example, are further subdivided into three categories based on their selectivity for particular glutamate analogues; NMDA receptors (NMDARs), AMPA receptors (AMPARs) and kainate receptors (KARs) (Watkins and Jane 2006). Similarly, metabotropic GluRs (mGluRs) can also be divided into Group I, Group II and Group III based on receptor structure, agonist specificity and physiological activity (Watkins 2006).

1.4.2.1.4 Glutamate receptor signalling and cation flux

All three iGluR subtypes form non-specific cation channels that are highly permeable to sodium (Na^+) and potassium (K^+) ions. Opening of these channels upon ligand binding allows Na^+ to enter the cell producing an inward Na^+ current. This increase in intracellular Na^+ can in turn activate nearby voltage-gated ion channels causing an even greater influx of Na^+. If a sufficient number of channels are activated to raise the membrane potential beyond the required threshold, an action potential will be initiated. Thus, iGluRs facilitate the conduction of action potentials and are largely responsible for the self-propagating nature of excitatory synaptic transmission.

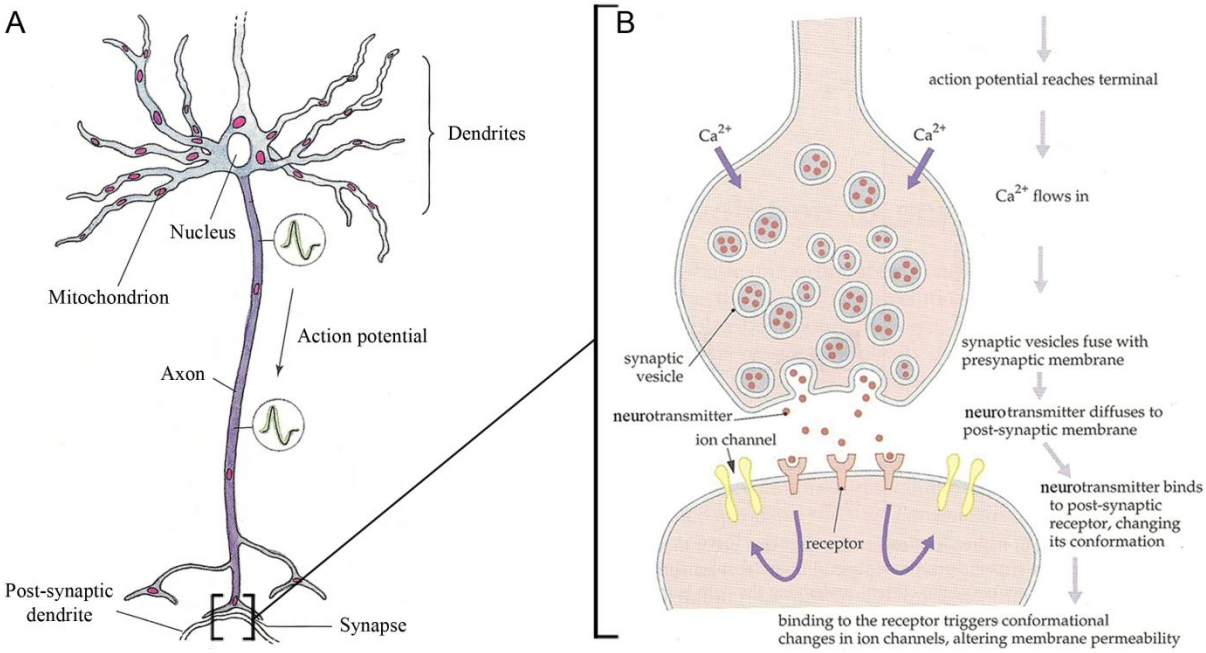


Figure 1.3 – Neurons and synapses
(A) "Schematic of Neuron" from the book *Evolving Brains* by John Morgan Allman. Copyright © 1998 by Scientific American Library. Reprinted by permission of Henry Holt and Company, LLC. **(B)** The anatomy of a synapse. This diagram describes the events occurring during synaptic transmission. Adapted from *Biology 2nd edition* by Knox, Ladiges, Evans and Saint. Copyright © 2001. Reprinted by permission of McGraw-Hill Australia.

The channels formed by iGluRs are, to a lesser degree, also permeable to Ca^{2+}, though the ionic selectivity varies greatly between receptor subtypes. NMDAR channels have the greatest permeability to Ca^{2+} and hence are the major pathway of Ca^{2+} entry into excitable neurons (Dingledine *et al.* 1999). Some inward flow of Ca^{2+} does occur through non-NMDAR channels, however the permeability of both AMPAR and KAR channels to Ca^{2+} is less than 3% that of NMDAR channels (Iino *et al.* 1990) so their contribution to Ca^{2+} influx is minor. Stimulation of Group I (but not Group II or Group III) mGluRs can also increase the amount of Ca^{2+} in the cytoplasm but through a different mechanism; rather than importing Ca^{2+} through the plasma membrane, activation of Group I mGluRs triggers a signalling cascade which results in mobilisation of Ca^{2+} from intracellular stores. Group I mGluRs are coupled to phospholipase C (PLC), an enzyme which cleaves phospholipids to produce the secondary messenger molecule inositol 1,4,5-trisphosphate (IP_3), and activation of these receptors causes IP_3 to be released into the cytoplasm where it binds to IP_3 receptors on the surface of the endoplasmic reticulum (ER) causing Ca^{2+} to be released into the cytosol (Conn *et al.* 2005). Thus, though the mechanism varies between receptor subtypes, another important outcome arising from the stimulation of (most types of) glutamate receptors is the generation of Ca^{2+} transients which are used by the cell for all manner of signalling pathways. Rising Ca^{2+} levels in the cytoplasm trigger Ca^{2+} influx into the nucleus where Ca^{2+} acts as a messenger that can regulate the expression of Ca^{2+}-sensitive genes.

1.4.2.1.5 Ca^{2+} signalling

Ca^{2+} is a complex, ubiquitous and versatile second messenger that is used in signalling cascades in all cell types. Ca^{2+} signalling plays a role in many diverse cellular processes from contraction, secretion, proliferation and apoptosis (Berridge *et al.* 2000). The cellular context of Ca^{2+} signalling determines the final outcome and different results, both qualitative quantitative, can be achieved depending on the speed, amplitude, frequency and spatio-temporal profile of the Ca^{2+} signal (Berridge *et al.* 2000). In the nucleus, Ca^{2+} transients can stimulate gene transcription through the activation of Ca^{2+}-sensitive transcription factors and many of the effects of Ca^{2+} signalling arise from changes in gene expression.

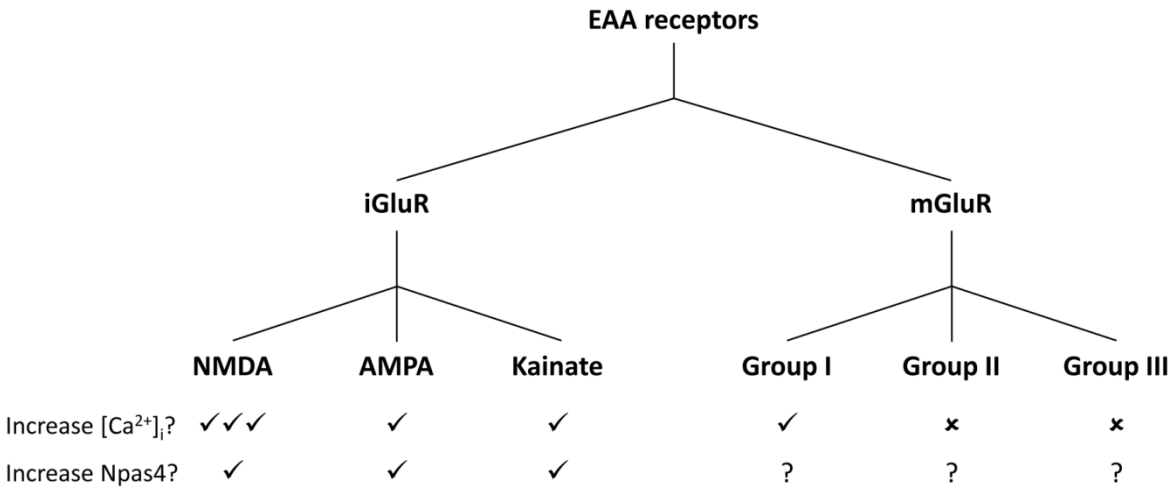


Figure 1.4 – Glutamate receptors
The EAA receptor family can be subdivided into two groups; iGluRs and mGluRs. These are then further divided into subgroups based on their agonist specificity and physiological properties. The permeability of each of the receptor subgroups to Ca^{2+} varies; NMDARs are highly permeable to Ca^{2+} while AMPARs, kainate receptors and Group I mGluRs have moderate Ca^{2+} permeability and Group II and Group III mGluRs do not affect intracellular Ca^{2+} (see Section 1.4.2.1.4). Activation of iGluRs results in up-regulation of Npas4, however the effect of mGluR activation on Npas4 expression has not been investigated (indicated by '?'). EAA - excitatory amino acid; iGluR - ionotropic glutamate receptor; mGluR - metabotropic glutamate receptor; NMDA - N-methyl-D-aspartic acid; AMPA - α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; $[Ca2^{+}]_i$ - intracellular calcium concentration.

1.4.2.1.6 Creb: an example of a Ca^{2+}-sensitive transcription factor

A classic example of a transcription factor that is regulated by Ca^{2+} signalling is the cyclic adenosine monophosphate (cAMP) response element-binding protein (Creb). Normally inactive in the basal state, the activity of Creb is crucially dependent on phosphorylation at serine residue 133 (Mayr and Montminy 2001). There are several independent pathways that can lead to phosphorylation of Creb including those modulated by cAMP-dependent kinases such as protein kinase A (PKA), however, in neurons the most commonly used pathway involves Ca^{2+} signalling. Increased intracellular Ca^{2+} causes Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMKIV) to become active leading to phosphorylation of Creb at serine 133 (Matthews *et al.* 1994). Once activated, Creb binds DNA at specific sites, called 'cAMP response elements' (CREs), which are located within the regulatory regions of target genes. Creb then recruits the co-activators Creb-binding protein (Cbp) and p300 to initiate transcription of these genes. Creb regulates the expression of a vast number of genes including the IEG *c-fos* as well as several pro-survival genes such as brain-derived neurotrophic factor (*Bdnf)* (Tao *et al.* 1998) and anti-apoptotic gene B-cell lymphoma 2 (*Bcl-2*) (Wilson *et al.* 1996). For this reason, Creb has been implicated in neurobiological processes such as activity-dependent neuronal survival and memory (Silva *et al.* 1998; Walton *et al.* 1999; Papadia *et al.* 2005).

1.4.2.2 Activity-dependent regulation of *Npas4* expression in neurons

Npas4 expression is rapidly, but transiently, induced in neurons in response to neural activity. This effect has been demonstrated both *in vivo* and *in vitro* using many different stimulators of excitatory synaptic activity. Global activation of GluRs using glutamate triggers *Npas4* expression (Ooe *et al.* 2009a), but so too does activation of specific iGluR subtypes by selective agonists such as NMDA (Zhang *et al.* 2007; Coba *et al.* 2008) or kainate (Ooe *et al.* 2009b). Indeed, blockade of NMDARs (Coba *et al.* 2008; Lin *et al.* 2008; Ooe *et al.* 2009a) or AMPARs (Lin *et al.* 2008) greatly inhibited the activity-dependent induction of *Npas4* indicating the importance of these channels in Npas4 signalling. Another method commonly used to stimulate neural activity experimentally is to remove inhibitory synaptic inputs from a neural circuit by blocking inhibitory GABA receptors. This instigates aberrant firing of neurons known as 'action potential bursting' which, in the context of a whole organism, can result in seizure. Correspondingly, application of different GABA receptor antagonists, such as pentylenetetrazol (Flood *et al.* 2004; Ooe *et al.* 2009a) or bicuculline (Lin *et al.* 2008; Zhang *et al.* 2009), is also able to elicit a robust increase in *Npas4* expression.

1.4.2.2.1 Neuronal depolarisation

Indeed, the cause of action potential firing is irrelevant as, irrespective of the stimulus, it is the actual depolarisation of the neuron itself which is sufficient to induce Npas4 expression. Experimentally, application of potassium chloride (KCl) can be used to mimic the effects of excitatory synaptic activity as it induces depolarisation of neurons without directly acting on glutamate receptors themselves. KCl treatment has been shown to stimulate *Npas4* expression both *in vivo* (Hester *et al.* 2007) and *in vitro* (Lin *et al.* 2008) indicating that it is not the activation of GluRs per se that induces *Npas4* expression, but rather the downstream consequences of glutamate signalling.

1.4.2.2.2 Nuclear Ca^{2+} signalling

More specifically, it was revealed that the intracellular events leading to *Npas4* expression after depolarisation involve nuclear Ca^{2+} signalling. When Ca^{2+} signalling was prevented either by blocking L-type voltage-gated Ca^{2+} channels or through use of Ca^{2+} chelators, the activity-dependent up-regulation of *Npas4* was almost completely abolished (Lin *et al.* 2008; Zhang *et al.* 2009). Indeed, *Npas4* up-regulation can be induced even in the absence of neural activity simply by raising cytosolic Ca^{2+} concentration which can be achieved by inhibiting organellar ion transporters such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps which move Ca^{2+} from the cytosol into the ER. Treatment of PC12 cells of the pheochromocytoma cell line (Greene and Tischler 1976) with thapsigargin (10µM), a SERCA inhibitor which prevents Ca^{2+} entry into the ER, was sufficient to produce a rapid and transient up-regulation of *Npas4* mRNA peaking at 30min after treatment and returning to basal levels by 120min (Ooe *et al.* 2009b).

The induction of *Npas4* was also found to be partially reliant on CaMKIV function (Zhang *et al.* 2009) suggesting that there may be targets of CamKIV that regulate Npas4 transcription. One obvious candidate is Creb, a Ca^{2+}-sensitive target of CaMKIV (Section 1.4.2.1.6), however the possibility that transcription of *Npas4* is regulated by Creb seems improbable given that the promoter region of the *Npas4* gene does not contain any CRE sites (Zhang *et al.* 2009). Similarly, no canonical downstream regulatory element (DRE) sites (GAGTCAAGG) (Carrion *et al.* 1998) are present in the *Npas4* genomic locus making it unlikely that *Npas4* expression is under the control of DRE-antagonist modulator (DREAM), a transcriptional repressor which is released from DNA upon binding of Ca^{2+} (Carrion *et al.* 1999). Thus, the precise molecular mechanisms controlling Ca^{2+}-dependent transcription of *Npas4* have yet to be elucidated.

In particular, one crucial question remains unanswered: given that Ca^{2+} is a ubiquitous second messenger present in all cell types, what are the factors that drive neuron-specific expression of Npas4? The answer to this question may lie within the first intron of the NPAS4 gene where four putative RE1-silencing transcription factor (REST) binding sites were recently identified using comparative genomics (Lindblad-Toh *et al.* 2011). REST is a transcriptional repressor that is expressed predominantly in non-neuronal cells where it silences expression of neuronal genes (Lunyak and Rosenfeld 2005). While this may be a compelling proposition, it remains to be experimentally demonstrated that NPAS4 expression is negatively regulated by REST in non-neuronal cells.

1.4.2.3 Excitotoxic brain injury and *Npas4* expression

Abnormally high levels of neuronal activity arising from excessive stimulation of GluRs can trigger activation of apoptotic signalling pathways ultimately resulting in programmed cell death of neurons - this pathological process is known as excitotoxicity (Ankarcrona *et al.* 1995). Unsurprisingly, the neuronal activity associated with excitotoxic injury can induce up-regulation of *Npas4* and, consequently, high levels of *Npas4* expression are seen in various types of brain injury that feature excitotoxic damage such as seizure and cerebral ischaemia.

1.4.2.3.1 Seizure

Seizure is defined as abnormal excessive neuronal activity in the brain (Fisher *et al.* 2005). In a mouse model of epilepsy, *Npas4* mRNA was found to be one of the most highly up-regulated transcripts 1 hour (h) after generalised convulsive seizure induced by administration of the GABA receptor antagonist pentylenetetrazol (Flood *et al.* 2004). Seizure induced moderate up-regulation of *Npas4* mRNA in the cortex and thalamus, however the greatest induction was observed in the DG and CA1 regions of the hippocampus (Flood *et al.* 2004), areas that are particularly sensitive to this form of brain insult. In a similar model where mice were injected with kainate (28mg/kg), increased Npas4 protein expression was detected in the hippocampus five days after seizure (Ooe *et al.* 2009b).

1.4.2.3.2 Cerebral ischaemia

Cerebral ischaemia, caused by insufficient blood flow to the brain, is characterised by two distinct regions of pathology; (1) the ischaemic core, which consists of tissue directly affected by the lack of blood flow and (2) the penumbra, which is the tissue surrounding the infact zone. Unlike the ischaemic core, which rapidly undergoes necrosis due to hypoxia, the penumbral tissue may survive for several hours after the ischaemic event due to collateral blood supply and can thus be salvaged

by early intervention. A significant element of the pathophysiology associated with ischaemic brain injury is related to excitotoxicity (Lipton 1999). Cerebral ischaemia rapidly leads to energy depletion within neurons as adenosine triphosphate (ATP) production by oxidative phosphorylation ceases. Without energy, ATP-dependent ion pumps and neurotransmitter transporters fail which leads to loss of membrane potential and uncontrolled glutamate release into the extracellular space (Dirnagl *et al.* 1999). Both of these events trigger repetitive peri-infarct depolarisation of neurons within the penumbra leading to increased levels of intracellular Ca^{2+} and induction of activity-regulated genes. Ischaemic gene expression can activate both cell survival programmes and pathogenic cascades, including pro-inflammatory and apoptotic pathways (Mitsios *et al.* 2006).

A microarray study of gene expression in the rat brain following stroke revealed that *Npas4* was one of the most profoundly up-regulated genes following 2h of middle cerebral artery occlusion (Shamloo *et al.* 2006). Temporal profiling showed that *Npas4* mRNA follows a biphasic expression pattern in response to focal cerebral ischaemia whereby robust expression was detected immediately after stroke at 0-1.5h of reperfusion before gradually decreasing to baseline levels between 3-6hr, then transiently increasing again between 9-12h before once more returning to basal levels over the next 12h (Shamloo *et al.* 2006). This distinctive pattern of expression was shared by a cluster of other genes which consisted mainly of IEGs such as *Arc* and *Egr1* but, interestingly, also included *Bdnf* (Rickhag *et al.* 2006) which is a direct target of Npas4 (Section 1.5.2.1.3).

Analysis of the spatial expression pattern of *Npas4* after focal ischaemia using ISH showed that immediately after reperfusion *Npas4* expression was elevated throughout the entire affected hemisphere in both the ischaemic core and the penumbra, with no change in the contralateral hemisphere (Shamloo *et al.* 2006). The induction of *Npas4* in the peri-infarct regions of the cingulate cortex and medial striatum was high and transient, while in the ischaemic core it was moderate but persisted for up to 12h after reperfusion (Shamloo *et al.* 2006). At the protein level, increased Npas4 immunoreactivty was detectable in layer V of the parietal cortex after 6h of reperfusion (Shamloo *et al.* 2006).

Global cerebral ischaemia, caused by clamping of the common carotid arteries for 10min, produced a much more sustained response in *Npas4* mRNA expression. Up-regulation of *Npas4* was most evident in the hippocampus where increased expression was observed between 12-24h after reperfusion (Shamloo *et al.* 2006). Furthermore, the increase in *Npas4* expression was directly

correlated with the duration of the ischaemic insult and, hence, the severity of damage (Shamloo *et al.* 2006). Expression of Npas4 protein was detected in surviving cells within the CA1 and CA2 regions of the rat hippocampus 10 days after transient global cerebral ischaemia (Ooe *et al.* 2009b) and likewise in the cortex and hippocampus (CA1 and CA3 regions) after chronic global ischaemia (Ooe *et al.* 2009a).

1.4.2.4 Learning and synaptic plasticity

Neural activity, in the form of experiences or sensory inputs, can modify the behaviour of neural circuits by modifying the connections between individual neurons in the brain – this process is known as synaptic plasticity (Chakraborty *et al.* 2007). These dynamic, activity-dependent modifications at synapses can alter the strength or efficacy of synaptic transmission between two neurons which, according to Hebbian theory, is the neurochemical basis for learning and memory (Bliss and Collingridge 1993; Neves *et al.* 2008). At a molecular level, synaptic plasticity is primarily driven by changes at the post-synaptic neuron that either increase or decrease the sensitivity of the neuron to incoming synaptic activity, such as changes in the size and number of dendritic spines or the number of neurotransmitter receptors that are expressed at the synapse. However, changes occurring at the pre-synaptic neuron (for example, changes in the number of neurotransmitter vesicles produced and the probability of their release) can also contribute to synaptic plasticity. These changes can contribute either to short-lived plasticity lasting only 30-60min or a long-term plasticity that persists for days or even weeks. Furthermore, these changes can increase or decrease the threshold of neural activity required to induce additional plastic changes, thus altering the ability of neurons to undergo further synaptic plasticity – a property termed 'metaplasticity' (Abraham and Bear 1996). It has recently been demonstrated that both learning and experimentally induced synaptic plasticity lead to an increase in *Npas4* expression (described below) which suggests that *Npas4* is involved in aspects of synaptic plasticity.

1.4.2.4.1 Long-term potentiation and long-term depression

There are various experimental protocols that can reproduce the phenomenon of synaptic plasticity *in vitro*. When these protocols result in plastic changes that enhance synaptic transmission, they are said to induce long-term potentiation (LTP) while, conversely, plasticity that dampens synaptic transmission is known as long-term depression (LTD). There are many forms of both LTP and LTD and the type of plasticity that is induced depends on several factors including; (1) the types of neurons forming the synapse, (2) the age of the animal, and (3) the mechanism used to trigger plastic changes.

The most extensively characterised forms of synaptic plasticity are the NMDAR-dependent LTP and LTD of neurons in the CA1 region of the hippocampus.

Both LTP and LTD consist of a transient early phase lasting 30-60min and a late phase lasting days to weeks that, unlike short-term plasticity, requires gene transcription and protein synthesis. Short-term plasticity is mediated by rapid changes occurring at the synapse such as trafficking of existing glutamate receptors to/from the synaptic membrane, while changes responsible for long-term plasticity include synthesis of additional GluRs and/or structural modifications of the dendritic cytoskeleton carried out by actin-binding proteins which can create new dendritic spines or modify the shape of existing spines through cytoskeletal reorganisation. One such actin-binding protein is developmentally regulated brain protein (Drebrin), which is a target of Npas4 (see Section 1.5.2.2.2).

Although LTP and LTD produce opposing effects on synaptic transmission, all synaptic plasticity is mediated by neural activity and therefore it is the pattern of electrical activity that determines which form of synaptic plasticity is induced. This is best illustrated by the different protocols used to induce LTP or LTD *in vitro*. Experimentally, LTP can be induced by high frequency tetanic stimulation or by using a 'paired protocol' (low frequency afferent stimulation paired with post-synaptic depolarisation) while LTD can be achieved with prolonged low frequency stimulation. Therefore, it is the GluRs on the post-synaptic cell (usually NMDARs) which decode the incoming neural activity and initiate pathways leading to either LTP or LTD via activation of the appropriate intracellular kinase signalling cascades. Indeed, although NMDARs mediate both types of plasticity, it has been demonstrated that different kinase networks are recruited depending on the type of stimulus (Coba *et al.* 2008). This suggests that NMDARs are able to modulate neural activity and translate the information encoded in different patterns of electrical activity into different cellular outcomes by recruiting a distinct set of kinases under each set of conditions. In other words, NMDARs and Ca^{2+} signalling are a focal point where both LTP and LTD converge (Figure 1.5). Furthermore, there may be some overlap between the kinase networks (and hence the gene expression programmes they control) that are activated in each scenario which allows for complex regulation of activity-dependent gene expression in which many different cellular outcomes can be produced by a common mechanism.

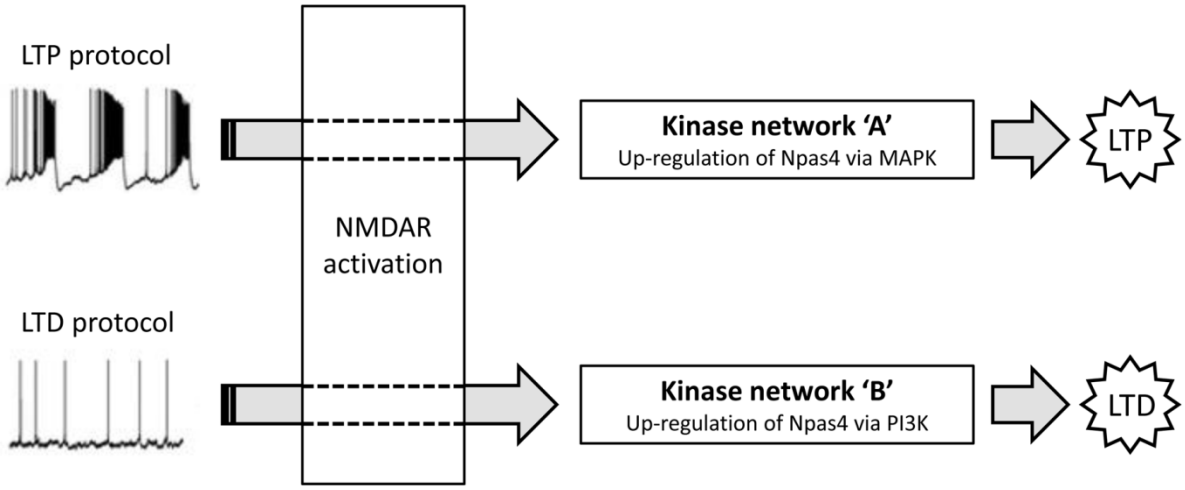


Figure 1.5 – Differential regulation of Npas4 expression by LTP and LTD
Both LTP and LTD induce up-regulation of Npas4 expression via activation of NMDARs, though different signalling pathways are used in each case. The pattern of incoming neural activity is decoded by NMDARs such that different kinase networks are activated by different stimulation protocols; LTP triggers one set of kinase cascades which results in up-regulation of Npas4 expression via the MAPK pathway, while LTD triggers a distinct set of kinase cascades which leads to up-regulation of Npas4 expression via the PI3K pathway. LTP and LTD have opposing effects on synaptic plasticity yet induction of Npas4 expression is common to both protocols which suggests that Npas4 target genes are involved in modulation of synaptic plasticity. LTP - long-term potentiation; LTD - long-term depression; NMDAR - NMDA receptor; MAPK - mitogen-activated protein kinase; PI3K - phosphatidylinositol 3-kinase.

Experimentally, these types of synaptic plasticity can also be induced chemically by directly acting on the biochemical machinery which underpins plasticity, thus avoiding the need for stimulation of individual neurons. Chemically induced LTP (Chem-LTP) can be achieved by treating neurons (in slice culture) with forskolin and rolipram in low magnesium (Mg^{2+}) conditions (Otmakhov *et al.* 2004) while chemically induced LTD (Chem-LTD) can be produced by bath application of NMDA (Lee *et al.* 1998).

1.4.2.4.2 Regulation of Npas4 by Chem-LTP and Chem-LTD

Gene expression programmes involved in LTP and LTD were investigated in a microarray study performed in mouse hippocampal slices and the results showed that *Npas4* was one of only 12 genes to be up-regulated by both Chem-LTP and Chem-LTD after 1h of stimulation (Coba *et al.* 2008). Though the magnitude of *Npas4* mRNA induction was similar in both Chem-LTP and Chem-LTD, the kinases mediating *Npas4* expression varied according to the protocol used (see Table 1.2).

Npas4 induction after Chem-LTD was partially blocked by pre-treatment with wortmannin, an inhibitor of phosphatidylinositol 3-kinases (PI3Ks), but was not significantly affected by treatment with U0126 which inhibits mitogen-activated protein kinase (MAPK) kinases (Coba *et al.* 2008). Conversely, *Npas4* induction after Chem-LTP was not affected by wortmannin treatment but was significantly reduced by pre-treatment with U0126 (Coba *et al.* 2008). KN-62, which inhibits Ca^{2+}/calmodulin-dependent protein kinases (Enslen *et al.* 1994) and SB203580, which inhibits the action of p38 kinases, failed to prevent *Npas4* induction in both protocols indicating that neither of those pathways is required for *Npas4* expression in the context of LTP or LTD (Coba *et al.* 2008). This indicates that while up-regulation of *Npas4* after LTP requires MAPK signalling, this pathway is dispensable for LTD-dependent *Npas4* induction which occurs largely through a PI3K-mediated mechanism (Figure 1.5).

1.4.2.4.3 Npas4 expression in the context of learning and memory

More recently, it was demonstrated that *Npas4* expression is induced by both formation and retrieval of fear memories. In rats, expression of *Npas4* was found to be transiently up-regulated in the lateral nucleus of the amygdala in a learning-dependent manner; in a Pavlovian fear conditioning model using tone-shock pairing, *Npas4* mRNA and protein expression were increased at 30min and 90min after training, respectively (Ploski *et al.* 2011). The increase in *Npas4* expression was specific to fear conditioning as a tone alone produced no change in expression while shock alone resulted in

only a mild increase in *Npas4* expression (Ploski *et al.* 2011). Retrieval of an auditory fear memory was also found to induce Npas4 protein expression; when a previously learned fear memory was reactivated by a tone, an increase in Npas4 protein expression was detected in the amygdala after 90min (Ploski *et al.* 2011).

1.4.3 Regulation of *Npas4* expression by neurotrophin signalling

1.4.3.1 Neurotrophins

Recently it has been demonstrated that neurotrophin signalling also regulates *Npas4* expression. The neurotrophins are a family of peptide growth factors that have diverse functions in the both the mature and developing CNS and are involved in the differentiation, maturation and survival of neurons, as well as processes such as synaptogenesis and synaptic plasticity (Henderson 1996; Huang and Reichardt 2001). Four members of the neurotrophin family, all highly related in both sequence and structure, have been identified in mammals; nerve growth factor (NGF), BDNF (see Section 1.5.2.1), neurotrophin-3 (NT3) and neurotrophin-4 (NT4). The neurotrophins are secreted factors that are synthesised as immature precursors called pro-neurotrophins before being processed by proteolytic cleavage, which can occur either intracellularly or extracellularly, to yield the mature form. Though it was first thought that pro-neurotrophins were merely inactive precursors, more recently it has been demonstrated that the pro-neurotrophins themselves can also act as signalling molecules with functions distinct to the mature neurotrophins (Lu *et al.* 2005).

1.4.3.2 Neurotrophin receptors

The neurotrophins form stable homodimers and exert their effect through binding of the tropomysin-related kinase (Trk) receptors which are a class of transmembrane proteins within the receptor tyrosine kinase (RTK) superfamily (Figure 1.6). Three Trk receptors exist in mammals and each receptor selectively binds the different neurotrophins with varying affinity; TrkA has highest affinity for NGF, TrkB is activated by both BDNF and NT4, while TrkC is highly specific for NT3 (Huang and Reichardt 2003).

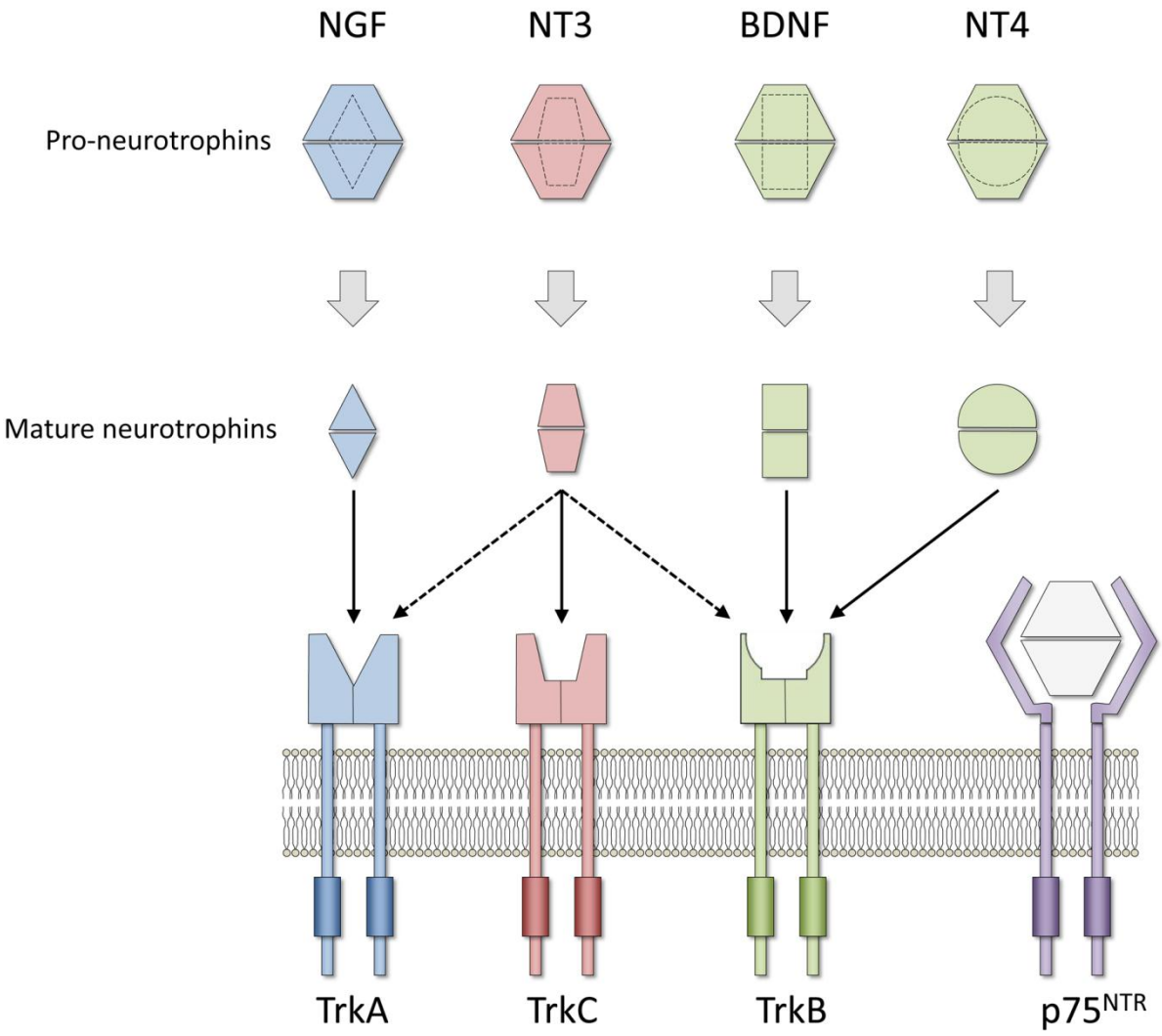


Figure 1.6 – Neurotrophins and neurotrophin receptors
TrkA has highest affinity for NGF but is also activated by NT3; TrkB is activated by both BDNF and NT4 but also has low affinity for NT3; TrkC is highly specific for NT3. The $p75^{NTR}$ binds all four of the neurotrophins, though it has weak affinity for the mature proteins and preferentially binds the unprocessed pro-neurotrophins. NGF - nerve growth factor; BDNF - brain-derived neurotrophic factor; NT3 - neurotrophin-3; NT4 - neurotrophin-4; Trk - tropomysin-related kinase; $p75^{NTR}$ - p75 neurotrophin receptor.

Upon binding of their cognate neurotrophins, Trk receptors undergo ligand-dependent dimerisation and subsequent auto-phosphorylation at tyrosine residues within the cytoplasmic domain. This allows adaptor proteins to be recruited which then trigger intracellular signalling cascades leading to many outcomes including gene regulation, neuronal survival and neurite outgrowth (Huang and Reichardt 2003). Alternative splicing of the Trk receptor genes can also give rise to naturally occurring truncated isoforms that lack tyrosine kinase activity and it is thought that these truncated isoforms modulate Trk receptor activity by acting as a dominant negative isoform (Eide *et al.* 1996) or by activating distinct signalling pathways (Baxter *et al.* 1997).

A further level of complexity is added to neurotrophin signalling as all of the neurotrophins are recognised by the non-selective p75 neurotrophin receptor ($p75^{NTR}$) which is structurally unrelated to the Trk receptors and instead belongs to the superfamily of tumour necrosis factor receptors. This receptor impacts upon neurotrophin signalling in several important ways (Hempstead 2002). Firstly, $p75^{NTR}$ can form heteromeric complexes with Trk receptors thereby changing their conformation and, hence, ligand binding properties (i.e. specificity and affinity for neurotrophins). Secondly, $p75^{NTR}$ indiscriminately interacts with all members of the neurotrophin family with equal affinity however, unlike the Trk receptors which only bind mature neurotrophins, it preferentially binds pro-neurotrophins in their unprocessed form. Lastly, in contrast to the Trk receptors, $p75^{NTR}$ lacks a catalytic kinase domain but instead contains an intracellular death domain which, upon activation of the receptor, can recruit factors that initiate signalling pathways distinct from those of Trk signalling, such as those that promote apoptosis. Thus, neurotrophin signalling can have vastly different, or even opposing, outcomes depending on the complement of neurotrophin receptors expressed by the target cell (ratio of Trk:$p75^{NTR}$ receptors) and the degree of pro-neurotrophins processing that occurs; while mature neurotrophins acting through canonical Trk receptors can promote neuronal survival, pro-neurotrophins acting through $p75^{NTR}$ can trigger apoptotic pathways (Lu *et al.* 2005).

1.4.3.3 Neurotrophin-dependent *Npas4* expression

It has been demonstrated that, in addition to activity-dependent nuclear Ca^{2+} signalling, *Npas4* mRNA is also regulated by a number of growth factor-mediated pathways. In PC12 cells, which express the TrkA receptor endogenously but lack TrkB receptors (Ip *et al.* 1993), treatment with the TrkA ligand Ngf (300ng/mL) induced up-regulation of endogenous *Npas4* mRNA while treatment with the TrkB ligand Bdnf (100ng/mL) had no effect on *Npas4* expression (Ooe *et al.* 2009a). The Ngf-mediated up-regulation of *Npas4* peaked at 30min after treatment and was blocked by pre-treating cells with

the PI3K inhibitor LY294002 while treatment with the MAPK kinase inhibitor U0126 had little effect (Ooe *et al.* 2009a). This indicates that Ngf signalling regulates *Npas4* expression specifically through activation of the PI3K pathway. Conversely, in primary cultures of rat cortical neurons (which do express endogenous TrkB receptors) treatment with Bdnf (100ng/mL) produced a rapid and robust induction of *Npas4* mRNA while Ngf (300ng/mL) had no effect (Ooe *et al.* 2009a). To demonstrate the specificity of Trk receptor involvement in *NPAS4* regulation, human neuroblastoma SH-SY5Y cells, which endogenously express neither TrkA nor TrkB receptors (Encinas *et al.* 1999), were transfected with expression vectors for either TrkA or TrkB which bestowed them with responsiveness to neurotrophins and consequently resulted in up-regulation of endogenous *NPAS4* mRNA after stimulation with either NGF or BDNF, respectively (Ooe *et al.* 2009a).

With regard to the intracellular signalling cascades that are responsible for neurotrophin-dependent *Npas4* induction, thus far only one pathway has been identified (Table 1.2). PI3K/Akt signalling downstream of the Ngf/TrkA interaction leads to increased *Npas4* expression via activation of the *Npas4* promoter as was demonstrated by experiments which showed that Ngf-dependent expression of a luciferase reporter gene under the control of a 5kb segment of the *Npas4* promoter could be blocked by pre-treatment with LY294002 (Ooe *et al.* 2009a).

Nevertheless, these findings are at odds with work published by others which showed that Npas4 did not respond to neurotrophin signalling (Lin *et al.* 2008). No change in Npas4 protein expression was visible in rat hippocampal neurons after treatment with Ngf (100ng/mL), Bdnf (50ng/mL), NT3 (50ng/mL) or NT4 (50ng/mL). This apparent discrepancy may be due to the difference in the concentration of growth factors and neuronal cell type used, or perhaps because *Npas4* mRNA and protein are regulated differently in response to neurotrophins. Nevertheless, further investigation is needed to unequivocally resolve this issue.

1.4.3.4 Other growth factors

Interestingly, induction of Npas4 seems to be specific to growth factors that act on neurotrophin receptors as no effect on Npas4 protein expression was observed when rat primary hippocampal neurons were treated with platelet-derived growth factor (100ng/mL), epidermal growth factor (100ng/mL), insulin-like growth factor 1 (100ng/mL) or the PKA-activating small molecule forskolin (10µM) (Lin *et al.* 2008).

Normal activation of Npas4			Effect of kinase signalling modifiers on Npas4 activation				Reference
Activation	**Cell Type**	**Stimulus**	**Treatment**	**Target**	**Pathway**	**Effect**	
↑ Npas4 mRNA after LTP	Mouse hippocampal slice	Forskolin (50μM) + Rolipram (100nM)	U0126 (20μM)	MEK1 and MEK2 inhibitor	MAPK/ERK	↓	(Coba *et al.* , 2008)
			Wortmannin (200nM)	PI3K inhibitor	PI3K/Akt	-	
			SB203580 (10μM)	MAPKAPK2 inhibitor	MAPK/p38	-	
			KN-62 (10μM)	CaMKII and CamKIV inhibitor	Ca^{2+}/CaMK	-	
↑ Npas4 mRNA after LTD	Mouse hippocampal slice	NMDA (20μM)	U0126 (20μM)	MEK1 and MEK2 inhibitor	MAPK/ERK	-	(Coba *et al.* , 2008)
			Wortmannin (200nM)	PI3K inhibitor	PI3K/Akt	↓	
			SB203580 (10μM)	MAPKAPK2 inhibitor	MAPK/p38	-	
			KN-62 (10μM)	CaMKII and CaMKIV inhibitor	Ca^{2+}/CaMK	-	
↑ Npas4 mRNA expression	PC12	NGF (300ng/mL)	LY294002 (10μM)	PI3K inhibitor	PI3K/Akt	↓	(Ooe *et al.* , 2009)
			U0126 (10μM)	MEK1 and MEK2 inhibitor	MAPK/ERK	-	
↑ Npas4 promoter activity	PC12	NGF (300ng/mL)	LY294002 (10μM)	PI3K inhibitor	PI3K/Akt	↓	(Ooe *et al.* , 2009)
			TPA	PKC activator	DAG/PKC	-	
			Chelerythrine chloride (10μM)	PKC inhibitor	PKC	-	
		-	Constitutively active Akt	#	PI3K/Akt	↑	
			Constitutively active PKA	#	cAMP/PKA	-	
↑ Npas4 transcriptional activity	PC12	NGF (300ng/mL)	U0126 (10μM)	MEK1 and MEK2 inhibitor	MAPK/ERK	↓	(Ooe *et al.* , 2009)
			PD98059 (10μM)	MAPKK inhibitor	MAPK/ERK	↓	
			LY294002 (10μM)	PI3K inhibitor	PI3K/Akt	-	
		-	Constitutively active MEK1	#	MAPK/ERK	↑	
			Constitutively active Akt	#	PI3K/Akt	-	
			Constitutively active PKA	#	cAMP/PKA	-	
↑ Npas4 mRNA	Mouse 1° hippocampal neurons	Bicuculline (50μM)	CaMBP4	Calmodulin	Ca^{2+}/CaM	↓	(Zhang *et al.* , 2009)
			Dominant negative CaMKIV	#	Ca^{2+}/CaMKIV	↓	

Table 1.2 – Regulation of Npas4 expression and activity by kinase signalling pathways
A summary of the effects of different kinases on Npas4 expression and activity. #These treatments affect more than one target.

1.4.4 Post-translational regulation of Npas4 expression and activity

The phosphorylation status of a protein often determines whether it is in an 'active' or 'inactive' state. Consequently phosphoregulation has developed as a commonly used mechanism of post-translational protein regulation in many aspects of cellular biology as it is rapid, reversible and binary in nature. Phosphoregulation of proteins is carried out by two classes of enzymes; kinases and phosphatases which attach or remove phosphate groups from proteins, respectively. These enzymes participate in complex signalling pathways that frequently begin extracellularly at the surface of the cell membrane where binding of a ligand to its receptor triggers an intracellular cascade ultimately resulting in changes in gene expression in the nucleus.

There is evidence that the expression and activity of Npas4 is regulated by different sets of kinases under different conditions (summarised in Table 1.2). For example, as described earlier, it seems that the PI3K/Akt pathway is important for the up-regulation of *Npas4* mRNA that is observed after NMDA-induced Chem-LTD (Coba *et al.* 2008) or stimulation with NGF (Ooe *et al.* 2009a). However, in contrast, *Npas4* mRNA induction after Chem-LTP did not require PI3K/Akt signalling, but instead was dependent on the MAPK pathway (Coba *et al.* 2008). Furthermore, the MAPK signalling pathway downstream of Ngf/TrkA signalling has also been shown to regulate the activity of the Npas4 protein on a post-translational level. Activity of the Npas4 C-terminal TAD was decreased in a one-hybrid reporter assay in the presence of MAPK kinase inhibitors U0126 (10µM) or PD98059 (10µM) while over-expression of a constitutively active form of the MAPK kinase MEK1 was sufficient to increase transcriptional activity of Npas4 (Ooe *et al.* 2009a). Activation of the TrkA receptor by Ngf induces MAPK-dependent phosphorylation of the Npas4 protein which is prevented by treatment with MAPK kinase inhibitors U0126 (10µM) or PD98059 (10µM) (Ooe *et al.* 2009a). This post-translational modification is hypothesised to confer increased transcriptional activity, though the phosphorylation site has not been identified. Thus, Ngf signalling increases both expression of *Npas4* mRNA and activity of the Npas4 protein via two different kinase pathways.

One group reported that Npas4 activity may be regulated at a post-translational level specifically in the context of cerebral ischaemia as several immunoreactive species of different sizes were detected upon immunoblotting of whole-tissue homogenates prepared from rat brain at various time points following focal cerebral ischemia using antibodies generated to the Npas4 protein (Shamloo *et al.* 2006). Specifically, a 200kDa immunoreactive species present in the contralateral hemisphere was seen to be down-regulated in the ipsilateral hemisphere after cerebral ischaemia, while a

simultaneous increase in a smaller 100kDa species was observed. The same observation was noted with two different polyclonal antibodies (generated to either the N-terminal or C-terminal portion of the Npas4 protein, respectively) however the identity of this 200kDa species is unknown and, furthermore, it has not been reported in any other studies in which the size of the Npas4 protein was investigated.

The authors speculate that under normal conditions Npas4 may exist in a complex bound by chaperone molecules where it is sequestered until an appropriate signal, for instance one resulting from cerebral ischaemia, directs the dissociation of Npas4 from this complex yielding the 100kDa active form. However, these results are unconvincing as the increase in the 100kDa species after ischaemia was not always proportional to the decrease in the 200kDa species. Furthermore, the presence of many spurious, and likely non-specific, signals on the immunoblots indicates that the antibodies used were not specific to the Npas4 epitope and, as such, any conclusions drawn from unidentified signals are dubious. It cannot be ruled out that the 200kDa signal merely represents cross-reactivity of the anti-Npas4 antibody to a protein of that size which, coincidentally, is down-regulated in response to cerebral ischemia.

1.4.5 *Npas4* expression in response to psychological stress

1.4.5.1 Psychological stress

Recently, it has been reported that *Npas4* expression is down-regulated by various forms of psychological stress, both chronic and acute. In biological terms, stress can be defined as any condition that perturbs the physiological or psychological homeostasis of an organism (Kim and Diamond 2002). One of the key events in the re-establishment of homeostasis after stress is the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, a cascade of signalling events that leads to elevated levels of glucocorticoids in the bloodstream. In response to stress, cells of the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing factor (CRF) which triggers release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland which in turn stimulates the production of glucocorticoids from the adrenal cortex. Glucocorticoids are a class of steroid hormones that are involved in many metabolic processes that are critical for adaptation to stress such as gluconeogenesis, immunosuppression and fat catabolism, however, prolonged exposure to high levels of glucocorticoids can be damaging (Sapolsky *et al.* 1985). The brain is an organ that is particularly sensitive to stress and the mechanism by which it responds to this challenge differs depending on whether the stress is acute or chronic (Choi *et al.* 2008).

1.4.5.2 Acute psychological stress

The physiological response to acute stress is rapid and involves a series of coordinated processes, both genomic and non-genomic, that together enable the organism to adapt to the stressful stimulus. Activation of the HPA axis occurs immediately and elevated levels of glucocorticoids are detectable in the bloodstream within minutes. In rodents, plasma concentrations of corticosterone, the primary glucocorticoid produced in response to stress, peak within 30min following the onset of stress (Imaki *et al.* 1995; Girotti *et al.* 2006) although the levels of corticosterone in the brain do not peak until 20min later (Droste *et al.* 2008). Tight regulation of circulating corticosterone levels is achieved through auto-inhibition of the HPA axis. Corticosterone represses the transcription of CRF creating a negative feedback loop such that levels of plasma corticosterone are restored to baseline within 60min of the the onset of stress (Imaki *et al.* 1995).

1.4.5.2.1 IEG expression in response to acute psychological stress

In addition to production of steroid hormones, acute stress also stimulates rapid up-regulation of IEGs in the brain via *de novo* mRNA synthesis. Transcription is initiated immediately and mRNA expression of IEGs reaches a peak at approximately 30min after the onset of stress (Cullinan *et al.* 1995; Imaki *et al.* 1995; Girotti *et al.* 2006). The induction of IEGs after stress is unrelated to HPA axis activation and occurs via an independent mechanism and therefore glucocorticoids are not involved in the up-regulation of IEGs (Helmreich *et al.* 1996). Nevertheless, there is some interaction between the two systems as, in addition to its auto-inhibitory effects, corticosterone also negatively regulates transcription of IEGs (Herman *et al.* 1992; Imaki *et al.* 1995).This repression results in a rapid decline in IEG expression such that mRNA levels are restored to baseline within 60min of the onset of stress (Imaki *et al.* 1995; Girotti *et al.* 2006). While the functional role of IEG products in the context of acute stress is not known, their expression has proven useful in highlighting areas of the brain that are affected by particular stresses.

1.4.5.3 Chronic psychological stress

It is now well accepted that the adult brain is not a static organ, but one which has the capacity to reorganise itself structurally and adapt functionally in response to appropriate environmental factors – this is a property known 'neural plasticity'. One such experience is chronic psychological stress which can have profound and long-lasting effects on the brain by altering the nature of neuronal connections, especially when experienced during early life. Various experimental paradigms can be used to simulate chronic stress in animals (for instance, rearing them in social isolation or

subjecting them to repeated restraint stress) and these models may have relevance to human psychiatric disorders such as anxiety, post-traumatic stress disorder, obsessive-compulsive disorder and schizophrenia. Much work has been done using these models in order to gain a better understanding of the mechanisms underlying the adaptive changes that occur in the brain as a result of long-term stress.

The effects of chronic stress are varied and can be in the form of morphological, neurochemical, and/or behavioural changes. Studies investigating the effects of social isolation have revealed a plethora of outcomes including:

- Decreased hippocampal neurogenesis (Ibi *et al.* 2008)
- Fewer synaptic connections between hippocampal neurons (Varty *et al.* 1999)
- Atrophy of hippocampal neuron dendrites (Vyas *et al.* 2002; Silva-Gomez *et al.* 2003)
- Decreased expression of Bdnf in the hippocampus (Scaccianoce *et al.* 2006)
- Increased aggressive behaviour (Wongwitdecha and Marsden 1996)
- Impaired spatial learning and memory (Lu *et al.* 2003)

Similarly, chronic restraint stress was also found to result in decreased hippocampal neurogenesis (Pham *et al.* 2003), reduced expression of Bdnf in the hippocampus (Smith *et al.* 1995) and atrophy of apical dendrites of hippocampal pyramidal neurons (Watanabe *et al.* 1992).

It is hypothesised that many of the changes stemming from chronic stress are mediated by changes in gene expression in plastic areas of the brain. One group of genes touted as being likely candidates for instigating these changes are the IEGs as they provide a molecular link between external stimuli (conveyed as neuronal activity) and cellular changes driven by gene transcription (Vendrell *et al.* 1993).

1.4.5.3.1 Repression of IEGs in response to chronic psychological stress

While acute stress leads to an induction of IEGs, prolonged or repetitious stress is associated with habituation and a concomitant suppression of IEG activation (Melia *et al.* 1994). Decreased basal expression of IEGs has been observed in the prefrontal cortex after social isolation (Levine *et al.* 2007) as well as in the hypothalamus following chronic restraint stress (Girotti *et al.* 2006). The mechanisms underlying down-regulation of IEGs after chronic stress are not known, however there are several hypotheses. Given that many IEGs are activity-regulated, it has been suggested that reduced

expression of these genes reflects a decrease in the amount of excitatory synaptic transmission occurring in the corresponding brain regions (Silva-Gomez *et al.* 2003; Levine *et al.* 2007). This seems like a plausible explanation in the case of social isolation where rodents are deprived of stimuli such as social interaction and new scents for an extended period of time. An alternative hypothesis proposes that, rather than a reduction in neuronal activity, there is an 'uncoupling' of the stimulus/IEG response such that the same level neural activity no longer induces IEG expression. According to this hypothesis, inhibition of sensory processing results in 'stress desensitisation' such that the threshold required for activation of IEGs is gradually raised as a result of repeated exposure to the same stress (Girotti *et al.* 2006).

1.4.5.4 The effect of psychological stress on *Npas4* expression

Several studies have shown that psychological stress inhibits *Npas4* expression. In mice that had experienced four weeks of either social isolation (Ibi *et al.* 2008) or chronic restraint stress (Yun *et al.* 2010), there was a significant reduction in *Npas4* mRNA expression in the hippocampus. These data are consistent with observations that many other IEGs are also down-regulated by chronic stress (Melia *et al.* 1994; Girotti *et al.* 2006; Levine *et al.* 2007). As will be discussed later in Section 1.5.2, Npas4 directly regulates transcription of *Bdnf* (Lin *et al.* 2008; Pruunsild *et al.* 2011) and *Drebrin* (Ooe *et al.* 2004), a gene involved in dendritic spine plasticity (Ivanov *et al.* 2009), and thus it is tempting to speculate that some of the molecular and neuroanatomical changes associated with chronic stress (see Section 1.4.5.3) may arise as a direct result of decreased expression of Npas4 target genes.

Surprisingly, it has been reported that *Npas4* expression is also decreased following exposure to acute stress. Naïve mice exposed to a single episode of restraint stress lasting either 2h or 6h exhibited lower levels of *Npas4* mRNA expression in the hippocampus compared to control animals (Yun *et al.* 2010). This finding seems counter-intuitive given that most IEGs are up-regulated in response to acute stress (Cullinan *et al.* 1995), however, it should be noted that measurement of *Npas4* expression occurred 2h and 4h after the onset of restraint stress - well after the time expression of most IEGs has returned to baseline levels (Cullinan *et al.* 1995; Imaki *et al.* 1995; Girotti *et al.* 2006). If it is assumed that *Npas4* responds to acute stress in a manner similar to other IEGs, then it is possible that *Npas4* expression had already peaked within 30min of the onset of stress and had already been down-regulated by the time expression was measured. Thus, a more comprehensive analysis of the temporal expression profile of *Npas4* must be undertaken before any conclusions can be drawn about the effect of acute stress on *Npas4* expression. In the same study,

the authors showed that a single subcutaneous injection of corticosterone (10mg/kg) had a similar effect and that *Npas4* mRNA expression was reduced in the hippocampus 4h after injection (Yun *et al.* 2010). This result, however, is not surprising as glucocorticoids are known to inhibit transcription of IEGs (Herman *et al.* 1992; Imaki *et al.* 1995) and thus administration of corticosterone alone, without the associated stimulus of stress to induce IEG expression, could be expected to result in repression of IEGs.

1.4.6 *Npas4* expression in response to cellular stress

1.4.6.1 Oxidative stress

A cell is considered to be under oxidative stress when the normal redox status of the cell is disrupted such that it is shifted to a more oxidized state which causes pathology. Oxidative stress is characterised by the intracellular accumulation of oxygen species such as nitric oxide and superoxide (O_2^-) which under normal circumstances are not damaging (in fact, they are important in many biological processes, such as host defence from microbial invasion), however, when in excess they react to spontaneously form the highly toxic molecule peroxynitrite (Pacher *et al.* 2007). Under normal conditions, scavenging enzymes with antioxidant properties, such as the superoxide dismutases (SODs), remove O_2^- molecules before they can accumulate to pathological levels. The deleterious effects of oxidative stress on cellular function are diverse and affect virtually all aspects of cell biology as they encompass oxidation of proteins, lipids and DNA. One consequence of oxidative stress is that Ca^{2+} signalling is also disrupted. Oxidative stress causes an increase in cytoplasmic Ca^{2+} via influx from the extracellular environment and/or release from intracellular stores (Ermak and Davies 2002) which consequently can have effects on gene expression.

SIN-1 is a compound that, when in solution, spontaneously liberates both nitric oxide and O_2^- and is therefore often used to experimentally induce oxidative stress in cultured cells. Recently, it was shown that treatment of PC12 cells with 1μM SIN-1 resulted in increased *Npas4* mRNA expression which peaked at 3h after treatment and could be blocked by co-treatment with 1mg/mL SOD (Ooe *et al.* 2009b). While this peak in *Npas4* expression occurs later than what is seen for other stimuli, the authors speculate that the delay in *Npas4* induction may be because generation of oxidative radicals from SIN-1 in culture medium requires a few hours. Another compound used to induce oxidative stress in the CNS is 6-hydroxydopamine (6-OHDA), an analogue of the natural catecholamine neurotransmitter dopamine. Several factors contribute to the cytotoxicity of 6-OHDA; firstly, 6-OHDA inhibits SOD activity (Perumal *et al.* 1989) and, secondly, oxidation of 6-OHDA itself produces

hydrogen peroxide, O_2^- and hydroxyl radicals (Cohen and Heikkila 1974). Treatment of rats with 6-OHDA induced up-regulation of Npas4 protein in the CA1 and CA3 regions of the hippocampus as well as the cortex (Ooe *et al.* 2009a). While these data could, as the authors suggest, indicate that Npas4 expression is induced by oxidative stress, it is also possible that it is not the reactive oxygen species *per se* that cause Npas4 up-regulation, but rather a secondary effect of oxidative stress, such as increased intracellular Ca^{2+}. These two possibilities could be distinguished by repeating the experiment in the presence of pharmacological agents that block Ca^{2+} channels on the ER and cell membranes, thus preventing a change in cytoplasmic Ca^{2+} concentration under oxidative conditions.

1.4.6.2 Osmotic stress

An isotonic state, where concentrations of solutes are equal on both sides of the cell membrane, is essential for proper functioning of virtually all cellular processes and disruption of this balance leads to osmotic stress. When the concentration of solutes outside the cell is much greater than the intracellular concentration, the cell is said to be under hyperosmotic stress while, conversely, when the concentration of solutes is higher in the cytoplasm than the surrounding solution, the cell is said to be under hypoosmotic stress. Sorbitol is an organic osmolyte which has a sugar alcohol structure that resembles glucose, however, unlike glucose, is not readily absorbed by cells and thus when introduced into the medium of cultured cells induces hypertonic stress. One cellular response to hypertonic stress is the osmotic efflux of water from the cell which results in an increase in the concentration of all intracellular constituents, including Ca^{2+}.

PC12 cells subjected to hyperosmotic stress by treatment with sorbitol (1M) showed an increase in *Npas4* mRNA expression, however, unlike other stimuli that induce *Npas4* expression, the up-regulation was slightly delayed beginning 2h after treatment and reaching a peak at 4h post-treatment (Ooe *et al.* 2009b). Hyperosmotic stress is known to induce Ca^{2+} transients in mammalian cells (Dascalu *et al.* 1995; Erickson *et al.* 2001) however they occur within seconds to minutes and thus are unlikely to be the cause of *Npas4* up-regulation in this case. Another consequence of hyperosmotic stress is the activation of various kinase signalling cascades, such as the MAPK pathway (Itoh *et al.* 1994), which in turn stimulate expression of IEGs (Cohen 1997). The expression of prototypical IEGs *c-fos* and *Egr1* was increased in canine kidney cells that were subjected to hyperosmotic conditions (100mM NaCl) with mRNA levels peaking between 2-6h after stimulation (Cohen *et al.* 1991). This delayed induction is strikingly similar to that seen for *Npas4* in

response to sorbitol treatment and therefore perhaps a similar mechanism is responsible for induction of Npas4 expression under hyperosmotic challenge.

1.4.7 The effect of drugs of abuse on *Npas4* expression

In a study investigating the effect of rewarding doses of various drugs of abuse on gene expression in the mouse striatum, it was found that *Npas4* mRNA was up-regulated in a pattern typical of other IEGs. *Npas4* expression was induced by acute administration of cocaine, methamphetamine, morphine and heroin, but not nicotine or ethanol, and this general pattern of response was shared by other IEGs including *c-fos*, *Arc* and *Egr1* (Piechota *et al.* 2010). Almost all drugs of abuse are known to increase the expression of IEGs and, though the pharmacology of each drug is different, GluR activation appears to be a common mechanism shared by most classes of drugs (Harlan and Garcia 1998).

1.4.7.1 Psychostimulants: cocaine and methamphetamine

Administration of either cocaine (25mg/kg) or methamphetamine (2mg/kg) induced rapid up-regulation of *Npas4* mRNA in the striatum, though the duration of this up-regulation varied; after cocaine injection, *Npas4* mRNA levels had returned to baseline with 2hr, whereas expression remained elevated 4h after methamphetamine injection (Piechota *et al.* 2010). Both of these drugs are known to stimulate expression of IEGs, such as *c-fos*, in the striatum (Graybiel *et al.* 1990) and, furthermore, it has been shown that this induction is dependent on NMDAR signalling. Blockade of NMDAR activation using a selective antagonist prevented induction of *c-fos* expression after treatment with cocaine (Torres and Rivier 1993) or methamphetamine (Konradi *et al.* 1996). While the mechanisms underlying psychostimulant-dependent *Npas4* induction are not known, it is possible that these drugs stimulate *Npas4* expression via activation of NMDARs.

1.4.7.2 Opioids: morphine and heroin

Treatment with the opioids morphine (20mg/kg) or heroin (10mg/kg) also caused up-regulation of *Npas4* expression though, unlike the psychostimulant drugs, the effect was not immediate; increased mRNA expression was not observed until 4h after opioid injection though by 8h it had already returned to baseline levels (Piechota *et al.* 2010). Initially, it might seem counter-intuitive that *Npas4* expression is induced by opiates as they are potent CNS depressants that antagonise the action of NMDARs, inhibit the action of voltage-gated Ca^{2+} channels and generally decrease the excitability of target neurons that express opioid receptors (McCleane and Smith 2007; Trescot *et al.* 2008).

However, by repressing the excitability of one specific population of neurons in the brain that are responsive to the drug, they can also have indirect trans-synaptic effects on other neuronal populations that are distal to the site of action and do not express opioid receptors. For example, if the neurons expressing opioid receptors are inhibitory GABAergic neurons that project onto glutamate-releasing neurons, then, by repressing the inhibitory effect that GABAergic neurons impose on the network, opiates can indirectly stimulate glutamatergic synaptic transmission through disinhibition. Hence, like pyschostimulant drugs, opioid use is also associated with an increase in IEG expression in the striatum which is mediated by signalling through GluRs (Liu *et al.* 1994; Garcia *et al.* 2003). This may provide an explanation for the increase in *Npas4* expression seen after opioid treatment, though, if this were the case, it is still unclear why there is a delay of 4h before the onset of *Npas4* mRNA transcription.

1.4.7.3 Other drugs of abuse: nicotine and ethanol

Interestingly, there did not seem to be a significant change in *Npas4* expression after treatment with either nicotine (1mg/kg) or ethanol (2g/kg) (Piechota *et al.* 2010), even though both of these drugs have previously been reported to induce expression of other IEGs (Harlan and Garcia 1998). Ethanol is a depressant having diverse effects on CNS function depending on factors such as dose and route of administration (Pohorecky and Brick 1988). Nevertheless, a large number of studies have been published citing an increase in IEG expression following ethanol treatment (Vilpoux *et al.* 2009). Nicotine is a CNS stimulant and, like cocaine and methamphetamine, has been shown to induce *c-fos* expression in the striatum through an NMDAR-dependent mechanism (Kiba and Jayaraman 1994). However, it should be noted that mRNA levels of *c-fos* and other IEGs were also not significantly altered in response to nicotine or ethanol in the study conducted by Piechota *et al.* indicating that perhaps the treatment paradigm used with these drugs was not one which was conducive to IEG expression in the striatum.

1.4.8 Circadian regulation of *Npas4* expression

It has been shown that expression of *Npas4* in the pars tuberalis of the anterior pituitary is higher at night than during the day and that this elevated nocturnal expression is dependent upon melatonin signalling (Unfried *et al.* 2010). This suggests that, in this part of the brain, *Npas4* expression may be regulated by circadian rhythm, though the physiological relevance of this is unclear. In this section, the possible molecular mechanisms underlying circadian regulation of *Npas4* in the pars tuberalis, and the biological significance that this holds, will be discussed.

1.4.8.1 Circadian rhythm

Circadian rhythm refers to the innate ability of organisms to maintain a constant pattern of biochemical, physiological and behavioural processes coordinated to the daily 24h light/dark cycle via an internal timing mechanism. Because of their importance, circadian rhythms have been used throughout evolution and have been found in plants, animals, fungi and even bacteria. In animals, the periodic oscillations generated by the circadian rhythm regulate many vital physiological processes such as body temperature, sleep/wake cycle and endocrine function.

1.4.8.1.1 The biological clock of the suprachiasmatic nuclei

In mammals, regulation of the circadian rhythm occurs in the hypothalamus within a collection of specialised neurons called the suprachiasmatic nuclei (SCN). The 'master clock' of the SCN is entrained to the 24h day by light entering through the retina and it coordinates the timing of slave clocks in other tissues such as the kidneys and liver (Reppert and Weaver 2002). The periodicity of the SCN master clock can be generated, and maintained, internally even in the absence of external cues. In a series of elegant experiments, it was demonstrated that each of the neurons in the SCN is able to autonomously maintain its own circadian period without intercellular communication involving action potentials (Schwartz *et al.* 1987; Welsh *et al.* 1995). This is because the time-keeping mechanism is genetically based and is therefore intrinsic to the cell.

In mammals, the intracellular clock mechanism which generates circadian rhythm is controlled by a set of genes collectively known as the 'clock genes' (Herzog *et al.* 1998). Two bHLH PAS transcription factors, Clock and Bmal1 (see Table 1.1), form the positive regulatory arm of the clock mechanism. Clock and Bmal1 dimerise to form a transcriptional complex that directs expression of a number of Clock-controlled genes (CCGs), including the three Period genes (*Per1*-*Per3*) and two Cryptochrome genes (*Cry1* and *Cry2*). The Per and Cry proteins form the negative arm of the clock mechanism by dimerising to form a complex that translocates to the nucleus and inhibits the activity of the Clock/Bmal1 dimer. In the absence of Clock/Bmal1, the Per and Cry proteins are rapidly degraded which allows levels of Clock/Bmal1 to rise once more, thus completing the cycle. In this way, a complex and self-perpetuating regulatory system is established involving both positive and negative feed-back loops and culminating in sinusoidal mRNA and protein expression of Clock genes (and hence their target genes) with an oscillation frequency of approximately 24h (Reppert and Weaver 2001).

1.4.8.1.2 Clock output and circadian control of melatonin release

In order for the circadian rhythm of the SCN to be imposed on other parts of the body, the rhythmic fluctuations in Clock gene expression must be converted into a signal which can be readily transmitted to other circadian effectors throughout the body. Evidence suggests that rhythmic synaptic transmission is likely to be the principal mode of output from the SCN; whole-cell patch-clamp recording of neurons in SCN brain slices showed that the spontaneous firing rate of SCN neurons follows a clear circadian rhythm with the highest firing rate seen during the subjective day (Schaap *et al.* 1999). It is hypothesised that the rhythmic oscillation of Clock target genes drives changes in the cell biology of SCN neurons (for example, changes in membrane potential or ion channel expression) that are responsible for creating these rhythmic oscillations in SCN neuronal firing (Reppert and Weaver 2001). Projections from the SCN then convey this information to other areas of the brain where these oscillating electrical signals produce neurochemical or physiological changes resulting in the expression of circadian behaviours. One of the regions receiving input from the SCN via a polysynaptic efferent pathway is the pineal gland, the major site of melatonin synthesis and secretion. There is evidence that the SCN precisely regulate the timing of melatonin release by modulating the amount of noradrenergic signalling reaching the pineal gland (Kalsbeek *et al.* 1999). Thus, one of the primary ways in which the SCN exert their effect on the physiology of the organism is by establishing a daily, rhythmic pulse of melatonin release.

1.4.8.2 Melatonin and melatonin receptor signalling

Melatonin (5-methoxy-N-acetyltryptamine) is a neurohormone that is produced in the pineal gland from the precursor serotonin (Borjigin *et al.* 1999). Light entering the eyes leads to suppression of melatonin production and therefore melatonin secretion is high during the night and low during the day (Brainard *et al.* 1997). Because the duration of nocturnal melatonin secretion is directly proportional to the length of darkness, it provides the organism with essential information regarding photoperiod (and, therefore, seasonal cycles) making it an extremely important chronobiological regulator. Melatonin acts through the neuroendocrine system to regulate many biological processes related to circadian rhythm, such as sleep/wake cycle, body temperature and glucocorticoid production, as well as processes related to seasonal behaviour such as coat growth and reproductive activity (Arendt and Skene 2005). One of the many functions of melatonin is to act as a zeitgeber (or 'time giver') which entrains peripheral circadian oscillators to the SCN master clock. In this capacity, melatonin functions as a messenger hormone that translates the circadian output of the SCN into a neuroendocrine signal that can be transmitted to peripheral tissues expressing melatonin receptors.

1.4.8.2.1 Melatonin receptors

The many functions of melatonin are mediated by signalling through high affinity melatonin receptors. Melatonin receptors are seven-transmembrane domain proteins that form a subfamily within the G protein-coupled receptor superfamily. In mammals, there are two canonical melatonin receptors named MT1 and MT2. Expression of melatonin receptors can be found in many parts of the brain though there is considerable inter-species variation in the distribution of their expression (Morgan *et al.* 1994). The pars tuberalis of the anterior pituitary is considered to be the primary site of melatonin action as it has the highest concentration of MT1 receptors of all tissues and it is the only tissue in which MT1 expression was detected in all mammalian species tested (Morgan *et al.* 1994). In the human brain, MT1 expression has also been detected in the SCN, cortex, cerebellum, thalamus, amygdala, striatum, substantia nigra and, in lower amounts, the hippocampus (Jockers *et al.* 2008). MT2 is primarily expressed in the retina (Reppert *et al.* 1995) and is thought to participate in light-dependent functions (von Gall *et al.* 2002). In some species, melatonin receptor expression has also been detected in a number of peripheral tissues such as the spleen and reproductive organs (Morgan *et al.* 1994). In regions where they are co-expressed, melatonin receptors can form oligomeric MT1/MT2 complexes changing their pharmacological activity and adding further complexity to melatonin signalling (Jockers *et al.* 2008).

1.4.8.2.2 Melatonin-dependent circadian regulation of gene expression in the pars tuberalis

Melatonin acts as a pivotal link between the SCN central clock and peripheral clocks; the recurring nocturnal pulse of melatonin drives circadian expression of clock genes within peripheral tissues that express melatonin receptors. Due to its high concentration of MT1 receptors, the pars tuberalis of the anterior pituitary is a major target of melatonin signalling in the brain and melatonin has been shown to drive circadian expression of several clock genes in the rodent pars tuberalis (Messager *et al.* 1999; Lincoln *et al.* 2002; Johnston *et al.* 2005). In mice lacking functional MT1 receptors, rhythmic expression of *Clock*, *Bmal1*, *Per1* and *Cry1* was completely abolished in the pars tuberalis, however, this effect was not seen when MT2 receptor function was disrupted indicating that circadian regulation of clock gene expression in the pars tuberalis is dependent on signalling though the MT1 receptor (von Gall *et al.* 2005). There is evidence that, in addition to clock genes, melatonin can also regulate the expression of early response genes in the pars tuberalis (Morgan 2000), though the mechanism underlying this phenomenon is unknown.

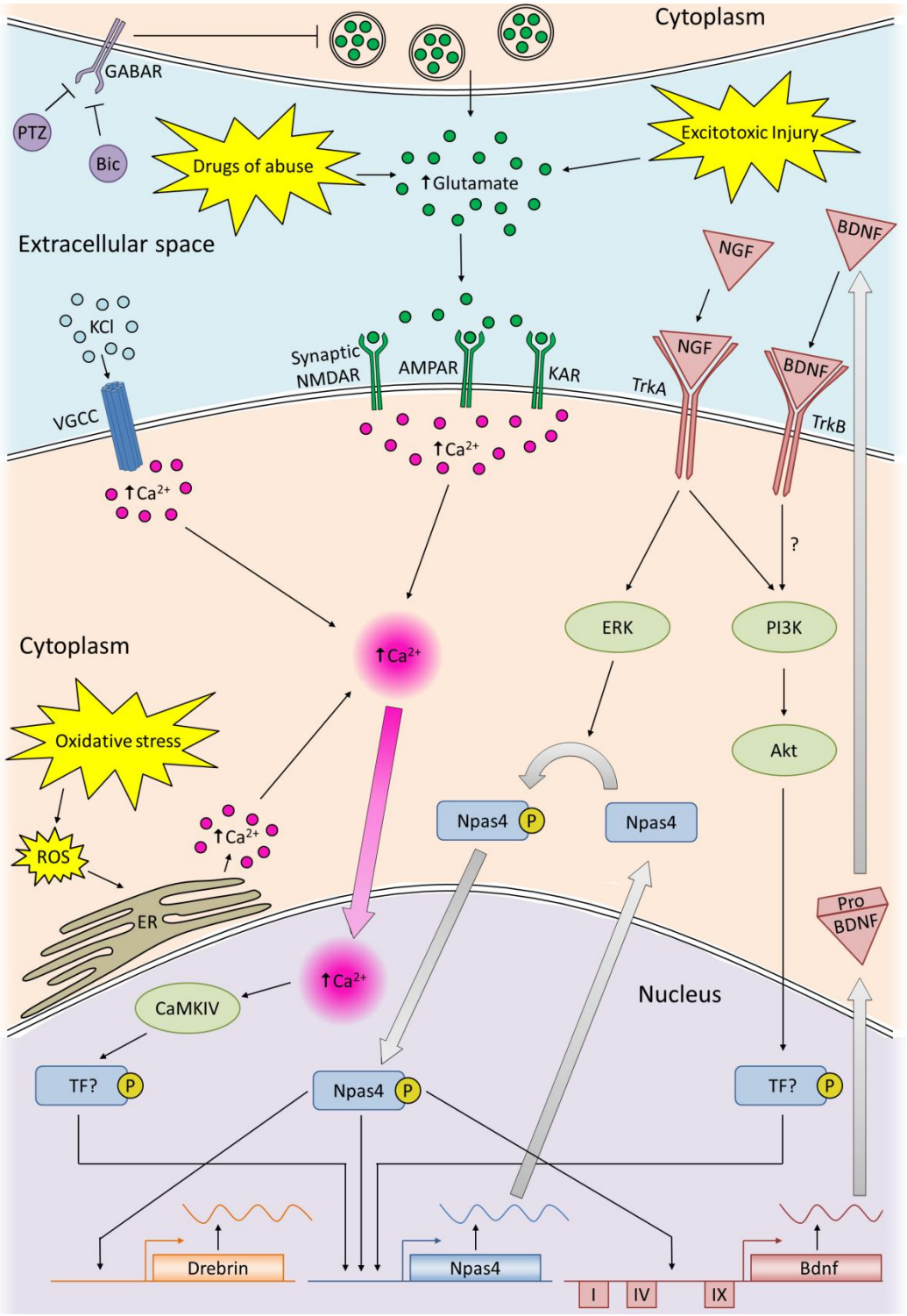


Figure 1.7 – Regulation of Npas4 expression and activity

A schematic summarising some of the factors that influence Npas4 expression and activity (see text for details). GABAR - GABA receptor; PTZ - pentylenetetrazol; Bic - bicuculline; KCl - potassium chloride; VGCC - voltage-gated calcium channel; NMDAR - NMDA receptor; AMPAR - AMPA receptor; KAR - kainate receptor; NGF - nerve growth factor; BDNF - brain-derived neurotrophic receptor; Trk - tropomysin-related kinase; ROS - reactive oxygen species; ER - endoplasmic reticulum; Ca^{2+} - calcium; ERK - extracellular signal-regulated kinase; PI3K - phosphatidylinositol 3-kinase; Akt - protein kinase B; CaMKIV - Ca^{2+}/calmodulin-dependent protein kinase type IV; TF? - unknown transcription factor; P - phosphate group.

1.4.8.3 Circadian expression of *Npas4* in the pars tuberalis

Recently, evidence has emerged which suggests that *Npas4* expression in some parts of the brain may be regulated by circadian rhythm. In mice, *Npas4* mRNA expression in the pars tuberalis was found to be significantly higher at mid-subjective night than at mid-subjective day (Unfried *et al.* 2010). Being nocturnal, mice are more active at night than during the day and thus it is possible to reason that the increase in *Npas4* expression observed at night is merely due to increased physiological and/or neural activity, as has been reported for the IEG *c-Fos* in other parts of the brain (Grassi-Zucconi *et al.* 1993). However, this appears not to be the case as the night-time peak in *Npas4* mRNA expression was abolished in MT1-deficient mice (Unfried *et al.* 2010) suggesting that the nocturnal increase in *Npas4* is dependent on signalling through the MT1 receptor. As only two time points were selected, more detailed analysis is needed to determine whether *Npas4* expression in the pars tuberalis oscillates in a true 24h circadian rhythm, however, if so, this raises two interesting possibilities; (1) that *Npas4* expression may also be under the control of circadian melatonin signalling in other parts of the brain where MT1 receptors are expressed (such as the SCN), and (2) that *Npas4* expression displays seasonal variation like other genes that are regulated by melatonin in the pars tuberalis, such as prolactin (Morgan 2000).

1.4.8.3.1 Possible mechanisms involved in circadian expression of Npas4

The mechanisms underlying MT1-dependent *Npas4* induction have not yet been elucidated, though several possibilities exist. One possibility is that stimulation of MT1 receptors can directly influence *Npas4* transcription through the action of intracellular signalling cascades. While there is evidence that MT1 signalling can generate Ca^{2+} transients in the cell through the mobilisation of Ca^{2+} from intracellular stores (Brydon *et al.* 1999), this is at odds with data published by others which suggest that melatonin prevents elevation of intracellular Ca^{2+} by inhibiting Ca^{2+} influx (Vanecek and Klein 1992; Slanar *et al.* 2000; Nelson *et al.* 2001). As the effect of melatonin signalling on intracellular Ca^{2+} levels is not clear, it is difficult to speculate on the feasibility of this hypothesis and further experimentation is needed to clarify this issue. Alternatively, melatonin signalling may cause nocturnal up-regulation of *Npas4* in the pars tuberalis via an indirect mechanism. Rhythmic melatonin signalling drives circadian expression of clock genes in the pars tuberalis (von Gall *et al.* 2005), which in turn confers a circadian pattern of expression to their target genes. It is possible, therefore, that *Npas4* could be, either directly or indirectly, regulated by the clock genes. Interestingly, in mice lacking Per1, rhythmic expression of *Npas4* in the pars tuberalis persists, though the amplitude of expression is reduced during both day and night compared to wildtype mice

(Unfried *et al.* 2010). This suggests that the molecular clock mechanism may contribute to baseline *Npas4* expression, but is not essential for its circadian oscillation.

Interestingly, there was no change in *Npas4* mRNA expression in the pars tuberalis 2.5h after mice were given a single subcutaneous injection of melatonin (1mg/kg) during the daytime (Unfried *et al.* 2010). While this may seem to conflict with the notion that *Npas4* is regulated by melatonin, it is in keeping with the observations of others which suggest that the effects of melatonin on IEG expression are phase-specific. In a similar study performed in rats, subcutaneous injection of melatonin (100µg/kg) at night produced a robust up-regulation of both *c-fos* mRNA (45min after injection) and protein (1h and 2h after injection) while the same treatment given during the day failed to increase *c-fos* expression (Kilduff *et al.* 1992). Furthermore, the authors found that the up-regulation of *c-fos* expression in response to melatonin injection was specific to the SCN and no such induction was observed in the pars tuberalis (Kilduff *et al.* 1992). This suggests that melatonin-mediated expression of IEGs in the SCN differs to that in the pars tuberalis and is worth investigating in the context of *Npas4* expression.

1.4.9 Post-transcriptional regulation of *Npas4* expression

Npas4 expression is highly restricted, both temporally and in regard to cell type, which suggests a complex and manifold regulatory mechanism. In particular, the short half-life of the *Npas4* mRNA suggests that there may be regulation of Npas4 occurring at the post-transcriptional level. One class of post-transcriptional regulators are microRNAs (miRNAs), short single-stranded RNA molecules that modulate gene expression by modifying the stability of target mRNAs are (see Section 4.1.1). Therefore, the possibility that *Npas4* is regulated by miRNAs was investigated using bioinformatics prediction. The miRNA target prediction software TargetScan was used to analyse the highly conserved 3' UTR of the mouse *Npas4* mRNA and a total of nine putative miRNA binding sites were identified (Figure 1.8). These sites are well conserved across mammalian species and among them are represented six different miRNA families. What is known about the expression and function of these miRNA families is summarised in Table 1.3. Only two of the miRNA families have members that are expressed in CNS cell types though their expression is only found in non-neuronal cells; miR-29 is expressed predominantly by astrocytes (Smirnova *et al.* 2005) while miR-335/335-5p is expressed in neurospheres (Sathyan *et al.* 2007). The remaining miRNA families are not expressed in the CNS at all which raises the possibility that these miRNA families contribute to the neuron-specific expression of *Npas4* by repressing *Npas4* expression in non-neuronal cell types.

microRNA family	Expression		Functions
miR-29	Bone	- Osteoblasts	- Positively regulates osteoblast differentiation
	Cancer	- Down-regulated in aggressive B-cell chronic lymphocytic leukemia	- Can act as either an oncogene or tumour suppressor
	CNS	- Minimal expression in neural tissue during embryonic and perinatal periods - Elevated expression in brain two weeks after birth - Higher expression in astrocytes than in neurons (ex vivo primary cultures)	- Unknown
	Immune system	- Down-regulated in natural killer cells, CD4(+) T cells and CD8(+) T cells upon intracellular bacterial infection	- Supresses immune response to intracellular pathogens
	Liver	- Down-regulated upon activation of primary cultured hepatic stellate cells	- Regulates collagen expression - associated with liver fibrosis - Supresses activation of hepatic stellate cells
	Lungs	- Post-natal and adult lung - Interstitial cells of the alveolar wall, pleura, alveolar duct - IMR-90 cells (human fetal lung fibroblasts) - Reduced expression in fibrotic lungs	- Regulates collagen expression - associated with pulmonary fibrosis
	Muscle	- Expressed in myoblast cells (C2C12)	- Regulates collagen expression - associated with cardiac fibrosis - Involved in myogenesis
miR-146	Cancer	- Down-regulated in metastatic breast cancer cell lines (MDA-MB-435) - Down-regulated in adult T-cell leukaemia	- Suppresses breast and pancreatic cancer metastasis
	Cartilage	- Down-regulated in chondrocytes in late-stage osteoarthritis - Up-regulated in synovial tissues in rheumatoid arthritis	- Involved in osteoarthritic pathogenesis
	Immune system	- Monocytes, bone marrow-derived-macrophages	- Controls Toll-like receptor signalling
	Lungs	- Expressed in alveolar epithelial cells	- Unknown
	Muscle	- Up-regulated in myoblast cells (C2C12) in response to stretch	- Involved in muscle differentiation
	Pancreas	- Up-regulated in pancreatic β cells after treatment with palmitate	- Increases apoptosis
miR-203	Cancer	- Up-regulated in bladder cancer - Up-regulated in ovarian cancer - Up-regulated in pancreatic ductal adenocarcinoma - Up-regulated in renal cell carcinoma - Hypermethylated (down-regulated) in hematopoietic tumours - Hypermethylated in cervical cancer - Hypermethylated (down-regulated) in lymphoma/acute lymphoblastic leukaemia - Down-regulated in cutaneous T-cell lymphoma - Down-regulated in prostate tumours/bone metastatic prostate cancer cell lines - Down-regulated in massive macronodular adrenocortical disease - Down-regulated in colorectal cancer - Down-regulated in oral and eosophageal squamous cell carcinoma - Up-regulated in head and neck squamous cell carcinoma (HNSCC) cell line upon UV irradiation	- Tumour suppressor; inhibits cell proliferation, metastasis
	Cartilage	- Up-regulated in rheumatoid arthritis synovial fibroblasts	- Unknown
	Digestive system	- Up-regulated in jejunum four days after oral infection with E. papillata parasite	- Unknown
	ES cells	- Up-regulated during epithelial differentiation of human embryonic stem cells	- Unknown
	Mouth	- Expressed in saliva - Up-regulated in oral lichen planus - Up-regulated in P. gingavitis infection	- Unknown
	Skin	- Expressed primarily in suprabasal epithelial cells - Up-regulated in keratinocytes upon epidermal differentiation - Up-regulated in psoriasis - Up-regulated by p53 in response to DNA damage in foreskin keratinocytes	- Promotes epidermal differentiation of keratinocytes by repressing 'stemness' - Restricts proliferation by promoting exit from cell cycle

microRNA family	Expression		Functions
miR-224	Cancer	- Up-regulated in hepatocellular tumours and hepatocellular carcinoma cell lines (HepG2) - Up-regulated in pancreatic ductal adenocarcinoma - Up-regulated in Wnt associated medulloblastoma - Down-regulated in oral tumours - Down-regulated in methotrexate-resistant colon cancer cell line (Caco-2) - Expressed by peri-neural cancer cells (prostate cancer)	- Increases apoptosis - Increases proliferation and migration - Increases non-anchored growth of non-transformed cells
	Liver	- Expressed in foetal liver - Up-regulated in liver cirrhosis	- Unknown
	Placenta	- Expressed in placental villi, trophoblast layer - Up-regulated in trophoblast cell line in response to hypoxia	- Unknown
miR-335/335-5p	Adipose tissue	- Up-regulated in white adipose tissue in obesity	- Unknown
	Bone	- Up-regulated in osteoblasts, hypertrophic chondrocytes	- Regulates bone development
	Cancer	- Up-regulated in pediatric acute myeloid leukaemia - Up-regulated in colonic cancer - Up-regulated in astrocytoma cell lines - Up-regulated in osteosarcoma cell line (U2OS) in response to DNA damage - Hypermethylated (down-regulated) in metastatic breast cancer - Down-regulated in adrenocortical carcinoma - Down-regulated in clear cell renal carcinoma - Down-regulated in gastric cancer - Down-regulated in drug-resistant ovarian cancer cell lines	- Tumour suppressor; activates p53 to limit cell proliferation, neoplastic transformations - Inhibits metastasis; decreases cell invasiveness - Pro-apoptotic
	CNS	- In neurospheres, down-regulated by high doses of ethanol, up-regulated by low doses	- Pro-apoptotic, anti-mitogenic miRNA
	Liver	- Down-regulated during hepatic stellate cell activation - Up-regulated in obesity	- Inhibits cell migration
	Lungs	- Highly expressed in neonatal mouse lung and foetal human lung	- Unknown
	Muscle	- Up-regulated in myofibres in a mouse model of Duchenne muscular dystrophy - Up-regulated in patients with myotonic dystrophy type 1 - Up-regulated during myogenic differentiation of myoblast cell line (C2C12)	- Regenerative miRNA
	Pancreas	- Up-regulated in islet cells by glucose treatement	- Unknown
	Placenta	- Expressed in placental villi, trophoblast layer - Up-regulated in trophoblast cell line in response to hypoxia - Up-regulated in placentas from women with severe preeclampsia	- Unknown
	Testis	- Down-regulated in rat epididymis from postnatal day 7-49	- Involved in epididymis development
mir-340/340-5p	Cancer	- Down-regulated in malignant mesothelioma - Down-regulated in aggressive breast cancer - Up-regulated in gastric cancer	- Tumour suppressor; inhibits cell migration, invasion

Table 1.3 – Expression and function of miRNA families having putative binding sites within the *Npas4* 3' UTR
A summary of the data published regarding the expression and functions of the different miRNA families that may potentially regulate *Npas4* expression.

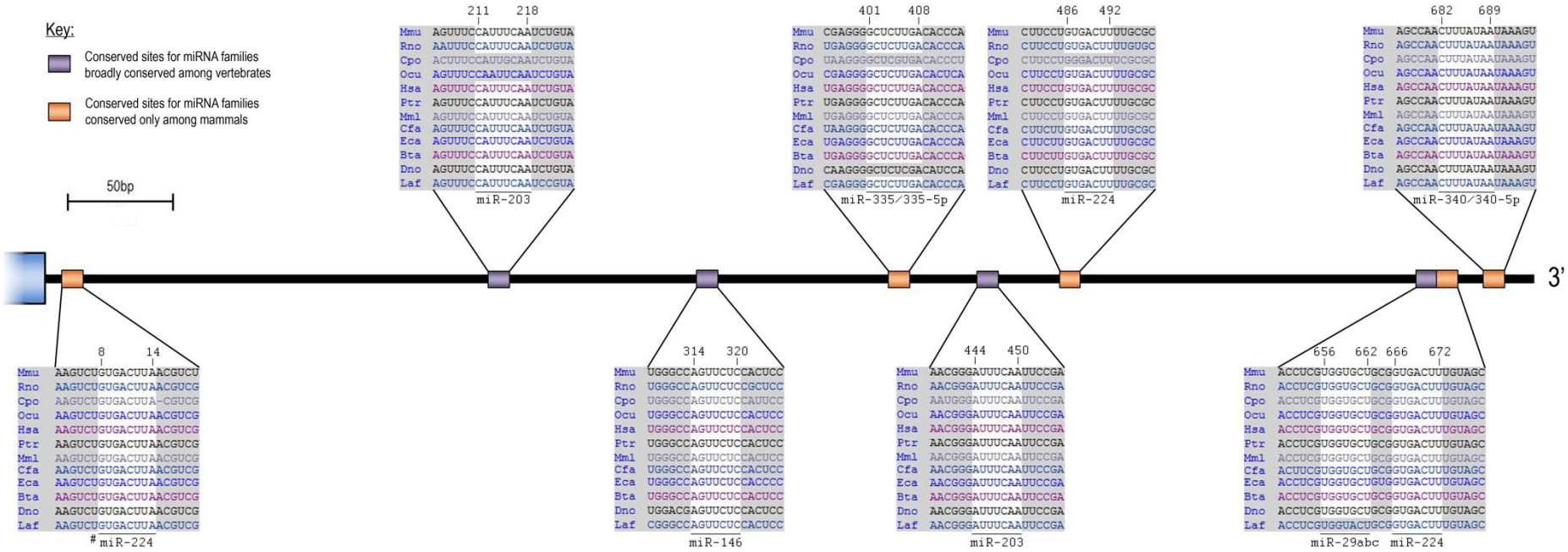


Figure 1.8 – Putative miRNA recognition sites within the 3' UTR of the mouse *Npas4* mRNA

Schematic representation of the mouse *Npas4* 3' UTR drawn to scale showing highly conserved sequences that correspond to known consensus miRNA binding sites. The 3' UTR of the mouse *Npas4* mRNA was analysed for putative miRNA binding sites using TargetScan Release 5.2 (www.targetscan.org). Regions containing putative miRNA binding sites are enlarged to show the sequence conservation between mammalian species. A white background indicates conservation with the mouse seed sequence. The nucleotide positions of the mouse *Npas4* mRNA sequence are marked (the +1 position being the first nucleotide of the 3' UTR following the STOP codon). For miRNA families conserved in vertebrates, the probability of preferentially conserved targeting (P_{CT}) is as follows: miR-29abc, $P_{CT} = 0.43$; miR-203, aggregate $P_{CT} = 0.33$; miR-146, $P_{CT} < 0.1$. Species abbreviations are: Mmu - mouse, Rno - rat, Cpo - guinea pig, Ocu - rabbit, Hsa - human, Ptr - chimpanzee, Mml - rhesus, Cfa - dog, Eca - horse, Bta - cow, Dno - armadillo, Laf - elephant. #This site is predicted to be too close to the open reading frame to be a functional miRNA binding site.

1.5 Regulation of gene expression by Npas4

1.5.1 Npas4 binding at enhancers

1.5.1.1 DNA regulatory elements: promoters and enhancers

There are several types of DNA regulatory elements that are bound by proteins which control gene transcription. Promoters are regulatory regions located at the 5' end of a gene locus immediately upstream of the transcription start site and they are the site for assembly of the transcription machinery (i.e. RNA polymerase transcriptional complex and co-activators) which is recruited to the promoter by transcription factors so that mRNA synthesis can be initiated from the transcription start site (Smale and Kadonaga 2003). While promoters generally contain the primary regulatory elements required for gene transcription, in some cases other distally located regulatory elements, such as enhancers and silencers, may modulate transcriptional regulation by either increasing or suppressing transcription, respectively.

Enhancers are regulatory regions that augment basal transcription and are located distal to the genes they regulate, sometimes up to several kb removed from the transcription start site (Bulger and Groudine 2011). Unlike promoters, they can be located either upstream or downstream of the genes they regulate and can be either intragenic or extragenic (Bulger and Groudine 2011). A subcategory of enhancers that are responsive to neuronal activity was recently characterised (Kim *et al.* 2010). These neuronal activity-dependent enhancers (NADEs) are defined as enhancers that are bound by the transcriptional co-activator Cbp exclusively after membrane depolarisation and have a pattern of histone modification that makes the DNA accessible for protein binding (Kim *et al.* 2010).

1.5.1.2 Activity-dependent binding of Npas4 at enhancers

Genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-Seq) in primary cultures of mouse cortical neurons showed that Npas4 binds NADEs in an activity-dependent manner (Kim *et al.* 2010). Indeed, unlike other activity-regulated transcription factors, such as Creb, which have moderate levels of DNA binding even in the absence of neural activity, binding of Npas4 to DNA seems to be completely reliant on neural activity as it is virtually undetectable on NADEs prior to neuronal stimulation but was found at more than 28,000 sites across the genome after KCl stimulation (Kim *et al.* 2010). Furthermore, while binding of Npas4 at canonical promoter sites is also slightly increased after neuronal depolarisation, its binding is heavily biased towards enhancer elements. Approximately 11% of Npas4 binding peaks were located within promoters (i.e. within 1kb

of annotated transcription start sites) while 22% were found to be within NADEs (Kim *et al.* 2010). Interestingly, the remaining 67% of Npas4 binding peaks occurred in regions of the genome that were undefined raising the possibility that Npas4 may also bind to another type of, as of yet, undiscovered regulatory element.

Npas4 binds to both intragenic and extragenic enhancers and does so within 100bp of the centre of the conserved enhancer domain which is defined by a pattern of histone modification that makes DNA accessible for protein binding (Kim *et al.* 2010). A search for DNA motifs matching the consensus Npas4 binding sequence within a 300bp window encompassing Npas4 binding peaks revealed that such a motif was present in only 27% of peaks when they occurred at NADEs as compared to 66% of peaks located within promoters (Kim *et al.* 2010). Given that the incidence of such motifs appearing at random is 14%, the figure for NADEs seems rather low, however, these figures are likely to underestimate the true number of Npas4 recognition sites present within Npas4 binding peaks as the search only included perfect matches to either GCGTG or TACGTG, thus excluding the preferred Npas4 binding sequence (TCGTG) and other variants thereof that are also bound by Npas4 (see Section 1.2.5). Nevertheless, the occurrence of these motifs in NADEs is still much lower than in promoters and, given that there were no significant differences in peak size between promoters and NADEs, this suggests that the Npas4 protein may bind to promoters and NADEs in different ways.

Overall, these data may reflect a specific role for Npas4 in enhancer function in the context of neural activity. It is now known that RNA polymerase II is recruited to NADEs where it mediates bi-directional transcription of a class of short, non-coding enhancer RNAs (eRNAs) and that this promotes increased transcriptional activity at nearby promoters (Kim *et al.* 2010). Thus, one possibility is that Npas4 binds NADEs in response to neuronal activity where it subsequently recruits Cbp or other co-factors that allow transcriptional machinery to be assembled at enhancer loci for the initiation of eRNA transcription.

1.5.2 Npas4 target genes

Much can be learnt about the function of a transcription factor by studying the genes that it regulates. In a DNA microarray experiment performed in E16 mouse hippocampal neurons, hundreds of genes were differentiatlly expressed as a result of reduced Npas4 expression either at steady-state or after neuronal depolarisation (Lin *et al.* 2008). Differentially regulated genes included those coding for ion channels, kinases and phosphatases, various classes of transcription factors, G-protein

signalling molecules as well as genes involved in pathways that modulate synaptic functions such as ubiquitination, trafficking and receptor endocytosis (Lin *et al.* 2008). The diversity of genes present in this pool suggests that Npas4 may be involved in a wide range of cellular processes

Indeed, several unrelated functions have already been described for Npas4 (discussed in Section 1.6) which itself is regulated by many diverse stimuli. Thus, it may be possible that Npas4 selectively regulates different subsets of target genes in a context-dependent manner to achieve specific functions. However, while many putative target genes have been identified, as of yet there have been very few genes that have been experimentally verified as being direct targets of Npas4.

1.5.2.1 Brain-derived neurotrophic factor (Bdnf)

1.5.2.1.1 Structure of the Bdnf gene

Bdnf is the most abundant and widely distributed neurotrophin (see Section 1.4.3.1) in the mammalian CNS with particularly high levels of expression in the hippocampus, cortex, cerebellum and spinal cord (Hofer *et al.* 1990; Conner *et al.* 1997; Aid *et al.* 2007; Pruunsild *et al.* 2007). The genetic structure of the mammalian *Bdnf* locus is complex with both the rodent (Aid *et al.* 2007) and human (Pruunsild *et al.* 2007) genes consisting of nine exons, with each exon having its own promoter. This multitude of transcriptional initiation sites, in combination with alternative splicing and two different poly-adenylation sites, allows for in excess of 20 unique *Bdnf* transcripts to be produced. Interestingly, all of the *Bdnf* mRNAs produce essentially the same protein as the most 3' exon, which encodes the pro-Bdnf peptide, is common to all transcript variants and they differ only the inclusion or exclusion of the eight most 5'exons which are essentially non-coding and have limited contribution to the coding sequence. However, as each promoter can be independently regulated by different combinations of transcription factors, this allows for unique spatial, temporal and stimulus-driven expression of each transcript which gives rise to an exquisitely complex *Bdnf* expression pattern.

1.5.2.1.2 Activity-dependent expression of Bdnf

Synaptic activity mediated by non-NMDA glutamate receptors induces rapid up-regulation of rodent *Bdnf* mRNA in cultured neurons (Zafra *et al.* 1990) and in neurons of the cortex and hippocampus *in vivo* (Lindefors *et al.* 1992). However, neuronal depolarisation selectively stimulates transcription only of certain *Bdnf* transcripts that are under the control of activity-regulated promoters, with the most responsive of those being promoters I, IV and IX (Aid *et al.* 2007; Pruunsild *et al.* 2011). It was

further shown that the responsiveness of *Bdnf* promoter IV to neuronal activity was mediated by intracellular Ca^{2+} signalling (Tao *et al.* 1998; Tao *et al.* 2002; Chen *et al.* 2003).

1.5.2.1.3 Regulation of Bdnf by Npas4

There is some evidence that Npas4, in conjunction with Arnt2, regulates the activity-dependent transcription of *Bdnf*. In a DNA microarray study performed in primary neuron cultures, acute knock-down of Npas4 expression using RNA interference (RNAi) resulted in a reduction in both basal and activity-dependent *Bdnf* mRNA expression (Lin *et al.* 2008). Furthermore, up-regulation of *Bdnf* mRNA after depolarisation was severely compromised in primary neuron cultures derived from $Npas4^{-/-}$ mice (Lin *et al.* 2008) while, conversely, over-expression of both NPAS4 and ARNT2 in the human embryonic kidney HEK293 cell line resulted in strong, moderate or weak induction of the endogenous *BDNF* transcripts from promoter I, IV or IX, respectively (Pruunsild *et al.* 2011).

Analysis of the regulatory regions of the human *BDNF* gene revealed that several of the *BDNF* promoters contain putative NPAS4/ARNT2 recognition elements; CACGAC in promoter I (reverse complement orientation), TTCGTG in promoter IV, and CTCGTG in promoter IX (Pruunsild *et al.* 2011). Mutation of the NPAS4/ARNT2 responsive element within promoter I demonstrated that binding of the NPAS4/ARNT2 dimer to this site is crucial for activity-dependent transcription from human *BDNF* promoter I and a similar result was also observed with rat *Bdnf* promoter I (Pruunsild *et al.* 2011). In the case of human *BDNF* promoters IV and IX, although not essential for expression, NPAS4 and ARNT2 were required for full induction of a reporter gene under the control of these promoters following neuronal depolarisation which suggests that NPAS4 partly contributes to the activity-dependent regulation of BDNF exon IV and exon IX transcripts (Pruunsild *et al.* 2011).

The Npas4/Arnt2 binding sites in promoters I and IV are conserved in rat, however the site in rat promoter IX contains a single base change (CTGGTG) which disrupts the core CGTG sequence and may explain why the Npas4-mediated activity-dependent induction of the *Bdnf* exon IX transcript is considerably less than that of exon I and exon IV in rat (Pruunsild *et al.* 2011). Chromatin immunoprecipitation (ChIP) performed in primary cortical neuron cultures obtained from rats was used to demonstrate that endogenous Npas4 binds to *Bdnf* promoters I and IV with approximately equal affinity (but not the *Bdnf* coding region or 3' UTR) in an activity-dependent manner (Lin *et al.* 2008). A similar result was reproduced in a non-neuronal human cell line; when both NPAS4 and ARNT2 were co-transfected into HEK 293 cells, ChIP was used to show that they strongly associate

with the human *BDNF* regulatory sequence as a dimer at promoter I and, to a lesser extent, promoter IV (Pruunsild *et al.* 2011).

Therefore, biochemical data suggests that Npas4 is the primary transcription factor regulating the activity-dependent expression of the *Bdnf* exon I transcript in both humans and rodents, and that it partly regulates expression of transcripts from promoters IV and IX. Interestingly, given that Bdnf has been shown to up-regulate *Npas4* mRNA expression through TrkB receptor signalling (Ooe *et al.* 2009a), it is possible that Npas4 and Bdnf may mutually up-regulate each other in a positive feed-back loop such that rapid expression of both genes can be achieved in response to stimulation.

1.5.2.1.4 Bdnf function in the central nervous system

The central role of Bdnf in neuronal survival is well-documented (Alderson *et al.* 1990; Hyman *et al.* 1991; Ghosh *et al.* 1994; Lipsky and Marini 2007), however, Bdnf is a versatile peptide that plays a part in many other important neurobiological processes including: inhibitory synapse formation (Vicario-Abejon *et al.* 1998; Ohba *et al.* 2005; Kohara *et al.* 2007), activity-dependent synaptic plasticity (Waterhouse and Xu 2009), learning (Tyler *et al.* 2002), memory (Yamada and Nabeshima 2003; Lu *et al.* 2008) and neuroprotection (Marini *et al.* 1998; Hashimoto *et al.* 2002; Castren 2004; Wu *et al.* 2004). Interestingly, Npas4 has also been implicated in all of these processes (see Section 1.6) which raises the possibility that Npas4 carries out these functions via regulation of Bdnf expression. The link between Npas4 and Bdnf in each of these processes will be discussed in the relevant sections.

1.5.2.2 Developmentally regulated brain protein (Drebrin)

1.5.2.2.1 Expression and function of the Drebrin proteins

The Drebrins are actin-binding proteins that have roles in early synaptogenesis and synaptic function through modulation of dendritic spine morphology. In mammals, there are two main isoforms of Drebrin which arise from alternative splicing of the *dbn1* gene; the embryonic isoform Drebrin E which is ubiquitously expressed and is present in the developing brain, and the adult isoform Drebrin A which is expressed in mature neurons of the adult brain (Majoul *et al.* 2007). In the rodent brain, Drebrin A protein is expressed in many brain structures including the olfactory bulb, hippocampus, striatum, thalamus and cerebral cortex (Hayashi *et al.* 1996) and, moreover, the mRNA expression pattern of *Drebrin* was found to be similar to that of *Npas4* (Ooe *et al.* 2004). The Drebrin proteins localise mainly to the spines found on dendrites of post-synaptic excitatory neurons where

they interact with filamentous actin and associate with other post-synaptic protein complexes such as the post-synaptic density (Sekino *et al.* 2007).The Drebrins effect dynamic changes in dendritic spine morphology by regulating the shape and composition of the cytoskeleton. As alterations in spine morphology are intricately linked to synaptic plasticity, it is hypothesised that the Drebrin proteins may play an important role in higher cognitive processes which depend upon plasticity such as learning and memory. This is substantiated by the observation that DREBRIN protein expression is greatly reduced in the brains of the elderly and patients with cognitive disorders, such as Alzheimer's disease and Down's syndrome (Harigaya *et al.* 1996; Hatanpaa *et al.* 1999; Shim and Lubec 2002).

1.5.2.2.2 Regulation of Drebrin expression by Npas4

It has been proposed that expression of Drebrin may be regulated by Npas4, however, the data are somewhat inconsistent. For example, two separate microarray studies produced differing results; one study conducted in human neuroblastoma SK-N-MC cells found that over-expression of NPAS4 resulted in increased expression of *DREBRIN* mRNA and this result was subsequently validated by RT-PCR (Ooe *et al.* 2004), however, in another study, no change in *Drebrin* mRNA expression was seen in depolarised mouse hippocampal neurons after knock-down of Npas4 using RNAi (Lin *et al.* 2008). The uncertainty is compounded by the fact that there are some data (mainly those involving over-expression of Npas4 *in vitro*) which suggest that *Drebrin* may be a target of Npas4, while other data (mainly taken from *in vivo* observations) seem to contradict this. For instance, a number of putative NPAS4-responsive sites containing the CGTG core binding sequence were identified in the *DREBRIN* promoter region and it was subsequently shown by ChIP assay that the NPAS4 protein is able to bind to these sites when transgenically over-expressed in SK-N-MC cells (Ooe *et al.* 2004). The NPAS4/ARNT2 heterodimer was also able to drive expression of a luciferase reporter gene from a construct containing 2.7kb of the *DREBRIN* promoter fused to the luciferase coding sequence and this transcriptional activity was abolished when NPAS4/ARNT2 binding sites were mutated (Ooe *et al.* 2004). These findings are at odds with data obtained from *in vivo* studies where it has been demonstrated that there are some experimental conditions which induce *Npas4* expression that do not affect *Drebrin* expression, such as cortical spreading depression (CSD) (Hester *et al.* 2007), or in fact cause a down-regulation of *Drebrin* expression, such as cerebral ischaemia (Shamloo *et al.* 2006). Nevertheless, this does not exclude *Drebrin* as a potential Npas4 target gene; one possible explanation for these incongruent observations is that that Npas4 may regulate several different transcriptional programmes and that the subset of genes activated is determined by the cellular context of Npas4 expression.

1.5.2.3 Bcl-2–associated X protein (Bax)

In eukaryotic cells, programmed cell death is tightly regulated by the Bcl-2 family of proteins which includes both anti-apoptotic factors, such as Bcl-2, and pro-apoptotic factors, such as Bcl-2-associated X protein (Bax). Both Bax and Bcl-2 can homodimerise to form ion-conducting membrane channels that associate with organellar membranes and either promote or inhibit apoptosis, respectively (Basu and Haldar 1998). Moreover, Bax and Bcl-2 also mutually antagonise each other and are able to form heterodimers with properties different to either of the ion channels created by homodimerisation. Therefore, the ratio of Bcl-2:Bax within a cell determines cell survival; when Bcl-2 is in excess, Bcl-2 homodimers and Bcl-2/Bax heterodimers predominate and apoptosis is suppressed, whereas when Bax is in excess, Bax homodimers and Bcl-2/Bax heterodimers predominate and the cell is sensitised to apoptotic signals (Oltvai *et al.* 1993). Thus, ordinarily a homeostatic balance is maintained between Bax and Bcl-2 expression which ensures normal levels of cell death within tissues, however in some disease states, such as cancer, this equilibrium is disrupted leading to deregulation of apoptosis (Cory and Adams 2005).

Expression profiling of transcripts in the HeLa human cervical cancer cell line by microarray revealed that *BAX* mRNA expression was increased upon over-expression of Npas4 (Hester *et al.* 2007). This result was validated by experiments which showed that adenoviral over-expression of Npas4 resulted in BAX protein expression in non-neuronal cells (HeLa), a neuroblastoma hybrid cell line (F-11) and primary cerebellar granule neuron cultures (Hester *et al.* 2007). Bioinformatic analysis of the *BAX* promoter region revealed a number of putative NPAS4/ARNT2 recognition sites located upstream of the transcriptional start site and ChIP was used to show that the endogenous *BAX* promoter was bound by Npas4 when both Npas4 and Arnt2 were over-expressed in HeLa cells (Hester *et al.* 2007). Furthermore, the Npas4/Arnt2 heterodimer was able to drive expression of a luciferase reporter gene from constructs containing segments of the *BAX* promoter fused to the luciferase coding sequence, however this transcriptional activity was abolished when the core ATCGTG sequence within the NPAS4/ARNT2 binding elements was mutated (Hester *et al.* 2007). The possible role of Npas4 in modulating apoptosis via regulation of Bax expression is discussed in Section 1.6.4.

1.5.2.4 Na^+/H^+ Exchanger 9 (NHE9)

1.5.2.4.1 Expression and function of NHE9

NHE9 is an organellar Na^+/H^+ transporter of the solute carrier family 9 and is encoded by the gene *SLC9A9*. The NHE9 protein localises primarily to the membranes of recycling endosomes and is involved in regulating luminal pH by facilitating proton efflux (Ohgaki *et al.* 2011). The NHE9 transcript is widely expressed in adult tissues with particularly high expression in the heart, skeletal muscle and placenta, though mRNA was also detected throughout the brain and spinal cord (de Silva *et al.* 2003; Nakamura *et al.* 2005). In the developing mouse, mRNA expression was detected at E12.5 in the heart, somites and regions of the brain (de Silva *et al.* 2003). It has been associated with some neurodevelopmental disorders including attention deficit hyperactivity disorder (ADHD) (de Silva *et al.* 2003; Lasky-Su *et al.* 2008) and autism (Morrow *et al.* 2008).

1.5.2.4.2 SLC9A9 as a putative Npas4 target gene

Though it has not been shown that *SLC9A9* is a direct target of Npas4, RNAi knock-down of Npas4 in primary mouse hippocampal cultures resulted in increased expression of Nhe9 after membrane depolarisation (Morrow *et al.* 2008). This suggests that Npas4 may be involved, either directly or indirectly, in the activity-dependent regulation of Nhe9 expression, though this has yet to be verified.

1.5.2.5 Autoregulation of Npas4

There is some evidence that Npas4 may positively regulate its own expression. In a ChIP assay performed in primary cortical neuron cultures, endogenous Npas4 was found to associate with the *Npas4* promoter in an activity-dependent manner (Lin *et al.* 2008). In a reporter gene assay, the Npas4/Arnt2 heterodimer was able to drive transcription of a luciferase reporter gene from a construct containing 2.7kb of the *Npas4* promoter fused to the luciferase coding sequence (Ooe *et al.* 2004). Several putative Npas4/Arnt2 responsive elements were identified within the *Npas4* promoter region and mutation of the core CGTG sequence within these elements abolished reporter gene expression (Ooe *et al.* 2004). Furthermore, over-expression of Npas4 in SK-N-MC neuroblastoma cells caused up-regulation of the endogenous *NPAS4* mRNA (Ooe *et al.* 2004). It is possible that in the context of stimulus-dependent *Npas4* induction this auto-regulation creates a feed-forward loop enabling *Npas4* to be rapidly up-regulated on demand. This may provide an explanation for the immediate elevation *Npas4* expression in response to stimuli such as depolarisation.

1.5.2.6 Other putative Npas4 target genes

Expression studies in Npas4 null mice have identified a number of cellular stress response genes whose expression may be indirectly regulated by Npas4. When Npas4$^{-/-}$ mice were compared to wildtype mice, the mRNA expression of four cellular stress response genes (*Xbp-1*, *Grp78*, *Ern1* and *Gpx2*) was decreased in the brains of Npas4$^{-/-}$ mice though, interestingly, this reduction was only observed in older mice (30 weeks of age) and was not seen in mice that were only 15 weeks old (Ooe *et al.* 2009b). This indicates a failure of aged Npas4$^{-/-}$ mice to activate genes needed for cellular stress response, though the reason for this is unclear. Due to the delay in the reduction of gene expression, the authors suggest that these genes, all of which have roles in cell-stress tolerance (described below), are unlikely to be direct transcriptional targets of Npas4 themselves, but instead may be involved in processes that are downstream of Npas4 signalling, though this is unverified and remains to be demonstrated.

- X-box binding protein 1 (Xbp-1) – a transcription factor that is involved in the unfolded protein response (Glimcher 2010)
- Glucose-regulated protein 78 (Grp78) – a heat shock protein that modulates the unfolded protein response (Zhang and Zhang 2010)
- ER-nucleus signalling 1 protein (Ern1) – encodes a protein that is involved in the ER stress response (Tirasophon *et al.* 1998)
- Glutathione peroxidase 2 (Gpx2) – has been shown to inhibit oxidative stress-induced apoptosis in a p53-dependent manner (Yan and Chen 2006)

1.6 Npas4 function

Like other factors involved in stimulus-transcription coupling, Npas4 can be regarded as a response factor that is expressed and performs a specific function in response to a certain stimulus. To date, Npas4 has been implicated in several important neurobiological processes including inhibitory synapse formation, fear memory formation and various neuroprotective pathways. The multiplicity of Npas4 functions may arise from context-dependent regulation of different sets of target genes. It is therefore possible that other, as of yet, undiscovered functions exist. For instance, it has been predicted by computational analysis that Npas4 may interact with many other bHLH transcription factors to form regulatory networks controlling a diverse array of processes; it is predicted that Npas4 may be a primary regulator of modules controlling ER function, ion channel activity, protein heterodimerisation activity and eye development, whilst being a secondary regulator of modules

controlling neurotransmitter metabolism, carboxylic ester hydrozylase activity, cell-cell adhesion and intracellular non-membrane bound organelle function (Li *et al.* 2007). While this has yet to be verified experimentally, it suggests that Npas4 is likely to be a complex and multi-faceted transcription factor having many diverse roles in neurobiology.

1.6.1 Excitation/inhibition homeostasis in neuronal networks

In the CNS, levels of neural activity are finely balanced by opposing forces – excitatory synaptic signalling, which stimulates neuronal firing, and inhibitory synaptic signalling, which dampens neural activity. Maintenance of an appropriate balance between excitatory and inhibitory signals is vital for proper functioning of neural networks and disruption of this balance can cause neuropathology (see Section 1.7.1).

Recently, Npas4 was identified as being a factor that is important for development of inhibitory synapses as it was found to positively regulate the number of inhibitory synapses forming on excitatory neurons. Hippocampal neurons isolated from rats at E18 (a stage prior to onset of developmental synaptogenesis) and then transfected with *Npas4*-specific RNAi constructs developed significantly fewer inhibitory synapses than those transfected with control RNAi constructs both at the soma and dendrites (Lin *et al.* 2008). Conversely, over-expression of Npas4 increased the number of perisomatic and dendritic inhibitory synapses that were formed (Lin *et al.* 2008). Both pre-synaptic, glutamic acid decarboxylase (GAD65), and post-synaptic, $GABA_A$-receptor γ2 subunit ($GABA_A$-γ2), markers of GABAergic synapses were affected when Npas4 expression was manipulated, though a greater effect was seen on post-synaptic markers which suggests that Npas4 may regulate inhibitory synapse formation primarily by influencing the receptiveness of the post-synaptic neuron (Lin *et al.* 2008). This effect was specific to inhibitory synapses, as manipulation of Npas4 expression had no effect on the number of excitatory synapses that were formed (Lin *et al.* 2008).

By virtue of its role in inhibitory synapse formation, Npas4 is also a critical regulator of excitability in neuronal networks. Acute disruption of Npas4 expression in individual neurons within organotypic hippocampal slices obtained from post-natal conditional Npas4 knock-out mice revealed that Npas4 expression is crucial for excitatory/inhibitory homeostasis; disruption of Npas4 expression reduced the frequency of miniature inhibitory postsynaptic currents (mIPSCs) while simultaneously increasing the frequency of miniature excitatory postsynaptic currents (mEPSCs) recorded from CA1 pyramidal neurons (Lin *et al.* 2008). Conversely, neurons containing additional copies of the *Npas4* gene

exhibited an increase in mIPSC frequency and a concurrent decrease in frequency and amplitude of mEPSCs (Lin *et al.* 2008). Therefore, the net result of Npas4 activity in an intact neural network is increased synaptic inhibition and, thus, decreased neuronal excitability.

In constrast to slices in which Npas4 expression was reduced acutely, when hippocampal slices from post-natal Npas4$^{-/-}$ mice were used, no change in mIPSCs amplitude or frequency was observed (Lin *et al.* 2008). One possible explanation for this observation is that compensatory pathways may be activated during development to counteract the lack of Npas4 expression. Alternatively, this may be because Npas4 is absent in both pre-synaptic and post-synaptic neurons in brain slices derived from Npas4$^{-/-}$ mice, while in experiments performed on slices from conditional knock-out mice, disruption of Npas4 expression occurs only in post-synaptic neurons.

It was further demonstrated that Npas4 exerts its effect on inhibitory synapse formation partly through regulation of Bdnf expression. Knock-down of Bdnf using RNAi partially attenuated the effect of Npas4 over-expression on mIPSC frequency and completely reversed the effect on mISPC amplitude in hippocampal slice cultures (Lin *et al.* 2008). It was previously known that Bdnf was involved in activity-dependent inhibitory synapse formation (Huang *et al.* 1999; Bolton *et al.* 2000; Marty *et al.* 2000; Seil and Drake-Baumann 2000), however these findings were the first to implicate Npas4 in this process.

Taken together, these data suggest that Npas4 mediates excitatory/inhibitory homeostasis in CNS networks through regulation of inhibitory synapse formation and that this occurs largely via a Bdnf-mediated mechanism. Given that expression of *Npas4* is induced by excitatory neuronal activity, this may represent a self-regulating negative feed-back system which allows neuronal excitation to be modulated in response to activity. This is suitably demonstrated in the context of a whole animal by Npas4$^{-/-}$ mice where this homeostatic mechanism is perturbed; Npas4$^{-/-}$ display phenotypic traits characteristic of excitation/inhibition imbalance such as anxiousness, hyperactivity and susceptibility to seizure (Lin *et al.* 2008).

1.6.2 Synaptic plasticity

1.6.2.1 Learning and memory

Following the discovery that expression of *Npas4* is induced by learning (see Section 1.4.2.4.3), a functional role for Npas4 in fear memory formation and retention was formally demonstrated in a series of experiments in which *Npas4* expression was reduced specifically in the lateral nucleus of the amygdala using adeno-associated viral RNAi. Knock-down of Npas4 impaired fear memory formation and the retention of a fear memory following retrieval but had no effect on innate fear, sensory input (shock perception), locomotor behaviour or the expression of a previously learned fear memory (Ploski *et al.* 2011).

As discussed in Section 1.4.5, social isolation not only diminishes *Npas4* expression in the hippocampus, but it also results in impaired learning and memory. The experiments of Ploski and colleagues for the first time implicate Npas4 as a causal factor in memory impairment associated with chronic psychological stress. Together with the observation that *Npas4* is up-reglated in response to LTP and LTD (see Section 1.4.2.4.2), these findings also suggest that a broader role for Npas4 in synaptic plasticity which, though yet to be demonstrated formally, seems likely given that several Npas4 target genes have already been implicated in synaptic plasticity. There is a considerable amont of evidence that Bdnf is involved in activity-dependent synaptic plasticity (Waterhouse and Xu 2009) and thus, by extension, long-term memory (Yamada and Nabeshima 2003; Lu *et al.* 2008). Stimulation protocols that induce long-term LTP in hippocampal CA1 neurons also induce *Bdnf* mRNA expression (Patterson *et al.* 1992) and cleavage of pro-Bdnf into the mature form is crucial for LTP at hippocampal synapses (Pang *et al.* 2004). Moreover, mice lacking Bdnf showed an impairment of LTP induction in the CA1 region of the hippocampus (Korte *et al.* 1995) which could be restored by treatment with recombinant Bdnf (Patterson *et al.* 1996). The precursor form of Bdnf also participates in synaptic plasticity, though in contrast to the mature peptide, pro-Bdnf has been shown to enhance LTD in hippocampal slices by acting on the $p75^{NTR}$ (Woo *et al.* 2005). Similarly, it has been proposed that Drebrin plays an important role in synaptic plasticity by regulating dendritic spine morphology (Dun and Chilton 2010) and this is supported by work which shows that elimination of the Drebrin A isoform is associated with deficits in learning and memory (Kojima *et al.* 2010). Thus, it is tempting to speculate that Npas4 is involved in synaptic plasticity through the activity-dependent regulation of *Bdnf* and/or *Drebrin*, however, more research is needed to validate this hypothesis.

1.6.2.2 Putative role of Npas4 in neural plasticity associated with chronic drug use

Chronic use of addictive drugs can lead to adaptive changes in the brain that are responsible for producing some of the symptoms associated with drug abuse such as tolerance, dependence and addiction (Jones and Bonci 2005). Changes in IEG expression are proposed to play a role in the molecular mechanisms underlying drug-mediated plasticity (Hyman and Malenka 2001). While it is not known yet whether long-term use of addictive drugs leads to lasting changes in *Npas4* expression, based on the *Npas4* response to acute drug treatment (see Section 1.4.7) and its proposed roles in plasticity in other paradigms such as chronic stress (see Section 1.4.5.4), it seems likely that Npas4 may also be involved in plastic changes linked to chronic drug use, though this requires further investigation.

1.6.3 Neuroprotection

1.6.3.1 Activity-dependent neuronal survival

It is well established that synaptic activity is essential for survival of neurons, particularly during development of the CNS when neurons which have failed to make appropriate synaptic connections are selectively removed in a process known as 'naturally occurring cell death' (Oppenheim 1991; Mennerick and Zorumski 2000). More specifically, it has been shown that activity-dependent neuronal survival is largely mediated by activation of NMDARs as blockade of NMDARs during a critical period during CNS development dramatically increases the amount of apoptosis that occurrs (Ikonomidou *et al.* 1999). Activation of NMDARs can promote neuronal survival via two mechanistically distinct pathways (Papadia *et al.* 2005). The 'early phase' of neuroprotection, which is only effective during ongoing neural activity, is independent of transcription and does not require Ca^{2+} signalling. It is mediated solely by the PI3K/Akt pathway and may include processes such as post-translational inactivation of pro-apoptotic factors. The 'late phase' of neuroprotection, which can promote survival for a long time after activity has ceased, is dependent on nuclear Ca^{2+} signalling and gene expression resulting from activation of Ca^{2+}-sensitive transcription factors, such as Creb (Walton and Dragunow 2000) or myocyte enhancer factor 2 (Mao *et al.* 1999), which induce expression of pro-survival genes. Phosphorylation of Creb increases neuronal survival by suppressing apoptosis (Walton *et al.* 1999) and it has been shown that this effect is partly mediated by up-regulation of Bdnf (Bhave *et al.* 1999).

1.6.3.2 NMDAR-mediated neuroprotection

The great paradox of NMDAR signalling is that while NMDAR-mediated Ca^{2+} entry can promote cell survival, under some conditions it can also induce cell death (Hardingham and Bading 2003). Excessive activation of NMDARs can disrupt intracellular Ca^{2+} homeostasis and trigger break-down of the mitochondrial membrane potential (Alano *et al.* 2002). Crucially, the level of NMDAR activation determines neuronal fate; both insufficient neural activity (due to lack of functional synapses) and excessive neural activity (as occurs in excitotoxic injury) lead to neuronal degeneration via programmed cell death and thus physiological levels of NMDAR activity are required to provide any beneficial effect (Figure 1.9A). Consistent with this, the 'Ca^{2+} set-point hypothesis' was proposed which states that there exists an optimum concentration of intracellular Ca^{2+} (approximately 100-250nM) that allows neurons grown *in vitro* to survive in the absence of otherwise essential neurotrophic factors, while levels of Ca^{2+} significantly above or below this limit are toxic (Johnson *et al.* 1992).

Moreover, the source of the Ca^{2+} signal (i.e. the location of the NMDAR on the cell membrane) is also important in determining the final outcome of NMDAR signalling (Figure 1.9B). Synaptic and extrasynaptic NMDARs exert opposing effects on cell fate via activation of distinct and largely non-overlapping gene expression programmes; activation of NMDARs located the synapse promotes neuronal survival via up-regulation of pro-survival genes and down-regulation of pro-apoptotic genes, while activation of extrasynaptic NMDARs initiates apoptotic pathways (Zhang *et al.* 2007). For example, Ca^{2+} entry via synaptic NMDAR signalling leads to activation of Creb and induction of Bdnf expression while, conversely, Ca^{2+} entry via extrasynaptic NMDARs represses Creb activity (and thus inhibits Bdnf expression) and instead triggers mitochondrial dysfunction (Hardingham *et al.* 2002).

1.6.3.3 Npas4: an activity-dependent neuroprotective factor

There is gathering evidence that Npas4 participates in the activity-dependent neuroprotective response. In keeping with this, *Npas4* mRNA expression was found to be selectively induced by activation of synaptic, but not extrasynaptic, NMDARs (Zhang *et al.* 2007). Furthermore, a number of different groups have demonstrated that induction of Npas4 in neurons provides a protective effect against various stresses, though the mechanisms underlying this have not yet been completely elucidated.

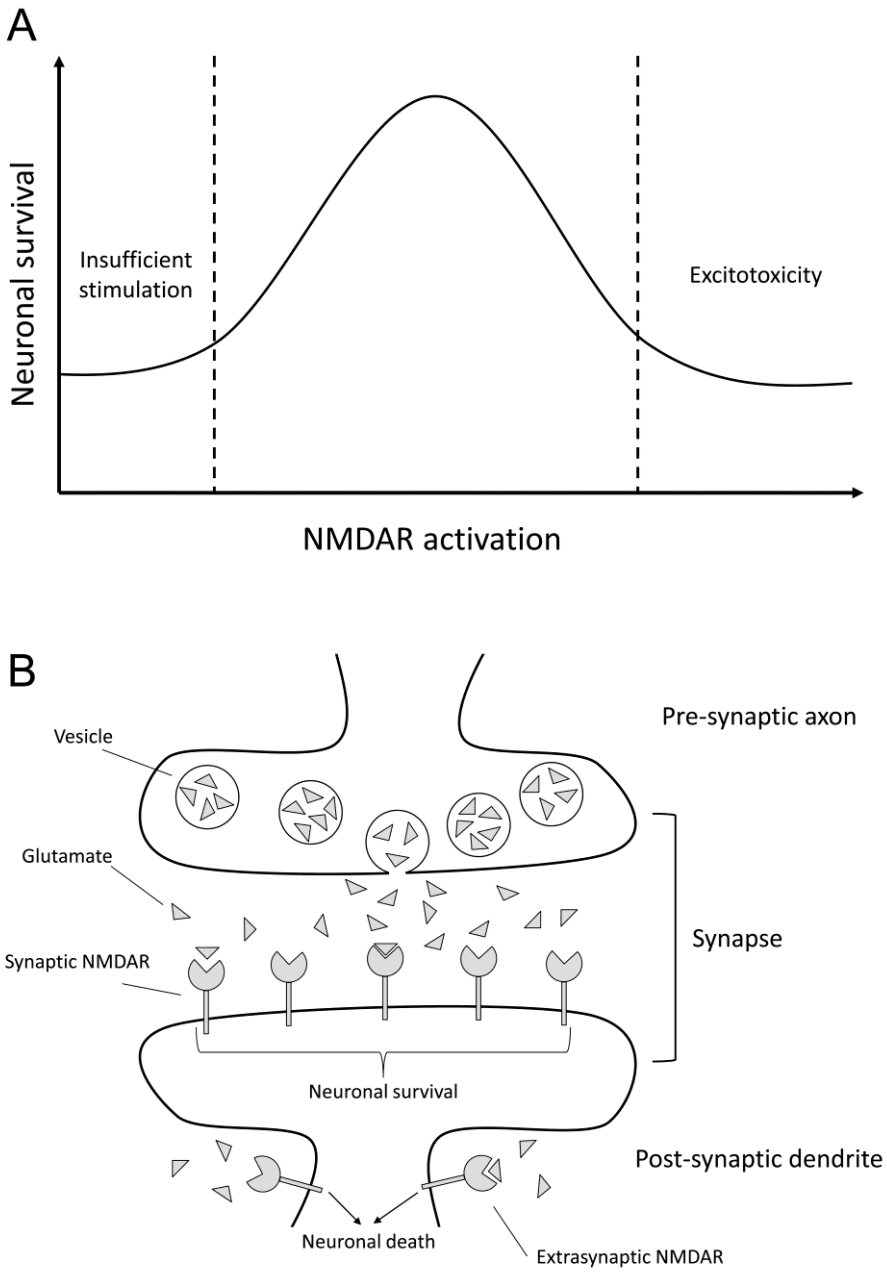


Figure 1.9 – NMDAR-mediated neuroprotection
(A) The level of NMDA receptor (NMDAR) activation determines neuronal fate; an optimal level of NMDAR activation exists and NMDAR stimulation within this range promotes neuronal survival while insufficient or excessive stimulation of NMDARs leads to cell death. This paradigm is intricately linked to the 'Ca^{2+} set-point hypothesis' (see Section 1.6.3.2 for details). **(B)** Equally, the location of the NMDARs on the plasma membrane also determines the outcome of NMDAR activation; stimulation of synaptic NMDARs promotes neuronal survival, while activation of extrasynaptic NMDARs triggers apoptotic pathways.

1.6.3.3.1 Npas4*-mediated neuroprotection in response to acute neurological challenge*

The neuroprotective effect of Npas4 in response to acute neurological challenge has been demonstrated *in vivo* using various animal models. In a rat model of seizure, neurons of the dorsal hippocampus that were transduced with adeno-associated virus to over-express Npas4 were protected from cell death induced by kainic acid treatment with a 92% inhibition of cell death relative to the contralateral hemisphere (Zhang *et al.* 2009). Conversely, mice lacking Npas4 proved to be more susceptible to brain damage when challenged with kainic acid. The treatment, which was not lethal to wildtype mice, resulted in death in more than one third of Npas4$^{-/-}$ mice and, furthermore, when compared to wildtype littermates Npas4$^{-/-}$ mice displayed markedly elevated levels of microglial activation in the hippocampus, as determined by glial fibrillary acidic protein (Gfap) immunoreactivity (Ooe *et al.* 2009b). This is suggestive of increased brain damage as activation of microglia is associated with the inflammatory response elicited by excitotoxic injury and the accompanying neuropathology which results (Mabuchi *et al.* 2000; Wang *et al.* 2005).

As further evidence of the protective function of Npas4, one group has suggested that following brain injury Npas4 expression can be found in surviving cells, but not dying cells. In the hippocampi of rats subjected to 10min of global ischaemia, few cells expressing Npas4 protein could be detected ten days after ischaemia while many degenerating neurons were detected by terminal deoxynucleotidyl transferase deoxyuracil triphosphate (dUTP) nick end labelling (TUNEL) and Fluoro-jade B staining (Ooe *et al.* 2009b). Given that Npas4 up-regulation in response to brain injury is rapid and likely to be transient, while this may, as the authors suggest, be an indication that Npas4 expression is associated with surviving cells rather than apoptotic cells, it is also possible that the lack of Npas4 expression is due to transient nature of Npas4 expression. This evidence is only circumstantial and direct co-localisation studies should be done to conclusively demonstrate this.

1.6.3.3.2 Involvement of Npas4 in preconditioning

One aspect of neuroprotection is a phenomenon known as 'preconditioning' - the ability of a brief period of depolarisation to provide neurons with tolerance to damage incurred by a subsequent stress. For example, potassium cyanide (KCN), a potent inhibitor of cellular respiration, is highly cytotoxic and triggers extensive cell death when applied to cultured F-11 cells, however, the amount of cell death can be greatly reduced by stimulation of cells with 50mM KCl for 90min prior to KCN treatment (Hester *et al.* 2007). The mechanisms underlying the protective effect of precondition are poorly understood though it is thought that induction of activity-dependent pro-survival genes plays

a part in this process. A role for Npas4 in this KCl-mediated neuroprotective paradigm can be inferred from experiments performed in F-11 cells which show that when induction of *Npas4* expression is prevented by RNAi, the protective effect of KCl preconditioning is greatly reduced (Hester *et al.* 2007). A similar result was observed in primary cultures of mouse hippocampal neurons preconditioned for 16h with 50µM bicuculline and subsequently challenged with either withdrawal of growth factors (insulin, transferrin and selenium) or treatment with the non-specific protein kinase inhibitor staurosporine (10nM); knock-down of Npas4 expression by adeno-associated viral RNAi abolished the neuroprotective effect of bicuculline-induced potential bursting after both growth factor withdrawal and staurosporine treatment (Zhang *et al.* 2009). Conversely, apoptosis induced by either growth factor withdrawal or staurosporine treatment was reduced by 30% and 70%, respectively, in hippocampal neurons cultures constitutively over-expressing Npas4 (Zhang *et al.* 2009).

There is evidence that the neuroprotective role of Npas4 also extends to excitotoxic damage caused by cerebral ischaemia. It is well-documented that preconditioning with a short period of sub-lethal ischaemia followed by several days of recovery provides increased resistance to neuronal damage caused by a subsequent, more severe, challenge (Kitagawa *et al.* 1990; Liu *et al.* 1992). A similar effect has also been observed in other types of brain insult, such as seizure (Sasahira *et al.* 1995). In this preconditioning phenomenon, it is hypothesised that factors induced by the initial insult, such as heat-shock proteins (HSPs) (Liu *et al.* 1992), may confer neuroprotection against an ensuing challenge. Consistent with Npas4 being involved in this process, expression of *Npas4* was elevated in the hippocampi of rats preconditioned with a brief episode of global ischaemia lasting 3min and, furthermore, when preconditioning was followed by a longer ischaemic challenge of 10min two days later, the resulting increase in *Npas4* mRNA expression was far more modest (Shamloo *et al.* 2006) suggesting that excitotoxicity had been reduced by preconditioning. While this is not direct evidence that *Npas4* expression during preconditioning is causative in reducing ischaemic damage, given the expression pattern of *Npas4* following cerebral ischaemia and its previously described roles in neuroprotection it is tempting to speculate that Npas4 may be involved in this neuroprotective paradigm. The involvement of Npas4 in preconditioning-induced neuroprotection could be further explored in mice lacking Npas4 expression.

1.6.3.3.3 **Npas4** ***in homeostatic neuroprotection***

There is also evidence that Npas4 promotes neuronal survival under normal conditions, that is, even in the absence of acute injury or challenge. Npas4$^{-/-}$ mice have a shortened lifespan and eventually

die due to the progressive accumulation of age-dependent neurodegeneration (Ooe *et al.* 2009b). At 30 weeks of age, Npas4$^{-/-}$ mice had increased levels of Gfap immunoreactivity and Fluoro-Jade B staining in the hippocampus and cerebral cortex when compared to wildtype mice (Ooe *et al.* 2009b). Furthermore, in the hippocampi of Npas4$^{-/-}$ mice there was also an increase in the number of TUNEL positive cells and microglia as detected by immunoreactivity for Iba1, a protein expressed specifically by microglia (Ooe *et al.* 2009b). It is hypothesised that without Npas4 expression neurons are unable to develop a tolerance to daily neuronal stress and are therefore more vulnerable to damage. It is likely that one element contributing to the neurodegeneration observed in Npas4$^{-/-}$ mice is oxidative stress as increased amounts of nitrotyrosine, a toxic compound produced by ROS, were detected in the brains of Npas4$^{-/-}$ mice (Ooe *et al.* 2009b). It is also possible that a perturbation of the homeostatic balance between excitatory and inhibitory inputs could affect neuronal survival in Npas4$^{-/-}$ mice. Drawing on work that describes the role of Npas4 in inhibitory synapse formation, an alternative hypothesis to explain these observations is that CNS neurons of Npas4$^{-/-}$ mice have fewer inhibitory synapses and hence the basal level of excitatory synaptic transmission in neural circuits is elevated above the threshold which is excitotoxic and that this causes cumulative damage over time.

The findings from *in vitro* RNAi experiments, however, are not so clear. One study reported that knock-down of Npas4 in cultured hippocampal neurons resulted in a slight increase in the basal level of cell death (Zhang *et al.* 2009). However this was not supported by another study where decreased expression of Npas4 was found to have no effect on the overall health or dendritic outgrowth of cultured cerebellar granule neurons (Lin *et al.* 2008). The reason for the discrepancy in these observations is not clear but may be due to differences in the neuronal populations that were used, the RNAi constructs used to achieve knock-down and/or the method of delivery (adenoviral delivery versus calcium phosphate transfection). Alternatively, it may be a function of the length of time the neurons were in culture; since no differences were observed between Npas4$^{-/-}$ and wildtype mice at an early age (Ooe *et al.* 2009b), it is evident that the effects of reduced Npas4 expression on neural health only become apparent over time and perhaps the same is true of neuronal viability *in vitro*.

1.6.3.3.4 Mechanisms involved in Npas4*-mediated neuroprotection*

One way in which Npas4 promotes neuronal survival is through stabilisation of mitochondrial membrane potential which makes the mitochondria more resilient to dysfunction. Both the rate and severity of NDMA-induced mitochondrial membrane break-down, as measured by rhodamine 123 fluorescence, were significantly reduced by adeno-associated viral over-expression of Npas4 in

mouse primary hippocampal neuron cultures exposed to 30μM NMDA (Zhang *et al.* 2009). Nevertheless, the factors downstream of Npas4 leading to enhanced mitochondrial stability have not yet been identified. Given that *Bdnf* has been shown to be a target gene of Npas4 (see Section 1.5.2.1.3), one possibility is that induction of Npas4 enhances neuronal survival via up-regulation of Bdnf in a manner similar to Creb (see Section 1.4.2.1.6).

1.6.4 Apoptosis

Paradoxically, it has also been reported that in some instances Npas4 expression can have a cytotoxic effect. One group found that sustained over-expression of Npas4 in cultured cells by adenoviral delivery led to a decrease in cell viability over several days of culture compared to cells infected with a control vector expressing green fluorescent protein (GFP) and, furthermore, this effect was purportedly due to Npas4-mediated up-regulation of the pro-apoptotic gene Bax (Hester *et al.* 2007). This cytotoxic effect was seen in primary mouse cerebellar granule neuron cultures and both neuronal (F-11) and non-neuronal (HeLa) cancer cell lines but was lessened by removal of the Npas4 C-terminal TAD (Hester *et al.* 2007) suggesting that Npas4-dependent transcription is required for this effect. These findings are not consistent with other data which show that Npas4 expression is neuroprotective and enhances cell survival (Ooe *et al.* 2009b; Zhang *et al.* 2009), nor have these observations been replicated by other groups. Indeed, these observations are even at odds with data published by the same group (Hester *et al.* 2007). Nevertheless, the authors do offer some explanations for these conflicting observations.

Hester *et al.* contend that the duration of *Npas4* expression is critical and that while transient expression of *Npas4* is beneficial, prolonged expression is detrimental. This seems a reasonable proposition given that all examples of normal physiological induction of *Npas4* that have been reported thus far have shown that, regardless of the stimulus, *Npas4* is only transiently expressed in the range of several hours before declining to basal levels (Shamloo *et al.* 2006; Hester *et al.* 2007; Lin *et al.* 2008; Ooe *et al.* 2009a). In this sense, constitutive over-expression of *Npas4* in cultured cells over a period of several days represents an artificial scenario which probably does not normally occur *in vivo* and thus care must be taken when interpreting any results derived from such experiments. Nevertheless, other groups have performed gain-of-function experiments employing long-term over-expression of *Npas4* without observing any deleterious effects (Lin *et al.* 2008) or, on the contrary, observing that cell survival was increased (Zhang *et al.* 2009). Thus, it would appear that the duration of *Npas4* over-expression alone does not adequately explain this observation.

Another possibility is that the disparity between these results may be attributable to differences in the cell type, method of gene delivery and/or the regulation of transgene expression. For instance, the expression construct employed by Hester *et al.* contained only the coding sequence of the rat *Npas4* gene fused to a sequence coding for a hemagglutinin tag and this fusion construct was expressed under the control of the cytomegalovirus (CMV) promoter (Hester *et al.* 2007). This type of construct may not be biologically relevant as the CMV promoter is a strong, constitutively active promoter that drives high levels of expression while the presence of an epitope tag may interfere with normal Npas4 function. In contrast, Lin *et al.* generated an Npas4 'mini-gene' construct containing the entire mouse genomic *Npas4* sequence including all exons and introns, both the 5' and 3' UTRs and the endogenous *Npas4* promoter region (Lin *et al.* 2008). This type of construct, in which all regulatory elements are preserved (particularly the 3' UTR which is well conserved among species and may contain important regulatory sequences, such as microRNAs binding sites; see Section 1.4.9), would enable appropriate spatial and temporal regulation of transgene expression which is comparable to that of the endogenous gene. Appropriately regulated expression may be crucial for a gene whose expression is so tightly controlled under normal circumstances. Nevertheless, while this may to some extent account for the conflicting observations, it cannot be the sole reason as Zhang *et al.* also constitutively expressed epitope-tagged Npas4 protein from a virally delivered construct using a similar CMV/chicken β-actin hybrid (CAG) promoter without any negative effects (Zhang *et al.* 2009).

Another possible explanation relates to the amount of *Npas4* transgene expression in each cell which is a function of the multiplicity of infection (MOI) used in viral gene delivery and the number of virions infecting each cell. Using a virus titre that gives a high MOI could potentially result in the same cell harbouring multiple copies of the experimental construct giving proportionally greater transgene expression as well as any problems that may arise from excessive expression. The infection efficiency reported by Zhang *et al.* in their 2009 publication was 80-95% of viable neurons, however no comparisons can be made between publications as the infection efficiency was not disclosed in the report published by Hester and colleagues in 2007. Nevertheless, over-expression of introduced proteins, particularly transcription factors which control many processes, is fraught with danger and can lead to unexpected results that are not biologically relevant in the context of normal physiology. Thus, care must be taken to separate meaningful results from *in vitro* artefacts produced by expression constructs. For instance, it is hypothesised that the cytotoxic effect of sustained Npas4 expression is due to direct transcriptional activation of the *Bax* promoter which contains CME variants, however, it is possible that over-expression of Npas4 to abnormally high levels leads to a

saturation of preferred Npas4/Arnt2 binding sites and results in aberrant binding to non-preferred sites that have only low to moderate affinity for Npas4/Arnt2. As of yet, binding of endogenous Npas4 to the *Bax* promoter has not been demonstrated. Furthermore, there may be other factors that contribute to cytotoxicity in this context. An overabundance of Npas4 protein may serve to sequester Arnt and/or Arnt2 proteins leaving them unable to partner other bHLH PAS factors that are vital to cellular functioning. Thus, without further supporting data, the role of Npas4 in apoptosis remains contentious.

1.6.5 A putative role for Npas4 in circadian regulation of *Bdnf*

While the significance of circadian expression of Npas4 in the pars tuberalis (see Section 1.4.8.3) is unknown, it is possible that Npas4 may contribute to the circadian gene expression profile in the pituitary by stimulating the transcription of certain target genes specifically at night. If this were the case, it would be expected that there may be increased expression of Npas4 target genes in the pituitary during the night. Indeed, one Npas4 target gene, *Bdnf* (see Section 1.5.2.1), has been shown to be expressed in a circadian manner in various parts of the brain. In rodents, expression of *Bdnf* mRNA is higher at night time in the SCN, hippocampus and frontal cortex (Bova *et al.* 1998; Berchtold *et al.* 1999; Baba *et al.* 2008). Regarding the pituitary, while it has been shown that *Bdnf* mRNA is expressed in the anterior pituitary in rats (Kononen *et al.* 1994; Hopker *et al.* 1997) it is not known whether this expression is subject to circadian oscillation. Nevertheless, the mechanisms underlying increased nocturnal *Bdnf* expression remain to be elucidated. One hypothesis states that increased neural activity is responsible for higher levels of Bdnf at night as the rise in *Bdnf* expression corresponds to the time when nocturnal animals (such as rodents) are most physically active and interacting with their surroundings. Indeed, this hypothesis has been supported by experiments which show that, in the SCN at least, *Bdnf* is not directly regulated by the circadian clock machinery at a transcriptional level, but rather by a mechanism that requires neural activity; treatment of cultured SCN slices with tetrodotoxin, a compound that selectively and reversibly blocks Na^+ channels (and thus inhibits the generation of action potentials), abolished rhythmic expression of *Bdnf* mRNA but did not affect clock gene cycling (Baba *et al.* 2008). Given that Npas4 regulates *Bdnf* expression (see Section 1.5.2.1.3) and displays rhythmic expression in the pars tuberalis (see Section 1.4.8.3), it is possible to put forward the hypothesis that *Bdnf* might also show Npas4-dependent rhythmic expression in the pars tuberalis.

1.7 NPAS4 in neurological disease

Due to its central roles in excitatory/inhibition homeostasis, neuroprotection and activity-dependent regulation of BDNF, NPAS4 has been linked to several disease states; however, there is little understanding about its role in this context. In most cases, it is not clear whether altered NPAS4 expression under pathological conditions is merely a secondary effect related to changes in neural activity or whether it is part of a purposeful protective mechanism employed by neurons to minimise damage caused by pathological states.

1.7.1 Diseases associated with an imbalance in excitatory/inhibitory synaptic homeostasis

There are many neurological diseases and psychiatric conditions that are characterised by a disruption of the balance between excitatory and inhibitory synaptic transmission that normally exists in the brain. These include; autism (Rubenstein and Merzenich 2003), anxiety (Crestani *et al.* 1999), epilepsy (Baulac *et al.* 2001), Fragile X Syndrome (Gibson *et al.* 2008), insomnia (Buhr *et al.* 2002) and schizophrenia (Wassef *et al.* 2003). Due to its role in inhibitory synapse formation, NPAS4 is thought to play a crucial role in the maintenance of excitatory/inhibitory homeostasis in neural networks (see Section 1.6.1) and thus dysregulation of NPAS4 function could have implications for these types of diseases. Consistent with this hypothesis, Npas4$^{-/-}$ mice display some neurological abnormalities related to aberrant or excessive excitatory neural activity such as anxiousness, hyperactivity and susceptibility to seizure (Lin *et al.* 2008).

1.7.1.1 Seizure

In a mouse model of epilepsy, *Npas4* was identified as one of the most profoundly up-regulated genes 1h following seizure (Flood *et al.* 2004). As described previously in Section 1.6.3.3.1, Npas4$^{-/-}$ mice are more vulnerable to post-seizure excitotoxicity than wildtype littermates (Ooe *et al.* 2009b), suggesting that up-regulation of *Npas4* during seizure serves an acute neuroprotective function. Many studies using various experimental models of seizure have shown that *Bdnf* mRNA and protein expression is transiently increased in the cortex and hippocampus following seizure (Ballarin *et al.* 1991; Ernfors *et al.* 1991; Isackson *et al.* 1991; Humpel *et al.* 1993; Katoh-Semba *et al.* 1999). Given the association between Npas4 and activity-dependent *Bdnf* expression (see Section 1.5.2.1.3), it is possible that Npas4 may be involved in *Bdnf* up-regulation in the context of seizure and that this contributes to the aforementioned neuroprotective effect of Npas4. This hypothesis could be tested by investigating the expression of *Bdnf* in Npas4$^{-/-}$ mice following seizure. Furthermore, given the role of Npas4 in inhibitory synapse formation, it is tempting to speculate that

Npas4 may also participate in a more long-term protection programme by increasing the number of inhibitory synapses in order to restore homeostasis in neuronal networks and prevent further seizures - a process which may also be mediated by Bdnf expression (see Section 1.5.2.1.4).

1.7.1.2 Autism and ADHD

Mutations affecting the *SLC9A9* gene, which is a putative target of Npas4 that encodes the Na^+/H^+ transporter NHE9 (see Section 1.5.2.4.2), have been linked to both ADHD (de Silva *et al.* 2003; Lasky-Su *et al.* 2008) and autism comorbid with epilepsy (Morrow *et al.* 2008). Given that *SLC9A9* expression appears to be regulated by Npas4 in an activity-dependent manner, perturbation of Npas4 expression may also be linked to these diseases via dysregulation of *SLC9A9* expression. Though it is not known precisely how mutations affecting NHE9 function would contribute to ADHD or autistic phenotypes, it is hypothesised that dysregulation of NHE9 after neural activity may contribute to disease susceptibility by effecting membrane polarity and hence excitability of neurons. Disruption of the normal flow of ions between cytoplasm and organelles can affect many aspects of cellular biology such as cell size, membrane polarity and protein trafficking/recycling. Proper functioning of the endomembrane system requires that the pH of endosomes be maintained within a narrow range and aberrant expression of NHE9 has been shown to perturb luminal pH balance. Over-expression of NHE9 in COS-7 cells resulted in alkalinisation of endosomes (Nakamura *et al.* 2005), while combined knock-down of NHE9 and the closely related NHE6 resulted in luminal acidification in HeLa cells (Roxrud *et al.* 2009). Therefore, misexpression of NHE9 may have adverse consequences for neuronal function as a result of changes in endosomal pH.

1.7.1.3 Tourette syndrome

Tourette syndrome (TS) is an inherited neuropsychiatric disorder characterised by hyperkinetic behaviour such as involuntary vocal or motor tics. Comorbidity with other neuropsychological disorders such as ADHD and obsessive-compulsive disorder is common among TS patients (Spencer *et al.* 1998). It has been postulated that the hyperactive behaviours associated with TS, known as tics, are due to aberrant excitatory stimulation of the motor cortex (Kulisevsky *et al.* 2001). This suggests a disinhibition of these areas arising from an imbalance in excitatory/inhibitory homeostasis. Consistent with this, TS patients showed markedly different distribution of inhibitory neurons in the basal ganglia, the area of the brain responsible for regulation of voluntary motor control, when compared to controls. In TS patients, a higher number of inhibitory projection neurons were found in the globus pallidus pars interna though there were fewer in the caudate and putamen (Kalanithi *et al.*

2005) which suggests that dysregulation of inhibitory synaptic connections in the basal ganglia may contribute to TS symptoms. Given the role of NPAS4 in excitatory/inhibitory homeostasis (Section 1.6.1), one might expect that alterations in NPAS4 activity may be implicated in this disorder. Indeed, an association between *NPAS4* expression and tic severity was observed in a cohort of teenage TS patients; in both medicated and unmedicated TS patients, *NPAS4* mRNA expression in the blood positively correlated with tic severity (Liao *et al.* 2010). It is not known whether increased expression of *NPAS4* in the blood reflects increased expression in the brain, or indeed in which areas of the brain it might be increased, however this does implicate *NPAS4* in TS pathology.

1.7.1.4 Cortical spreading depression

Cortical spreading depression (CSD) is a slowly propagating wave of depolarising cortical activity that is commonly associated with various types of brain injury including migraine, concussion, seizure (more specifically, the postictal state which follows a seizure) and cerebral ischaemia (Somjen 2001). There is considerable evidence that CSD is a neuroprotective phenomenon that simultaneously promotes brain repair and increases resistance to subsequent injury. In the short-term, CSD triggers rapid up-regulation of *Bdnf* mRNA in cortical neurons which is dependent on NMDAR activation and peaks within 2h of CSD (Kokaia *et al.* 1993). CSD also provies a longer lasting benefit by stimulating SVZ neurogenesis that persists for up to six days (Yanamoto *et al.* 2005). A recent study of the neuroprotective effect of CSD highlighted the importance of Bdnf in CSD-induced neuroprotection; wildtype rats preconditioned with prolonged CSD were more resistant to subsequent ischaemic challenge while $Bdnf^{+/-}$ mice received no benefit from the same treatment suggesting that CSD confers tolerance via a Bdnf-dependent mechanism (Yanamoto *et al.* 2004). The observation that expression of *Npas4* mRNA is rapidly up-regulated by experimentally induced CSD (Hester *et al.* 2007) raises the possibility that Npas4 is involved in CSD-mediated neuroprotection via regulation of *Bdnf* expression.

1.7.2 Cerebral ischaemia

The *Npas4* gene is highly up-regulated in response to cerebral ischaemia and, though the pattern of expression has been studied in detail (Section 1.4.2.3.2), the *role* of Npas4 expression is this context is unknown. Given that Npas4 has been implicated in neuroprotective mechanisms following excitotoxic brain damage (Ooe *et al.* 2009b; Zhang *et al.* 2009), it is reasonable to hypothesise that Npas4 may perform a similar function following ischaemic injury. One way in which Npas4 may contribute to post-ischaemic brain repair is through activity-dependent regulation of *Bdnf* expression.

There is considerable evidence that Bdnf promotes post-ischaemic recovery in the CNS. In a rat model of stroke, intravenous infusion with exogenous Bdnf 30min after middle cerebral artery occlusion was found to improve neurological deficit, decrease cortical infarct volume and reduce the number of apoptotic cells in the penumbra (Schabitz *et al.* 2000). Other studies using different Bdnf treatment regimens have found similar improvements in infarct size and motor recovery (Schabitz *et al.* 1997; Schabitz *et al.* 2004; Schabitz *et al.* 2007). Nevertheless, even without intervention there is still a small amount of spontaneous recovery that occurs and this is attributed to neural plasticity whereby motor control of affected limbs is acquired by other parts of the brain through relearning. Studies have shown that endogenous Bdnf mediates this plasticity-dependent spontaneous recovery (Ploughman *et al.* 2009), probably arising from enhanced LTP (Kiprianova *et al.* 1999), and that it is dependent on GluR activation (Clarkson *et al.* 2011).

In support of the notion that Npas4 activates activity-dependent transcription of *Bdnf* in response to cerebral ischaemia, the timing, location and mechanism of endogenous *Bdnf* induction all indicate that a role for Npas4 is possible. Studies performed in rodent models of stroke have shown that cerebral ischaemia induces rapid up-regulation of *Bdnf* mRNA expression in both the cortex and hippocampus and that this up-regulation is dependent on activation of GluRs (Comelli *et al.* 1992; Lindvall *et al.* 1992; Comelli *et al.* 1993). Furthermore, temporal analysis of *Bdnf* expression in a rat stroke model also supports a role for Npas4 in post-ischaemic *Bdnf* regulation (Rickhag *et al.* 2006). The role of Npas4 in ischaemia-induced brain repair requires further investigation and could be addressed directly by assessing post-ischaemic recovery in Npas4$^{-/-}$ mice.

1.7.3 Tauopathies: Alzheimer's disease and frontotemporal dementia

Tau proteins are microtubule-associated proteins that are abundantly expressed in neurons of the CNS and promote axonal microtubule assembly and stabilisation. There are six different isoforms of the tau protein that arise from alternative splicing of a single gene, microtubule-associated protein tau (*MAPT*), which is located on human chromosome 17 (Hernandez and Avila 2007). Mutations in the *MAPT* gene or hyperphosphorylation of the tau proteins can lead to the abnormal and irreversible aggregation of tau proteins into insoluble, toxic polymers called neurofibrillary tangles (NFTs). It is now accepted that NFTs cause synaptic dysfunction and, ultimately, neuronal cell death and consequently the term 'tauopathy' has been used to describe neurodegenerative diseases that are characterised by misfolding of tau proteins and intracellular NFTs (Spires-Jones *et al.* 2009). Cerebral atrophy, cognitive decline and dementia are symptoms that are commonly associated

tauopathies among which are included Alzheimer's disease, frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and progressive supranuclear palsy.

In order to study the molecular processes involved in NFT formation, transgenic mice carrying a mutation commonly found in FTDP-17 patients (Tau P301L) were generated as an animal model of FTDP-17 (Murakami *et al.* 2006). Many symptoms of the human FTDP-17 condition are mirrored in the transgenic mice ($TgTau^{P301L}$) including age-dependent formation of NFTs, neuronal cell loss, memory deficits and, as is seen in human families, considerable phenotypic variation among individuals of the same genotype (Murakami *et al.* 2006). Comparison of $TgTau^{P301L}$ mice showing extensive NFTs and neuronal cell loss with $TgTau^{P301L}$ mice that had only pretangles revealed that *Npas4* mRNA and protein expression were markedly up-regulated in brains containing NFTs (Wakasaya *et al.* 2011). Furthermore, these findings were confirmed in human patients where higher levels of NPAS4 protein expression were observed in the brains of individuals with Alzheimer's disease and frontotemporal dementia compared to control brains (Wakasaya *et al.* 2011).

These data suggest that increased Npas4 expression is specifically associated with NFTs, however the role of Npas4 in NFT pathophysiology is unknown. While the authors have speculated that Npas4 may participate in cascades leading to NFT formation, there is no evidence to support this hypothesis. It is possible that, instead of contributing to pathology, Npas4 is up-regulated in response to NFTs and provides a neuroprotective effect through the stimulation of *Bdnf* expression which has been shown to promote dephosphorylation of tau proteins (Elliott *et al.* 2005). Further research is needed to distinguish between these two possibilities.

1.7.4 Down's syndrome

Down's syndrome is a congenital condition caused by a chromosomal abnormality whereby an extra copy of genetic material from chromosome 21 is present. This may arise due to trisomy of chromosome 21 or, less frequently, a Robertsonian translocation where only part of chromosome 21 is present in three copies. Symptoms of Down's syndrome vary widely between individuals and, along with mental retardation which is the only symptom to appear in all cases of Down's syndrome, may include some or all of the following; short stature, characteristic cranofacial anomalies, infertility, muscle hypotonia and immune system perturbations. People with Down's syndrome also have a higher incidence of childhood leukaemias, are susceptible to epilepsy and have an increased risk of developing Alzheimer's disease (Kola and Hertzog 1998).

At the cellular level, Down's syndrome affects dendrite morphology and synaptic function and it is postulated that these changes may be the morphological basis of cognitive impairment associated with Down's syndrome. During the early post-natal period, children with Down's syndrome exhibit dendritic atrophy in the visual cortex (Becker *et al.* 1986) while a reduction in the number of dendritic spines present on hippocampal neurons was observed in adults (Ferrer and Gullotta 1990). Supporting these findings, decreased hippocampal dendritic spine density was also observed in a segmental trisomy mouse model of Down's syndrome (Belichenko *et al.* 2004; Kurt *et al.* 2004; Belichenko *et al.* 2007). These mice also exhibited defects in post-natal hippocampal synaptogenesis (Chakrabarti *et al.* 2007) as well as a failure to induce LTP (Siarey *et al.* 1999; Siarey *et al.* 2005). It is thought that this impairment of LTP is due to increased inhibitory synaptic transmission and, therefore, reduced activation of NMDARs (Kleschevnikov *et al.* 2004).

It is proposed that the symptoms associated with Down's syndrome are caused by a dosage imbalance of some (or all) of the genes located within a 'Down's syndrome critical region' (DSCR) on chromosome 21 (McCormick *et al.* 1989), though it's likely that multiple genes each contribute to part of the overall phenotype. One of the candidate genes implicated in the mental retardation phenotype associated with Down's syndrome is SIM2 (located at 21q22.2). Several lines of evidence support a role for SIM2 in Down's syndrome. Firstly, during development it is expressed in regions of the CNS that are affected in Down's syndrome (Rachidi *et al.* 2005). Secondly, in a segmental trisomy mouse model of Down's syndrome, expression of the orthologous murine gene (*Sim2*) was found to be increased by 50% in the fetal brain (Vialard *et al.* 2000). Finally, over-expression of *Sim2* in mice produced some of the symptoms associated with Down's syndrome including impaired learning and memory (Ema *et al.* 1999; Chrast *et al.* 2000).

Altered expression of NPAS4 has been proposed to be a causative factor in Down's syndrome pathogenesis in a model involving combined regulation of target genes by NPAS4 and SIM2 (Ooe *et al.* 2004). Given that both Npas4 and Sim2 are able to dimerise with partner factor Arnt2 and that there is some overlap in DNA sequence binding of the Npas4/Arnt2 and Sim2/Arnt2 heterodimers (Ooe *et al.* 2004) it is possible that these factors may compete for DNA binding at mutually recognised sites, such as the CME. As Npas4 has transcriptional activation activity and Sim2 is a repressor, these factors are predicted to exert opposing effects on transcription of common target genes which in the normal situation would result in appropriate transcription of these genes. In the case of Down's syndrome, it is hypothesised that this finely balanced expression of target genes would be disrupted

by a stoichiometric excess of SIM2 protein; excess SIM2 may sequester ARNT2, thus preventing formation of other functional heterodimers (i.e.NPAS4/ARNT2), and/or actively repress transcription of shared target genes.

One of the genes proposed to be affected in this model is DREBRIN (Ooe *et al.* 2004). Indeed, decreased expression of DREBRIN has been observed in the frontal and temporal cortex (but not cerebellum) of Down's syndrome patients (Shim and Lubec 2002) though the cause of down-regulation has not been established. While SIM2 has been shown to inhibit NPAS4/ARNT2-dependent transcription of DREBRIN in a reporter gene assay performed in a human neuroblastoma cell line (Ooe *et al.* 2004), whether this occurs *in vivo* is not clear. Furthermore, though the expression pattern of Drebrin in the adult rodent brain overlaps with that of Npas4 and Sim2 in some areas, such as the hippocampus (Ooe *et al.* 2004), the extent of overlapping expression during embryonic development, when the first signs of synaptic abnormality are seen, is not known. Though this model is appealing, currently no *in vivo* data are available regarding NPAS4 expression in Down's syndrome patients and therefore this is an area of research that should be pursued further.

1.8 Homologs of NPAS4 in invertebrate species

Often, insights into the function of a protein can be gained by examining the function of closely related protein in other species. Putative homologs of NPAS4 have been identified in both the fruit fly *D. melanogaster* and the nematode *Caenorhabditis elegans* (*C. elegans*) where they are known as Dysfusion (Dys) and C15C8.2, respectively (Jiang and Crews 2003; Ooe *et al.* 2004; Ooe *et al.* 2007). The corresponding homologous genes are similar to the human *NPAS4* gene in their exon-intron architecture and they encode bHLH PAS transcription factors having a domain structure similar to that of NPAS4 (Ooe *et al.* 2007). Based on amino acid homology within the bHLH domain, the human NPAS4 protein and the invertebrate Npas4-like factors are more closely to each other than to other bHLH PAS factors within the same species (Ooe *et al.* 2007) and hence these proteins cluster together to form a distinct subgroup within the bHLH PAS family (Jiang and Crews 2003) (Figure 1.10). This suggests that all of the Npas4-related genes are derived from a common ancestral gene which existed before the divergence of nematodes, insects and vertebrates. Nevertheless, although there is a reasonable degree of homology within the bHLH domains (57-58% amino acid identity), the similarity in the PAS domains is considerably less (only 49-52% and 30-33% amino acid identity in the PAS A and PAS B domains, respectively) and outside of these domains the protein sequences are poorly conserved (Ooe *et al.* 2004; Ooe *et al.* 2007). Therefore, rather than being true orthologs, it is

possible that the Npas4-like factors have acquired non-conserved functions throughout the course of evolution and the current evidence suggests that this is the case.

1.8.1 *D. melanogaster*: Dysfusion

Dys, also known as *Drosophila* NXF-like-factor (dNXFL), is the *D. melanogaster* homolog of the mammalian NPAS4 protein and, while there is some evolutionary conservation of biochemical function, the available evidence suggests that two proteins may have divergent functions.

1.8.1.1 Expression of *dys*

During *D. melanogaster* development, the *dys* gene is expressed in tracheal fusion cells (specialised cells located at the tips of tracheal tubules) from mid embryogenesis until larval hatching (Stages 12-17) where it controls the process of tracheal branch fusion (Jiang and Crews 2003; Jiang and Crews 2006). In addition to this, expression of *dys* can be found in a diverse collection of embryonic cell types including the epidermal leading edge, the foregut atrium, hind gut, anal pad and sections of the nervous system (Jiang and Crews 2003), though its function in these tissues has not been investigated.

1.8.1.2 Dys function

Disruption of *dys* function using either RNAi (Jiang and Crews 2003) or genetic means (Jiang and Crews 2006) results in widespread tracheal fusion defects; while tubule branching and migration are normal, by Stage 16 a complete lack of tracheal fusion is seen in the dorsal branch, the ganglionic branch and the lateral trunk. Although Dys is also expressed in fusion cells of the dorsal trunk, fusion of these cells is relatively normal (Jiang and Crews 2003; Jiang and Crews 2006) suggesting that other factors besides Dys are involved in tracheal fusion in the dorsal trunk. Animals lacking Dys expression do not survive to adulthood and die at the second or third instar larval stage (Jiang and Crews 2003; Jiang and Crews 2006) indicating that the *dys* gene is essential for normal development. It is presumed that tracheal defects are the cause of lethality, however as *dys* is also expressed in a variety of other embryonic tissues, the possible involvement of defects other cannot be excluded.

1.8.1.3 Dys target genes

In the trachea, Dys expression is specific to fusion cells where it regulates the expression of genes that mediate tracheal fusion (Jiang and Crews 2006). Several genes that are positively regulated by

Dys encode transmembrane proteins with putative roles in cell adhesion, such as *shotgun* and *CG13196*. Other positively regulated genes include *members only*, which encodes a nucleoporin protein, and *CG15252*, which is only expressed in dorsal trunk fusion cells and encodes a protein of unknown function. Interestingly, loss of *members only* results in tracheal fusion defects (Uv *et al.* 2000) which closely resemble the *dys* mutant phenotype. In addition to this, it has been proposed that Dys can enhance its own expression in a manner reminiscent of the mammalian NPAS4 protein (Ooe *et al.* 2004) via an autoregulatory module located in the 5' flanking region of the *dys* gene (Jiang *et al.* 2010). Dys also negatively regulates expression of the bHLH PAS factor trachealess (Trh) at the post-translational level (Jiang and Crews 2003; Jiang and Crews 2006); it has been shown that Dys expression is needed for efficient proteasome-dependent degradation of Trh by the F-box/WD repeat protein Archipelago (Mortimer and Moberg 2007). Trh, the ortholog of the mammalian Npas3 protein (see Section 1.9.1), is a master regulator of tracheal development (Isaac and Andrew 1996) and directly regulates expression of the fibroblast growth factor (FGF) receptor Breathless (Ohshiro and Saigo 1997). Over-expression of Dys results in aberrant expression of Dys target genes and, consequently, ectopic tracheal fusion events (Jiang and Crews 2006).

1.8.1.4 Biochemical properties of the Dys protein

There are many parallels between the Dys protein and its mammalian counterparts. The primary structure of the Dys polypeptide is highly similar to that of other NPAS4-related proteins although, in addition to the requisite bHLH and PAS domains, it also contains an N-terminal glutamine-rich region which is not present in either the mammalian NPAS4 proteins or the *C. elegans* C15C8.2 protein (Ooe *et al.* 2007). Like NPAS4, Dys does not homodimerise but can interact with Tgo to form a transcriptionally active heterodimer (Jiang and Crews 2007; Ooe *et al.* 2007). As with the NPAS4 proteins, the transactivation domain of Dys is localised to the C-terminal region of the protein and deletion studies have shown that the unique glutamine-rich region does not contribute to transcriptional activity (Ooe *et al.* 2007). Another common feature is the promiscuous DNA binding of the respective dimers. The Dys/Tgo complex can bind sequences of the form NCGTG and has an identical preference spectrum (TCGTG>GCGTG>ACGTG>CCGTG) to that of Npas4/Arnt (Jiang and Crews 2007; Ooe *et al.* 2007). This is a property that is unique among bHLH PAS factors, which usually have only one preferred consensus sequence (such as the Sim/Tgo and Trh/Tgo dimers which preferentially bind ACGTG), and the fact that it has been conserved throughout evolution suggests that this promiscuous DNA binding may be biologically important, though its significance is not yet known.

1.8.1.5 Regulation of *dys* expression

Recently, efforts have been made to identify the regulatory sequences that are responsible for the precisely controlled expression of *dys* as well as the upstream factors that control its expression. Analysis of the 5' flanking region of the *dys* gene allowed two distinct regulatory elements to be isolated which specifically promote *dys* expression in all tracheal fusion cells (Jiang *et al.* 2010). It is proposed that the more 5' element initiates early expression of *dys* as it is active from Stages 12-17 and contains putative binding sites for E-twenty six (ETS) and POU-homeodomain proteins (Jiang *et al.* 2010) while the more 3' element, which contains three putative Dys/Tgo TCGTG binding sites and is active only from Stages 14-17, is responsible for amplification of *dys* expression at a later stage of development through positive autoregulation (Jiang *et al.* 2010). The presence of a sequence similar to the ETS binding site consensus sequence raises the interesting possibility that Pointed, an ETS factor that mediates expression of the *branchless*/*breathless* (FGF/FGFR) pathway during tracheal development (Myat *et al.* 2005), is also responsible for initiation of *dys* expression in the developing trachea. In addition, although expression of *dys* in tracheal fusion cells of the dorsal branch and ganglionic branch was found to be dependent on the zinc finger transcription factor Escargot (Jiang and Crews 2003), no Escargot binding sites were identified in the *dys* 5' flanking region (Jiang *et al.* 2010) indicating that Escargot may regulate *dys* expression via an indirect mechanism. Considering that the regulatory elements and transcription factors controlling expression of the mammalian *NPAS4* genes have not yet been identified, perhaps a comparative genomics approach could be used to determine whether the regulatory motifs identified in the *dys* locus are also conserved in other NPAS4 homologs.

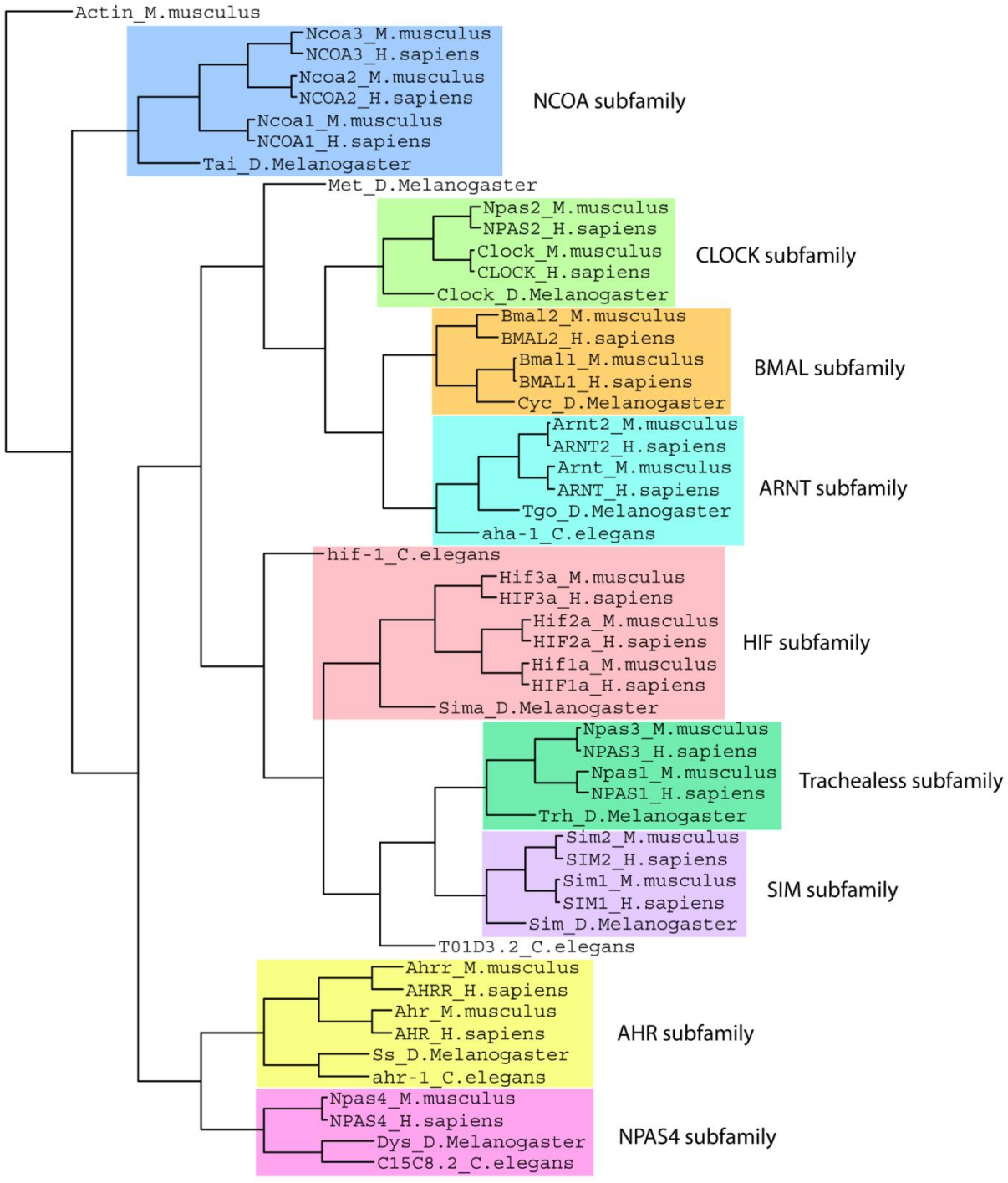


Figure 1.10 – Phylogenetic tree of bHLH PAS transcription factors across species
The evolutionary relationships among bHLH PAS transcription factors from different species were calculated using the Clustal Omega algorithm (http://www.clustal.org/omega/) and a phylogenetic tree constructed using the unweighted pair group method with arithmetic mean. The mouse actin protein was used to root the tree. Amino acid sequences can be found in Appendix 1.

1.8.2 *C. elegans*: C15C8.2

While there is a significant amount of published data relating to the expression, function and regulation of Dys, comparatively little is known about C15C8.2, the *C. elegans* homolog of NPAS4. Although there are currently no data concerning the function of the C15C8.2 protein, also referred to as cky-1 or *C. elegans* NXF-like-factor (cNXFL), it has been stated that it is expressed in the pharynx of the nematode (Jiang and Crews 2003) which raises the possibility that it may have a similar role to Dys in regulating the development of hollow tube-like structures. The non-conserved C-terminal portion of the C15C8.2 polypeptide is considerably shorter than that of other NPAS4-related proteins and this accounts for the overall smaller size of the C15C8.2 protein (676 amino acids in total) when compared to either Npas4 (802 amino acids) or Dys (921 amino acids). Nevertheless, despite this difference in length, it is apparent that the C15C8.2 protein has retained at least some the biochemical properties that are common to all of the NPAS4-related proteins that have been examined thus far. Like both Npas4 and Dys, the C15C8.2 protein does not homodimerise, but is capable of binding aryl hydrocarbon receptor associated protein (aha-1), the *C. elegans* ortholog of ARNT (Ooe *et al.* 2007). Furthermore, the C15C8.2/aha-1 heterodimer is able to bind the TCGTG DNA motif that is recognised by other NPAS4-related proteins and can drive expression of a reporter gene indicating that it functions as a transcriptional activator (Ooe *et al.* 2007). Therefore, while it seems as though some of the fundamental biochemical properties have been conserved among the NPAS4-related proteins, investigations into the regulation and function of the C15C8.2 protein are required to determine whether the similarities are merely mechanistic or also functional.

1.9 Neuronal PAS proteins

Npas4 is the fourth, and most recently discovered, of the neuronal PAS proteins, a subset of bHLH PAS transcription factors that are abundantly expressed in the brain. It is worth mentioning that classification of these factors as 'neuronal PAS' proteins was based purely on initial observations of their expression pattern (Zhou *et al.* 1997; Brunskill *et al.* 1999) rather than any functional or evolutionary relatedness and, therefore, although these proteins share a common nomenclature, they each have distinct, and mostly unrelated, biological functions. Nevertheless, when discussing Npas4, it would be remiss not to at least briefly mention the other neuronal PAS proteins.

1.9.1 Npas1 and Npas3

The Npas1 and Npas3 proteins are highly related (50.2% identity) at the amino acid level (Brunskill *et al.* 1999) making them the only two members of the neuronal PAS group that share significant homology. Furthermore, both proteins appear to be orthologs of the *D. melanogaster* Trh protein as demonstrated by extensive sequence homology (Zhou *et al.* 2009) and conservation of some functions; like Trh, both Npas1 (Levesque *et al.* 2007) and Npas3 (Zhou *et al.* 2009) have roles in lung development and, moreover, Npas3 has been shown to regulate expression of the FGF receptor (Pieper *et al.* 2005). Both proteins bind the common partner factor Arnt, however, while it has been demonstrated that Npas3 can either promote or repress gene expression *in vitro* (Zhou *et al.* 2009), Npas1 lacks a transactivation domain and instead has three C-terminal repression domains (Teh *et al.* 2006). There is evidence that the Npas1/Arnt dimer can bind the HRE and directly down-regulate the expression of a number of Hif target genes, such as erythropoietin (Ohsawa *et al.* 2005) and tyrosine hydroxylase (Teh *et al.* 2007), which suggests that these factors may collaboratively regulate gene expression in areas where both factors are present.

In addition to being expressed in the developing lung, both *Npas1* and *Npas3* are also expressed in the developing CNS where they are thought to be important for neurodevelopment (Zhou *et al.* 1997; Brunskill *et al.* 1999; Gould and Kamnasaran 2011). Inexplicably, although one group has reported that Npas3$^{-/-}$ mice die shortly after birth due to respiratory defects (Zhou *et al.* 2009), this differs to what has been reported by other laboratories; a number of groups have reported that Npas3$^{-/-}$ mice survive to adulthood albeit with some brain abnormalities which include impaired adult hippocampal neurogenesis and a subsequent reduction in hippocampal volume coupled with behavioural and neurosignalling defects (Brunskill *et al.* 2005; Pieper *et al.* 2005; Pieper *et al.* 2010). Indeed, Npas1$^{-/-}$;Npas3$^{-/-}$ double knock-out mice were generated and these animals also survived to adulthood but, similarly to Npas3$^{-/-}$ mice, they displayed a number of behavioural abnormalities reminiscent of psychosis (Erbel-Sieler *et al.* 2004). Thus, while results are varied, there is some evidence that these genes may be involved in important neurodevelopmental processes.

In humans, the *NPAS3* locus has been linked to a number of neurological conditions, particularly psychiatric illnesses (Pickard *et al.* 2006). In one family, a reciprocal chromosomal translocation which causes disruption of the *NPAS3* locus was associated with schizophrenia (Kamnasaran *et al.* 2003; Pickard *et al.* 2005) while a number of single nucleotide polymorphisms (SNPs) within the *NPAS3* gene are associated with an altered risk of schizophrenia (Pickard *et al.* 2009; Macintyre *et al.*

2010). Interestingly, variations in the *NPAS3* gene are also associated with increased or decreased responsiveness to the antipsychotic drug iloperidone (Lavedan *et al.* 2009). Genome-wide association studies have also linked allelic variants of the *NPAS3* gene to addiction (Liu *et al.* 2006) and smoking cessation (Uhl *et al.* 2008). The *NPAS3* gene has also been identified as a candidate gene for holoprosencephaly (Kamnasaran *et al.* 2005) and mental retardation associated with Sotos syndrome (Visser *et al.* 2010). Finally, it has also been proposed that NPAS3 may act as a tumour suppressor in astrocytes as NPAS3 expression was down-regulated in 80% of astrocytomas (Moreira *et al.* 2011).

1.9.2 Npas2

Npas2, which is also known as member of PAS superfamily 4 (MOP4), was found to be a gas-regulated paralog of the Clock protein and, as such, it forms part of the molecular machinery which governs circadian rhythm. More specifically, Npas2 controls the food-entrainable oscillator (a control mechanism which coordinates synchronisation of circadian behaviour with food intake) and thus is critical for adaptability to altered feeding schedules (Dudley *et al.* 2003; Wu *et al.* 2010). It has also been proposed that Npas2 may play a role in sleep homeostasis as $Npas2^{-/-}$ mice exhibit impaired long-term memory (Garcia *et al.* 2000) and disrupted sleeping rhythms (Dudley *et al.* 2003; Franken *et al.* 2006). Consistent with this, SNPs in the human *NPAS2* gene have been linked to various conditions that are associated with circadian dysfunction including fertility and seasonality (Kovanen *et al.* 2010), metabolic syndrome (Englund *et al.* 2009) and mood disorders such as unipolar major depression (Soria *et al.* 2010) and seasonal affective disorder (Partonen *et al.* 2007).

Like Clock, Npas2 partners Bmal1 to regulate the expression of clock-controlled genes such as *Per1*, *Per2*, *Cry1* and *Bmal1* itself (Reick *et al.* 2001). Indeed, the functional redundancy of these two proteins is such that Npas2 can substitute for the Clock protein in the 'master clock' of the SCN (DeBruyne *et al.* 2007) as well as the peripheral oscillator located in the liver (Bertolucci *et al.* 2008). Though first identified as a CNS-specific factor (Zhou *et al.* 1997), *Npas2* was later found to be expressed in many peripheral tissues including the liver, heart, stomach, spleen, kidney, testis (Yamamoto *et al.* 2004; Takeda *et al.* 2011) where, like all clock genes, its expression oscillates with a period of 24h (highest expression occurs at the end of the dark period). The PAS domains of the Npas2 protein bind heme, a prosthetic group that has a high affinity toward diatomic gases, and this allows Npas2 to function as a heme-based gas sensor. The ligand which controls the activity of Npas2 is carbon monoxide; binding of carbon monoxide to the heme group induces a structural change in the Npas2 protein which enables dimerisation with Bmal1 (Dioum *et al.* 2002; Uchida *et al.* 2005).

1.10 Project rationale and research aims

1.10.1 Project rationale

To date, most of the research relating to *Npas4* has focused on its expression in the adult brain and its role in neural function after birth. Little data exists regarding the expression of *Npas4* during development (see Section 1.3.5) and no developmental role has been described for Npas4 thus far. Two independent research groups (Lin *et al.* 2008; Ooe *et al.* 2009b) have generated Npas4 null mice, however no striking developmental abnormalities were reported; Npas4$^{-/-}$ mice do not show any gross morphological abnormality at birth, they are able to breed normally and homozygous Npas4 null mutants are present in the expected Mendelian ratios (Ooe *et al.* 2009b). While these observations suggest that the *Npas4* gene is not essential for embryogenesis, it does not exclude the possibility that Npas4 plays an accessory role during development. There are numerous examples of genes that have roles in important developmental processes but do not show a profound developmental phenotype when deleted, particularly when there may be compensation by other genes having similar functions. For instance, the sry-related high mobility group box 1 (*Sox1*) gene is specifically expressed in the neural plate during early CNS development where it is involved in maintenance of NPCs (see Section 3.1.1.2); however, Sox1$^{-/-}$ mice are viable, are born in the expected Mendelian ratios and show no signs of developmental abnormalities (Nishiguchi *et al.* 1998). This is likely due to functional redundancy between Sox1 and related proteins having overlapping functions (see Section 3.1.1.2).

Though experimental evidence is still lacking, several lines of evidence suggest that Npas4 may have a developmental role. Firstly, it is not uncommon for bHLH PAS transcription factors to have roles in both adulthood and development (see Table 1.1). For example, in addition to regulating the hypoxic response in adults, Hif1-α is also required for proper vascularisation of the embryo and consequently mice lacking Hif1-α die *in utero* due to vascularisation defects (Ryan *et al.* 1998). Indeed, some researchers have suggested a possible developmental role for Npas4 due to its similarity to other bHLH PAS proteins that are involved in regulating various aspects of embryonic development. Npas4 falls within a subset of bHLH PAS proteins that are restricted in their expression pattern and that are not dependent on pre-activation (such as ligand binding or protein stabilisation) to form transcriptionally active dimers. Other factors belonging to this subgroup include the *D. melanogaster* proteins Sim and Trh both of which have critical roles in development. Sim regulates the fate of precursor cells in the midline of the embryonic CNS and is required for their differentiation into mature neural cell types (Nambu *et al.* 1990; Nambu *et al.* 1991), while Trh is a master regulator of

tracheal cell fate determination in the developing embryo (Wilk *et al.* 1996). Moser *et al.* have proposed that since Npas4 shares a similar mode of action to these proteins it could also be important for embryonic development (Moser *et al.* 2004).

Secondly, consideration of evolutionarily related genes also supports of the notion that Npas4 may have a role during development. The *D. melanogaster* homolog of Npas4, *Dys*, is expressed during embryogenesis and, indeed, is essential for normal development (see Section 1.8.1). Given that Npas4 and Dys are descended from a common ancestral protein, it is possible that some aspects of the developmental expression or function of Dys are conserved in the mammalian Npas4 proteins.

Thirdly, several Npas4 target genes are known to be important for CNS development. The embryonic isoform of Drebrin, Drebrin E, plays a role in axonal growth during development (Mizui *et al.* 2009; Dun and Chilton 2010) while disruption of Bdnf signalling has profound consequences for neuronal survival resulting in death during the first few weeks of life (Ernfors *et al.* 1994; Alcantara *et al.* 1997).

Lastly, a computational analysis which examined networks of interacting bHLH transcription factors predicted that Npas4, together with NeuroD6, a neurogenic bHLH transcription factor important for neuronal differentiation and survival (Schwab *et al.* 2000; Uittenbogaard and Chiaramello 2005), may be part of a transcriptional regulatory module which is important in mouse brain development (Li *et al.* 2007).

1.10.2 Reseach aims

Based on these observations, it is possible to put forward the hypothesis that *Npas4* has a role in embryonic development. Accordingly, the primary objective of this thesis is to address this hypothesis by investigating both the expression and function of *Npas4* in a number of developmental model systems. In order to achieve this, several research aims have been devised:

1. To investigate the expression of *Npas4* during neural differentiation of embryonic stem (ES) cells.
2. To investigate the expression of *Npas4* in the developing mouse embryo.
3. To investigate the function of Npas4 in the context of development by using a loss-of-function approach in mouse ES (mES) cells.

2 – Materials and methods

2.1 Materials

2.1.1 Reagents

All reagents used were of molecular biology grade.

Reagent	Company	Catalogue #
Hybond™-N+ positively charged nylon membrane	Amersham	RPN 137B
ABI PRISM® Optical Adhesive Cover Starter Pack	Applied Biosystems® (Life Technologies™)	4313663
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems® (Life Technologies™)	4337455
MicroAmp® Optical 96-well Reaction Plate	Applied Biosystems® (Life Technologies™)	N801-0560
MicroAmp® Optical Caps	Applied Biosystems® (Life Technologies™)	4323032
MicroAmp® Optical Tubes	Applied Biosystems® (Life Technologies™)	N801-0933
SYBR® Green PCR Master Mix	Applied Biosystems® (Life Technologies™)	4309155
Cell Strainer (70µm)	BD Falcon™	352350
40% Acrylamide/Bis Solution (37.5:1)	BIO-RAD	161-0148
Precision Plus Protein™ Dual Xtra Standards	BIO-RAD	161-0377
Protein Assay Dye Reagent Concentrate	BIO-RAD	500-0006
Formamide (deionized)	Chemicon® (Merck)	S4117
Fluorescence mounting medium	Dako	S3023
Skim Milk Powder	Diploma	28510001
Dimethyl sulfoxide (DMSO)	Finnzymes (Thermo Fisher Scientific)	F-515
Phusion™ Flash High-Fidelity DNA Polymerase	Finnzymes (Thermo Fisher Scientific)	F-530
Custom oligonucleotides	GeneWorks	-
Horse Serum	Gibco® (Life Technologies™)	16050-098
1 Kb DNA Ladder	Invitrogen™ (Life Technologies™)	15615-016
1 Kb Plus DNA Ladder	Invitrogen™ (Life Technologies™)	10787-018
100 bp DNA Ladder	Invitrogen™ (Life Technologies™)	15628-019
BenchMark™ Pre-Stained Protein Ladder	Invitrogen™ (Life Technologies™)	10748-010
ProLong® Gold antifade reagent	Invitrogen™ (Life Technologies™)	P36934
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen™ (Life Technologies™)	10777-019
SuperScript™ III Reverse Transcriptase	Invitrogen™ (Life Technologies™)	18080-093
Trypan Blue Stain	Invitrogen™ (Life Technologies™)	15250061
UltraPure™ Agarose	Invitrogen™ (Life Technologies™)	15510-027
Chambered coverglass	Lab-Tek®	177402
Alkaline Phosphatase Detection Kit	Millipore™ (Merck)	SCR004
Immobilon-P PVDF Membrane (0.45 µm)	Millipore™ (Merck)	IPVH00010
Immobilon™ Western Chemiluminescent HRP Substrate	Millipore™ (Merck)	WBKL S00 50
Gel Loading Dye, Blue (6X)	New England Biolabs®	B7021S
Prestained Protein Ladder, Broad Range	New England Biolabs®	P7710S
SacI	New England Biolabs®	R0156S
SacII	New England Biolabs®	R0157S
SDS Blue Loading Buffer (3X)	New England Biolabs®	B7703S
Taq DNA Polymerase	New England Biolabs®	M0267L

Riboprobe® Combination System - SP6/T7 RNA Polymerase	Promega	P1460
DNeasy Blood and Tissue Kit	QIAGEN®	69504
Plasmid Midi Kit	QIAGEN®	12143
QIAprep® Spin Miniprep Kit	QIAGEN®	27106
QIAquick® Gel Extraction Kit	QIAGEN®	28704
Blocking Reagent for nucleic acid hybridization and detection	Roche	11 096 176 001
cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets	Roche	11 836 170 001
DIG Nucleic Acid Detection Kit	Roche	11 175 041 910
Digoxigenin-11-uridine-5'-triphosphate (DIG-11-UTP)	Roche	03 359 247 910
FuGENE® 6 Transfection Reagent	Roche	11 815 091 001
High Pure RNA Isolation Kit	Roche	11 828 665 001
NBT/BCIP Stock Solution	Roche	11 681 451 001
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich®	D9542
Ampicillin	Sigma-Aldrich®	A9393
Albumin from bovine serum	Sigma-Aldrich®	A7906
Ammonium persulfate	Sigma-Aldrich®	A3678
Deoxynucleotide (dNTP) Mix	Sigma-Aldrich®	D7295
Dithiothreitol (DTT)	Sigma-Aldrich®	D9163
DNA sodium salt from salmon testes	Sigma-Aldrich®	D1626
Ethidium bromide	Sigma-Aldrich®	E8751
Hybri-Max™ DMSO	Sigma-Aldrich®	D2650
IGEPAL® CA-630	Sigma-Aldrich®	I7771
MISSION® Non-Target shRNA Control Vector	Sigma-Aldrich®	SHC002
MISSION® pLKO.1-puro Control Vector	Sigma-Aldrich®	SHC001
MISSION® shRNA Bacterial Glycerol Stock (mouse Npas4)	Sigma-Aldrich®	SHGLY-NM_153553
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich®	T7024
Sodium deoxycholate	Sigma-Aldrich®	D6750
TWEEN® 20	Sigma-Aldrich®	P5927

Tissue culture reagents	**Company**	**Catalogue #**
Dulbecco's modified Eagle medium (DMEM)	Gibco® (Life Technologies™)	11965-118
Ham's F-12 Nutrient Mix	Gibco® (Life Technologies™)	11765-047
Neurobasal® Medium	Gibco® (Life Technologies™)	21103-049
B-27® Supplement (50X)	Gibco® (Life Technologies™)	17504-044
N-2 Supplement (100X)	Gibco® (Life Technologies™)	17502-048
Trypsin/EDTA	Gibco® (Life Technologies™)	15400-054
Qualified foetal bovine serum (FBS)	Invitrogen™ (Life Technologies™)	10099-141
Penicillin/Streptomycin	Sigma-Aldrich®	P4333
Puromycin	Sigma-Aldrich®	P8833

2.1.2 Equipment

Equipment	**Company**
CP1000 Processor	Agfa HealthCare
CURIX Ortho HT-G Film	Agfa HealthCare
ABI PRISM® 7000 Sequence Detection System	Applied Biosystems® (Life Technologies™)
FACSCanto™ Flow Cytometer	BD Biosciences
Mini-PROTEAN® 3 Multi-Casting Chamber	BIO-RAD

Mini-Sub® Cell GT electrophoresis apparatus	BIO-RAD
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell	BIO-RAD
Haemocytometer	BLAUBRAND®
5415C Centrifuge	Eppendorf
5415D Centrifuge	Eppendorf
5702 Centrifuge	Eppendorf
BioPhotometer	Eppendorf
Fujix Colour Digital Camera	Fujifilm Corporation
ImageQuant™ ECL	GE Healthcare
Pipetman® (P10, P20, P200, P1000)	Gilson®
MJ Research PTC-200 Thermo Cycler	Global Medical Instrumentation
Premium Glass Coverslips - Circles 15mm	HD Scientific Supplies
HERAcell® 150 Incubator	Heraeus®
SuperFrost® Microscope Slides	MENZEL-GLÄSER (Thermo Fisher Scientific)
SilkFit™ Powder-free Latex Exam Gloves	Mirella Research
SilkFit™ Powder-free Nitrile Exam Gloves	Mirella Research
ART® Aerosol Resistant Tips (ART 20, 200, 1000)	Molecular BioProducts (Thermo Fisher Scientific)
EMax® Endpoint ELISA Microplate Reader	Molecular Devices
Cryo 1°C Freezing Container	NALGENE®
Eclipse TE300 Inverted Microscope	Nikon Corporation
Eclipse TS100 Inverted Microscope	Nikon Corporation
CryoTubes™	Nunc (Thermo Fisher Scientific)
SZ1145 Stereo Microscope	Olympus
Nanosep® Centrifugal Devices with Omega™ Membrane	Pall Corporation
Parafilm®	Pechiney Plastic Packaging
CoolSNAP fx® Camera	Photometrics®
Hybridisation Oven	Ratek Instruments
AxioPlan2 Upright Epi-fluorescence Microscope	Zeiss

2.1.3 Media

HEK 293T medium

90% (v/v) DMEM
10% (v/v) FBS

ES cell medium

90% (v/v) DMEM
10% (v/v) FBS
2mM L-Glutamine
100µM β-mercaptoethanol
1X Leukaemia inhibitory factor (LIF)[#]
1X Penicillin/Streptomycin

N2B27 medium

50% (v/v) Neurobasal medium
25% (v/v) DMEM
25% (v/v) F12
0.5X N-2 supplement
0.5X B-27® supplement
50mg/mL BSA
25mg/mL Insulin
100µM β-mercaptoethanol
1mM L-Glutamate
1X Penicillin/Streptomycin

[#] Note: Conditioned medium containing LIF was obtained from COS cells transiently transfected with a LIF expression construct. Conditioned medium was collected, assayed for LIF activity and aliquoted in 1mL aliquots at 1500X and stored at -20°C.

2.1.4 Buffers and solutions

Detection buffer (pH 9.5 at 20°C)
0.1M Tris-HCl
0.1M NaCl

2X HEPES-buffered saline (HeBS), pH7.04
0.28M NaCl
0.05M HEPES
1.5mM Na_2HPO_4

Hybridisation buffer
50% (v/v) formamide
5X SSC
0.1% (v/v) Tween20
40µg/mL salmon sperm DNA[#]
[#]Heated for 5min at 80°C prior to addition to buffer

Lysogeny broth (LB), pH7
1% (w/v) NaCl
1% (w/v) amine
0.5% (w/v) yeast extract

Lysis buffer
50mM Tris-HCl, pH8.0
150mM NaCl
1% (v/v) NP-40
1% (w/v) Sodium deoxycholate
0.1% (w/v) Sodium dodecyl sulfate

Maleic acid buffer (pH 7.5 at 20°C)
0.1M Maleic acid
0.15M NaCl

4% Paraformaldehyde (PFA), pH7.4
4% (w/v) PFA
0.15M NaCl
1mM NaOH
10mM phosphate buffer, pH7.4

Phosphate buffer, pH7.4
0.1M Na_2HPO_4
0.1M NaH_2PO_4

Phosphate buffered saline (PBS), pH7.4
137mM NaCl
2.7mM KCl
4.3mM Na2HPO4
1.47mM KH2PO4

0.1% PBS Tween solution (PBST)
0.1% (v/v) Tween20 in PBS

20X Saline sodium citrate (SSC), pH7.0
3M NaCl
0.3M Tri-sodium citrate

Super optimal catabolite repression medium
20mM glucose
10mM NaCl
10mM $MgCl_2$
10mM $MgSO_4$
2.5mM KCl
2% (w/v) bactotryptone
0.5% (w/v) yeast extract

TAE buffer
40mM Tris
20mM acetic acid
1mM EDTA

TE buffer
10mM Tris-HCl, pH 7.5
0.1mM EDTA

Washing solution
50% (v/v) formamide
2X SSC
0.1% (v/v) Tween20

2.1.5 Vectors

pGEM®-T Easy Vector System I (Promega, A1360)
Mouse Npas4 expression vector (kindly provided by Assoc. Prof. Murray Whitelaw)

2.1.6 Cell lines

Cell Line	Species Cell Type	Reference
HEK 293T	Human embryonic kidney epithelial cell	ATCC® number: CRL-11268™
46C	Mouse ES cell	(Ying *et al.* 2003)
D3	Mouse ES cell	(Doetschman *et al.* 1985)
Envy	Human ES cell	(Costa *et al.* 2005)

2.1.7 Software

Adobe® Photoshop® Creative Suite (Adobe Systems Incorporated) - Figure assembly
FACSDiva (BD) - Flow cytometry
Prism® 5 (GraphPad) - Statistics and graphing
ImageJ - Neurite measurement, densitometry analysis, cell counting
NIS Elements F package 3.0 (Nikon Corporation) - Image acquisition
Neurite Tracer (ImageJ plug-in) - Neurite measurement
Photograb™ (Fujifilm Corporation) - Image acquisition
TargetScan (www.targetscan.org) – Analysis of Npas4 3' UTR and miRNA site prediction
V++™ software (Digital Optics™) - Image acquisition

2.2 Methods

2.2.1 Cell culture

Maintenance of HEK 293T cells

HEK 293T cells were cultured in HEK 293T medium on standard plastic tissue culture dishes. Medium was replaced every two days and cells were routinely passaged when approximately 80-90% confluent at a ratio of 1:10. Cells were cultured in a 37°C humidified HERAcell® 150 Incubator (Heraeus®) containing a mixture of 95% air/5% carbon dioxide (CO_2).

Maintenance of mES cells

The methods used to culture D3 and 46C mES cells were identical. Cells were cultured in ES cell medium on standard plastic tissue culture dishes without the use of feeder cells. Medium was replaced every two days and cells were routinely passaged every 3-4 days at a density of $8.5x10^5$ cells/100mm dish. Cells were cultured in a 37°C humidified HERAcell® 150 Incubator (Heraeus®) containing a mixture of 95% air/5% CO_2.

Dissociation of cells using Trypsin

When routinely passaging cells or harvesting cell samples for experiments, the protease enzyme Trypsin was used to detach cells from culture dishes. Briefly, medium was aspirated and cells washed once with sterile PBS after which they were incubated in 1mL of 0.05% Trypsin/EDTA in PBS for 1-2min at room temperature. An equal volume of medium containing 10% (v/v) FBS was added to inactivate the enzyme and the cell suspension was centrifuged at 1,000 revolutions per minute (rpm) for 2min in a 5702 Centrifuge (Eppendorf) to pellet the cells. This is equivalent to 140 times the earth's gravitational acceleration (g). The supernatant was removed and the cells were either resuspended in medium (for passaging) or the cell pellet was used for downstream experiments.

Cryopreservation of cell lines

Cryopreservation was used for long-term storage of cell line stocks. After dissociation and centrifugation as described above, cells were resuspended in a cryopreservation mixture [90% FBS (Invitrogen™), 10% Hybri-Max™ DMSO (Sigma-Aldrich®)] at approximately $5x10^6$ cells/mL. Cells were then immediately aliquoted (500µL) into CryoTubes™ (Nunc) and brought to -80°C using a Cryo 1°C Freezing Container (NALGENE®). Upon freezing, cells were stored at ultra-low temperatures (vapour phase nitrogen) until required. Frozen cells were recovered using the following method: cells were thawed at 37°C after which medium (pre-heated to 37°C) was added dropwise (1mL over 2-5min) to the thawed cells. The solution was then centrifuged at 1,000rpm (equivalent to 140g) for 2min in a 5702 Centrifuge (Eppendorf) to pellet the cells after which they were resuspended in 10mL of pre-heated medium. This step was repeated to remove traces of DMSO. Cells were then plated in medium and placed in a 37°C humidified HERAcell® 150 Incubator (Heraeus®) containing a mixture of 95% air/5% CO_2 and the following day the medium was replaced with fresh medium.

Neural differentiation of mES cells in N2B27 medium

One day prior to differentiation, mES cells were replated at high density ($3x10^6$ cells in a 100mm dish). The method used for neural differentiation of mES cells as a monolayer has been described previously (Ying and Smith 2003). Briefly, plastic tissue culture dishes were coated with sterile 0.1% (w/v) gelatin/PBS overnight at room temperature and the following day the gelatin was aspirated and the coating allowed to dry for 10-15min at room temperature. After dissociation with Tryspin, mES cells were replated onto 0.1% gelatin-coated dishes in N2B27 medium at a density of $1x10^4$ cells/cm^2. Cells were cultured in a 37°C humidified HERAcell® 150 Incubator (Heraeus®) containing a mixture of 95% air/5% CO_2 and N2B27 medium was replaced every two days.

Antibody		Company	Catalogue Number	Host Species	Clonality (Isotype)	Application	Dilution
Anti-Actin	1°	Sigma-Aldrich®	A 2066	Rabbit	Polyclonal	Immunoblot	1/5,000
Anti-Green fluorescent protein	1°	Rockland	600-101-215	Goat	Polyclonal	Immunoblot	1/2,500
Anti-Myc tag (9B11)	1°	Cell Signalling Technology®	2276	Mouse	Monoclonal (IgG2a)	Immunoblot	1/5,000
Anti-Npas4	1°	#	#	Rabbit	Polyclonal	Immunoblot	1/15,000
Anti-Goat IgG-HRP	2°	Rockland	605-4302	Rabbit	Polyclonal	Immunoblot	1/60,000
Anti-Mouse IgG-HRP	2°	Rockland	610-703-124	Donkey	Polyclonal	Immunoblot	1/60,000
Anti-Rabbit IgG-HRP	2°	Rockland	611-703-127	Donkey	Polyclonal	Immunoblot	1/60,000
Anti-βIII Tubulin	1°	Millipore™	MAB5564	Mouse	Monoclonal (IgG)	Immunocytochemistry	1/1,000
Anti-Nestin	1°	Abcam	Ab5968	Rabbit	Polyclonal (IgG)	Immunocytochemistry	1/800
Anti-Mouse IgG-Cy™2	2°	Jackson ImmunoResearch Laboratories	315-225-003	Rabbit	Polyclonal	Immunocytochemistry	1/200
Anti-Rabbit IgG-Cy™3	2°	Jackson ImmunoResearch Laboratories	711-165-152	Donkey	Polyclonal	Immunocytochemistry	1/200
Anti-Digoxigenin-AP, Fab fragments	1°	Roche	11 093 274 910	Sheep	Polyclonal (IgG)	In situ hybridisation	1/5,000

Table 2.1 – List of antibodies

Details of antibodies used in this study. #Anti-Npas4 antibody was a kind gift from Asst. Prof. Yingxi Lin.

Sox1$^+$ NPC enrichment

To enrich for Sox1$^+$ NPCs, mES cells were differentiated in N2B27 medium as described above but on Day 4 of differentiation medium was replaced with N2B27 medium containing puromycin at a final concentration of 0.5µg/mL. Puromycin selection was commenced on Day 4 once a substantial proportion of cells had reached the NPC stage and selection was maintained for either 48h or 96h at which time cells were harvested for immunoblotting using Trypsin. Control cultures were not treated with puromycin.

Noggin-induced neural differentiation of hES cells

The method used to achieve neural differentiation of hES cells that will be discussed in this thesis has been described previously (Dottori and Pera 2008). Briefly, routine culture of hES cells in an undifferentiated state dictates that they be grown on a feeder layer of mitotically inactivated mouse embryonic fibroblasts in the presence of foetal calf serum. Neural induction is initiated by treatment with recombinant Noggin (500ng/mL) for a period of 14 days which primes hES cells for neural differentiation. Following this, colonies are dissected and transferred to serum-free medium supplemented with epidermal growth factor (20ng/mL) and FGF (20ng/mL) causing cells to form free-floating cellular aggregates known as 'neurospheres' which contain a heterogeneous mixture of cell types. Terminal differentiation into neuronal and glial cell types can be triggered by plating neurospheres onto laminin substrates and withdrawal of growth factors from the medium (Pera *et al.* 2004; Dottori and Pera 2008). Differentiated hES cell samples kindly provided by Dr. Mirella Dottori.

2.2.2 Cell assays

Alkaline phosphatase staining

Undifferentiated mES cells were plated in 6-well plates ($9x10^3$ cells/cm^2) in ES medium and allowed to grow for five days before alkaline phosphatase staining. The Alkaline Phosphatase Detection Kit (Millipore™) was used to assay for alkaline phosphatase activity according to the manufacturer's instructions and colonies were viewed microscopically. Only colonies in which 100% of cells were stained were scored as being alkaline phosphatase positive. n=3.

Counting of Nestin$^+$ cells

Counting of Nestin$^+$ cells was done manually using ImageJ software by a blinded experimenter and the value expressed as a percentage of total cells counted. For each experiment, four fields were counted per cell line and a total of four independent experiments were performed. n=4.

Flow cytometry

Control and Npas4 KD mES cells were differentiated in N2B27 medium 35mm dishes (as described in Section 2.2.1) and beginning on Day 3 were analysed by flow cytometry at 24h intervals for a period of seven days. Prior to flow cytometry, cells were dissociated using Trypsin, resuspended in PBS and the suspension was passed through a 70µm cell strainer (BD Falcon™) to remove any aggregates. Cells were then analysed for GFP expression using a FACSCanto™ Flow Cytometer (BD Biosciences) using the following settings: Blue laser (Filter D), Forward scatter 5V, Side scatter 400V, FITC 375V. Data were analysed using FACSDiva™ (BD Biosciences) software. Cells were first gated using side and forward scatter to remove any cell debris or doublets and subsequently were gated to exclude the negative control D3 mES cell line from the GFP positive pool. 10,000 events were counted per run. n≥3.

Growth rate assay

Undifferentiated mES cells were plated in 6-well plates ($9x10^3$ cells/cm^2) in ES medium and the number of living cells was counted at 24h intervals over a period of four days using the vital stain trypan blue. Briefly, once cells from one well were dissociated using Trypsin, the cells were resuspended in 1mL PBS and the total number of live cells/well was determined by means of the dye exclusion method using the Trypan blue dye and a haemocytometer. To minimise variability, the mean of two independent counts was used for each experiment. n=4.

Neurite measurement

The length of βIII tubulin$^+$ neurites was measured using the NeuriteTracer software (ImageJ plugin). This method of automated neurite measurement has been described previously (Pool *et al.* 2008). For each experiment, four fields were counted per cell line and a total of three independent experiments were performed. To measure the overall neurite complexity of the neuronal network, the mean total neurite length per field was calculated. To measure the mean neurite length per cell, this value was divided by the number of nuclei present in that field (as determined by DAPI staining). n=3.

2.2.3 Immunocytochemistry

Control and Npas4 KD mES cells were differentiated in N2B27 meium in 24-well plates as described in Section 2.2.1 and were fixed after either four or ten days of differentiation for analysis of Nestin or βIII tubulin protein expression, respectively. Briefly, N2B27 medium was removed and cells were washed twice with PBS before being fixed in 4% PFA for 20min at room temperature. To permeabilise the cells and to block non-specific antigens, cells were then incubated in blocking solution [10% (v/v) horse serum in 0.1% PBST] for 60min at room temperature after which this was replaced with fresh blocking solution containing the appropriate primary antibody (see Table 2.1). Cells were incubated with the primary antibody overnight at 4°C with gentle agitation and the following day were washed four times in 0.1% PBST for 5min. Following the last wash, cells were incubated with the appropriate secondary antibody (see Table 2.1) diluted in blocking solution for 2h at room temperature and were then once again washed four times in 0.1% PBST for 5min. Cells were then incubated in 0.1% PBST containing 300nM DAPI for 10min at room temperature after which they were washed twice in 0.1% PBST. After the final wash, PBS was added to the wells and the cells viewed under fluorescence microscopy using an Eclipse TE300 inverted microscope (Nikon Corporation). Images were acquired using a CoolSNAP *fx*® camera (Photometrics®) and V++™ software (Digital Optics™). n≥3.

2.2.4 Immunoblotting

Immunoblotting

After cells were harvested using Trypsin (see Section 2.2.1), lysis buffer containing protease inhibitor cocktail (Roche) was added to the cell pellet (volume of lysis buffer varied with size of pellet) and the cells were placed on ice for 10min. Cells were then disrupted mechanically using a plastic mortar before centrifugation at 13,000rpm (equivalent to 15,600g) for 10min in a 5415D Centrifuge (Eppendorf) to pellet cell debris. A Bradford protein assay (Bradford 1976) was performed to quantify total protein content in lysates using the Protein Assay Dye Reagent Concentrate (BIO-RAD) and colorimetric changes were measured using an EMax® Endpoint ELISA Microplate Reader (Molecular Devices). An equal amount of protein was loaded for each sample analysed in the experiment and, if necessary, volumes were adjusted by addition of lysis buffer to ensure volumes loaded were also equal. Prior to loading, samples were diluted 2:1 in 3X SDS Blue Loading Buffer (New England Biolabs®) and the reducing agent DTT was added to a final concentration of 40mM. Samples were then heated for 2min at 95°C and briefly centrifuged before loading. Polyacrylamide gels with a stacking gel of 3.75% and a resolving gel of 10% were cast using the Mini-PROTEAN® 3 Multi-Casting Chamber system (BIO-RAD) and proteins were separated using electrophoresis at 200V for 40-60min.

Following electrophoresis, proteins were transferred onto a 0.45µm Immobilon-P PVDF membrane (Millipore™) using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD) for 70min at 10 volts (V). Membranes were then blocked for 30min at room temperature (approximately 22°C) in blocking solution [5% (w/v) skim milk powder, 1% (w/v) BSA, 1% (v/v) FBS in 0.1% PBST] and rinsed in 0.1% PBST. Next, membranes were incubated with the appropriate primary antibody diluted in 0.1% PBST (see Table 2.1) overnight at 4°C with gentle agitation after which they were washed four times in 0.1% PBST for 5min. Following this, membranes were incubated with the appropriate horse radish peroxidase-conjugated secondary antibody (see Table 2.1) diluted in 0.1% PBST for 2h at room temperature and again washed four times in 0.1% PBST for 5min. After blotting membranes on a paper towel, Immobilon™ Western Chemiluminescent HRP Substrate (Millipore™) was then applied according to the manufacturer's instructions and chemiluminescence was detected either using the ImageQuant™ ECL imaging system (GE Healthcare) or by exposure to CURIX Ortho HT-G autoradiography film (Agfa HealthCare) and development using a CP1000 Processor (Agfa HealthCare). Developed films were scanned to produce digital images for analysis.

Semi-quantitative analysis of protein expression using densitometry

Chemiluminescent signal intensity was quantified by densitometry analysis using ImageJ software. Briefly, the area under the Npas4 signal peak was compared between samples after normalisation to the density of the β-actin loading control. The Empty vector control sample was given an arbitrary value of 1.

2.2.5 *In situ* hybridisation

Riboprobe synthesis by *in vitro* transcription

The sequences of the sense and antisense riboprobes used in detection of Npas4 mRNA expression have been used previously in ISH (Flood *et al.* 2004). The 991bp segment of Npas4 cDNA corresponding to the probe sequence had been cloned into the multiple cloning site of the pGEM®-T Easy vector (Promega) in a 5'-3' orientation where it was flanked by pUC/M13 primer binding sites. The primers used to amplify the probe sequence can be found in Table 2.2. After sequencing (see Section 2.2.6) using pUC/M13 primers (Table 2.2) confirmed that the sequence of the probe was correct, 50ng of plasmid DNA was used as a template to amplify a linear DNA fragment containing the Npas4 probe sequence by PCR using pUC/M13 primers. 10µg of PCR product was digested overnight (see Section 2.2.6) at 37°C with either SacI (1U/µL) for the sense probe or SacII (1U/µL) for the antisense probe and separated from undigested DNA by 1% agarose gel electrophoresis

(see Section 2.2.6). Digested PCR fragments of approximately 1kb were visualised by ethidium bromide staining under UV light then excised and purified using QIAquick® Gel Extraction Kit (QIAGEN®) according to the manufacturer's instructions. 3.5µg of purified DNA was used as template to make a DIG-labelled riboprobe using *in vitro* transcription. A UTP:DIG-11-UTP ratio of 2:1 was used in the following 20µL labelling reaction:

1X Reaction buffer

250µM NTP mix (250µM ATP, 250µM CTP, 250µM GTP, 163µM UTP)

87.5µM DIG-11-UTP

10mM DTT

40U RNase inhibitor

36U RNA polymerase (Antisense - SP6; Sense - T7)

3.5µg template DNA

After mixing, the reaction was allowed to proceed for 2h at 37°C after which the reaction was stopped by addition of 1µL 500mM EDTA. Following this, unbound DIG-11-UTP was removed using a Nanosep® Centrifugal Device (Pall Corportation) according to the manufacturer's instructions and the eluted probe (40µL) was aliquoted and stored at -20°C until use.

Assessment of probe sensitivity by dot blot

Serial dilutions (ranging from 10ng/µL to 0.01pg/µL) of both the sense and antisense probes were tested for sensitivity using a dot blot. For each dilution in the series, 1µL of diluted probe was spotted onto a dry Hybond™-N+ positively charged nylon membrane (Amersham) which was then incubated for 2h at 80°C. The membrane was then rinsed in washing buffer (0.1% Tween20 in maleic acid buffer) and incubated in blocking solution [1% (w/v) blocking reagent, 10% (v/v) horse serum in maleic acid buffer] for 30min at room temperature after which time this was replaced with fresh blocking solution containing the anti-DIG antibody (1/5,000) and incubated for a further 30min at room temperature. The membrane was then washed twice in washing buffer for 15min and then allowed to equilibrate in detection buffer for 5min at room temperature. Following equilibration, the detection buffer was replaced with the detection solution composed of 2% (v/v) NBT/BCIP stock solution in detection buffer. The colour reaction was allowed to develop overnight at room temperature in the dark and the following day the reaction was stopped by washing the membrane in TE buffer. The minimum amount of probe that could be detected was 3pg.

Hybridisation

Sterile glass coverslips were placed inside a 12-well plate (1/well) coated with 0.1% gelatin/PBS. D3 mES cells were differentiated on the glass coverslips in N2B27 medium (as described in Section 2.2.1). On Day 4 of neural differentiation, N2B27 medium was removed and cells were washed twice with PBS before being fixed in 4% PFA for 20min at room temperature. Following this, cells were again washed twice with PBS and dehydrated through a series of ascending methanol/PBS washes (25%, 50%, 75%, 100%). Cells were immersed once more in 100% methanol and left overnight at -20°C. The following day, cells were rehydrated with a series of descending methanol/PBS washes (75%, 50%, 25%, 0%). Cells were then permeabilised by washing in 0.1% PBST for 30min at room temperature after which they were again fixed in 4% PFA for 20min at room temperature. This was followed by three washes of 5min each in 0.1% PBST at room temperature and then one 5min wash in a 1:1 mixture of hybridisation buffer/PBS. Subsequently, cells were incubated in hybridisation buffer for 60min at 65°C (prehybridisation) after which this was replaced with fresh hybridisation buffer containing the riboprobe (175ng/mL). Before addition of probe to the hybridisation buffer, the probe was diluted in an equal volume of nuclease-free water, heated for 2min at 80°C and then cooled for 5min on ice. Hybridisation was allowed to proceed overnight at 65°C in a humidified chamber. The following day, cells were washed three times in Washing Solution for 30min at 65°C and then twice in maleic acid buffer for 30min at room temperature. After this, cells were incubated in blocking solution [1% (w/v) blocking reagent, 10% (v/v) horse serum in maleic acid buffer] for 60min at room temperature after which this was replaced with fresh blocking solution containing the anti-DIG antibody (1/5,000). Cells were incubated with the anti-DIG antibody overnight at 4°C with gentle agitation and the following day were washed three times in maleic acid buffer for 20min at room temperature, followed by two washes of 10min each in detection buffer at room temperature. Coverlips were placed face-up onto a microscope slide and detection solution, composed of 2% (v/v) NBT/BCIP stock solution in detection buffer, was added to the slides (500µL/slide). A strip of parafilm was placed on each slide to prevent evaporation of the detection solution and the colour reaction was allowed to develop overnight at room temperature in the dark. Once colour development was complete, cells were washed three times in PBS, fixed in 4% PFA for 20min at room temperature and washed twice more in PBS. Following this, coverslips were placed upside-down onto a drop of mounting medium which has been placed onto a microscope slide. The mounting medium was allowed to cure overnight at room temperature and the following day, slides were sealed with nail polish and were viewed microscopically using AxioPlan2 microscope (Zeiss). Images were taken using a Fujix colour digital camera (Fujifilm Corporation). n=2.

2.2.6 Molecular techniques

Agarose gel electrophoresis

Electrophoretic separation of nucleic acids was performed in a Mini-Sub® Cell GT electrophoresis apparatus (BIO-RAD). Samples were prepared with DNA loading buffer at a final concentration of 1X and loaded on a 1% (w/v) agarose gel in 1X TAE buffer containing 0.5µg/mL ethidium bromide. Gels were run at 60-90V for 20-45min after which they were photographed under ultra violet (UV) light using the ImageQuant imaging system (GE Healthcare).

Bacterial transformation

DH5α *Escherichia coli* (*E. coli*) were made competent using the rubidium chloride method described previously (Hanahan 1983). Chemically competent cells were stored at -80°C and thawed on ice immediately prior to transformation. Approximately 10-100ng of plasmid DNA was added to 50µL of competent cells and the mixture incubated on ice for 30min before heat shocking at 42°C for 30s. The mixture was then returned to ice for 2min before 450µL of super optimal catabolite repression medium was added. Cells were then incubated at 37°C for at least 1h. Bacterial cultures were then plated on selective agar media [LB containing 1.5% (w/v) bactoagar supplemented with 100mg/mL ampicillin] which permit the growth of only transformed cells and incubated overnight at 37°C.

Isolation of genomic DNA

Three days after passaging (when approximately 80% confluent), undifferentiated ES cells were dissociated using Trypsin and $5x10^6$ cells were harvested for purification of genomic DNA (gDNA). Isolation of gDNA was performed using the DNeasy Blood and Tissue Kit (QIAGEN) according to the manufacturer's instructions and gDNA was eluted in a final volume of 200µL in elution buffer. Integrity of gDNA was inspected by 1% agarose gel electrophoresis (as described above).

Plasmid purification

Purification of plasmid DNA was performed using either a QIAprep® Spin Miniprep Kit (QIAGEN®) or a Plasmid Midi Kit (QIAGEN®) according to the manufacturer's instructions.

Restriction enzyme digestion of DNA

Restriction enzymes were used according to the manufacturer's instructions. Briefly, 10µg of DNA was digested in a 20µL reaction containing 20 units of the appropriate restriction enzyme and buffer. All digests were incubated overnight at the appropriate temperature.

Reverse transcription

The maximum allowable volume of RNA (17µL) was combined with 500ng Oligo(dT)15 Primers (Promega), 500ng Random Primers (Promega) and 1µL 10mM deoxynucleoside triphosphates (dNTPs) and the mixture was heated for 10min at 70°C then cooled for 5min on ice. To this were added 10X SuperScript™ III reaction buffer, DTT, SuperScript™ III reverse transcriptase enzyme (Invitrogen™) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen™) in a final volume of 30µL in the following reaction:

1X SuperScript™ III reaction buffer

2,000U SuperScript™ III reverse transcriptase enzyme

40U RNaseOUT™ Recombinant Ribonuclease Inhibitor

16.5ng/µL Oligo(dT)15 Primers

16.5ng/µL Random Primers

6.7mM DTT

0.3mM dNTPs

17µL template RNA

Negative control samples (-RT) contained RNA template but the reverse transcription enzyme was substituted for water. After mixing, the reaction was allowed to proceed for 2.5h at 50°C at which time the reaction was stopped by incubation at 70°C for 15min.

RNA extraction

RNA was isolated from cell and tissue samples using the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction. Included in the protocol is a DNaseI treatment step to remove traces of gDNA. Integrity of RNA was inspected by 1% agarose gel electrophoresis (as described above).

Sequencing

DNA was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) according to the manufacturer's instructions except that the final reaction volume was half of that described in the protocol. Briefly, 20µL reactions were performed with 400-800ng of double-stranded DNA used as a template and approximately 100ng of either the forward or reverse pUC/M13 primer (see Table 2.2). Reactions were performed using an MJ Research PTC-200 Thermo Cycler (Global Medical Instrumentation). Cycling conditions were: 25 cycles of 96°C for 30s, 50°C for 15s and 60°C for 4min. Samples were then precipitated with 80µL 75% isopropanol for at least 15min at room temperature before the sequencing product was pelleted by centrifugation for 20min at 13,000rpm (equivalent to 15,600g) in a 5415D Centrifuge (Eppendorf). The supernatant was then removed after which the pellet was washed in 250µL of 75% isopropanol and pelleted for another 10min before the supernatant was removed and the pellet dried on a 95°C heating block for approximately 5min. Sequencing analysis was performed at the Institute of Medical and Veterinary Science (Frome Road, Adelaide).

Transient transfection of HEK 293T cells

HEK 293T cells were seeded onto a 6-well plate and, once approximately 70% confluent, were transiently transfected using the lipid-based transfection reagent FuGENE® 6 according to the manufacturer's instructions. Briefly, a FuGENE® 6/DNA mixture with a FuGENE® 6:DNA ratio of 3:2 (3µL FuGENE® 6 + 2µg plasmid DNA in a final volume of 100µL) was prepared and incubated for 15min at room temperature after which time it was added to the HEK 293T medium (100µL/well).

Plasmid DNA consisted of 1µg Npas4-Myc expression vector plus 1µg small hairpin RNA (shRNA) vector. Where no shRNA vector was transfected (Npas4-Myc only), 1µg of non-specific competitive plasmid DNA was added to bring the total amount of plasmid DNA to 2µg so as to maintain transfection efficiency between experimental groups. In untransfected cells, a mock transfection was performed where 3µL FuGENE® 6 was diluted in water containing no DNA and added to cell medium. 24h after transfection, whole cell lysates were collected for immunoblotting; cells were first washed once with PBS before lysis buffer was added to lyse cells (200µL/well).

2.2.7 Polymerase chain reaction

RT-PCR

Sequences of primers used in polymerase chain reactions (PCRs) can be found in Table 2.2. Equal amounts of cDNA were used as template in RT-PCR experiments. Negative control reactions (-) contained water in place of template cDNA. As conditions were optimised individually for each set of primers, the reaction conditions varied according to the primers used (see Table 2.2), however the general format of RT-PCR experiments was as follows:

Taq DNA polymerase	Reaction conditions		
1X reaction buffer	Denaturation	95°C 2min	
$MgCl_2$[#]	Denaturation	95°C 30s	X[#] cycles
400µM dNTPs	Annealing	X°C[#] 1min	
Forward primer[#]	Extension	72°C 1min 20s	
Reverse primer[#]	Extension	72°C 3min	
1,000U Taq DNA polymerase	Cooling	12°C ∞	
Template cDNA			
Water to 25µL			

Phusion™ Flash	Reaction conditions		
1X reaction buffer	Denaturation	98°C 10s	
Forward primer[#]	Denaturation	98°C 1s	X[#] cycles
Reverse primer[#]	Annealing	X°C[#] 5s	
Template cDNA	Extension	72°C 15s	
Water to 20µL	Extension	72°C 1min	
	Cooling	12°C ∞	

[#] Note: These parameters are specific to the primers used and can be found in Table 2.2.

All thermal cycling reactions were performed using an MJ Research PTC-200 Thermo Cycler (Global Medical Instrumentation). PCR products were visualised by ethidium bromide staining under UV light after 1% agarose gel electrophoresis (see Section 2.2.6) and images captured using the ImageQuant imaging system (GE Healthcare).

Primer set	Application	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing temperature	Final concentration	DNA Polymerase	Cycles	Amplicon size	
								cDNA	gDNA
Mouse *Npas4*	RT-PCR	TCATGAGTCTTGCCTGCATC*	GAGGGACTTGGAGGTGTTGA*	58°C	500nM	Phusion™ Flash	35	347bp	1,385bp
	qRT-PCR	AGCATTCCAGGCTCATCTGAA	GGCGAAGTAAGTCTTGGTAGGATT	60°C	0.3pM	AmpliTaq Gold®	40	82bp	82bp
	ISH probe PCR	TATGAGAAGTTGCCCCCAAG	CGGTGAGGAAGTGAGACTCC	58°C	60nM	Taq (1mM $MgCl_2$)	35	991bp	1,649bp
Mouse *β-actin*	RT-PCR/gDNA PCR	TGTGATGGTGGGAATGGGTCAG*	TTTGATGTCACGCACGATTTCC*	54°C	600nM	Taq (2.5mM $MgCl_2$, 5% DMSO)	35	511bp	965bp
	qRT-PCR	ACGGCCAGGTCATCACTATTG	CCAAGAAGGAAGGCTGGAAAA	60°C	0.3pM	AmpliTaq Gold®	40	72bp	72bp
Mouse *Pou5f1*	RT-PCR	CCCAGGCCGACGTGG	GATGGTGGTCTGGCTGAACAC	61°C	400nM	Taq (1.5mM $MgCl_2$)	37	65bp	250bp
Mouse *Nanog*	RT-PCR	CAGAAAAACCAGTGGTTGAAGACTAG	GCAATGGATGCTGGGATACTC	61°C	400nM	Taq (1.5mM $MgCl_2$)	37	81bp	507bp
pUC/M13	ISH probe PCR	GTTTTCCCAGTCACGAC	CAGGAAACAGCTATGAC	58°C	400nM	Taq (1mM $MgCl_2$)	35	~1100bp	
shRNA contruct	gDNA PCR	GAGAGAGATGGGTGCGAGAG	CCAAGAACCCAAGGAACAAA	58°C	400nM	Taq (0.5mM $MgCl_2$)	25	542bp	

Table 2.2 – List of primers

Primer sequences and PCR conditions used for each primer pair are shown as well as the expected amplicon length. All oligonucleotides were ordered from GeneWorks and were reconstituted as a 10X stock in TE buffer. For *Npas4* primers, the locations of the primer binding sites are shown in Figure 1.2A (RT-PCR) and Figure 4.1C (ISH). *As coding sequences of these genes are highly conserved between mouse and human, the primers used for amplification of mouse cDNA templates were also used in RT-PCR analysis of human cDNA samples. Nucleotides which have been underlined indicate mismatches between the primer sequence and the human cDNA sequence.

gDNA PCR

180ng of gDNA was used as template in a PCR reaction similar to the one described above using the primers shown in Table 2.2. PCR products were visualised by ethidium bromide staining under UV light after 1% agarose gel electrophoresis (see Section 2.2.6) and images captured using the ImageQuant imaging system (GE Healthcare).

Quantitative RT-PCR

Sequences of primers used in quantitative RT-PCR (qRT-PCR) can be found in Table 2.2. All cDNA samples were diluted to a uniform concentration of 50ng/µL and 50ng of cDNA was used as template in qRT-PCR experiments. All qPCR reactions were performed using SYBR® Green PCR Master Mix (Applied Biosystems®) containing the AmpliTaq Gold® DNA polymerase according to the manufacturer's instructions. The reaction was as follows:

1X SYBR® Green PCR Master Mix

7.5pmol Forward primer

7.5pmol Reverse primer

50ng Template cDNA

Water to 25µL

After mixing, thermal cycling (40 cycles) was performed using an ABI PRISM® 7000 Sequence Detection System driven by ABI PRISM SDS v1.1 software (Applied Biosystems®) with an annealing temperature of 60°C. Each experiment consisted of three technical replicates (i.e. samples were loaded in triplicate) and each experiment was performed three times (i.e. three biological replicates). Samples were loaded on a MicroAmp® Optical 96-well Reaction Plate (Applied Biosystems®). *β-actin* was used as an internal reference to control for loading and to facilitate relative quantification using the comparative *C*t method (Schmittgen and Livak 2008). *β-actin* was selected as an internal reference because transcript levels remain relatively stable during N2B27 differentiation of mES cells (see Figure 3.1A). Primers directed to the *Npas4* transcript were similar in reaction efficiency to the *β-actin* internal reference primers. n=3.

2.2.8 Mouse embryo collection

Uteri of pregnant female mice (B6CBAF1) were collected at the indicated times and the embryos dissected from the decidua under a SZ1145 Stereo Microscope (Olympus) in cold PBS. Vaginal plugs were used to determine the age of a litter; the presence of a vaginal plug was taken to indicate that

mating had occurred the previous night and matings are assumed to have taken place at mid-subjective night. Upon dissection, embryos from the same litter were pooled and RNA was extracted immediately as described in Section 2.2.6. All animals were housed and treated in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The University of Adelaide Animal Ethics Committee approved all experiments prior to commencement.

2.2.9 Lentiviral transduction

Production of virus

HEK 293T cells were used as a packaging cell line for virus production. Replication incompetent lentiviral particles harbouring shRNA expression vectors were generated by co-transfection of HEK 293T cells with the shRNA contructs (which contain the *cis* viral elements) and expression vectors containing the *trans* (protein coding) viral elements *gag*, *pol*, *tat* and *rev*. HEK 293T cells were seeded onto a 6-well plate and, once approximately 50% confluent, were transiently transfected with a cocktail of viral packaging vectors (listed below) together with the appropriate shRNA or control construct using the calcium phosphate ($CaPO_4$) transfection method.

Plasmid	Amount
shRNA/control construct	3µg/well
pHCMVRev	60ng/well
pHCMVGagpol	360ng/well
CMV-G	150ng/well

Briefly, plasmid DNA was added to a solution of 250mM calcium chloride ($CaCl_2$) and the DNA/$CaCl_2$ mixture was gradually added to an equal volume of 2X HeBS over five seconds (s). The DNA/$CaCl_2$/HeBS mixture was vortexed for a further 25s and then allowed to stand for 90s at room temperature before being added dropwise to the HEK 293T medium (120µL/well). Eight hours after transfection, the medium was replaced with fresh medium which was left for a further 40h before being collected; this conditioned medium contained the virus particles. The virus titre was determined empirically.

Transduction of 46C mES cells

Preliminary experiments using an enhanced yellow fluorescent protein (eYFP) expression construct confirmed that purification of the virus was not necessary and that adequate transduction (as determined by both fluorescence microscopy and flow cytometry) could be achieved in the absence of Polybrene simply by using the conditioned medium once it had been passed through a 0.2µm filter.

Furthermore, no adverse effects (i.e. toxicity or spontaneous differentiation) were observed as a result of adding conditioned medium to mES cells.

For viral transduction, 46C mES cells were seeded onto a 24-well plate coated with 0.2% gelatin/PBS ($8.5x10^5$ cells/well) and 24h later virus was added to the ES cell medium. A range of virus titres (ranging from 0-16µL of filtered conditioned medium) were tested in preliminary experiments to determine the appropriate titre for isolation of single mES cell colonies and 2.5µL of filtered conditioned medium was found to be optimal. Medium was changed 24h after addition of virus and after a further 24h mES cells were dissociated using Trypsin and replated onto a 100mm dish coated with 0.2% gelatin/PBS to facilitate isolation of single colonies.

Antibiotic selection and picking of mES cell clones

Two days after re-plating, ES cell medium was replaced with selective ES cell medium containing 3µg/mL puromycin. Selection with puromycin was maintained for 10 days (medium changed every other day) to ensure all untransduced cells were eliminated. Once clonally derived resistant colonies were large enough to visualise with the naked eye, single colonies were picked and carefully transferred to a 24-well/plate coated with 0.2% gelatin/PBS using a dilute Trypsin solution and a P1000 pipette (one colony/well). For each shRNA construct, 12-24 clones were picked. Prior to picking, colonies were microscopically inspected and only colonies with a healthy appearance and typical mES cell morphology were selected. All clones were expanded and divided into two fractions; half of the cells were processed for gDNA and RT-PCR analysis and the remainder were frozen for subsequent retrieval.

2.2.10 Other

Microscopy

Light microscopy: For analysis of mES cell morphology, brightfield images were captured using an Eclipse TS100 Inverted Microscope (Nikon Corporation) and Digital Sight DS-2M camera (Nikon Corporation). The software used for image acquisition was NIS Elements F package 3.0 (Nikon Corporation). For analysis of ISH and alkaline phosphatase staining, brightfield images were captured using an AxioPlan2 microscope (Zeiss) and a Fujix colour digital camera (Fujifilm Corporation). The software used for image acquisition was Photograb™ (Fujifilm Corporation).

Fluorescence microscopy: Epifluorescence images were captured using an Eclipse TE300 Inverted Microscope (Nikon Corporation) and a CoolSNAP fx® Camera (Photometrics®). The software used for image acquisition was V++™ (Digital Optics™).

Statistical analysis

All graphing and statistical analyses were performed using Prism® 5 (GraphPad). Results were considered significant when $P < 0.05$. When stated, the 'n value' is the number of independent experiments performed.

3 – *Npas4* expression during neural differentiation of ES cells and early embryonic development

3.1 Introduction

Little data exists as to the expression or function of *Npas4* during embryonic development. Both of these aspects were explored using a number of model systems. *In vitro* differentiation of ES cells represents a reductionist approach to developmental biology as it recapitulates, to a certain extent, the events occurring in the embryo during the early stages of development but without the complexity of a whole organism (Rathjen and Rathjen 2001; Rodda *et al.* 2002). On this basis, the expression of *Npas4* was investigated in ES cell models of both mouse and human development using neural differentiation paradigms unique to each species; adherent differentiation of mES cells in N2B27 medium and Noggin-induced differentiation of human ES (hES) cells as neurospheres, respectively. Both of these models will be discussed below with particular attention being paid to key developmental genes that are pertinent to this thesis. Finally, these *in vitro* data were complemented by direct analysis of *Npas4* expression in the whole mouse embryo during early development.

3.1.1 Embryonic stem cells

During the early stages of mammalian development, the totipotent single-celled zygote undergoes a series of rapid cleavages to produce a multi-cellular ball of cells known as the morula. Further cell divisions give rise to the blastocyst which consists of a layer of trophoblast cells surrounding an inner cell mass and a fluid-filled blastocyst cavity. The cells of the inner cell mass (also known as an embryoblast) are pluripotent and will produce all of the embryonic tissues while the trophoblast cells will go on to form extraembryonic tissues (such as the placenta) and do not contribute to tissues of the embryo proper. Cells derived from inner cell mass of the pre-implantation blastocyst can be cultured *in vitro* as a homogeneous population of undifferentiated cells termed ES cells. Like their *in vivo* counterparts, ES cells are pluripotent and, under the appropriate conditions, retain the ability to differentiate into any embryonic or adult cell populations. Furthermore, in the undifferentiated state they have the ability to (theoretically) self-renew indefinitely which makes them attractive as a potentially unlimited source of tissue for cell replacement and transplantation therapies. Indeed, ever since the isolation of the first hES cell line (Thomson *et al.* 1998), there has been much optimism within the field of stem cell biology about the therapeutic potential of ES cells. To this end, much

effort has been invested in trying to establish appropriate conditions for directed differentiation of ES cells to desired cell types and, while much progress has been made, obtaining pure populations of differentiated progeny remains an elusive goal.

Potential clinical applications aside, ES cells have also proven to be a valuable tool in basic research. Ever since the isolation of the first stable mES cell lines over 30 years ago (Evans and Kaufman 1981; Martin 1981), the study of ES cells and their differentiation into other cell types has contributed extensively to our knowledge of the biological processes underlying mammalian development. Differentiation of ES cells *in vitro* recapitulates many of the fundamental cellular and molecular events occurring during the early stages of embryogenesis (Rathjen and Rathjen 2001; Rodda *et al.* 2002) making them a useful model in which complex processes can be studied in a simplified and much more accessible way without the complexity of a whole organism. Many aspects of early development can be studied using ES cells including cell progression during differentiation, morphogenesis and signalling mechanisms involved in lineage specification and patterning. In addition to this, the ease with which ES cells can be genetically manipulated and their ability to contribute to chimeras have provided a platform for generating transgenic mice which have proved an invaluable resource for researchers.

3.1.1.1 Neural differentiation of mES cells

While ES cells have the ability to produce almost any cell type, spontaneous differentiation, which is the default pathway triggered by withdrawal of pluripotency-promoting factors, is uncontrolled, disorganised and leads to a heterogeneous mixture of cell types derived from many different lineages. Harnessing the differentiation potential of ES cells to generate homogenous neural cell populations remains one of the major goals of the stem cell field. It is hoped that by achieving this we may not only gain further insights into the cellular and molecular mechanisms regulating neural differentiation but, at a clinical level, it is hoped that these ES cell-derived populations may one day be used in cell replacement therapies to treat conditions associated with loss of specific cell populations, such as Parkinson's disease which is characterised by the loss of the dopaminergic neurons of the substantia nigra (Shulman *et al.* 2011).

Over the last 30 years or so, many different strategies have been employed to achieve neural differentiation of ES cells with varying degrees of success (Stavridis and Smith 2003; Zhang 2006). Several techniques involving either multi-cellular aggregation (Bain *et al.* 1996; Rathjen *et al.* 2002)

or co-culture with feeder cells (Kawasaki *et al.* 2000) have proven effective in stimulating efficient neural conversion of mES cells; however, these studies have been limited in their usefulness due to the use of undefined media containing unknown factors. Recently, a method was devised in which mES cells can be differentiated towards a neural fate as an adherent monolayer in a completely defined N2B27 medium (Ying and Smith 2003) and it is this technique that was used in the present study (see Section 2.2.1). While this method achieves bulk conversion of cells to a neural lineage, the conversion is not synchronous meaning that differentiating cultures contain a heterogeneous mixture of cells that are staggered at various stages of neural differentiation (i.e. cells in some colonies may be delayed/advanced in their differentiation with respect to others). Nevertheless, the differentiation proceeds in a linear fashion and over several days the majority of cells transition through a number of discrete, identifiable stages. In the first stage, pluripotent ES cells are first converted to NPCs, a multipotent population of cells that is committed to producing cells of neural lineage. This is an intermediate population that exists only transiently before undergoing further differentiation to ultimately produce either neurons or glia which occurs in the final stage of differentiation.

3.1.1.2 SoxB1 proteins in embryonic development and NPC maintenance

The SRY-related high mobility group box (Sox) proteins are a large family of transcription factors that contain a well conserved high mobility group (HMG) DNA-binding domain. More than 20 Sox proteins have been identified in mammals and these are subdivided into nine groups based on amino acid sequence homology (Guth and Wegner 2008). The SoxB1 subgroup is comprised of three members, Sox1, Sox2 and Sox3, which share more than 90% amino acid identity in the HMG domain and have overlapping expression patterns and functions. In vertebrates, all of the SoxB1 proteins are expressed during embryogenesis and have important roles in early development, particularly in maintenance of proliferative stem cell populations (Guth and Wegner 2008). Both *Sox2* and *Sox3* are expressed in the pre-gastrulation epiblast (Wood and Episkopou 1999) where Sox2 functions as a key regulator of pluripotency (Avilion *et al.* 2003). In contrast, expression of *Sox1* is induced at a later stage of development which coincides with the neural induction of ectoderm (Wood and Episkopou 1999) when it is expressed specifically in the neural plate (Pevny *et al.* 1998). At this time, the expression of *Sox2* and *Sox3* also becomes restricted to the neuroectoderm and all of the *SoxB1* genes continue to be expressed in the neural tube along the entire anterior/posterior axis (Wood and Episkopou 1999). Initially, the SoxB1 proteins are expressed in all cells of the neural tube but later their expression becomes confined to the ventricular zone (VZ) where self-renewing NPCs are located (Tanaka *et al.* 2004). In the chick embryo, it has been shown that the SoxB1 proteins function to

maintain the cells of the VZ in a regionally unspecified NPC state by inhibiting cell cycle exit and neuronal differentiation (Graham *et al.* 2003). This NPC-specific expression is maintained throughout ontogeny with *SoxB1* genes being expressed in the neurogenic zones of the adult brain which further strengthens the notion that expression of SoxB1 proteins defines NPC identity (Pevny and Placzek 2005). Thus, while all *SoxB1* genes are involved in stem cell maintenance, *Sox1* is unique as, unlike *Sox2* and *Sox3* which are expressed in earlier pluripotent populations, it one of the first genes to be expressed specifically in the neurectoderm which has made it useful as a marker of neural induction.

3.1.1.2.1 The 46C Sox1::GFP mES cell line

During neural differentiation of mES cells *in vitro*, Sox1 is transiently expressed by a population of multipotent NPCs (Ying and Smith 2003; Suter *et al.* 2009) and isolation of the Sox1 positive ($Sox1^+$) cells eliminates teratoma-forming pluripotent cells leaving a population of self-renewing neurally committed precursor cells that can differentiate into neurons and glia (Chung *et al.* 2006). A transgenic mES cell line has been developed to exploit the NPC-specific expression of *Sox1* and this cell line was used in the present study. The 46C mES cell line (Ying *et al.* 2003) is a transgenic green fluorescent protein (GFP) knock-in cell line that has been engineered in such a way that, in one of the alleles, the open reading frame of the *Sox1* gene has been replaced with a dual reporter/selection cassette containing the sequence coding for enhanced GFP (eGFP) followed by an internal ribosome entry site (IRES)-linked puromycin N-acetyl-transferase (*Pac*) gene which confers resistance to the antibiotic puromycin. In this way, both the *eGFP* and *Pac* genes are under the control of the endogenous *Sox1* promoter and will be expressed concurrently in cells which express *Sox1* (Figure 3.2A). This allows living *Sox1*-expressing cells to be visualised microscopically using the vital reporter eGFP or to be selected from a mixed culture using antibiotic resistance.

3.1.1.3 Neural differentiation of hES cells

Like their murine counterparts, hES cells also have the potential to differentiate into neural cells *in vitro*. However, given the species-specific differences between the two pluripotent cell populations, different methods must be used to derive neural cells from hES cells. In view of the finding that addition of exogenous Noggin to hES cells cultures prevents differentiation towards extraembryonic cell types and instead directs them towards a neural lineage (Pera *et al.* 2004), a variety of neural differentiation protocols have been developed which involve treatment of hES cells with Noggin; these include differentiation in suspension culture (Itsykson *et al.* 2005), adherent culture (Gerrard *et al.* 2005; Yao *et al.* 2006; Sonntag *et al.* 2007), or a mixture of adherent and aggregate phases

(Dottori and Pera 2008). While the differentiation protocols vary, addition of Noggin to hES cells has proven to be a very effective method for producing populations of cells highly enriched for NPCs. The method used to achieve neural differentiation of hES cells that will be discussed in this thesis has been described previously (Dottori and Pera 2008) and is described in more detail in Section 2.2.1.

3.1.1.3.1 Noggin

Noggin is a polypeptide morphogen that has important roles during embryogenesis, particularly in the process of neural induction. In the developing mouse embryo, *Noggin* mRNA is first detected at E7.5 in the node, a developmental structure equivalent to Spemann's organiser (Beddington 1994), and is later expressed in the notochord and dorsal somites (McMahon *et al.* 1998). It was first identified in Xenopus as a dorsalising factor that is secreted by Spemann's organiser and which has potent neural inducing properties (Smith and Harland 1992). Noggin binds to the bone morphogenetic proteins (BMPs) with high affinity and consequently is able to disrupt BMP signalling (Zimmerman *et al.* 1996). The BMPs are a family of growth factors within the transforming growth factor β superfamily that have important roles in both embryogenesis and in adulthood. During embryonic development, BMP4 acts on ectodermal cells instructing them assume an epidermal fate at the expense of neurectoderm formation (Wilson and Hemmati-Brivanlou 1995). Thus, through graded inhibition of BMP signalling, Noggin prevents the conversion of neurectoderm to epidermis and in doing so specifies the region of surface ectoderm destined to become the neural plate in the post-gastrulation embryo (Lamb *et al.* 1993). Due to the compensatory activity of other BMP antagonists, such as chordin (Piccolo *et al.* 1996) and follistatin (Hemmati-Brivanlou *et al.* 1994), Noggin is not essential for neural induction, though it is required for normal growth and patterning of the neural tube (McMahon *et al.* 1998).

3.2 Results

3.2.1 *Npas4* expression during neural differentiation of mES cells

3.2.1.1 Temporal expression profile of *Npas4* mRNA during neural differentiation of mES cells

To characterise the temporal expression profile of *Npas4* during neural differentiation of mES cells, semi-quantitative RT-PCR was used to assay for *Npas4* mRNA expression at various time points throughout N2B27 differentiation. 46C mES cells (see Section 3.1.1.2.1) were differentiated according to the N2B27 method (see Section 2.2.1) and cell samples were collected at 48h intervals

over the course of ten days. Undifferentiated mES cells (Day 0) were also analysed to determine whether *Npas4* is expressed by pluripotent mES cells.

Expression of *Npas4* mRNA was not detected in undifferentiated mES cells using 35 cycles of PCR; however, *Npas4* was found to be transiently up-regulated during N2B27 differentiation (Figure 3.1A). Though low levels of *Npas4* mRNA expression were detected after only two days of differentiation, peak expression was reached between Days 4-6 before declining again to baseline levels by Day 10. When the change in *Npas4* expression during neural differentiation of mES cells was quantified using qRT-PCR (Figure 3.1B), it was found that the highest expression of *Npas4* occurred at Day 4 when a 10-fold induction was observed relative to undifferentiated mES cells (Day 0).

3.2.1.2 *In situ Npas4* mRNA expression during neural differentiation of mES cells

The spatial expression pattern of *Npas4* during neural differentiation of mES cells was investigated using ISH (Figure 3.1C). After four days of differentiation in N2B27 medium, *Npas4* mRNA expression in differentiating mES cultures was analysed using a DIG-labelled antisense probe (see Section 2.2.5). A strong signal was observed in virtually all colonies with most of the cells in each colony showing staining. In negative control experiments where a sense probe was used, no signal was detected in any colonies.

3.2.1.3 Npas4 expression in NPC-enriched versus heterogeneous populations

The distinctive temporal expression profile of *Npas4* during neural differentiation of mES cells suggested that *Npas4* may be expressed by a transient progenitor cell type. The period of peak *Npas4* expression corresponds to the phase of differentiation marked by the proliferation of NPCs (Ying *et al.* 2003) and therefore the possibility that *Npas4* is expressed by NPCs was investigated using the transgenic 46C mES cell line which allows $Sox1^+$ NPCs to be purified from a heterogeneous population (discussed in Section 3.1.1.2.1).

Homogeneous populations of $Sox1^+$ NPCs were generated using puromycin selection (see Section 2.2.1) and Npas4 protein expression in these cultures was compared to that of untreated control cultures. A schematic of the experimental paradigm is shown in Figure 3.2B. Immunoblotting using an anti-Npas4 antibody was used to determine Npas4 protein expression at both Day 6 (Figure 3.2C) and Day 8 (Figure 3.2D). The same blots were re-probed using an antibody to GFP to verify enrichment of $Sox1^+$ cells, while an antibody to the β-actin protein served as a loading control.

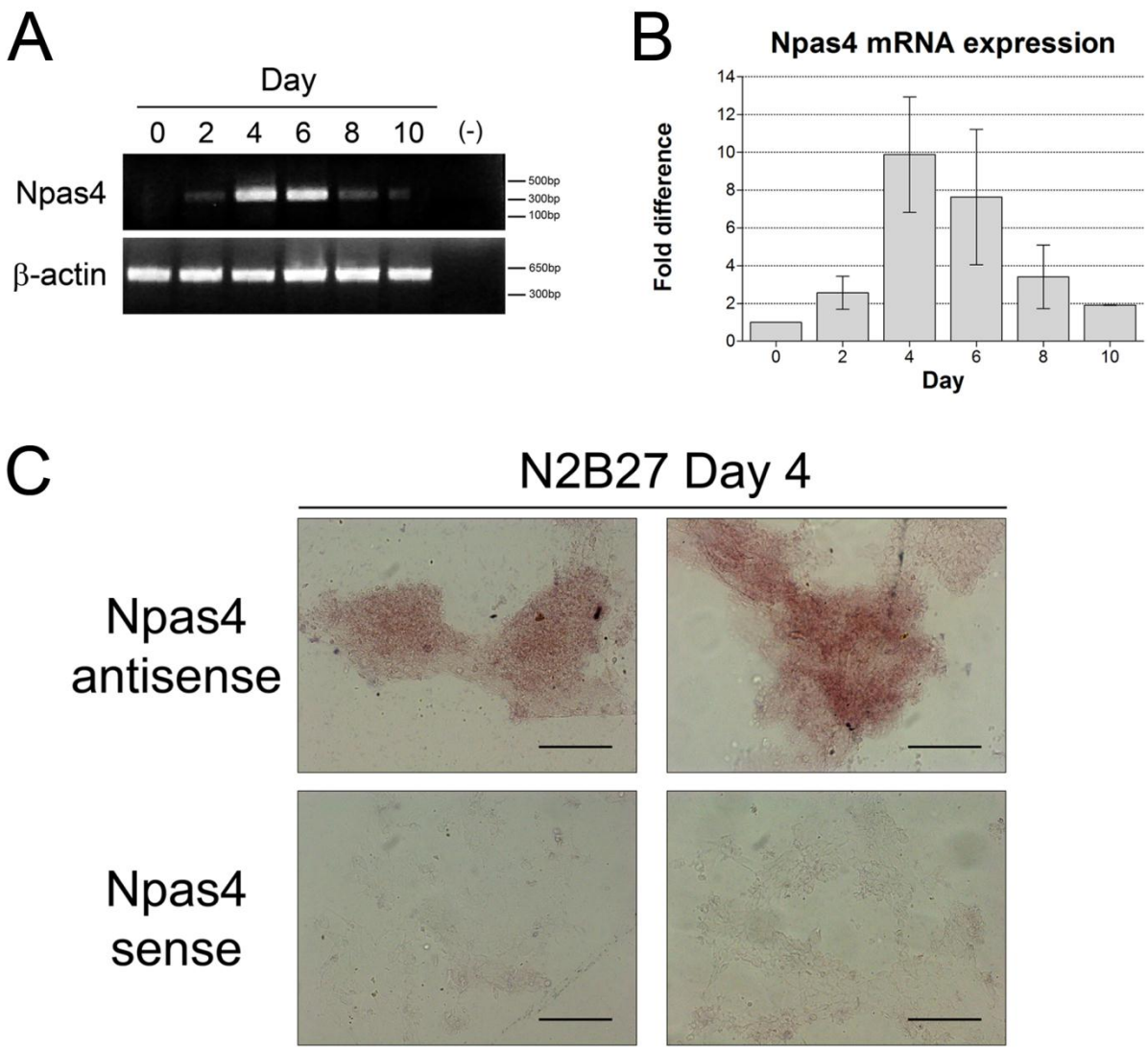


Figure 3.1 – *Npas4* mRNA expression during neural differentiation of mES cells
(A) Semi-quantitative RT-PCR analysis was used to determine the temporal expression profile of *Npas4* mRNA during N2B27 differentiation of mES cells (n=3). Primers to housekeeping gene *β-actin* were used as a loading control. The negative control reaction (-) contained water in place of template cDNA. **(B)** qRT-PCR was performed on the same samples as in (A) to quantify changes in *Npas4* mRNA expression during N2B27 differentiation. *Npas4* expression at each time point has been normalised to *β-actin* expression which was constant throughout differentiation [see (A)]. Fold changes in *Npas4* expression are relative to Day 0 which was given an arbitrary value of 1. Mean values and standard deviations of three independent experiments (n=3) are displayed. **(C)** ISH analysis of Npas4 mRNA expression at Day 4 of N2B27 differentiation of mES cells. Two representative images of differentiating colonies from two independent experiments (n=2) are shown. Top panel - *Npas4* antisense probe; Bottom panel - *Npas4* sense probe. Scale bar = 100µm.

At both Day 6 and Day 8, Npas4 immunoreactivity was lower in lysates collected from NPC-enriched cultures than in those obtained from heterogeneous cultures (Figure 3.2C-D). Two bands were detected using an anti-Npas4 antibody; a higher molecular weight (MW) species, which corresponds to the expected size of the Npas4 protein, and a previously unreported lower MW species which may represent a development-specific isoform of Npas4 (discussed in Section 3.3.3). The difference in Npas4 expression between the two cultures became more pronounced with continued antibiotic selection; after two days of selection the amount of Npas4 immunoreactivity detected in NPC-enriched lysates was considerably less than in control cultures (Figure 3.2C) while Npas4 immunoreactivity was barely detectable after four days of selection (Figure 3.2D). Conversely, GFP immunoreactivity was greater in NPC-enriched cultures than in heterogeneous cultures and this difference became more pronounced with prolonged selection (Figure 3.2C-D).

3.2.2 *NPAS4* mRNA expression during neural differentiation of hES cells

To determine whether the pattern of *Npas4* expression observed during neural differentiation of mES cells was conserved in other mammalian species, the temporal profile of *NPAS4* expression was investigated during Noggin-induced neural differentiation of hES cells (see Section 2.2.1) using semi-quantitative RT-PCR (Figure 3.3). Samples corresponding to four stages of differentiation were analysed; undifferentiated hES cells, Noggin-treated hES cells, and neurospheres derived from Noggin-treated hES cells that were cultured for either one or two weeks.

It was found that *NPAS4* mRNA expression was transiently up-regulated during neural differentiation of hES cells in a manner strikingly similar to that observed in the mES cell model. Using 35 cycles, a single PCR product was amplified from the cDNA sample derived from Noggin-treated hES cells, while no PCR products were amplified in samples derived from undifferentiated hES cells or neurospheres (Figure 3.3). Though the primers that were used contained some nucleotide mismatches to the human cDNA sequences (see Table 2.2), they successfully generated PCR products of the expected size (*Npas4* primers - 348bp; *β-actin* primers - 511bp). A plasmid carrying the mouse *Npas4* cDNA sequence was used as template DNA in the positive control reaction.

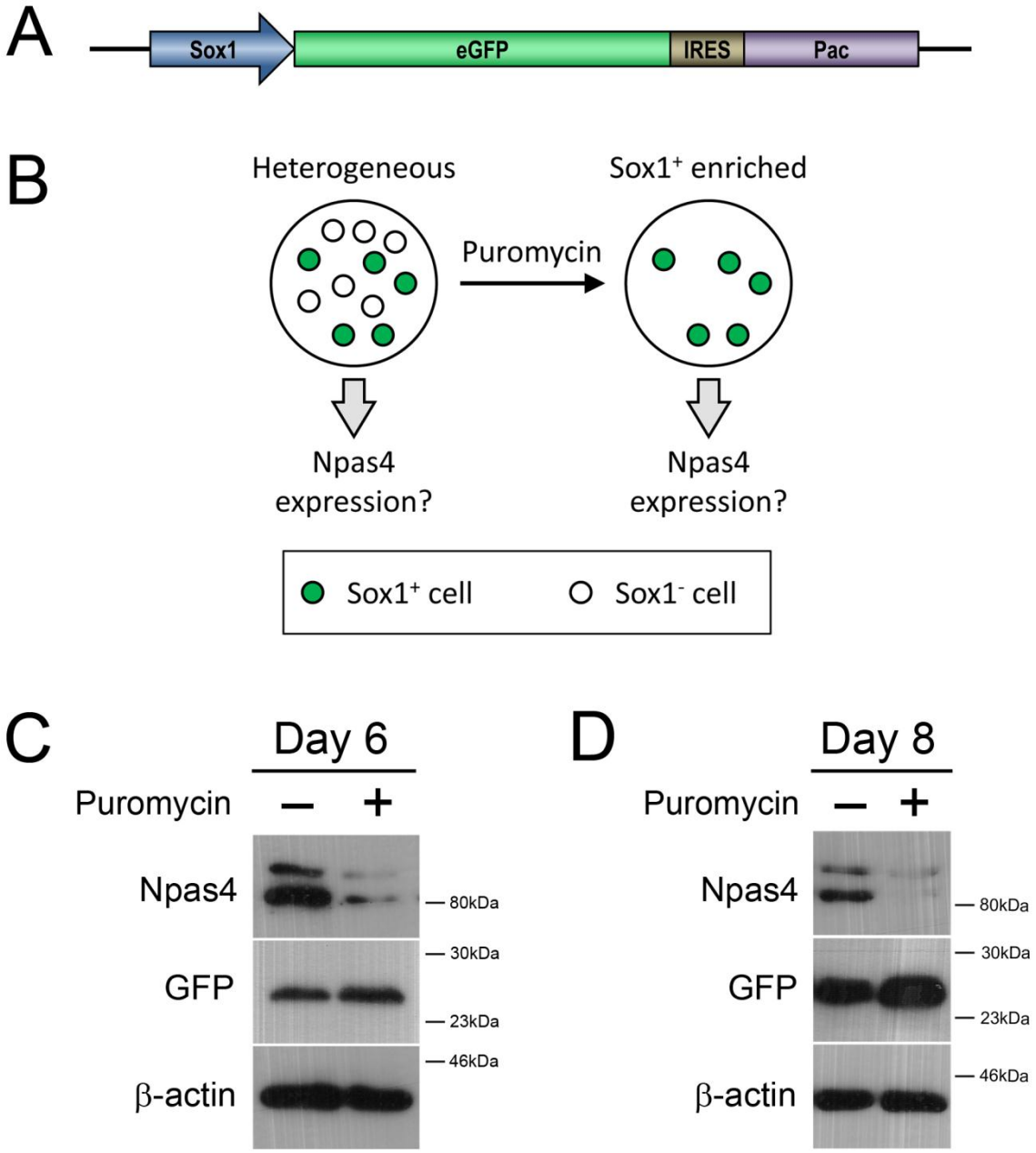


Figure 3.2 – Analysis of Npas4 expression in cultures enriched for Sox1$^+$ NPCs
(A) Schematic representation of the *Sox1* locus on chromosome 8 of the transgenic 46C mES cell line (not to scale). Sox1 – endogenous *Sox1* promoter; eGFP – coding sequence of the enhanced green fluorescent protein; IRES - internal ribosome entry site; Pac - puromycin N-acetyl-transferase coding sequence. **(B)** Schematic showing the experimental approach used in the Sox1 enrichment experiment. Neural differentiation of mES cells in N2B27 medium normally produces a heterogeneous population of cells at various stages of differentiation, however if the 46C mES cell line is used, puromycin selection can be used to eliminate cells which do not express Sox1 thus enriching for Sox1-expressing neural precursor cells. **(C) - (D)** Npas4 protein expression was compared in heterogeneous N2B27 cultures and those enriched for Sox1-expressing neural progenitors (n=3). To enrich for Sox1$^+$ cells, puromycin (0.5µg/mL) was added to cultures on Day 4 and lysates were collected either after two days (C) or four days (D) of selection. Npas4 protein expression was assessed using an anti-Npas4 antibody. The same membranes were probed with an antibody to GFP to determine Sox1 enrichment. β-actin served as a loading control.

3.2.3 *Npas4* mRNA expression in the developing mouse embryo

Given that *Npas4* mRNA was found to be transiently up-regulated in two independent *in vitro* models of development, the expression of *Npas4* mRNA was investigated in the whole mouse embryo to determine whether a similar pattern of expression also occurs during murine embryonic development *in vivo*. RT-PCR was used to analyse the temporal profile of *Npas4* mRNA expression at various stages of embryogenesis ranging from E5.5 to E9.5 (Figure 3.4A).

Npas4 mRNA expression was detected at several developmental timepoints, although there was some variation in *Npas4* expression between litters (Figure 3.4B). A PCR product of the expected size was observed in samples collected from E7.5 embryos (1/4 litters, 40 cycles) and E9.5 embryos (2/3 litters, 36 cycles). However, no PCR product could be detected after 40 cycles in samples collected from E6.5 embryos (0/2 litters) or E5.5 embryos (0/1 litter).

3.3 Discussion

3.3.1 *Npas4* expression in the context of embryonic development

Both *in vivo* and *in vitro* models of embryonic development were used to investigate the expression of *Npas4* in a developmental context and, when taken together, the findings described above provide the first detailed description of *Npas4* expression during early embryogenesis. In an *in vitro* model of early murine development, *Npas4* was found to be transiently expressed during neural differentiation of mES cells and these data were substantiated by similar findings a hES cell model. That a similar expression pattern was reproduced in two independent ES cell models systems across two mammalian species validates these results and suggests that *Npas4* expression in the context of development may be evolutionarily conserved in mammals and, thus, biologically important. These data were then corroborated by experiments performed *in vivo* which showed that *Npas4* mRNA is expressed in the developing mouse embryo between E7.5-E9.5.

3.3.1.1 Variability of *Npas4* expression during embryogenesis

When the temporal profile of *Npas4* mRNA expression in the developing mouse embryo was analysed by RT-PCR, there was some variability in *Npas4* expression between litters (Figure 3.4B). For instance, the earliest incidence of *Npas4* mRNA expression in the mouse embryo occurred at E7.5, though this was observed in only one of four litters sampled. Similarly, *Npas4* expression was detected in only two of three litters sampled at E9.5. There are several possible reasons for this

variability. Firstly, when using animal models in research there is always an element of inherent biological variability, particularly in regards to the progression of embryonic development from one litter to the next. Accordingly, litters collected at the same gestational age may not always correspond to the same developmental stage (Downs and Davies 1993). Furthermore, although the time of collection was consistent between litters, the timing of the mating cannot be precisely controlled (see Section 2.2.7). The combination of these two elements may account for slight differences in staging between litters.

Another factor to consider is intralitter variability, as rarely are all embryos from a given litter at precisely the same developmental stage. For example, at E7.5, extensive variation is observed and embryos can be found at developmental stages ranging from the mid-streak stage to the early allantoic bud stage (Downs and Davies 1993). Thus, when all embryos from a single litter are pooled, a mixed RNA sample is created in which the level of expression of a particular RNA species will be the mean expression across all embryos in that litter. This may lead to some RNA species being misrepresented in litters where developmentally delayed or advanced embryos are present. In future experiments, variability could be minimised by staging embryos immediately upon dissection and pooling embryos according to developmental stage rather than gestational age.

Nevertheless, this variability suggests that the period of embryonic development between E7.5-E9.5 may represent the cusp of *Npas4* induction (i.e. a stage of development when expression of *Npas4* mRNA is at the borderline of the detection threshold) and is therefore very sensitive to minute changes in the timing of embryo collection. Together with the observation that *Npas4* mRNA expression was not detected in samples collected prior to E7.5 (neither at E5.5 nor at E6.5), these data suggest that *Npas4* mRNA is first expressed in the developing mouse embryo between E7.5-9.5. A proposed temporal expression profile of *Npas4* during embryonic development is illustrated in Figure 3.5A. Later embryonic stages were not investigated and therefore it is not known whether *Npas4* expression is maintained until birth or is only transiently expressed as was seen in ES cell models. More research is needed to answer these questions.

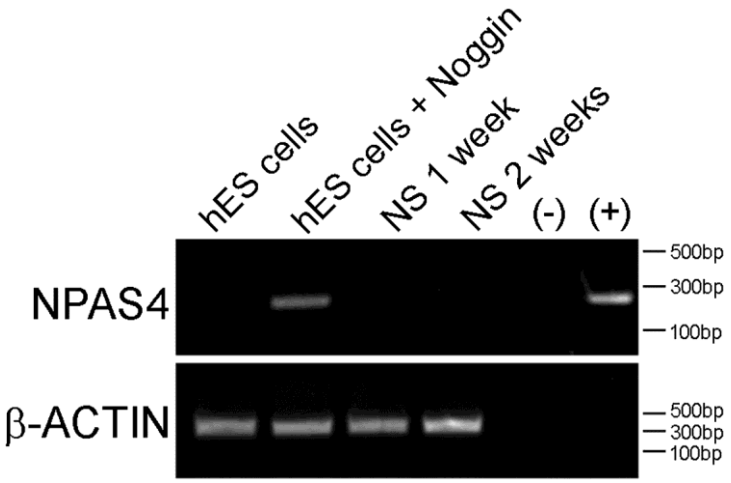


Figure 3.3 – *NPAS4* mRNA expression during Noggin-induced neural differentiation of hES cells
Semi-quantitative RT-PCR analysis was used to determine the temporal expression profile of *NPAS4* mRNA during Noggin-induced neural differentiation of hES cells (n=1). Primers to housekeeping gene *β-ACTIN* were used as a loading control. NS – neurospheres. The negative control reaction (-) contained water in place of template cDNA. A plasmid containing the mouse *Npas4* cDNA sequence was used as a positive control (+).

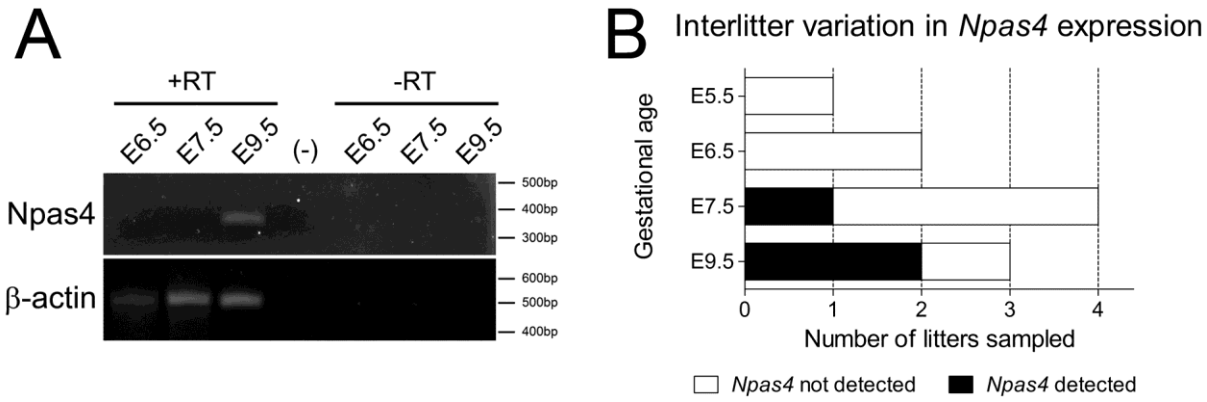


Figure 3.4 – *Npas4* mRNA expression during mouse embryonic development
(A) RT-PCR analysis was used to determine *Npas4* mRNA expression at various stages of mouse embryonic development ranging from E6.5-E9.5. RNA was isolated from mouse embryos at the indicated development stage and this was used as a template for reverse transcription in a reaction containing either the reverse transcription enzyme (+RT) or water (-RT). The PCR negative control reaction (-) contained water in place of template cDNA. Primers to housekeeping gene *β-actin* were used as a positive control for cDNA synthesis. The number of litters analysed at each time point is shown in (B). **(B)** Interlitter variation in *Npas4* expression at various stages of development. The proportion of samples in which *Npas4* expression could be detected by RT-PCR is indicated for each collection time point.

3.3.2 Which cells express *Npas4* in the context of embryonic development?

Before speculating on the identity of the *Npas4*-expressing cell population seen during neural differentiation of ES cells, it is important to understand how differentiation progresses in each of the differentiation systems used. During N2B27 differentiation of mES cells, NPCs first start to appear after two days of differentiation as mES cells begin to lose pluripotent status; however, significant neural induction is not achieved until Day 4 when around 70-80% of cells are neurally committed as determined by expression of the NPC marker *Sox1* (Ying and Smith 2003). The relative percentage of NPCs remains stable until Day 8 when it begins to decline as the NPCs terminally differentiate into either neurons or glia. Neuronal cells first begin to appear after five days of differentiation and the number of neurons in culture steadily increases as more NPCs undergo differentiation. By Day 12, approximately 30% of the cells in culture are Tau-expressing neurons with the remainder being predominantly glia and undifferentiated NPCs (Ying and Smith 2003). As with practically all differentiation protocols, the neural conversion is not 100% effective and a small percentage of contaminating non-neural cells (usually of the mesodermal lineage) will also be generated, though they make up a relatively small percentage of total cells.

In Noggin-induced neural differentiation of hES cells, Noggin treatment efficiently converts pluripotent hES cells into a population of early NPCs having properties similar to embryonic neuroectoderm. Upon neural induction with Noggin, expression of pluripotency factors octamer-binding transcription factor 4 (OCT4) and NANOG is gradually down-regulated (Pera *et al.* 2004; Gerrard *et al.* 2005) and after 14 days of Noggin treatment, although some undifferentiated hES cells remain, the neuroectodermal marker paired box gene 6 (PAX6) is expressed by the majority of cells (Pera *et al.* 2004; Denham and Dottori 2009). Though neurospheres also contain NPCs, the population of NPCs found within neurospheres is distinct to those present in Noggin-treated hES cell cultures and represents a more mature precursor population. Neurosphere NPCs have characteristics more similar to the neuroepithelial cells of the neural tube and express PAX3 and PAX7 instead of PAX6 (see Figure 3.5B).

3.3.2.1 Npas4^{+} cells are not pluripotent ES cells

The findings presented here demonstrate that *Npas4* is expressed during the early stages of neural differentiation and, though the identity of the *Npas4*$^{+}$ cell type was not explicitly established, the data can be used to make several deductions regarding the nature of the *Npas4*-expressing cells. Firstly, it is possible to immediately exclude several cell populations as candidates based on the

available evidence. Both the *in vivo* and *in vitro* data suggest that developmental expression of *Npas4* cannot be attributed to pluripotent cell types. Firstly, the *Npas4* expression seen in the post-gastrulation mouse embryo at E7.5-E9.5 occurs several days after the pluripotent cells of the ICM have differentiated into the three germ layers of the embryo. Secondly, although *Npas4* expression was not investigated in the E4 blastocyst, when homogeneous populations of undifferentiated ES cells were analysed for *Npas4* mRNA expression, none was detected in either mouse or human ES cells.

3.3.2.2 Npas4^{+} cells are not neurons

Similarly, it is unlikely that *Npas4* expression in these developmental models can be ascribed to neurons for the following reasons:

(1) Very little neurogenesis occurs in the embryo at the time that *Npas4* expression was detected between E7.5-E9.5 (the bulk of embryonic neurogenesis occurs between E11-E17).

(2) There are no neurons present in Noggin-treated hES cell cultures at the time that *Npas4* expression was detected (neuronal differentiation does not occur until after the neurosphere stage - well after *Npas4* expression has been down-regulated).

(3) Peak *Npas4* expression during N2B27 differentiation of mES cells precedes neuronal differentiation (indeed, neurogenesis coincides with the down-regulation of *Npas4* expression; see Section 3.1.1.1).

Thus, in all three model systems, *Npas4* expression occurs prior to the formation of neurons which renders this the first detailed account of *Npas4* expression in non-neuronal cells.

Given that virtually all reports of *Npas4* expression published thus far have stated that *Npas4* is expressed in neurons, it may seem counter-intuitive that down-regulation of *Npas4* expression coincides with the formation of neurons during neural differentiation of mES cells. However, it should be noted that although neurons are present in culture as early as Day 5 (Ying *et al.* 2003), at this time the neuronal networks are composed of immature neurons lacking functional synapses. Work from our laboratory has shown that there is no spontaneous neural activity occurring in mES cell-derived neuronal circuits at this early stage of differentiation (personal communication Lauren Sandeman, Kylie Ellis) and hence one would not expect to find expression of activity-dependent genes during this time. Interestingly, it was observed that *Npas4* expression re-emerged after extended culture of mES cell-derived neurons past Day 25 (data not shown) and this expression may

be due to the spontaneous action potential firing which occurs upon maturation of neurons in culture.

3.3.2.3 *Npas4* may be expressed by a transient NPC population

Both the timing of *Npas4* expression (which occurs after differentiation of pluripotent cells yet before neurogenesis) and its transience during ES cell differentiation *in vitro* are consistent with the possibility that *Npas4* is expressed by an intermediate population of NPCs. In both the mouse and human ES cell neural differentiation systems, peak *Npas4* expression coincided with the period of differentiation marked by the proliferation of NPCs and, while there are other cell types present in the culture at this time, the evidence suggests that *Npas4* may be expressed by a NPC population. For example, as discussed in Section 2.2.1, treatment of hES cells with Noggin for 14 days produces a heterogeneous mixture of cells; the majority of cells are $PAX6^+/SOX1^-$ cells, though some residual undifferentiated hES cells are present in addition to a small number of more advanced $PAX6^+/SOX1^+$ NPCs (Dottori and Pera 2008). Given that *NPAS4* expression was not detected in a homogeneous population of undifferentiated hES cells, it is unlikely that the *NPAS4* expression seen in Noggin-treated cultures can be ascribed to residual undifferentiated hES cells which therefore suggests that it occurs in either, or both, of the NPC types. Similarly, batch differentiation of mES cells in N2B27 medium is not synchronous but staggered (see Section 3.1.1.1); once differentiation has commenced, the population will be heterogeneous as different colonies are in various phases of differentiation and consequently the emergence of a transient NPC population over time will follow a standard curve. Thus, both the timing and the shape of the *Npas4* expression profile during N2B27 differentiation of mES cells (Figure3.1A-B) support the notion that *Npas4* expression occurs in a transient population of NPCs.

3.3.2.4 Npas4 is not expressed in $Sox1^+$ NPCs

For this reason, it was somewhat unexpected to find that expression of Npas4 protein was markedly reduced in differentiating mES cell cultures that had been enriched for $Sox1^+$ NPCs using antibiotic selection. This suggests that Npas4 is not expressed by $Sox1^+$ NPCs and, moreover, that expression of Npas4 and Sox1 is mutually exclusive. As a logical extension of this hypothesis, one would expect that Npas4 expression would be correspondingly increased in the $Sox1^-$ pool. Though analysis of the $Sox1^-$ population is not possible when using antibiotic selection, by using the 46C Sox1::GFP reporter mES cell line it is possible to use flow cytometry to separate the $Sox1^+$ and $Sox1^-$ fractions and examine them in isolation. While this experiment was attempted, it was not successful due to technical

difficulties and hence antibiotic selection was used. Thus, although likely, it remains to be confirmed that Npas4 is expressed exclusively by $Sox1^-$ cells. In future, other methods could be used to conclusively demonstrate segregation of Npas4 and Sox1 expression; for example, co-localisation studies could be performed using immunocytochemistry to determine whether Npas4 and Sox1 proteins are co-expressed within the same cell during neural differentiation of mES cells.

While $Sox1^+$ NPCs do not appear to express Npas4, this does not discount the possibility that Npas4 is expressed by other NPC types. Many NPC types exist and each is characterised by a unique gene expression profile; as cells progress through neural differentiation, they pass through discrete stages marked by expression of particular marker genes and hence specific NPC populations can be identified on the basis of their gene expression profile (see Figure 3.5B).

The observation that Npas4 and Sox1 do not appear to be simultaneously expressed in the same cell raises two possibilities: (1) cells express these genes sequentially and transition from expression of one gene to another, or (2) the $Npas4^+$ and $Sox1^+$ populations represent two completely different non-overlapping cell lineages. Without additional data it is difficult to distinguish between these two possibilities. However, given that cells progress through the differentiation process in a somewhat linear manner, it would seem that the first option is more likely. This then raises the question; does *Npas4* expression precede or follow expression of *Sox1*?

It is difficult to directly compare the timing of Npas4 expression with that of Sox1 given that Sox1 expression is measured indirectly via GFP, which is a more stable protein than the endogenous Sox1 protein (Chung *et al.* 2006). Nevertheless, the timing of *Npas4* expression during neural differentiation of mES cells suggests that *Npas4* is expressed prior to *Sox1*; while many cells (>70%) continue to express Sox1 at Day 8 [(Ying *et al.* 2003) and Figure 5.1A], *Npas4* mRNA expression is already approaching baseline levels by this time (Figure 3.1A-B). Curiously, this seems to be at odds with what is observed in the mouse embryo; Sox1 is expressed from E7.5 (Pevny *et al.* 1998) while expression of *Npas4* is first detected at some time between E7.5-E9.5 which indicates that *Npas4* expression at the very least coincides with, or possibly even follows, *Sox1* expression. The reason for this apparent discrepancy is not known, though it is accepted that *in vitro* models do not always faithfully replicate the intended paradigm and thus this may represent one such situation. To resolve this issue, the relative timing of *Sox1* and *Npas4* expression must be investigated further in both murine embryogenesis and neural differentiation of mES cells.

3.3.2.5 *Npas4* expression in relation to other NPC marker genes

To better understand which cells express *Npas4* during neural differentiation of ES cells, it is important to consider how the timing of *Npas4* expression relates to that of other marker genes expressed at this time. *Pax6*, for example, is a well-characterised marker gene that can be used as a point of reference when trying to determine the identity of a cell population. Pax6 is a highly conserved transcription factor which contains both a paired domain and a homeodomain and consequently is a member of the paired box family of proteins which have important roles during development. Pax6 is expressed in the developing CNS and, although known as a master regulator of eye development, also has roles in neural tube patterning, neurogenesis and neuronal migration (Simpson and Price 2002). During early embryogenesis, *Pax6* expression demarcates the forebrain and hindbrain vesicles in the primordium of the brain (Schwarz *et al.* 1999). In the neural tube, Pax6 expression is localised ventrally where it specifies the identity of a population of radial glial NPCs which give rise to ventral interneurons and motor neurons (Ericson *et al.* 1997). In adult brain, Pax6 continues to be expressed in radial glial cells of the SVZ and the SGZ of the DG where it participates in postnatal neurogenesis (Osumi *et al.* 2008).

In the developing mouse embryo, Sox1 expression is first detected in the neural plate at E7.5 (Pevny *et al.* 1998) while *Pax6* is not expressed until the neural tube has formed at E8 (Walther and Gruss 1991). This sequential expression is recapitulated during neural differentiation of mES cells where expression of Sox1 precedes Pax6 expression (Suter *et al.* 2009). Interestingly, the order of marker gene expression is reversed in rodents and primates and the converse is seen during neural differentiation of both human and rhesus ES cells where PAX6 is expressed before SOX1 (Pankratz *et al.* 2007). Whether this signifies an inherent difference between primate and rodent neural development or is simply an *in vitro* quirk which is peculiar to culture of primate ES cells *ex vivo* is not known.

Nevertheless, in the initial stages of neural induction of hES cells, most NPCs are $PAX6^+/SOX1^-$ with very few $SOX1^+$ cells being detected after 14 days of Noggin treatment (Denham and Dottori 2009). Continued culture in the presence of Noggin eventually leads to up-regulation of SOX1 resulting in a population of $PAX6^+/SOX1^+$ NPCs (Gerrard *et al.* 2005; Yao *et al.* 2006) that also express NESTIN at later stages (Gerrard *et al.* 2005; Sonntag *et al.* 2007). A schematic which depicts the progression of cells during the process of Noggin-induced neural differentiation is shown in Figure 3.5B. The $PAX6^+/SOX1^-$ NPCs represent a transient cell population having a distinctively anterior regional

identity. As these cells express forebrain markers such as OTX2 and LHX2 but do not express the hindbrain markers HOXB4 or HOXC8, this unique population of cells has been termed 'primitive anterior neuroectoderm' (PAN) and has been likened to the neural epithelium of the embryo, which is the earliest neurally specified tissue (Pankratz *et al.* 2007). Transfer of cells to the neurosphere stage promotes further differentiation into a population of more mature NPCs which is accompanied by a down-regulation of PAX6 and concomitant up-regulation of dorsal neural markers PAX3 and PAX7 (Denham and Dottori 2009).

In this system, *NPAS4* mRNA expression was found to be up-regulated once hES cells were primed for neural differentiation by treatment with Noggin for 14 days; at this stage of differentiation the majority of cells in culture are $PAX6^+/SOX1^-$ PAN cells and very few $PAX6^+/SOX1^+$ cells are present (Dottori and Pera 2008). If the mutually exclusive expression of Npas4 and Sox1 is conserved in humans, then it's likely that *NPAS4* is expressed by the $PAX6^+/SOX1^-$ PAN cells. This raises the possibility that, unlike PAX6 and SOX1 which are expressed by more than one NPC type, expression of *NPAS4* is unique to the $PAX6^+/SOX1^-$ cell type. If so, *NPAS4* could be used as a marker of PAN cells. This hypothesis could be tested using immunocytochemistry to determine whether NPAS4 is expressed by $PAX6^+/SOX1^-$ PAN cells during neural differentiation of hES cells.

3.3.2.6 Spatial expression of *Npas4* during embryogenesis

An understanding of the spatial expression of *Npas4* in the mouse embryo between E7.5-E9.5 may assist in the identification of the $Npas4^+$ cell type and may validate or reject the notion that *Npas4* is expressed in the neural epithelium of the developing embryo. To this end, ISH analysis of whole mount embryos was attempted a number of times using a probe specific to the *Npas4* mRNA but was not successful due to technical difficulties. Due to the low level of *Npas4* expression, an extended incubation time was necessary for the colour reaction to develop, though this also caused background staining to become visible which masked the *Npas4* expression pattern. On account of this, it may prove less problematic to use sectioned embryos or to use fluorescent immunohistochemical analysis using an anti-Npas4 antibody. Alternatively, characterisation of the endogenous spatial and temporal developmental expression pattern of *Npas4* may require the creation of a transgenic reporter mouse in which the regulatory elements controlling *Npas4* expression in the embryo drive the expression of reporter genes (i.e. *lacZ* or *GFP*). If a fluorescent reporter is used, it would also be possible to definitively conclude whether or not the $Npas4^+$ population is pluripotent; $Npas4^+$ cells could be isolated using flow cytometry and injected into a

mouse blastocyst to see whether they are able to contribute to a chimeric animal, and hence whether they are pluripotent. Before this can be achieved, however, the regulatory elements of the *Npas4* promoter sequence must be defined as to date they have not been characterised.

3.3.3 Development-specific isoform of Npas4?

In whole cell lysates collected from differentiating mES cells, two bands were detected when blots were probed with an anti-Npas4 antibody; a higher MW species of approximately 100kDa and a lower MW species of approximately 85kDa that was more strongly expressed. Although the predicted MW of the Npas4 protein has been reported as 87.4kDa (Moser *et al.* 2004), the experimentally observed size of the protein in neuronal lysates has been in the region of 100-120kDa (Moser *et al.* 2004; Shamloo *et al.* 2006; Hester *et al.* 2007; Zhang *et al.* 2009; Ploski *et al.* 2011; Pruunsild *et al.* 2011). This corresponds to the size of the higher MW species and suggests that this signal corresponds to full-length Npas4 protein, though it also raises questions about the identity of the lower MW species.

One possibility is that this second signal may signify cross-reactivity of the antibody with non-specific proteins having structural similarity to Npas4. While this is possible, the antibody has previously been shown to be very specific for the rodent Npas4 protein in lysates collected from rat hippocampal neurons (Lin *et al.* 2008). Moreover, both species responded similarly to puromycin treatment with changes in expression of the higher MW species being mirrored in the lower MW species making this an unlikely explanation. This raises the possibility that this lower MW species represents another isoform of the Npas4 protein, for instance a shortened splice variant or a cleavage product arising from proteolysis of the full-length Npas4 protein. As this has not been observed in other systems, this may represent an isoform of Npas4 that is expressed specifically in a developmental context. This type of ontogeny-specific splicing has been observed in other genes, such as *Drebrin* (see Section 1.5.2.2.1), which has both an embryonic and an adult isoform.

3.3.4 Ca^{2+}-mediated neural induction: is Npas4 the missing link?

It has been shown that an increase in nuclear Ca^{2+} leads to up-regulation of *Npas4* expression (see Section 1.4.2.2.2), though to date this has only been described in post-mitotic neurons. The observation that *Npas4* expression during early development does not occur in neurons (see Section 3.3.2.2) raises the obvious question: what triggers *Npas4* expression in the embryo in the absence of neural activity? One possibility is that the inherent Ca^{2+} signalling that is an integral part

of embryonic development stimulates *Npas4* expression in non-neuronal cells during embryogenesis. In a number of vertebrate species, Ca^{2+} signalling has been implicated as a key regulator of neural development and, in particular, the process of neural induction (Webb *et al.* 2005; Leclerc *et al.* 2006; Moreau *et al.* 2008). It was first hypothesised that Ca^{2+} signalling may be involved in the molecular mechanisms underlying neural induction when it was noted that the density of L-type Ca^{2+} channel expression in ectodermal cells correlates with the acquisition of neural competence in both the salamander (Leclerc *et al.* 1995) and Xenopus (Drean *et al.* 1995) embryo. Imaging techniques which allowed the complex patterns of Ca^{2+} signalling to be visualised in the live Xenopus embryos confirmed that the timing, location and intensity of Ca^{2+} transients in the embryo is consistent with a possible role in neural induction (Leclerc *et al.* 2000). An unquestionable role for Ca^{2+} signalling in nervous system development was established when it was demonstrated that increased intracellular Ca^{2+}, arising from activation of L-type Ca^{2+} channels, is both necessary and sufficient to trigger the process of neural induction in the salamander (Morgan *et al.* 1994; Leclerc *et al.* 1997). Due to the inaccessibility of mammalian embryos, investigations into the role of Ca^{2+} signalling in neural development have thus far been limited to lower vertebrates; however, as many aspects of embryogenesis are evolutionarily conserved, it is reasonable to hypothesise that similar mechanisms may also be at work in mammals (Aruga and Mikoshiba 2011).

While it is now evident that Ca^{2+} signalling is crucial for neural induction, the molecular mechanisms underlying this process are not known. It is hypothesised that Ca^{2+} signalling regulates neural induction via changes in expression of early pro-neural genes and that this is mediated by extracellular signalling molecules which drive neural induction, such as Noggin (Aruga and Mikoshiba 2011). Several observations support this hypothesis. Firstly, in the Xenopus embryo it has been shown that Ca^{2+} transients can induce expression of *Zic3* (Leclerc *et al.* 2003), a primary regulator of neural development (Nakata *et al.* 1997). Secondly, in the salamander, Noggin has been shown to directly increase intracellular Ca^{2+} via activation L-type Ca^{2+} channels (Leclerc *et al.* 1997). Lastly, addition of Noggin to explants of prospective neuroectoderm isolated from the animal cap was sufficient to induce Ca^{2+}-dependent expression of the Fos protein (Leclerc *et al.* 1999). By extension, it is possible that Noggin may similarly regulate the expression of other Ca^{2+}-sensitive IEGs, such as *Npas4*, in the developing embryo and that these genes are involved in regulating developmental processes.

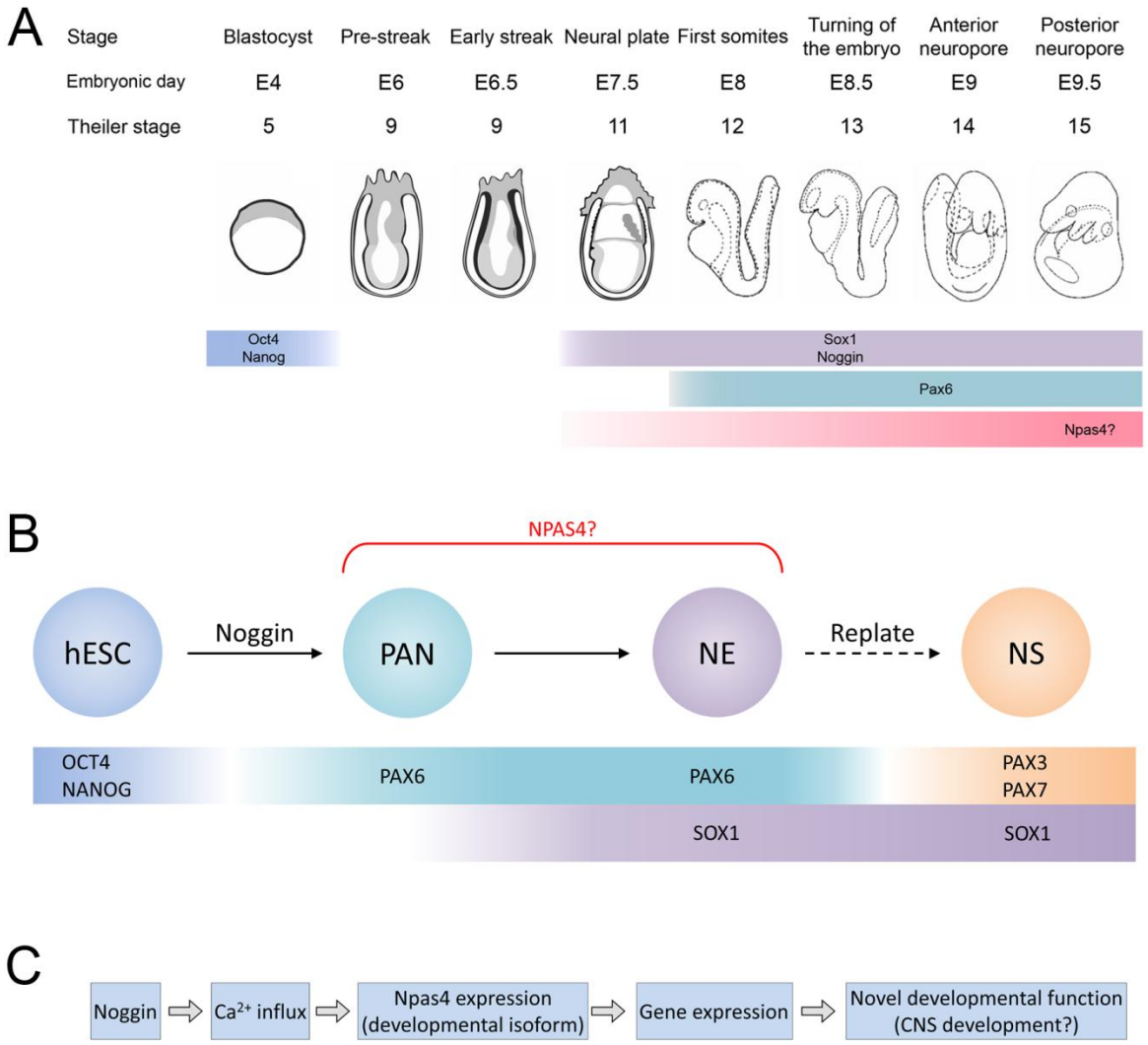


Figure 3.5 – Proposed model for the embryonic expression of *Npas4*
(A) Timeline of mouse embryonic development. Key stages in mouse embryonic development are shown with the corresponding gestational age and Theiler stage indicated above. Expression of relevant marker genes is also indicated below. Proposed period of *Npas4* expression during development is also marked. The graded shading of the *Npas4* bar indicates that the cusp of *Npas4* expression occurs at some time between E7.5 and E9.5. Images adapted from the eMouse Atlas Project (http://www.emouseatlas.org) **(B)** Schematic representation of Noggin-induced neural differentiation of hES cells. Expression of marker genes at each stage of differentiation is indicated below. Proposed range of *NPAS4* expression is indicated above. hESC - human embryonic stem cell; PAN – primitive anterior neuroectoderm; NE – definitive neuroectoderm; NS – neurosphere. **(C)** Proposed model for the expression and function of Npas4 during development.

The *in vitro* models of development used in this study are consistent with a possible role for Noggin in the induction of *Npas4* during development; *NPAS4* expression was observed in Noggin-treated hES cells and, although the protocol used for neural differentiation of mES cells does not require addition of exogenous Noggin, expression of endogenous *Noggin* mRNA increases from Day 1-5 (Ying *et al.* 2003) in a manner similar to *Npas4.* The hypothesis that Noggin induces *Npas4* expression in a Ca^{2+}-dependent manner could be tested by performing Noggin-induced neural differentiation of hES cells in the presence of Ca^{2+} channel blockers to determine whether this prevents up-regulation of Npas4 expression. If Noggin were able to directly induce *Npas4* mRNA expression via Ca^{2+} entry without the need for *de novo* protein synthesis, this would place *Npas4* as one of the first genes to be up-regulated in neurally specified tissue. This is in keeping with the observation that *Npas4* appears to be expressed by an early NPC population (see Section 3.3.2.5).

3.3.5 A novel function for *Npas4* in embryonic development?

The expression of *Npas4* during early embryonic development described here differs to other examples of *Npas4* expression in several significant ways. Firstly, while the majority of published data regarding *Npas4* are concerned with its expression in neurons, this is the first detailed account of *Npas4* expression in non-neuronal cells (see Section 3.3.2). Secondly, unlike other *Npas4* induction paradigms in which it acts as a stimulus-response gene and is down-regulated within a few hours of stimulation, in these developmental models *Npas4* expression remains up-regulated for several days (Figure 3.1A-B). Thirdly, these data provide evidence that a previously unreported atypical Npas4 isoform may be expressed exclusively in the context of development (see Section 3.3.3). Given these differences, it is possible that the function of *Npas4* in non-neuronal cells during early development is distinct to that seen in neurons of the adult brain (Figure 3.5C).

Before speculating on the possible role of *Npas4* during development, it is necessary to have an understanding of the events occurring in the embryo at the time of *Npas4* expression. E6.5 sees the formation of the primitive streak and the onset of gastrulation, the process which will give rise to the three germ layers of the embryo; the ectoderm, mesoderm and endoderm (Tam and Loebel 2007). By E7.5, gastrulation is well underway and development of the nervous system begins. In the first stage of neural induction, a region of the ectoderm becomes specifically defined as the neuroectoderm as a result of diffusible signals, such as Noggin (see Section 3.1.1.3.1), which emanate from the underlying mesoderm. This sheet of neuroectoderm, called the neural plate, will later fold onto itself to form the neural tube through a process known as primary neurulation. At E7.5, which

corresponds to Theiler stage 11, the neural plate is defined anteriorly and laterally and this coincides with induction of *Sox1* expression in the neuroectoderm. Soon after, the head process develops in conjunction with the formation of the neural groove in the midline of the neural plate, an event which marks the first step in the folding of the neural plate onto itself. At E8 (Theiler stage 12), the neural groove deepens and the first somites begin to appear. At around the seven somite stage, approximately E8.5, the first neural tube closure event (Closure 1) occurs at the hindbrain/cervical boundary when the lateral edges of the neural folds make contact at the dorsal midline and fuse together (Copp *et al.* 2003). Neural tube closure proceeds bilaterally from this site along the anterior/posterior axis. Two more neural tube closure events follow; one at the anterior neuropore, the future brain region (Closure 2), which occurs at approximately E9 (Theiler stage 14) and another at the posterior neuropore, located at the caudal extremity of the spine (Closure 3), which occurs at approximately E10 (Theiler stage 16) (Copp *et al.* 2003).

Taking into consideration the events occurring in the embryo at the time of *Npas4* expression (Figure 3.5A), it is unlikely that *Npas4* is involved in the process of gastrulation as this event precedes the earliest detectable expression of *Npas4*. Rather, it appears that *Npas4* expression in the embryo is first detected shortly after neural induction has commenced and coincides with the onset of neurulation. This raises the intriguing possibility that *Npas4* may be involved in development of the nervous system, perhaps playing a role in patterning of the neural tube. Further research will be needed to clarify the role of *Npas4* in the embryo.

Nevertheless, a recent finding that Ca^{2+} signalling modulates cell fate choice during neural differentiation of mES cells may provide clues regarding the function of *Npas4* in this context. In 2007, Yamada *et al.* demonstrated that a transient increase in intracellular Ca^{2+} at a critical period of differentiation preferentially directs differentiation of mES cells towards a neuronal fate (Yamada *et al.* 2007), though the mechanisms underlying this phenomenon were not elucidated. It is conceivable that Ca^{2+} signalling during differentiation may promote neuronal differentiation via activation of Ca^{2+}-sensitive pro-neural genes. Though evidence in support of such a hypothesis is limited, it is tempting to speculate that Ca^{2+}-mediated induction of *Npas4* may be involved in this process given that *Npas4* is transiently expression during differentiation and its expression is dependent on Ca^{2+} signalling. If this hypothesis were correct (i.e. if *Npas4* expression during mES cell differentiation is required for neuronal differentiation), one prediction stemming from this hypothesis is that blocking *Npas4* induction would inhibit neuronal differentiation (see Chapter 5).

3.4 Summary

In two distinct differentiation models across two species it was demonstrated that *Npas4* mRNA is transiently up-regulated during neural differentiation of ES cells. In both systems, expression of *Npas4* coincided with proliferation of NPCs in culture suggesting that *Npas4* may be expressed this cell population. Furthermore, it was shown that expression of Npas4 and Sox1 is mutually exclusive during neural differentiation of mES cells raising the possibility that Npas4 expression is upstream of Sox1 in the transcriptional programme. A previously undescribed Npas4 immunoreactive species was observed in lysates collected from differentiating mES cells raising the possibility that a development-specific isoform of Npas4 exists. Finally, analysis of *Npas4* expression *in vivo* showed that *Npas4* mRNA is expressed in the developing mouse embryo between E7.5-E9.5.

4 – Generation of Npas4 knock-down mES cell lines

4.1 Introduction

The distinctive temporal profile of *Npas4* expression observed during neural differentiation of ES cells (see Chapter 3) suggested a possible role for Npas4 in this process. A loss-of-function approach was used to study the function of *Npas4* in the context of neural differentiation. Lentiviral transduction was used to create stable mES cell lines in which the expression of Npas4 during differentiation was reduced by RNAi. These Npas4 knock-down mES cell lines were then compared to control mES cell lines to determine the effect of decreased Npas4 expression on neural differentiation. This chapter describes the generation and validation of the Npas4 knock-down mES cell lines used in this study.

4.1.1 RNA interference

Gene expression can be modulated at multiple levels. At the mRNA level, stability of transcripts can be regulated by a phenomenon known as RNAi whereby short, single-stranded RNA molecules suppress gene expression in a sequence-specific manner by binding to complementary sites found on target mRNAs. Silencing of gene expression can occur either via direct degradation of the mRNA or by repression of translation. This post-transcriptional regulatory mechanism was first described in the nematode (Fire *et al.* 1998) but is well-conserved throughout evolution and is found in plants, invertebrates and mammals (Rodriguez *et al.* 2004).

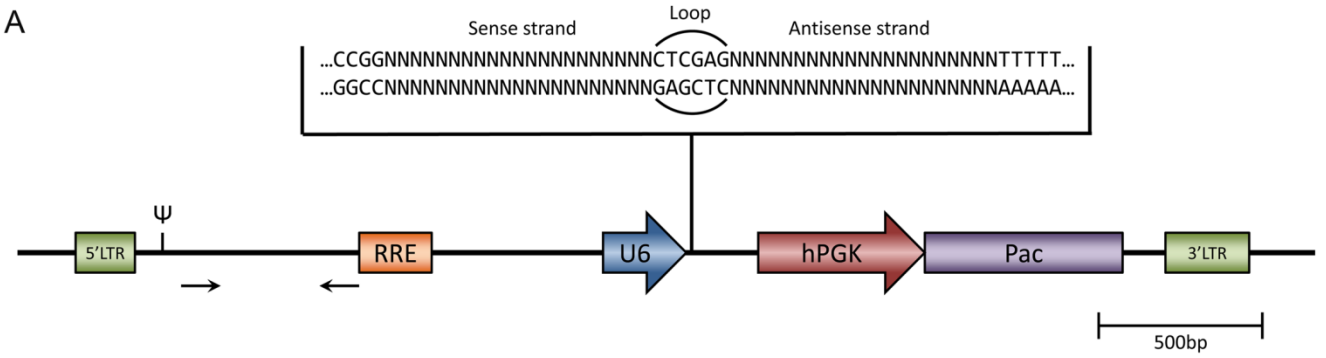
RNAi is mediated by a class of endogenous non-protein-coding RNAs called miRNAs. Genes encoding miRNAs are found throughout the genome, often within introns of protein coding genes (Rodriguez *et al.* 2004), and are transcribed largely by RNA polymerase II (Lee *et al.* 2004). Initially, miRNAs are transcribed as immature precursor molecules called pre-miRNAs which have a complex secondary structure often containing numerous hairpin loops and double-stranded regions. In the cytoplasm, these double-stranded RNAs are recognised by the endoribonuclease Dicer which cleaves the precursor miRNAs into short, double-stranded RNAs of 21-25 nucleotides termed small interfering RNAs (siRNAs). These siRNAs are then bound by Argonaute and other proteins to form a ribonucleic/protein complex called the RNA-induced silencing complex (RISC). Further processing occurs in which the RNA duplex is separated so that only one strand is incorporated into the complex; this strand becomes the guide strand which directs binding of the RISC to target mRNAs containing

sequences that are complementary to the guide strand (usually located within the 3' UTR). Total complementarity results in cleavage and subsequent degradation of the target mRNA while partial complementarity generally prevents translation from occurring.

This naturally occurring silencing process, thought to have originated as an anti-viral defence mechanism, is exploited by researchers to suppress gene expression in experimental systems. Introduction of exogenous shRNAs which mimic precursor miRNAs activates the RNAi/RISC pathway and enables gene expression to be down-regulated with a high degree of specificity. Unlike knock-out animals carrying null mutations where expression of a gene is abolished, the efficacy of RNAi-mediated gene silencing is variable and often only results in a partial reduction of gene expression (termed 'knock-down'). Though the reasons for this variability are not completely understood, it is recognised that RNAi knock-down efficiency is related to the sequence of the targeting siRNA and thus it is necessary to trial several siRNAs before selecting the ones which give satisfactory results. In accordance with this, several *Npas4*-specific shRNA expression vectors were tested for their ability to reduce Npas4 protein expression and subsequently the most suitable vectors were introduced into mES cells to create stable, clonally-derived Npas4 knock-down mES cell lines using lentiviral transduction.

4.1.2 Lentiviral transduction

Retroviruses are enveloped viruses that belong to the retroviridae family. The single-stranded RNA genome of retroviruses is reverse-transcribed into double-stranded DNA within the host cell and is subsequently integrated into the host cell genome by the integrase enzyme (Anson 2004). Lentiviruses are a subclass of the retroviridae family that are able to infect non-dividing cells. The ability of lentiviruses to permanently integrate large amounts of genetic material into eukaryotic cells has been exploited for both therapeutic applications, such as gene therapy (Federico 1999), and in the field of molecular biology for modification of a cell's genome. Genetic material of interest is packaged into virions that are capable of infecting eukaryotic cells and delivering the genetic material to the host cell chromosome but are incapable of producing more virus particles. This approach was used to introduce *Npas4* shRNA expression vectors into 46C mES cells (for details, see Section 2.2.9).



B

siRNA	Sequence	Target
Control	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT	CAACAAGATGAAGAGCACCAA
Npas4 #1	CCGGCGACAGTATCTACGATATCATCTCGAGATGATATCGTAGATACTGTCGTTTTT	CGACAGTATCTACGATATCAT
Npas4 #2	CCGGCGTTTCTGAAAGTGTCCTAATCTCGAGATTAGGACACTTTCAGAAACGTTTTT	CGTTTCTGAAAGTGTCCTAAT
Npas4 #3	CCGGCCTGGATCTTAAACCCTGGAACTCGAGTTCCAGGGTTTAAGATCCAGGTTTTT	CCTGGATCTTAAACCCTGGAA
Npas4 #4	CCGGCCTGATAATTTATTCCTGGAACTCGAGTTCCAGGAATAAATTATCAGGTTTTT	CCTGATAATTTATTCCTGGAA

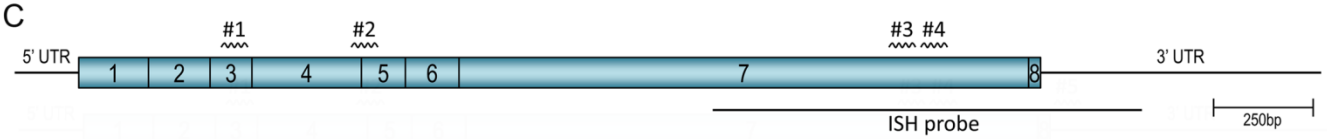


Figure 4.1 – *Npas4* shRNA constructs

(A) Schematic representation of the lentiviral constructs used to express shRNAs drawn to scale. Constructs differ only in the sequence coding for the shRNA which consists of a 21bp targeting sequence and a complementary antisense sequence separated by a short loop. LTR – Long terminal repeat; Ψ - Psi packaging element; RRE – Rev response element; U6 – U6 promoter; hPGK - Human phosphoglycerate kinase eukaryotic promoter; Pac - puromycin N-acetyl-transferase coding sequence. Primer binding sites are indicated by arrows (See Table 2.2). **(B)** Control and *Npas4* shRNA sequences and target sites. Complementary sense and antisense sequences which bind to form a hairpin structure are underlined. **(C)** Schematic representation of the mouse *Npas4* mRNA drawn to scale with the location of siRNA binding sites indicated above. Exons are numbered 1-8. UTR - Untranslated region. The region recognised by the mouse *Npas4 in situ* hybridisation (ISH) probe is also indicated (see Table 2.2 for primer sequences).

4.2 Materials and methods

4.2.1 *Npas4* and control shRNA vectors

The general structure of the shRNA vectors used in this study is shown in Figure 4.1A. Expression of the shRNAs was driven by the U6 promoter. The *U6* gene is transcribed by RNA polymerase III and encodes a non-protein-coding nuclear RNA that forms part of the spliceosomal complex involved in processing of eukaryotic pre-mRNAs (Kunkel *et al.* 1986). RNA polymerase III is a multisubunit transcriptional complex that synthesises a wide range of small, highly expressed, structural RNAs (Dieci *et al.* 2007) and for this reason the *U6* promoter is commonly used in the field of molecular biology in the expression of non-protein-coding RNAs. The shRNAs expressed by the vectors are 57 nucleotides in length and consist of a short 21 nucleotide sense sequence which is separated from a complementary antisense sequence of the same length by a short 6 nucleotide loop. The antisense component is the sequence which, after intracellular processing, will become the mature siRNA that recognises and binds to a complementary sequence located on the target mRNA. Immediately 3' of the antisense sequence is a run of five thymines which acts as a transcription termination signal for RNA polymerase III (Kunkel *et al.* 1986).

The shRNA vectors contained all the necessary *cis* elements required for viral transduction such as the Psi RNA packaging element (Ψ), the Rev response element (RRE) and the flanking long terminal repeats (LTRs) which define the integration fragment. During viral infection of a eukaryotic cell, the entire sequence between the 5'LTR and the 3'LTR is inserted into the host genome; the size of the integration fragment produced by these shRNA vectors was approximately 3,500bp. Also contained within the integration cassette was a selectable marker gene used to select for cells carrying the vector. Expression of *Pac*, which confers resistance to the antibiotic puromycin, was driven by the constitutively active human phosphoglycerate kinase (*hPGK*) eukaryotic promoter.

Four *Npas4* shRNA vectors, each targeting different regions of the *Npas4* mRNA, were tested. The vectors, numbered #1-#4, were identical, differing only in the hairpin sequence. Complete shRNA sequences are listed in Figure 4.1B. The locations of the siRNA binding sites on the *Npas4* mRNA are shown in Figure 4.1C with siRNA #1 binding most 5' and siRNA #4 binding most 3'. Additionally, two negative control vectors were tested in order to ensure that any effect on Npas4 expression is due to expression of the siRNAs rather than other confounding factors; (1) an empty vector which does not produce an RNA hairpin, and (2) a control siRNA vector which produces a 'non-targeting' siRNA that has at least five nucleotide mismatches to any known human or mouse mRNA and hence should not

recognise the *Npas4* mRNA. In every other respect, the control vectors were identical to the *Npas4* shRNA vectors. The empty vector acts as a control for the transfection process and for the introduction of foreign DNA into cells while the 'non-targeting' control shRNA vector, in addition to this, also controls for the effects of siRNA expression and activation of the RISC/RNAi pathway.

4.2.2 Npas4 expression vector

Transgenic expression of Npas4 in mammalian cells was achieved using a bicistronic DNA vector allowing constitutive expression of an epitope-tagged Npas4 protein together with an antibiotic resistance marker gene (Figure 4.2A). The vector contained the cDNA sequence coding for the mouse Npas4 protein in which the stop codon had been removed and replaced with two copies of the sequence coding for a Myc epitope placed in tandem. The Myc sequences were positioned in frame and were followed by a stop codon such that translation of the resulting mRNA would yield the mouse Npas4 protein fused to two C-terminal Myc tags. Additionally, an IRES was situated 3' of the stop codon and this was followed by the coding sequence of the *Pac* gene allowing both the Npas4 and Pac proteins to be synthesised concurrently from the same mRNA. Expression of the vector was driven by the constitutive human elongation factor-1 alpha (*EF1α*) promoter which regulates transcription of the *EEF1A1* gene *in vivo*. *EEF1A1* encodes the Elongation factor 1-alpha 1 protein which is involved in extension of the polypeptide chain during translation of mRNA (Browne and Proud 2002).

4.3 Results and discussion

4.3.1 Evaluation of *Npas4* shRNA vectors in HEK 293T cells

Before stable shRNA-expressing mES cell lines were created, all four shRNAs were tested for their ability to reduce the expression of recombinant, epitope-tagged Npas4 protein expressed in HEK 293T cells. The HEK 293T cell line was selected for these experiments as these cells do not express Npas4 protein endogenously (Figure 4.3B). To determine the effectiveness of each shRNA in reducing Npas4 protein expression, HEK 293T cells were transiently co-transfected with the Npas4 expression vector and each of the *Npas4* shRNA expression vectors individually (Figure 4.2B). In place of *Npas4* shRNA vectors, control cultures were transfected with either the empty vector or the control shRNA vector. The positive control group received only the Npas4 expression vector, while the negative control group consisted of untransfected HEK 293T cells. To ensure that transfection efficiency was equal between experimental groups, an equal amount of DNA was transfected each

time using the same transfection volume and incubation time (for details, see Section 2.2.6). Cell lysates were collected 24h after transfection after which the proteins were separated by SDS-PAGE and transferred to a membrane which was probed with an anti-Myc antibody to determine relative amounts of recombinant Npas4 expression. The same blots were also probed with an antibody directed against the constitutively expressed β-actin protein which served as a loading control.

No signal was detected with an anti-Myc antibody in untransfected cells, while a signal of approximately 100kDa was observed in lysates collected from cells transfected with the Npas4 expression vector alone (Figure 4.2B). This is the expected size of the recombinant Npas4 protein and is consistent with data published by others in which recombinant Npas4 expression was detected by immunoblot (Moser *et al.* 2004; Shamloo *et al.* 2006; Hester *et al.* 2007; Zhang *et al.* 2009; Ploski *et al.* 2011; Pruunsild *et al.* 2011). Together, these data strongly suggest that the signal detected using the anti-Myc antibody corresponds to the full-length Myc-tagged Npas4 protein. No other signals were observed indicating that the antibody is specific to the Myc epitope.

When compared to the positive control group, expression of Npas4 protein was reduced in all groups co-transfected with *Npas4* shRNA vectors, although to varying degrees (Figure 4.2B). Notably, siRNAs #1 and #3 were most effective and in these groups Npas4 protein expression was almost completely abolished. Knock-down was less effective in cells co-transfected with either shRNA #2 or #4, though Npas4 protein expression was still considerably diminished compared to the positive control. Co-transfection with the empty vector did not result in decreased Npas4 expression when compared to the positive control making it unlikely that reduced Npas4 expression seen in Npas4 shRNA groups was due to cell handling, the transfection process or the introduction of foreign DNA. In contrast, co-transfection with the control shRNA vector consistently resulted in a minor decrease in Npas4 expression when compared to the positive control or empty vector control.

The reason for this reduction in Npas4 expression in cells transfected with the control shRNA vector is not clear, however there are several possibilities. It is possible that activation of the RISC/RNAi pathway by any shRNA within the cell, regardless of sequence, in some way induces down-regulation of Npas4 protein either directly or indirectly, though this seems unlikely as this has not been reported by other groups using control siRNA vectors (Hester *et al.* 2007; Lin *et al.* 2008; Zhang *et al.* 2009). Alternatively, it is possible that although the control siRNA is not specific for the *Npas4* mRNA, it may inadvertently bind to regions of the *Npas4* transcript with low affinity resulting in partial

knock-down of Npas4 expression. An alignment of the *Npas4-Myc* cDNA sequence and the sequence targeted by the control siRNA was performed to determine the level of homology between these sequences and to identify potential target sites within the *Npas4* mRNA that could be bound by the control siRNA (Figure 4.2C). The sequence coding for the Myc peptide was included in the analysis in the event that the fusion of the *Npas4* and *Myc* coding sequences created a site that could be recognised by the control siRNA. The sequence targeted by the control siRNA was found to have moderate homology to a region of the *Npas4* cDNA sequence located between positions 1,870 and 1,890. In this region, a base-pair match was found in 12/21 nucleotide positions (four of which are G/C pairings) with a maximum run of seven consecutive base-pairs.

Thus, although there are more than five mismatches between the control siRNA and the *Npas4* cDNA sequence (as stipulated by the manufacturer), it may be possible that there is enough homology between the sequences to allow low affinity binding of the control siRNA to the *Npas4* mRNA resulting in partial knock-down of Npas4 expression. Employing a control shRNA of a different sequence may eliminate this problem and hence may be informative in distinguishing between 'off-target' effects (unintended knock-down of Npas4 expression due to sequence homology) and non-specific effects on Npas4 expression arising from activation of the RISC/RNAi pathway. Nevertheless, given that this control shRNA vector was shown to be an unsatisfactory control, it was not used in subsequent experiments and only the empty vector control was used.

4.3.2 Stable genomic integration of shRNA expression constructs in 46C mES cells

Once the ability of each siRNA to reduce Npas4 expression had been determined in HEK 293T cells, two *Npas4* shRNA vectors were selected to be used in lentiviral transduction of 46C mES cells (see Section 2.2.9). *Npas4* shRNA vectors #2 and #3 were selected as: (1) they target distinct areas of the *Npas4* transcript, and (2) the degree of Npas4 knock-down was not equal in these two cell lines which would enable detection of any Npas4 dose-dependent phenotypes. If a spectrum of Npas4 phenotypes exists, there may be a correlation between level of Npas4 knock-down and phenotype severity. In the same way, 46C mES cells carrying an empty vector were also generated.

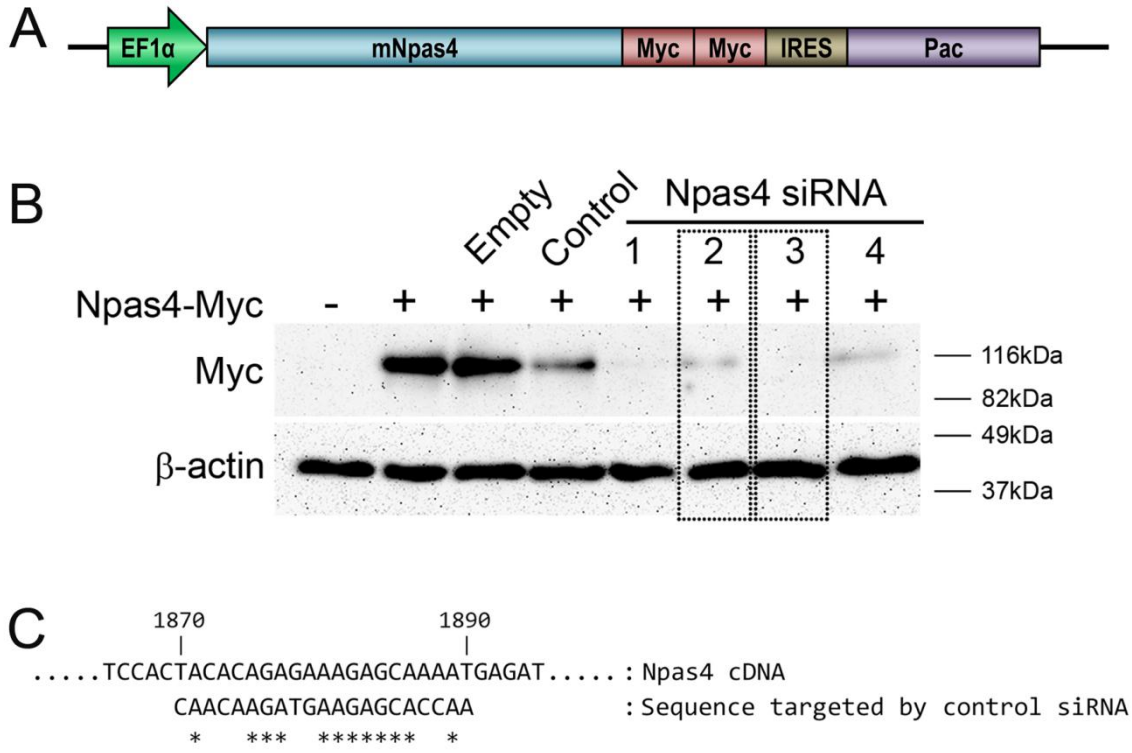


Figure 4.2 – Testing of shRNA constructs
(A) Schematic representation of the Npas4-Myc expression construct. EF1 α – elongation factor-1 alpha promoter; mNpas4 – mouse Npas4 cDNA sequence; Myc – c-Myc epitope sequence; IRES - Internal ribosome entry site; Pac - puromycin N-acetyl-transferase coding sequence. Not drawn to scale. **(B)** Each siRNA was tested for its ability to knock-down Npas4 protein expression by immunoblotting in HEK 293T cells (n=3). Cells were either untransfected or transiently transfected with a recombinant Myc-tagged mouse Npas4 expression construct either alone or together with an shRNA expression construct and lysates were harvested 24h after transfection. An anti-Myc antibody was used to assess Npas4 expression while β-actin served as a loading control. siRNA #2 and #3 were selected for further experiments. Empty - Empty vector control; Control - 'Non-targeting' control siRNA. **(C)** Alignment of the mouse *Npas4* cDNA sequence with the sequence targeted by the control siRNA. *Npas4* cDNA postitions are numbered. Identity is indicated by an asterisk.

The 46C mES cell line (see Section 3.1.1.2.1) was chosen as the host cell line to facilitate detection of possible phenotypes affecting NPC production or *Sox1* expression. Although the 46C mES cell line does already inherently carry the *Pac* gene within its genome, it is under the control of the endogenous *Sox1* promoter and therefore is not actively expressed in undifferentiated mES cells. Thus, undifferentiated mES cells carrying the shRNA vectors could be selected owing to their resistance to puromycin. As the mES lines were clonally derived, when cultured they consisted of a homogenous population; each cell contained the same number of viral insertions in the same chromosomal locations and therefore expression of the transgenic vector was equal in all cells. The mES cell clone expressing *Npas4* siRNA #3 was the first clone to be isolated and thus will hereafter be referred to as the Knock-down 1 (KD1) cell line, while similarly the mES cell line expressing *Npas4* siRNA #2 will be referred to as Knock-down 2 (KD2) cell line (see Appendix 2).

To verify that the cells selected using puromycin did indeed carry the shRNA expression contructs and that they were stably integrated into the host genome, gDNA was isolated from each of the puromycin-resistant mES cell lines after ≥10 passages and used as a template in a PCR using primers directed against a segment of DNA unique to the integration fragment. The location of the primer binding sites is shown in Figure 4.1A. The segment of DNA amplified by these primers is 542bp in length and, as it is common to all of the lentiviral vectors, it is expected that the size of the PCR product will be identical in each of the gDNA samples containing an insert.

An amplicon of approximately 542bp was detected in gDNA samples from all three transduced cell lines (Empty, KD1 and KD2) confirming that the viral vector was stably integrated in the genome of these cells (Figure 4.3A). No signal was detected in the negative control sample where gDNA template was substituted with water. Likewise, no signal was observed in gDNA isolated from the untransduced parental 46C ES cell line which suggests that the PCR product observed in samples isolated from transduced cells is not due to non-specific amplification of an endogenous sequence present in the mouse genome. To ensure that intact gDNA had been successfully isolated from each of the cell lines, the same gDNA samples were subjected to a second PCR in which primers to *β-actin* gene were used. Using these primers, a signal was observed in all mES cell gDNA samples, but not in the negative control, indicating that the lack of signal seen in the 46C sample was not due a lack of template gDNA.

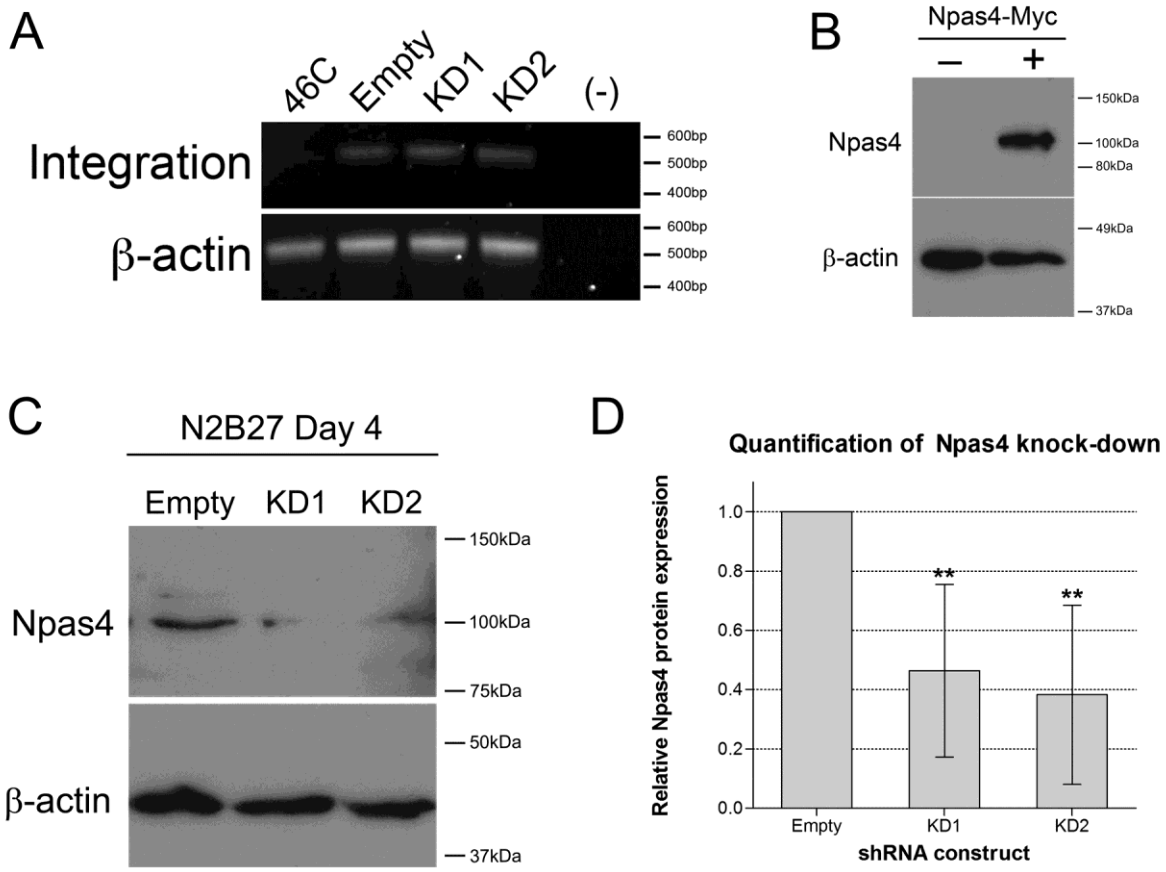


Figure 4.3 – Knock-down of endogenous Npas4 protein during neural differentiation of mES cells **(A)** Integration of shRNA constructs into the mES cell genome was verified by PCR using primers specific to the viral integration region (see Figure 4.1A for primer locations; see Table 2.2 for primer sequences). gDNA was isolated from mES cell lines that were virally transduced with shRNA expression constructs and also from the untransduced 46C parental mES cell line. Primers to housekeeping gene *β-actin* were used as a positive control for gDNA isolation **(B)** Validation of anti-Npas4 antibody specificity in HEK 293T cells. Cells were either untransfected (-) or transfected (+) with a recombinant Myc tagged mouse Npas4 expression construct and lysates were collected 24h after transfection. Blots were probed with an anti-Npas4 antibody and β-actin served as a loading control. **(C)** Knock-down of endogenous Npas4 protein in differentiated mES cells (n=3). Lysates were harvested after four days of differentiation in N2B27 medium and blots were probed with an anti-Npas4 antibody. β-actin served as a loading control. **(D)** Knock-down of Npas4 protein in differentiating mES cells was quantified by densitometry analysis of western blot exposures using the image analysis software ImageJ. Npas4 protein expression in each sample was normalised to β-actin expression and is relative to Npas4 expression in the Empty vector control line which was given an arbitrary value of 1. Mean values and standard deviations of at least four independent experiments (n≥4) are shown. ** $P < 0.01$.

4.3.3 Knock-down of endogenous Npas4 protein during neural differentiation of mES cells

Immunoblotting was used to assess relative amounts of Npas4 protein expression in each of the transduced mES cell lines (Figure 4.3C). As *Npas4* is not expressed in undifferentiated mES cells (Figure 3.1), to verify knock-down of endogenous Npas4 protein it was necessary to differentiate mES cell lines until they reach a stage when Npas4 is expressed. Therefore, whole cell lysates were collected from mES cells that had been differentiated in N2B27 medium for four days and the samples were analysed for Npas4 expression. A signal of approximately 100kDa was detected in the protein sample obtained from the Empty mES cell line using the anti-Npas4 antibody. The intensity of this signal was significantly reduced in samples obtained from both the KD1 and KD2 mES cell lines. This suggests that expression of the endogenous Npas4 protein is reduced in the *Npas4* shRNA mES cell lines compared to the Empty mES cell line during N2B27 differentiation. Interestingly, the lower MW Npas4 immunoreactive species (see Section 3.3.3) was not observed after only four days of differentiation, indicating that perhaps it only becomes expressed from Day 6 onwards in this differentiation system.

To quantify the efficiency of Npas4 knock-down in each mES cell line, relative densitometry analysis was used to compare the level of Npas4 protein expression in the Empty mES cell line to that of the Npas4 KD mES cell lines (Figure 4.3D). When analysed using a one-way analysis of variance (ANOVA), the level of Npas4 knock-down in each of the Npas4 KD mES cell lines was statistically significant ($P < 0.01$). Interestingly, though *Npas4* shRNA #3 was more effective at reducing Npas4 protein expression than *Npas4* shRNA #2 in transiently transfected HEK 293T cells (Figure 4.2B), the converse was observed in stable mES cell lines carrying these constructs; it was found that the reduction in Npas4 expression was more pronounced in the KD2 mES cell line (62% reduction versus Empty vector control) than in the KD1 mES cell line (54% reduction versus Empty vector control). It is possible that this apparent inconsistency can be explained by the unpredictable effect that genomic integration has on transgene expression. The degree to which a transgenic construct is expressed in a host cell depends on several factors including: (1) the number of genomic integration events, and (2) the location of integration sites (i.e. whether the construct is inserted into a transcriptionally active region of euchromatin or a silenced region of heterochromatin). Therefore, although *Npas4* shRNA #2 was less effective at reducing Npas4 protein expression in transfected HEK 293T cells, the superior knock-down seen in the KD2 mES cell line may be indicative of enhanced shRNA expression in this cell line due to either a greater number of integration events and/or integration at a more

transcriptionally favourable site. Nevertheless, in both Npas4 KD mES cell lines the difference in Npas4 protein expression was highly statistically significant when compared to Empty mES cell line.

To verify the specificity of the antibody and to ensure that the signal observed corresponded to the Npas4 protein, the anti-Npas4 antibody was tested in HEK 293T cells expressing recombinant Npas4 protein (Figure 4.3B). A signal of approximately 100kDa was observed in HEK 293T cells transfected with the Npas4 expression vector but was absent from untransfected cells. It is to be expected that the MW of the recombinant Npas4 protein is slightly greater than that of the endogenous protein due to the presence of a double Myc tag. This strongly suggests that the antibody is specific for the Npas4 protein and is able to recognise both recombinant and endogenous Npas4 protein.

4.3.4 Assessment of pluripotent phenotype of transduced mES cells

The ability of lentiviruses to integrate DNA into the genome of the host cell raises the possibility of insertional mutagenesis or oncogene activation via disruption of key protein coding and/or regulatory sequences. If the inserted fragment disrupts the expression of genes that are critical to normal mES cell biology, this may lead to spurious phenotypes not related to knock-down of Npas4 expression. Therefore, to ensure that the mES cells had not been adversely affected by the process of viral transduction in a way which may affect interpretation of results, several aspects of the normal pluripotent mES phenotype were compared between transduced mES cell lines and the untransduced parental 46C mES cell line. In addition, comparison of the Empty vector mES cell line with the Npas4 KD mES cell lines was used to investigate whether expression of *Npas4*-specific shRNAs has an affect on the normal growth of mES cells.

First, the morphology of the transduced mES cell lines was examined. When cultured in the presence of LIF *in vitro*, undifferentiated mES cells form colonies with a distinctive amorphous morphology. This characteristic morphology is lost upon differentiation into other cell types making it an easily identifiable feature of the mES pluripotent phenotype. Therefore, to determine whether mES cell morphology had been affected by the lentiviral transduction process, the morphology of mES cell colonies from different cell lines was compared over many passages. No difference in morphology was observed between either: (1) the 46C parental mES cell line and the transduced mES cell lines, or (2) the Empty mES cell line and the Npas4 KD mES cell lines (Figure 4.4A). This indicates that neither lentiviral transduction nor expression of *Npas4* shRNAs affected the gross morphology of mES cell colonies.

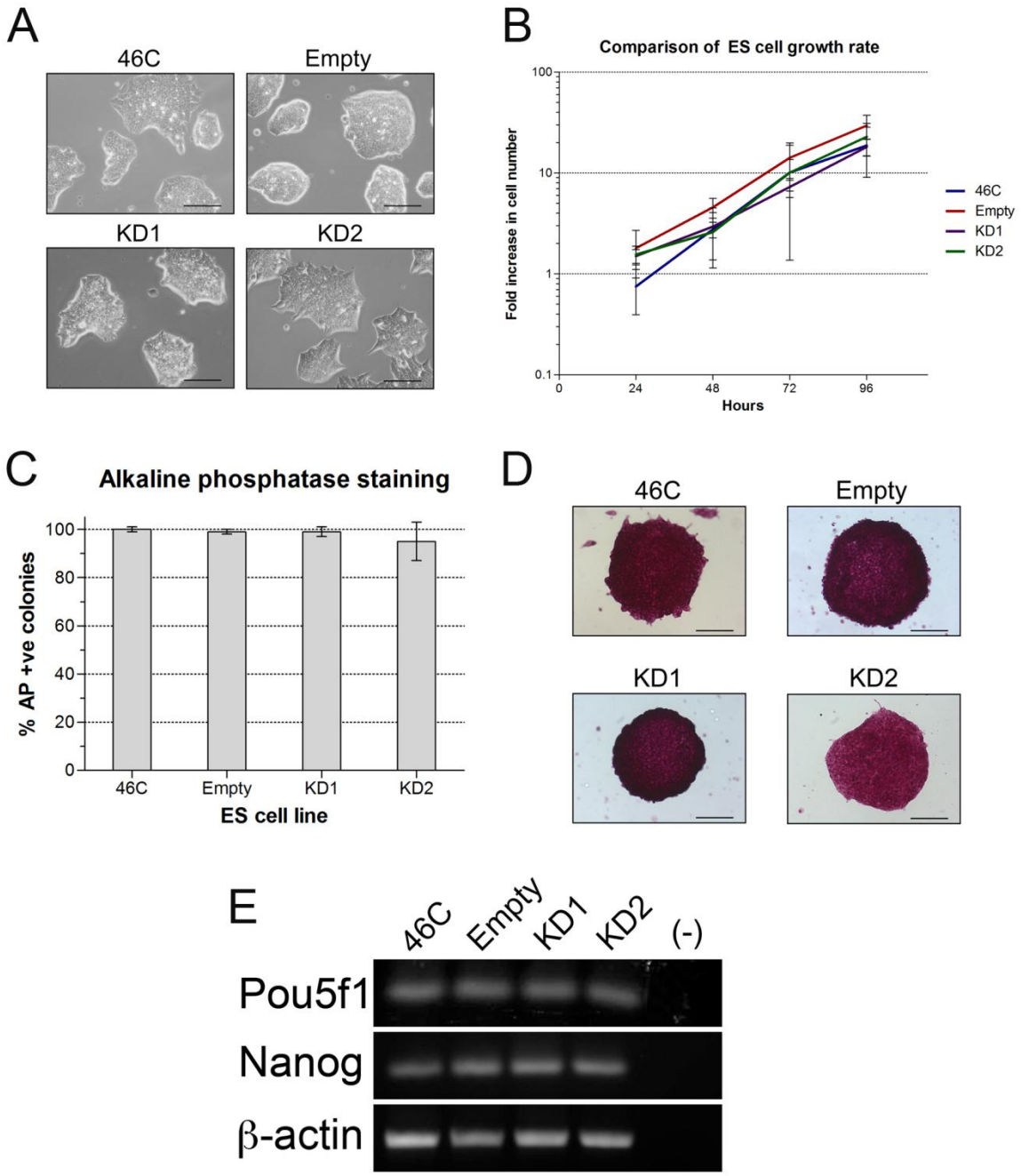


Figure 4.4 – Assessment of pluripotent phenotype of transduced mES cell lines
(A) Comparison of the morphology of undifferentiated shRNA mES cell lines and the parental 46C mES cell line. Scale bar = 100µm. **(B)** Comparison of growth rate between shRNA mES cell lines and the parental 46C mES cell line. $9x10^3$ cells/cm^2 were plated on Day 0 and total viable cells were counted at 24h intervals using the trypan blue vital stain. Means and standard deviations of four independent experiments are shown (n=4). **(C)** Percentage of undifferentiated mES cell colonies expressing alkaline phosphatase (AP) in each cell line as determined by an AP detection assay, shown in (D). Means and standard deviations of three independent experiments are shown (n=3). **(D)** Representative images of Fast Red Violet stained colonies from each mES cell line. Scale bar = 100µm. **(E)** Expression of pluripotency genes in mES cell lines. RT-PCR was used to determine mRNA expression of pluripotency genes *Pou5f1* and *Nanog* in each mES cell line (n=3).

Another important feature of pluripotent mES cells is their ability to self-renew. Under normal growth conditions, undifferentiated mES cells divide at a constant rate with a doubling time of approximately 12-24h depending on the cell line (Udy *et al.* 1997). Mutations that disrupt the function of genes involved in cell cycle control are likely to alter the proliferative state of the mES cells and, as such, would become manifest as changes in growth rate (i.e. hyperplasia or arrested division). When the growth rate of different mES cell lines was compared over four days of growth, there was no statistically significant difference between: (1) the 46C parental mES cell line and any of the virally transduced mES cell lines, or (2) the Empty mES cell line and the Npas4 KD mES cell lines (Figure 4.4B). This indicates that neither lentiviral transduction nor expression of *Npas4* shRNAs affected the normal growth rate of undifferentiated mES cells.

Finally, transduced mES cell lines were analysed for the expression of several key pluripotency genes. Undifferentiated mES cells can be identified by their unique gene expression profile, or 'molecular fingerprint'; in addition to having high levels of alkaline phosphatase activity, they are also characterised by the expression of classic pluripotency marker genes, such as *Pou5f1* and *Nanog* (Palmqvist *et al.* 2005). The *Pou5f1* gene encodes a protein called Oct4 which promotes self-renewal of undifferentiated ES cells (Nichols *et al.* 1998) and, similarly, *Nanog* also encodes a transcription factor that is important in sustaining pluripotency (Chambers *et al.* 2003). These two genes, known as the 'master regulators' of pluripotency, work in concert to maintain mES cells in a pluripotent state and differentiation of mES cells into other cell types is accompanied by the down-regulation of these genes.

The level of alkaline phosphatase activity in transduced mES cell lines was compared to that of the 46C parental mES cell line using Fast Red Violet staining (Figure 4.4C). Representative images of alkaline phosphatase positive colonies from each mES cell line are shown in Figure 4.4D. In order for a mES cell population to be considered pluripotent, the accepted standard is for greater than 95% of colonies to be positive for alkaline phosphatase and this was the case for all mES cell lines tested. No significant difference in alkaline phosphatase activity was observed between: (1) the 46C parental mES cell line and any of the virally transduced mES cell lines, or (2) the Empty mES cell line and the Npas4 KD mES cell lines. RT-PCR was used to confirm the expression of the pluripotency markers *Pou5f1* and *Nanog* in undifferentiated mES cell lines (Figure 4.4E). Both *Pou5f1* and *Nanog* mRNAs were expressed by all mES cell lines tested. No signal was detected in the negative control reaction in

which cDNA template was substituted for water. RT-PCR using primers directed to the *β-actin* gene was performed using the same cDNA as a loading control.

These data indicate that the transduced mES cell lines retained the expression of a number of key pluripotency marker genes in the undifferentiated state. By extension, this suggests that the differentiation potential of mES cells was unaltered by either lentiviral transduction or the expression of shRNAs that target the *Npas4* mRNA. Nevertheless, the ultimate indicator of pluripotency is the ability of a cell population to give rise to cells of all three germ lineages; ectoderm, mesoderm and endoderm. Injection of pluripotent cells into nude mice results in the formation of teratomas and therefore this could be used to definitively demonstrate the pluripotency of transduced mES cells.

4.4 Summary

Lentiviral delivery was used to introduce *Npas4* shRNA expression constructs into 46C mES cells. The constructs were stably integrated into the mES cell genome and two clonally derived mES cell lines expressing different *Npas4* shRNAs were generated. In the same way, 46C mES cells carrying an empty vector were also generated. Immunoblotting confirmed that, in comparison to the Empty vector control mES cell line, expression of endogenous Npas4 protein was significantly reduced in the *Npas4* shRNA mES cell lines upon neural differentiation in N2B27 medium. Likewise, it was also demonstrated that the efficiency of Npas4 knock-down was slightly greater in the KD2 mES cell line than in the KD1 mES cell line. In the undifferentiated state, transduced mES cell lines were found to maintain characteristics of mES cells such as morphology and growth rate. They also retained expression of several key pluripotency marker genes; namely *Pou5f1*, *Nanog* and alkaline phosphatase. While none of these methods conclusively demonstrates adherence to a normal mES cell phenotype in isolation, when taken together, these data strongly suggest that the normal growth and differentiation potential of mES cells was not adversely affected by viral transduction and genomic integration of introduced DNA. Furthermore, although the siRNAs directed against the *Npas4* transcript are expressed constitutively under the *U6* promoter, these data indicate that expression of these siRNAs does not interfere with normal mES cell biology, which is to be expected given that *Npas4* is not expressed by undifferentiated mES cells (see Chapter 3). By extension, it can be concluded that *Npas4* is dispensable for normal mES cell growth and pluripotency.

5 – The effect of reduced Npas4 expression during neural differentiation of mES cells

5.1 Introduction

In order to determine whether reduced Npas4 expression affects neural differentiation of mES cells, the Npas4 knock-down ES cell lines generated previously (see Chapter 4) were compared to the Empty vector control ES cell line in their ability to undergo neural differentiation in N2B27 medium. Given the expression pattern of *Npas4* during neural differentiation and early embryonic development, Npas4 KD ES cell lines were analysed for changes in a number of key aspects likely to be affected by reduced Npas4 expression, namely formation of NPCs and, consequently, neuronal differentiation.

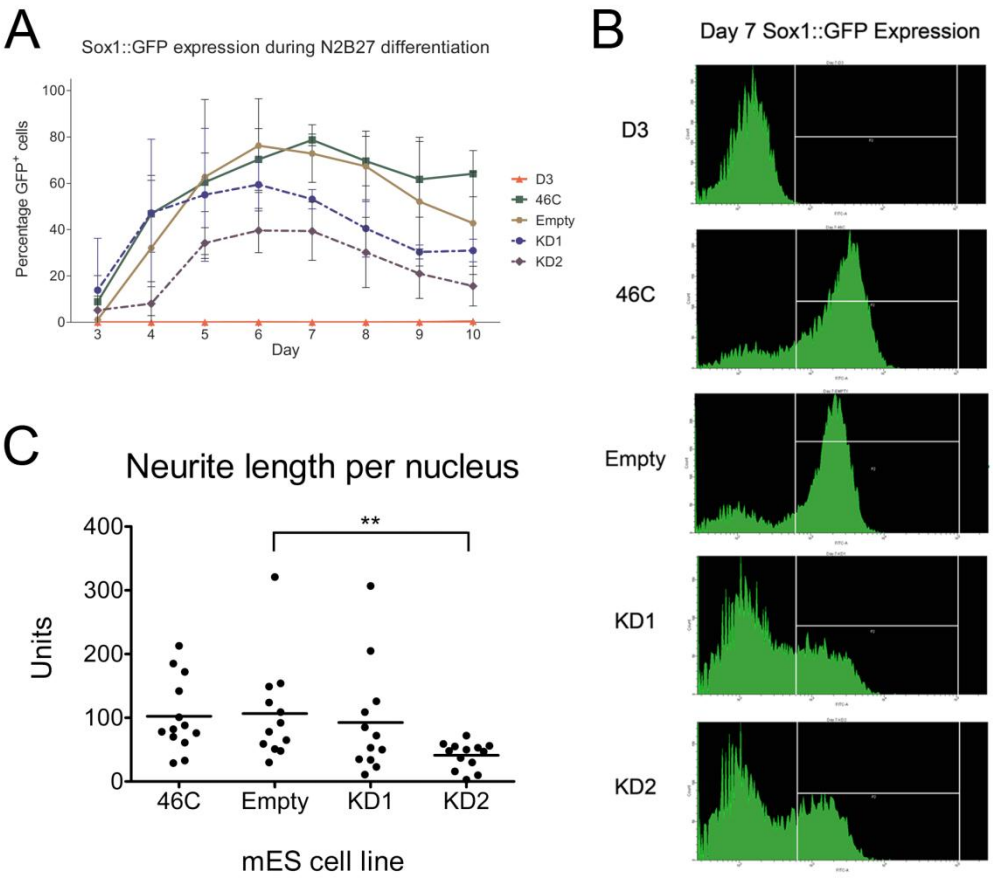
5.2 Results

5.2.1 Analysis of NPC formation in Npas4 knock-down cultures

5.2.1.1 *Sox1* expression

To investigate the effect of reduced Npas4 expression on neural differentiation of mES cells, the formation of NPCs was assessed in both Npas4 KD and Empty vector control mES cell lines. The 46C mES cell line (see Section 3.1.1.2.1) was used as the background for the experimental mES cell lines to facilitate analysis of NPC formation; transgenic expression of GFP in $Sox1^+$ NPCs derived from these cells means that neural conversion can be readily monitored via GFP fluorescence. Flow cytometry was used to evaluate the occurrence of $Sox1^+$ NPCs over the course of the mES cell neural differentiation in N2B27 medium (Figure 5.1A). Beginning on Day 3, when *Sox1* expression first becomes evident, cells were analysed at 24h intervals for a period of seven days with 10,000 events being counted at each time point. As a negative control, the wildtype D3 mES cell line (Doetschman *et al.* 1985) was also differentiated under conditions identical to those used for the other mES cell lines. As the D3 mES cell line does not carry the gene coding for GFP in its genome, any fluorescence observed in these cells line would represent background auto-fluorescence. Therefore, the threshold for positive GFP fluorescence was set such that 99% of D3 cells were excluded and only cells whose fluorescence intensity was greater than this level were scored as GFP^+.

Figure 5.1 – The effect of reduced Npas4 expression during neural differentiation of mES cells
(A) Temporal analysis of Sox1::GFP expression during N2B27 differentiation of mES cells. Npas4 KD and control mES cell lines were differentiated in N2B27 medium and flow cytometry was used to assess Sox1::GFP expression at 24h intervals over a period of seven days beginning at Day 3. At each time point cells were trypsinised and $1x10^4$ cells were counted to determine the percentage of GFP^+ cells. Each point represents the mean of at least three independent experiments (n≥3) with error bars showing standard deviations. **(B)** Sox1::GFP expression at Day 7 of neural differentiation. Representative fluorescence histograms are shown for each mES cell line. The y-axis shows the number of cells counted while the x-axis shows GFP fluorescence intensity. The gate used to count GFP^+ cells is shown. **(C)** Comparison of average neurite length per cell of mES cell derived neuronal cultures after ten days of differentiation in N2B27 medium. For each field, the the total neurite length was measured and divided by the number of nuclei in the field to give an estimate of the averge neurite length per cell. Each dot represents the average neurite length per nucleus for one field while the bars show the mean of three independent experiments (n=3). When analysed using an unpaired t test, there was no statistically significant difference in the mean neurite length per nucleus between 46C and Empty vector control cultures (P = 0.8752) or Empty vector control and KD1 cultures (P = 0.6782). There was, however, a statistically significant difference between Empty vector control and KD2 cultures (** P < 0.01). **(D)** Immunocytochemical analysis of Nestin expression during neural differentiation. Representative images of cultures after four days of differentiation in N2B27 medium are shown for each mES cell line (n≥4). An antibody specific to the Nestin protein was used to detect Nestin expression (red) and cells were counterstained with DAPI to visualise nuclei (blue). Scale bar = 100µm. **(E)** Immunocytochemical analysis of βIII tubulin expression after ten days of neural differentiation in N2B27 medium. Representative images of mES cell derived neuronal cultures are shown for each mES cell line. An antibody specific to the βIII tubulin protein was used to detect βIII tubulin expression (green) and cells were counterstained with DAPI to visualise nuclei (blue). Scale bar = 100µm.


A
Sox1::GFP expression during N2B27 differentiation
Percentage GFP+ cells
Day
D3
46C
Empty
KD1
KD2
B
Day 7 Sox1::GFP Expression
D3
46C
Empty
KD1
KD2
C
Neurite length per nucleus
Units
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46C
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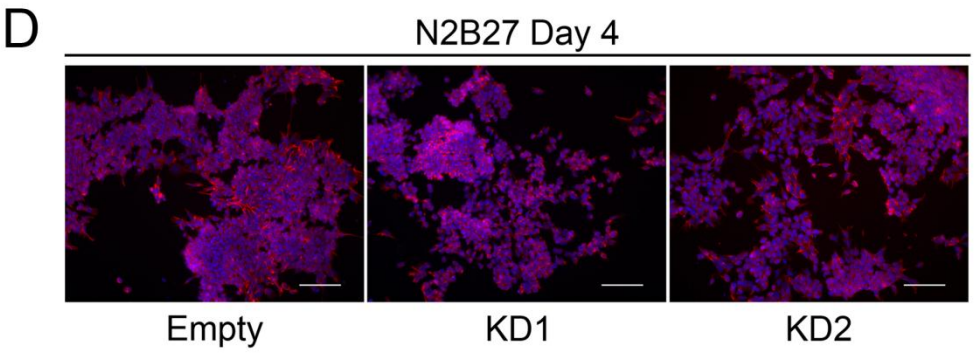
KD2
mES cell line


D
N2B27 Day 4
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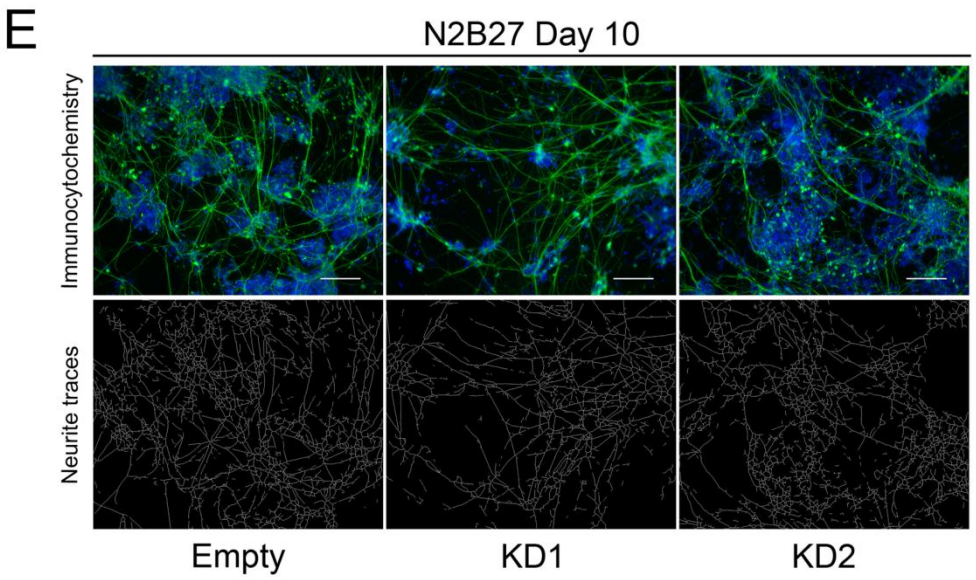
E
N2B27 Day 10
Immunocytochemistry
Neurite traces
Empty
KD1
KD2

The Sox1::GFP expression profile of the Empty vector control mES cell line was comparable to that of the 46C parental mES cell line and no statistically significant difference was detected when the two cell lines were compared using a two-way ANOVA. In both cell lines, the proportion of GFP^+ cells steadily increased between Days 3-5 reaching a peak of approximately 80% GFP^+ cells at around Day 6-7 before gradually declining once more. Analysis of Npas4 KD mES cell cultures revealed that the Sox1::GFP expression profiles for these cell lines were similar in shape to those of the control mES cell lines; however, the maximum percentage of GFP^+ cells attained was considerably lower. The highest percentage of GFP^+ cells recorded in KD1 cultures was 60% (Day 6), while in KD2 cultures this figure was only 40% (Days 6-7). Over the course of the differentiation period, the difference in the percentage of GFP^+ cells generated between the Empty vector control mES cell line and both the Npas4 KD1 and KD2 mES cell lines was statistically significant when tested using a two-way ANOVA ($P < 0.0001$). Representative histogram plots of GFP fluorescence intensity recorded on Day 7 are shown in Figure 5.1B. These data suggest that reduced Npas4 expression during neural differentiation of mES cells results in a decrease in the proportion of cells that express *Sox1*.

5.2.1.2 Nestin expression

To further characterise the effect of reduced Npas4 expression on formation of NPCs, expression of another NPC marker gene, *Nestin*, was investigated. The *Nestin* gene encodes an intermediate filament protein whose expression is specific to CNS progenitor cells (Lendahl *et al.* 1990), however, Nestin is expressed at a later stage of differentiation than Sox1 (Tanaka *et al.* 2004) and thus $Nestin^+$ cells represent a more mature type of NPC. After four days of differentiation in N2B27 medium, cells were fixed and analysed for Nestin expression using immunocytochemistry. Representative images of Nestin expression at Day 4 are shown for each mES cell line in Figure 5.1D. The percentage of cells expressing Nestin was counted manually and compared between mES cell lines. In the Empty vector control mES cell line, approximately 87% of cells were $Nestin^+$ after four days of differentiation and there was no statistically significant difference between the Empty vector control and the 46C parental mES cell line when analysed using an unpaired t test (Appendix 3). The percentage of $Nestin^+$ cells observed in Npas4 KD cultures was slightly lower; as was the case with Sox1 expression, the reduction in Nestin expression was more pronounced in the KD2 mES cell line (66% $Nestin^+$ cells) than in KD1 cultures (77% $Nestin^+$ cells), however, when compared to the Empty vector control mES cell line using an unpaired t test, neither of these differences were statistically significant (Appendix 3). These data suggest that reduced Npas4 expression during neural differentiation of mES cells does not significantly affect expression of Nestin at Day 4.

5.2.2 Analysis of neurite sprouting in Npas4 knock-down cultures

Given that the proportion of cells expressing Sox1 was diminished in Npas4 KD cultures, neurite sprouting was investigated in order to ascertain whether neuronal differentiation was also affected by reduced expression of Npas4 as a direct consequence of there being fewer $Sox1^+$ NPCs. The extent of neurite sprouting in Npas4 KD and Empty vector control mES cell lines was analysed using immunocytochemical techniques. After 10 days of differentiation in N2B27 medium, cells were fixed and immunocytochemistry was used to analyse the expression of Class III β-tubulin (βIII tubulin), a specialised type of microtubule protein whose expression is specific to immature neurons (Easter *et al.* 1993; Liu *et al.* 2007). For each mES cell line, randomly selected fields were photographed and the automated digital image analysis programme NeuriteTracer (Pool *et al.* 2008) was used to measure the extent of neurite sprouting (see Section 2.2.2). Figure 5.1E shows representative images of βIII tubulin expression at Day 10 for each mES cell line (top panel) and the corresponding neurite traces generated by the image analysis software (bottom panel).

Two aspects of neurite sprouting were investigated: (1) the overall extent of neurite sprouting in the culture as a whole (measured by calculating the mean neurite length per field), and (2) the extent of neurite sprouting in individual neurons that comprise the network (measured by calculating the mean neurite length per cell). In both cases, the Empty vector control mES cell line was comparable to the 46C parental mES cell line and there was no statistically significant difference between these two groups when analysed using an unpaired t test (Figure 5.1C; Appendix 4). When the overall extent of neurite sprouting in the Npas4 KD cultures was compared to the Empty vector control mES cell line, a slight decrease in mean absolute neurite length per field was observed in each of the Npas4 KD mES cell lines. Consistent with previous results, the effect was more severe in the KD2 line (78% of Empty vector control) than in the KD1 line (89% of Empty vector control), however, neither of these differences were statistically significant when analysed using an unpaired t test (Appendix 4). Similarly, when the degree of neurite sprouting was measured at the level of individual cells, a decrease in mean neurite length per cell was observed in both the KD1 and KD2 mES cell lines (Figure 5.1C). Once again, the reduction was more pronounced in the KD2 line (39% of Empty vector control) than in the KD1 line (87% of Empty vector control). While this difference was statistically significant in the case of the Npas4 KD2 mES cell line ($P < 0.01$), the difference did not reach statistical significance for the Npas4 KD1 mES cell line when analysed using an unpaired t test ($P = 0.6782$). These data suggest that although reduced Npas4 expression did not significantly affect

the total extent of neurite sprouting during neural differentiation of mES cells, reduction of Npas4 expression below a putative threshold results in a decrease in the mean neurite length per cell.

5.3 Discussion

5.3.1 Validity of experimental mES cell lines

There were no statistically significant differences between the Empty vector control mES cell line and the 46C parental mES cell line in any of the parameters that were analysed, namely the percentage of $Sox1^+$ cells formed, the percentage of $Nestin^+$ cells at Day 4, the overall extent of neurite sprouting at Day 10 or the mean neurite length per cell at Day 10. In conjunction with other data (see Section 4.3.4), this further demonstrates the similarity between the two cell lines and suggests that the Empty vector control mES cell line was not adversely affected by the lentiviral transduction process. Thus, given that making comparisons to the Empty vector control mES cell line is equivalent to comparing to wildtype mES cells, these results validate the Empty vector control mES cell line as a suitable control.

Similarly, the same phenotypes were consistently observed in both of the Npas4 KD mES cell lines, though the phenotypes observed in the KD2 cell line were consistently more severe than those of the KD1 cell line (see Table 5.1). This may be due to the increased efficacy of Npas4 knock-down in the KD2 mES cell line (Figure 4.3D) which would indicate that the phenotype of Npas4 knock-down is dose-dependent. Nevertheless, that the same phenotypes were reproduced in two indepent Npas4 KD mES cell lines expressing different shRNAs suggests that these phenotypes were indeed due to decreased expression of Npas4 and not non-specific 'off-target' effects.

mES cell line	Npas4 protein expression^ (Day 4)	% $Sox1^+$ cells (maximum)	% $Nestin^+$ cells (Day 4)	Overall neurite sprouting^ (Day 10)	Mean neurite length/cell^ (Day 10)
Empty	100%	80%	87%	100%	100%
KD1	46%**	60%****	77%	89%	87%
KD2	38%**	40%****	66%	78%	39%*
$Npas4^{-/-}$	0%	?	?	?	?

Table 5.1 – Summary of Npas4 knock-down phenotypes
Comparison of mES cell lines and the phenotypes observed during neural differentiation. Statistical significance is represented by asterisks; * $0.01 \leq P < 0.05$, ** $0.001 \leq P < 0.01$, *** $0.0001 \leq P < 0.001$, **** $P \leq 0.0001$. ^Denotes relative values given as a percentage of the Empty vector control mES cell line value. All other values are absolute.

5.3.2 The effect of reduced Npas4 expression on formation of NPCs

In comparison to the Empty vector control mES cell line, fewer Sox1$^+$ cells were present in Npas4 KD cultures which suggests that knock-down of Npas4 expression results in a decrease in the proportion of Sox1$^+$ cells generated during neural differentiation. The severity of the phenotype was related to the level of Npas4 knock-down with a more marked reduction in Sox1$^+$ cells observed in the KD2 cell line than in the KD1 cell line. Gates for GFP fluorescence were positioned in such a way as to exclude the negative control D3 mES cell line which does not carry a GFP reporter cassette, thus eliminating the possibility that the fluorescence that was measured was due to background auto-fluorescence.

There are several possible explanations that could account for the reduced percentage of Sox1$^+$ cells observed in Npas4 KD cultures (summarised in Figure 5.2). If the total number of cells is unchanged, then a reduction in the proportion of cells expressing Sox1 could signify either; (1) that fewer NPCs are present in Npas4 knockdown cultures (which may be due to decreased survival or proliferation of NPCs), or (2) that the number of NPCs is unchanged and it is only the expression of Sox1 in NPCs which has been reduced. To better discriminate between these two possibilities, cultures were analysed for expression of another NPC marker gene, *Nestin*. While both Sox1 and Nestin are expressed by NPCs, Nestin is downstream of Sox1 and thus Nestin$^+$ cells represent a more mature type of NPC. In the ventricular zone of the mouse embryonic spinal cord, expression of Nestin in NPCs is directly regulated by the SoxB1 transcription factors (Tanaka *et al.* 2004) and, accordingly, these proteins are expressed sequentially in the developing CNS; Sox1 expression begins at E7.5 (Pevny *et al.* 1998) while expression of *Nestin* is not detected until E7.75 (Dahlstrand *et al.* 1995). From this point onwards, both proteins are co-expressed in CNS NPCs until the cells exit the cell cycle following the final round of mitosis at which time both proteins are down-regulated (Lendahl *et al.* 1990; Pevny and Placzek 2005). Thus, given that the majority of NPCs express both Sox1 and Nestin, one might expect that if knock-down of Npas4 expression during neural differentiation of mES cells resulted in a decrease in the number of NPCs produced, then the expression both Sox1 and Nestin would be affected. Alternatively, if knock-down of Npas4 expression did not affect NPC formation but merely caused a reduction in Sox1 expresion in NPCs, then it is possible that the expression of Nestin would be unaffected.

Indeed, while there was a significant decrease in the percentage of Sox1$^+$ cells generated in Npas4 KD mES cell lines, the decrease in the percentage of Nestin$^+$ cells at Day 4 was only modest. Once again, the reduction was more substantial in the Npas4 KD2 mES cell line, though the difference did not

reach statistical significance in either cell line. While these data seem to suggest that the formation of NPCs was not affected in Npas4 KD mES cell lines, it should be noted that the expression of Nestin was only investigated at a single time point during the differentiation period and, as such, it is possible that these experiments did not uncover the full extent of the reduction in Nestin$^+$ cells. For example, after four days of differentiation, there is only a minor difference in the percentage of Sox1$^+$ cells observed between control and Npas4 KD cultures, however this difference becomes more pronounced as differentiation proceeds. In the same way, perhaps a greater difference in the percentage of Nestin$^+$ cells would also be observed at a later stage of differentiation had those time points been investigated. Further temporal analysis of Nestin expression during N2B27 differentiation is needed before the possibility that formation of Nestin$^+$ NPCs is compromised in Npas4 KD cultures can be excluded. Alternatively, it is possible that NPC formation per se is not affected by knock-down of Npas4 expression, but rather that there is merely a reduction in the level of Sox1 expression in NPCs. If so, this would suggest that Npas4 acts upstream of Sox1 in the transcriptional programme during neural differentiation and that in wildtype cells Npas4 positively regulates Sox1 expression. Given that expression of Npas4 and Sox1 was found to be mutually exclusive during neural differentiation of mES cells (Figure 3.2C-D), it is unlikely that Npas4 directly activates transcription of *Sox1* in this context, but rather that Npas4 may promote expression of *Sox1* via an intermediate transcription factor.

If knock-down of Npas4 selectively reduces expression of Sox1, then Nestin expression may be only mildly affected due to functional redundancy between SoxB1 members. Sox1, Sox2 and Sox3 have all been shown to activate the *Nestin* enhancer (Tanaka *et al.* 2004) and thus other SoxB1 proteins may compensate for the reduced expression of Sox1 in the Npas4 KD mES cell lines. The functional redundancy that exists between SoxB1 members is particularly evident in targeted deletion studies in which a single SoxB1 gene is disrupted; in regions of the CNS where multiple SoxB1 proteins are expressed, only mild phenotypes are observed in single SoxB1 mutants while in areas of unique expression more severe phenotypes are seen (Guth and Wegner 2008). For instance, even in mice completely lacking Sox1 expression, no difference in Nestin expression was observed in the cortex at E14.5 when compared to wildtype mice which the authors speculated may be due to compensation by other SoxB1 proteins (Elkouris *et al.* 2011). Furthermore, there is a distinct population of NPCs in which Nestin expression is not regulated by the SoxB1 group proteins at all (Tanaka *et al.* 2004) and thus a reduction of Sox1 expression would not interfere with Nestin expression in this population.

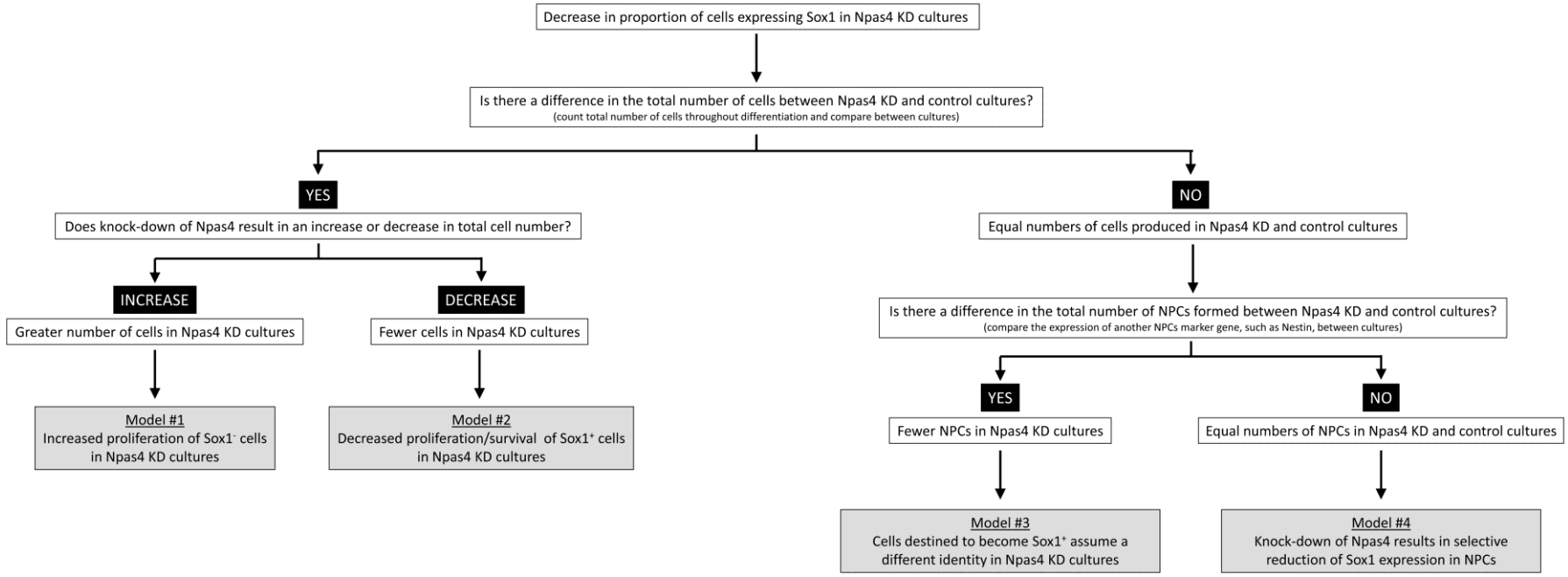

Figure 5.2 – Flowchart outlining the possible mechanisms responsible for the reduction in the percentage of Sox1$^+$ cells seen during neural differentiation of Npas4 KD cultures

Analysis of the possible underlying causes of decreased Sox1 expression yields four models which could explain the Npas4 knock-down phenotype. The available data suggest that Model #4 may be the most likely (see text).

Drawing on all of the available evidence, it is possible to construct a hypothetical model which describes the interplay between Npas4, Sox1 and Nestin during neural differentiation of mES cells (Figure 5.3). During differentiation of wildtype mES cells, Npas4 is expressed by a population of early NPCs (possibly PAN cells) where it activates transcriptional cascades that culminate in the expression of Sox1 in a more advanced population of Npas4^{-} NPCs. In concert with Sox2 and Sox3, Sox1 then induces and maintains expression of Nestin in late NPCs. In Npas4 knock-down cultures, reduced Npas4 expression in PAN cells results in a corresponding decrease in Sox1 expression in early NPCs; however, Nestin expression in late NPCs is unaffected due to the functional redundancy exhibited by SoxB1 members.

Alternatively, it is possible that the level of Npas4 knock-down was not sufficient to produce a transcriptionally relevant change in the expression of downstream target genes that would result in a statistically significant decrease in Nestin expression. The results of this study suggest that the dosage of Npas4 expression may be an important factor in determining the phenotype; several Npas4 KD mES cell lines of varying effectiveness were generated and in each of the aspects examined, the severitiy of the phenotype was commensurate with the level of knock-down. Unlike targeted genomic disruption, RNAi results in only partial knock-down leaving a low level of basal Npas4 expression which may result in a mild phenotype that is difficult to detect. Even the most effective Npas4 knock-down mES cell line (KD2), expression of the Npas4 protein was approximately 38% of that found in the Empty vector control mES cell line and therefore it is possible that differentiation of mES cells derived from Npas4$^{-/-}$ mice may accentuate subtle phenotypes and possibly expose other phenotypes that are not detectable in Npas4 KD mES cell lines (Table 5.1).

5.3.3 The effect of reduced Npas4 expression on neuronal maturation

When the extent of neurite sprouting at Day 10 was compared between Npas4 knock-down and control mES cell lines, a small decrease in the overall neurite length was observed in Npas4 KD cultures; as with other phenotypes, the reduction was more substantial in the Npas4 KD2 mES cell line, though the difference did not reach statistical significance in either cell line. When the mean neurite length per cell was considered, a decrease was seen in both Npas4 KD mES cell lines, though the difference was only statistically significant in the KD2 mES cell line.

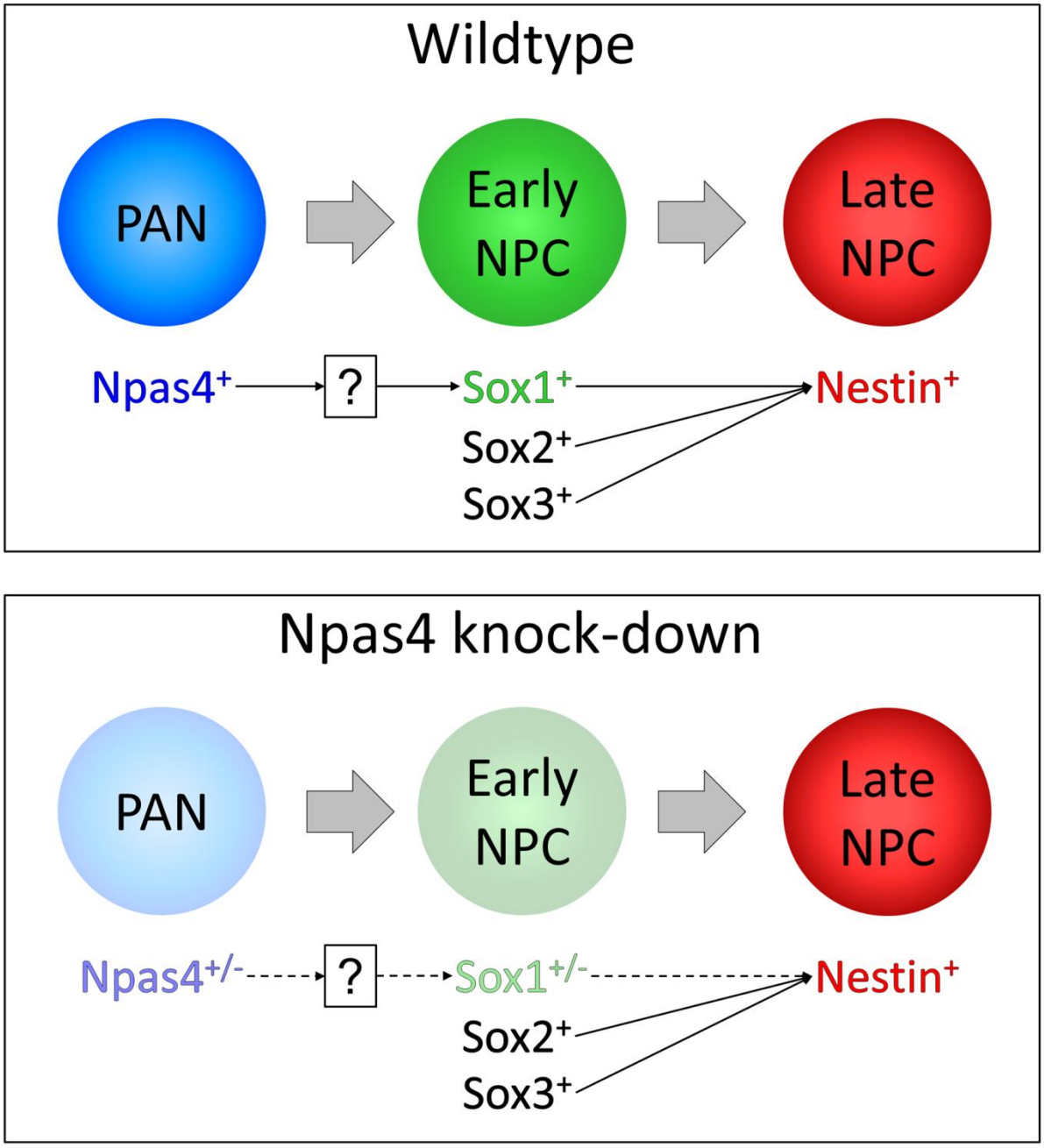

Figure 5.3 – Proposed model of the interplay between Npas4, Sox1 and Nestin during neural differentiation of mES cells

During neural differentiation of wildtype mES cells, Npas4 is expressed by a population of early neural progenitor cells (NPCs), possibly primitive anterior neuroectoderm (PAN) cells, where it activates transcriptional cascades that culminate in the expression of Sox1 in a more advanced population of Npas4$^-$ NPCs. In concert with Sox2 and Sox3, Sox1 then induces and maintains expression of Nestin in late NPCs. In Npas4 knock-down cultures, reduced Npas4 expression in PAN cells results in a corresponding decrease in Sox1 expression in early NPCs; however, Nestin expression in late NPCs is unaffected due to the functional redundancy exhibited by SoxB1 members.

The reason that the reduction in mean neurite length per cell was significant in only one Npas4 KD mES cell line and not the other may be due to small differences in population composition that become amplified as differentiation proceeds. For instance, both the Npas4 KD mES cell lines showed a slight reduction in the number of Nestin$^+$ cells observed at Day 4, though the reduction was more pronounced in the Npas4 KD2 mES cell line. If every Nestin$^+$ NPC gives rise to several βIII tubulin$^+$ progeny as a result of cell division, then a small difference in the number of Nestin$^+$ cells would become magnified when the population of βIII tubulin$^+$ neurons is considered. Thus, while the decrease in the number of Nestin$^+$ NPCs formed was not significant for either Npas4 KD mES cell line, it is possible that the more severe reduction in Nestin$^+$ cells in the KD2 mES cell line translated into a statistically significant difference in βIII tubulin$^+$ neurons while the more modest reduction in Nestin$^+$ cells in the KD1 mES cell line did not. Again, this is consistent with the KD2 mES cell line having a more severe phenotype and suggests that the Npas4 phenotype is dose-dependent.

When interpreting the significance of the Npas4 KD2 phenotype, it should also be noted that because DAPI is a non-selective DNA-binding molecule that labels all nuclei, the measurements of neurite length per nucleus do not represent the mean neurite length per neuron, but instead represent the mean neurite length per cell (namely both neurons and glia). Thus, the apparent decrease in neurite length may, in fact, be due to an alteration in the ration of neurons:glia produced during differentiation. Accordingly, there are several possible explanations for this observation (illustrated in Figure 5.4 and Figure 5.5):

(1) Model #1: Reduced Npas4 expression results in decreased neuronal survival and, thus, an increase in the proportion of glial cells present in Npas4 KD cultures

(2) Model #2: Reduced Npas4 expression skews neural differentiation of mES cells to glial cell types

(3) Model #3: Reduced Npas4 expression results in impaired neurite sprouting/neuronal maturation

Of these three possible models, Model #2 seems the most favourable as it in keeping with available data. An increase in the number of glial cells produced satisfies the observation that mean neurite length per cell is reduced in Npas4 KD cultures despite there being no change in the overall neurite length. In addition, this model suggests that expression of Npas4 in NPCs during the early stages of differentiation (rather than in neurons themselves) can influence the mean neurite length per cell which is consistent with the early expression of Npas4 seen in this differentiation system. Nevertheless, more research is needed to understand the role of Npas4 in neurogenesis.

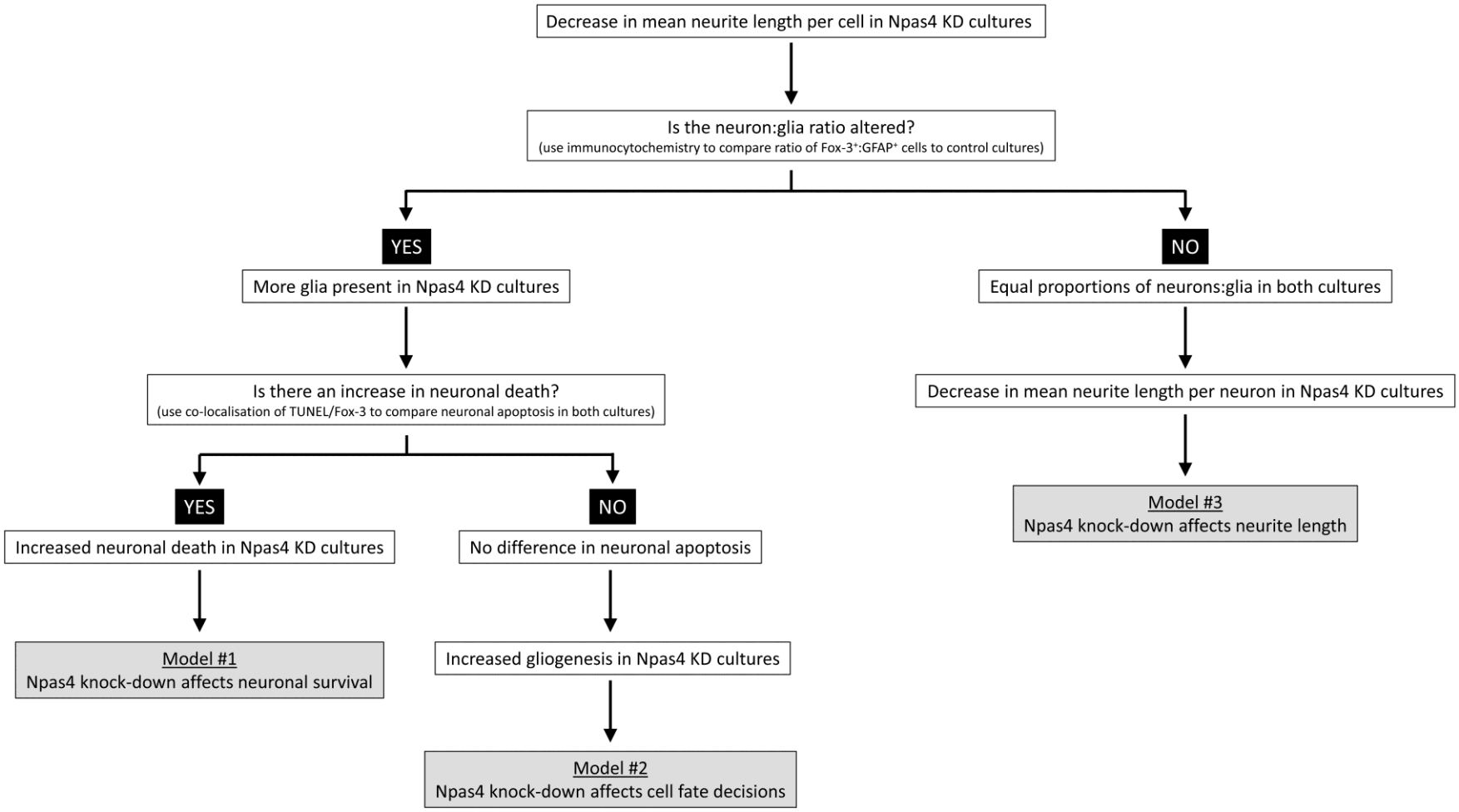

Figure 5.4 – Flowchart outlining the possible mechanisms underlying the decrease in mean neurite length per cell seen during neural differentiation of Npas4 KD cultures

Analysis of the possible causes responsible for a decrease in neurite length yields three models which could explain the Npas4 knock-down phenotype. The three models are illustrated schematically in Figure 5.5.

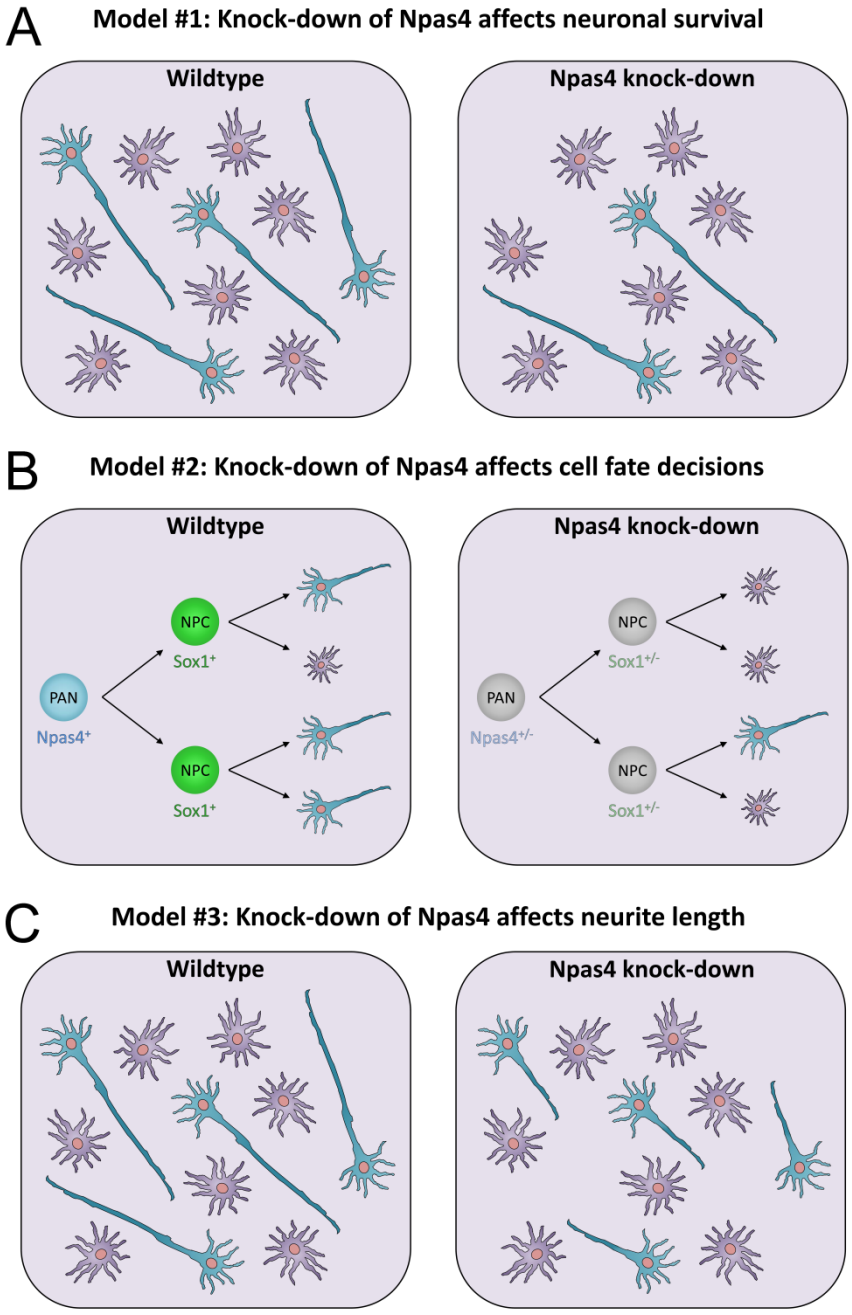

Figure 5.5 – Three possible models that explain the decrease in mean neurite length per cell seen during neural differentiation of Npas4 knock-down cultures
(A) Model #1 – In this model, neither the ratio of neurons:glia produced nor the neurite length is affected by knock-down of Npas4; however, neuronal survival is compromised leading to a decrease in the percentage of neurons in culture. **(B)** Model #2 – In this model, reduced expression of Npas4 alters the differentiation potential of NPCs such that differentiation is skewed in favour of glial cells. This results in an increase in the proportion of glial cells in culture while neuronal survival and neurite outgrowth are unaffected. **(C)** Model #3 – In this model, there is no difference between Npas4 knock-down and wildtype cultures in the ratio of neurons:glia produced; however, decreased Npas4 expression affects neuronal maturation resulting in a reduction in neurite length. PAN - primitive anterior neuroectoderm cell; NPC - neural progenitor cell.

5.4 Summary

The Npas4 KD and Empty vector control mES cell lines were assessed for their ability to undergo neural differentiation in N2B27 medium. The Empty vector control mES cell line was found to be equivalent to 46C parental mES cell line in every aspect examined, thus confirming it as a suitable negative control. Knock-down of Npas4 expression affected several aspects of neural differentiation and these phenotypes were consistently reproduced in both Npas4 KD mES cell lines thereby confirming the specificity of the knock-down effect. Notably, the KD2 mES cell line consistently yielded a more severe phenotype than the KD1 line which is likely due to the increased efficacy of Npas4 knock-down in KD2 line. Firstly, the proportion of cells expressing Sox1 during differentiation was significantly lower in both Npas4 KD cultures than in the Empty vector control mES cell line. In contrast, no significant difference in Nestin expression was observed at Day 4. Together, these data suggest that knock-down of Npas4 expression does not impact on the formation of NPCs but instead results in a selective reduction in Sox1 expression in early NPC populations. Consequently, it can be deduced from these data that Npas4 acts upstream of Sox1 in the transcriptional programme and, furthermore, that Npas4 may indirectly regulate Sox1 expression via an intermediate transcription factor.

A second independent phenotype was also observed in Npas4 KD cultures relating to the extent of neurite sprouting after ten days of differentiation in N2B27 medium. Although there was no significant difference between mES cell lines in the overall neurite length when cultures were analysed as a whole, there was a significant reduction in the mean neurite length per cell in the KD2 cell line. A minor reduction in the mean neurite length per cell was also seen in the KD1 mES cell line, though this difference was not statistically significant. Nevertheless, these data suggest that knock-down of Npas4 may affect either neuronal maturation or the ratio of neurons:glia produced during neural differentiation of mES cells.

6 – Summary and conclusions

6.1 Summary of results

6.1.1 *Npas4* expression in a developmental context

In this study, two experimental paradigms were used to investigate the expression of the bHLH PAS transcription factor Npas4 during the early stages of development. In the first, differentiation of ES cells was used as an *in vitro* model of early embryogenesis and the expression of *Npas4* was investigated during neural differentiation of both human and murine ES cells. These studies were complemented by direct analysis of *Npas4* expression *in vivo* in the developing mouse embryo.

It was found that *Npas4* is not expressed by pluripotent mES cells but is transiently expressed during the early stages of neural differentiation. This was supported by work performed in hES cells where a similar finding was observed suggesting that this is not a species-specific phenomenon, but is conserved across mammalian species. Both the timing and transient nature of *Npas4* expression during neural differentiation suggest that *Npas4* expression may occur in a population of early NPCs; however, the identity of the *Npas4*$^{+}$ population has yet to be definitively determined. Nevertheless, it was found that Npas4 was not co-expressed with Sox1 thus excluding this population of NPCs.

In the developing mouse embryo, *Npas4* mRNA expression was detected by RT-PCR between E7.5-9.5 thus validating the findings obtained in ES cells *in vitro* and providing the first direct evidence for *Npas4* expression during development. Due to the limitations of this technique, spatial information regarding Npas4 expression in the embryo is still lacking. Thus, further investigation of *Npas4* expression during embryogenesis is required including a more detailed temporal analysis to precisely identify the period during which Npas4 is expressed in conjunction with techniques which allow for visualisation of *Npas4* expression in the embryo.

6.1.2 Function of Npas4 in development

The function of Npas4 in the context of development was investigated using a loss-of-function approach in which Npas4 expression was reduced in mES cells undergoing neural differentiation. Lentiviral transduction was employed to create two independent stable Npas4 knock-down mES cell

lines expressing *Npas4*-specific siRNAs as well as an Empty vector control mES cell line which was used to control for off-target effects.

Knock-down of Npas4 expression affected two aspects of neural differentiation; the expression of the neuroectoderm marker *Sox1* and neuritogenesis. When compared to the Empty vector control mES cell line, fewer $Sox1^+$ cells were observed in Npas4 KD cultures. Despite this, the percentage of cells expressing Nestin was not significantly affected by Npas4 knock-down. This may be somewhat surprising given that expression of Nestin is directly regulated by Sox1 (Tanaka *et al.* 2004), however it is possible that functional compensation by other SoxB1 proteins minimises the effect on Nestin expression (see Figure 5.3) which would suggest knock-down of Npas4 expression results in a selective decrease in Sox1 expression but does not affect the number of NPCs formed. When the effect of reduced Npas4 expression on neurite sprouting was examined, no difference was found between Npas4 KD and Empty vector control mES cell cultures in the total neurite length, however, at the level of individual cells, a decrease in mean neurite length per cell was observed in Npas4 KD cultures.

That similar phenotypes were observed in both Npas4 knockdown mES cell lines validates these results and suggests that the effect is specific to reduced Npas4 expression and not due to 'off-target' effects. Furthermore, it is unlikely that the phenotypes were caused by non-specific integration effects; extensive characterisation of transduced mES cell lines showed that they were not fundamentally altered by the lentiviral transduction process as they retained expression of several key pluripotency genes and maintained a normal ES cell morphology and growth rate.

6.2 Discussion

6.2.1 Developmental expression of *Npas4*

The work described here provides the first detailed account of *Npas4* expression in the context of development. Until now, there had been little evidence to suggest that *Npas4* might be expressed in the embryo either in the literature (see Section 1.3.5) or from other sources. One such resource is the Allen Brain Atlas (http://www.brain-map.org/), which is a publicly available database containing ISH gene expression maps for a large number of genes that are expressed in the brain (Jones *et al.* 2009). While this database does contain a catalogue of *Npas4* expression maps at various stages of ontogeny, no *Npas4* mRNA expression was detected at any of the embryonic stages that were

investigated (E11.5, E13.5, E15.5 and E18.5). Given that in the present study *Npas4* expression was detected in the embryo between E7.5-E9.5 using RT-PCR, this may signify that *Npas4* is only transiently expressed during development and is down-regulated by E11.5 or, alternatively, it may be a reflection of the sensitivity of the respective techniques used to assess *Npas4* expression. The former seems likely based on the transient nature of *Npas4* expression seen in two different *in vitro* models of mammalian development (neural differentiation of both mouse and human ES cells).

The precise timing of *Npas4* expression in the mouse embryo, particularly in relation to other genes, is a matter which requires further investigation as the data obtained from *in vitro* experiments differ somewhat to the observations of *Npas4* expression *in vivo*. The data concerning the expression of *Npas4* during neural differentiation of mES cells suggest that *Npas4* is expressed prior to *Sox1*, while the observation that *Npas4* is first expressed in the mouse embryo at some time between E7.5-E9.5 suggests that *Npas4* is expressed parallel to, if not after, *Sox1* which is first expressed at E7.5. The reason for this apparent discrepancy is unclear, however, it is important to recognise that while *in vitro* differentiation of ES cells generally provides a reliable model of the early stages of embryogenesis, there is not always a direct correlation between the events occurring in the embryo and those occurring in the culture dish. For this reason, more data are required concerning the temporal and spatial expression of *Npas4* during embryogenesis. Nevertheless, adhering to the current hypothesis that Noggin stimulates developmental expression of *Npas4* via Ca^{2+} influx (see Section 3.3.4), one would therefore expect Npas4 to be expressed shortly after the emergence of Noggin expression which occurs at E7.5. Thus, the *in vivo* data (Figure 3.4) are in keeping with this hypothesis.

6.2.2 Identity of the *Npas4*$^+$ cell population during development

Although it was demonstrated in this study that *Npas4* is expressed during the early stages of development, the identity of the *Npas4*$^+$ population was not definitively characterised. As discussed in Section 3.3.2, the data suggest that the *Npas4*-expressing cells are neither neurons nor pluripotent cell types, but are likely to be an early NPC type. Experiments using neural differentiation of hES cells indicate that the *NPAS4*$^+$ cells may correspond to PAN cells, while similar work in a mES cell model suggests that *Npas4* may be expressed prior to *Sox1*, a gene considered to be the first definitive marker of committed neuroectoderm cells (see Section 3.1.1.2). Together, these data raise the possibility that the *Npas4*$^+$ population represents an early NPC cell type, one that perhaps occurs

before *Sox1* becomes expressed. If these cells are indeed committed to a neural fate, then we may hypothesise Npas4 to be a marker of the earliest CNS cells to be formed during development.

This is a plausible hypothesis given the expression of *Npas4* seen in developmental models *in vitro* and what is known about *Npas4* expression in response to stimuli (see Section 1.4.1). *Npas4* is an IEG whose expression can be rapidly up-regulated in response to stimuli and thus is it perfectly placed to link cellular changes, such as Ca^{2+} signalling, to changes in gene expression. Using these data, it is possible to put forward a hypothesis regarding the expression and function of *Npas4* during development; in the early embryo, secretion of Noggin by the notochord triggers Ca^{2+} influx in overlying cells of the neural plate (the prospective neuroectoderm) via activation of Ca^{2+} channels which leads to induction of *Npas4* in these cells. *Npas4* expression then regulates transcription of target genes involved in CNS development, such as *Sox1*. In this way, *Npas4* could act as a direct link between neural induction signalling (i.e. Noggin-induced Ca^{2+} influx) and neurodevelopmental transcriptional programmes (illustrated in Figure 3.5C).

As Npas4 is expressed at an early stage of differention, there is also the possibility that the *Npas4*$^{+}$ cell population is not yet committed to a neural fate but instead represents a late pluripotent cell population that is distinct to undifferentiated ES cells, such as the early primitive ectoderm-like (EPL) cells that have been described by others (Rathjen *et al.* 1999). While this seems unlikely given that EPL cells correspond to a cell population that is found in the embryo between E5.5-6 and *Npas4* expression was not detected in the embryo at this time (Figure 3.4), the possibility cannot be discounted. Analysis of *Npas4* expression during differentiation of ES cells to other germ lineages (i.e. mesoderm or endoderm) may reveal whether induction of *Npas4* is specific to neural differentiation or is a hallmark of all kinds of ES cell differentiation. Interestingly, one of the miRNAs that was identified as a putative regulator of *Npas4* expression (see Section 1.4.9) was miR-203, a miRNA that is involved in epidermal differentiation of hES cells. It is thought that miR-203, which is highly up-regulated during the early stages of differentiation, promotes differentiation of hES cells into keratinocytes by preventing neural differentition (Nissan *et al.* 2011). If *Npas4* is indeed a bonafide target of mir-203, then it is possible to speculate that miR-203 may inhibit neural differentitation via down-regulation of *Npas4* expression.

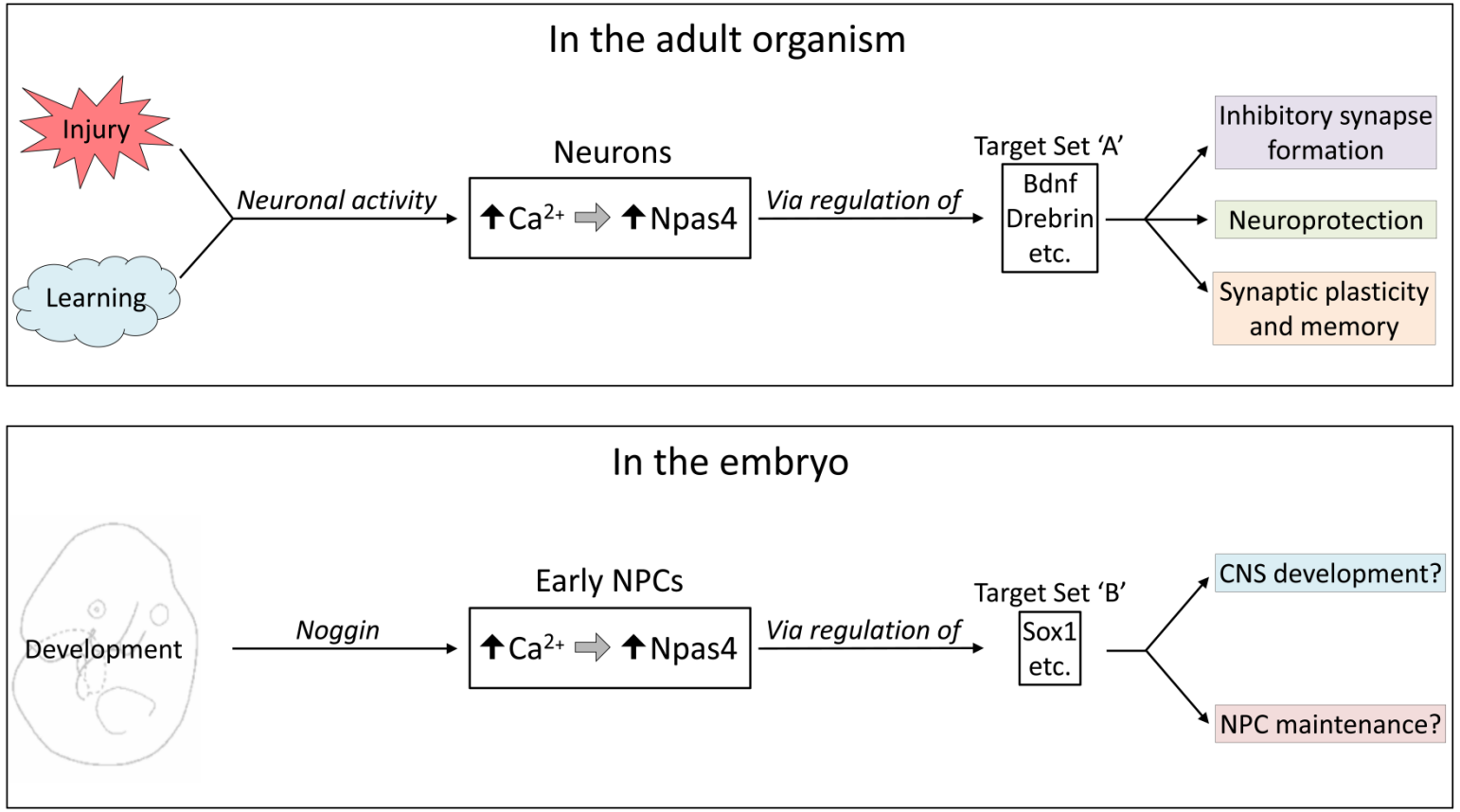

Figure 6.1 – Proposed model for the dual function of *Npas4*

In the adult brain, *Npas4* is up-regulated in neurons in response to an activity-dependent increase in nuclear Ca^{2+} which can occur either as a result of excitotoxic injury or normal neurophysiological processes such as learning. Once activated, Npas4 participates in diverse aspects of neurobiology including inhibitory synapse formation, neuroprotection and synaptic plasticity via regulation of target genes such as *Bdnf* and *Drebrin*. In the developing embryo, Noggin-induced Ca^{2+} signalling stimulates up-regulation of *Npas4* in a population of early neural progenitor cells (NPCs) where it plays a role in CNS development. The genes regulated by Npas4 during development may differ to those regulated by Npas4 in neurons and may include the NPC maintenance gene *Sox1*.

6.2.3 Developmental role for Npas4

While several roles have been described for Npas4 in the adult brain (see Section 1.6), the possible function of Npas4 during early development has not been explored. The work described here provides the first evidence which suggests that Npas4 may have a developmental role that is unrelated to its function in the adult organism (see Section 3.3.5). Such a proposition is not unreasonable when one considers that other bHLH PAS transcription factors also have context-dependent functions throughout ontogeny (see Table 1.1). The Ahr, for example, is differentially expressed throughout ontogeny and is involved in both developmental and adult physiology. In the adult rat, expression of the Ahr is found primarily in the lung, kidney, liver and thymus with lower levels of expression seen in the heart and spleen (Carver *et al.* 1994), while in the mouse embryo expression has been detected in the heart, liver, somites, neuroepithelium, branchial arches, adrenal glands, ectoderm, bone and muscle (Abbott *et al.* 1995). Aside from the well characterised role in mediating the response to xenobiotic compounds that has been described in the adult (Mimura and Fujii-Kuriyama 2003), the Ahr has also been implicated in embryonic development. Mice homozygous for an Ahr null allele have decreased body weight and small livers (Fernandez-Salguero *et al.* 1995; Schmidt *et al.* 1996) which is suggestive of developmental defects caused by lack of Ahr expression. Therefore, it is conceivable that, like other bHLH PAS factors, Npas4 may also have dual functions; one in the embryo in the context of development and another in the adult brain.

Although no developmental phenotype has been reported in either of the Npas4$^{-/-}$ mouse lines generated (Lin *et al.* 2008; Ooe *et al.* 2009b), this does not discount the possibility that Npas4 plays an accessory role in developmental processes. There are numerous examples of genes that have roles in important developmental processes but do not show a profound developmental phenotype when deleted, particularly when there may be compensation by other genes having similar functions. For instance, the largely overlapping expression patterns and partial functional redundancy of SoxB1 proteins means that targeted disruption of individual SoxB1 genes produces only a mild phenotype restricted to the areas of unique expression. Thus, although Sox1 is specifically expressed in the neural plate during early CNS development where it is involved in maintenance of NPCs (see Section 3.1.1.2), Sox1$^{-/-}$ mice are viable, are born in the expected Mendelian ratios and show no signs of developmental abnormalities (Nishiguchi *et al.* 1998).

The absence of a reported Npas4$^{-/-}$ developmental phenotype is not evidence of its absence. Occasionally, subtle phenotypes can be overlooked when mutant mice are first analysed, as was the case when the first Gfap$^{-/-}$ mice were generated. Initial reports declared that Gfap$^{-/-}$ mice develop normally and that no gross neurological, behavioural or structural CNS abnormalities were detected from which the authors concluded that Gfap is not essential for the morphogenesis of the CNS (Gomi *et al.* 1995; Pekny *et al.* 1995; Shibuki *et al.* 1996). Contrary to this, it was later discovered that Gfap$^{-/-}$ mice develop late-onset myelination defects and have several phenotypes associated with abnormal astrocyte structure and function including increased permeability of the blood-brain barrier and hydrocephalus associated with white matter loss which was present in 50% of mice (Liedtke *et al.* 1996).

The data gathered from *in vitro* models of development in the present study are consistent with the notion that developmental phenotypes associated with disruption of Npas4 function may be subtle; reduced Npas4 expression during neural differentiation of mES cells was seen to have an effect on an early Sox1^{+} NPC population, though this was corrected at a later stage of differentiation in a more mature Nestin^{+} NPC population (see Figure 5.3). These obervations suggest that decreased Npas4 expression may affect one part of neural differentiation in isolation without having downstream consequences. In a whole animal, this may mean that normal developmental processes are not profoundly disrupted by loss of Npas4 expression.

Significantly, work published by Lin and collegues has alluded to the possibility that compensatory pathways may be activated in Npas4$^{-/-}$ mice. While no major differences in mIPSC frequency were observed in organotypic slices prepared from either wildtype or Npas4$^{-/-}$ mice, when Npas4 expression was acutely disrupted (either using RNAi in wildtype mice or Cre-mediated recombination in Npas4$^{flx/flx}$ mice) a marked difference in mIPSC frequency was detected (Lin *et al.* 2008). This suggests that compensatory pathways may become activated in the absence of Npas4 expression which could potentially mask the full extent of the Npas4$^{-/-}$ phenotype in the whole animal. Furthermore, these observations raise the prospect that additional Npas4-related phenotypes may be identified in conditional Npas4$^{flx/flx}$ mice by inactivating Npas4 function at a stage of development when compensatory pathways would normally already have been activated in Npas4$^{-/-}$ mice. For this reason, the simplified nature of *in vitro* models may provide an ideal system for investigating phenotypes associated with altered Npas4 expression as individual pathways can be dissected

without the complexity of a whole organism. Often this reductionist approach allows subtle changes to be uncovered which would otherwise be masked by other factors *in vivo*.

As discussed in Section 3.3.5, the function of Npas4 during development may be quite different to that seen in neurons of the adult brain, particularly if the embryonic expression of Npas4 occurs in a progenitor cell type as the data suggest. From this perspective, the observation that *Npas4* is also expressed in the adult testes (see Section 1.3.1) is interesting as a high proportion of the cell types found within the testes are progenitor cells, such as the spermatogonia which give rise to gametes through the process of spermatogenesis (Yoshida 2010). Currently, it is not known which cell types in the testes express *Npas4*. While the testes are highly innervated by neurons projecting from the spinal cord, the soma of these neurons are located within the spinal cord (Gerendai *et al.* 2000). Thus, given that the Npas4 protein is localised to the nucleus, it is unlikely that the expression of Npas4 seen in the testes can be ascribed to the peripheral neurons which innervate the testes. It is possible, then, to put forward the hypothesis that Npas4 has two functions (one in neurons and one in certain progenitor cell populations) and that in the testes Npas4 is expressed by a progenitor cell type where it has a similar function to that seen during development. A role for Npas4 in testis function is supported by the observation that Npas4$^{-/-}$ mice breed poorly (personal communication, Yingxi Lin).

6.2.4 Regulation of *Sox1* expression by Npas4

One of the major findings presented in this thesis is that reduced expression of Npas4 during neural differentiation of mES cells results in a reduction in the percentage of cells expressing *Sox1*. As the percentage of Nestin^{+} NPCs was unaffected, this suggests that knock-down of Npas4 selectively decreases *Sox1* expression rather than affecting the number of NPCs formed. Together, these data suggest that Npas4 acts upstream of Sox1 to positively regulate its expression; however, as Npas4 and Sox1 are not co-expressed in the same cells during neural differentiation of mES cells (see Figure 3.2C-D), it is likely that Npas4 does not directly regulate *Sox1* expression but does so via an intermediate transcription factor.

Several microarray studies have been performed in order to identify genes that may be regulated by Npas4, and therefore one may ask, "Why was *Sox1* not identified as a candidate gene that may be regulated by Npas4 earlier?" These microarray studies were carried out in a variety of different cell types including HeLa cells (Hester *et al.* 2007), SK-N-MC cells (Ooe *et al.* 2004) and E16 mouse hippocampal neurons (Lin *et al.* 2008). Two of these studies involved over-expression of Npas4 (Ooe

et al. 2004; Hester *et al.* 2007), though the complete list of candidate genes whose expression was affected by increased Npas4 expression was not disclosed. In the third study, which involved knock-down of Npas4 expression using RNAi in E16 mouse hippocampal neurons (Lin *et al.* 2008), expression of *Sox1* was not found to be altered by knock-down of Npas4 though this is unsurprising given that *Sox1* is not normally expressed in neurons. When using knock-down of gene expression to identify potential downstream targets, the transcriptome of the cell population that is selected for the microarray study will define the pool of candidate targets. If Npas4 is expressed in other cell populations besides neurons (such as NPCs, for example), then in order to identify targets of Npas4 in these cells, it will be necessary to use the appropriate cell line in microarray experiments. Furthermore, if an alternative isoform of Npas4 is expressed during development (see Section 3.3.3), it may regulate a completely different set of target genes to the canonical Npas4 isoform.

If Npas4 does indeed regulate expression of *Sox1*, then it would be expected that in the Npas4$^{-/-}$ mouse there would be some evidence of disrupted Sox1 function. Strikingly, there is some similarity between the Npas4$^{-/-}$ and Sox1$^{-/-}$ mice; both knock-out mice display a hyperexcitability phenotype. One of the features of the Sox1$^{-/-}$ phenotype is enhanced synaptic activity in the olfactory cortex which manifests as spontaneous epileptiform discharges and ultimately results in spontaneous limbic seizures beginning at 4-6 weeks of age (Malas *et al.* 2003). Given that Npas4$^{-/-}$ mice display a similar hyperexcitability phenotype and are also prone to seizures (Lin *et al.* 2008), it is tempting to speculate that there may be a common underlying mechanism (i.e. perhaps the spontaneous seizure phenotype observed in Npas4$^{-/-}$ mice is partly due to a reduction in *Sox1* expression).

6.3 Future experiments

The work presented in this thesis provides evidence that Npas4 is expressed, and may have a functional role, during mammalian embryonic development. This is a novel proposition as Npas4 was previously thought to be important only for the proper functioning of the adult brain. As such, this study is the first step in a new direction for Npas4 research, a platform that will provide the foundation for future studies in which the role of Npas4 in embryonic development can be explored further. Clearly, there are many questions that still remain unanswered and there is much work to be done before we have a complete understanding of the significance of Npas4 expression in the context of development. This section will cover some of the more pressing questions that need to be addressed, as well as the experimental methodology that could be used to achieve this.

Though it was demonstrated that Npas4 is expressed both during neural differentiation of ES cells and in the mouse embryo during development, one of the limitations of this study is that the identity of the Npas4-expressing cells was not conclusively established. In addition, the timing of developmental Npas4 expression *in vivo* needs to be defined with a greater level of precision. With regard to developmental Npas4 expression *in vivo*, both of these issues could be addressed simultaneously using either ISH or immunohistochemistry of whole mount staged embryos to analyse the spatiotemporal pattern of Npas4 expression throughout development. The latter could be performed in conjunction with known marker proteins (such as Sox1, Noggin and Pax6) which would allow Npas4 expression to be defined in the context of other important developmental players.

An alternative approach may be to create a transgenic Npas4 reporter mouse line in which the Npas4 open reading frame is replaced with coding sequence for a reporter such as GFP or β-galactosidase. In these animals, expression of the reporter gene would be under the control of endogenous Npas4 regulatory elements and, in this way, the expression pattern of Npas4 during development (and even in adulthood or disease) could be visualised via expression of the reporter. This approach may be particularly useful in overcoming issues of sensitivity in tissues where Npas4 is lowly expressed, such as the embryo.

Regarding neural differentiation of ES cells *in vitro*, where a heterogeneous mixture of cells is produced, *Npas4* expression ought to be examined in relation to various marker genes so that the $Npas4^{+}$ cell population can be characterised according to the marker genes it expresses (or does not express). For instance, in Noggin-induced differentiation of hES cells, dual-colour immunofluorescence could be used to test the hypothesis that NPAS4 is expressed by $PAX6^{+}/SOX1^{-}$ PAN cells by assessing co-expression of NPAS4 with PAX6 and SOX1 at Day 14.

Another issue which has yet to be explored relates to the molecular mechanisms underlying Npas4 expression in these developmental model systems. As discussed in Section 3.3.4, current evidence suggests that Noggin-induced Ca^{2+} signalling may provide a plausible explanation for the Npas4 expression observed in these systems. This hypothesis could be tested *in vitro* by performing neural differentiation of ES cells in the presence of an L-type Ca^{2+} channel blocker (i.e. nifedipine, isradipine) or activator (i.e. Bay K8644) to determine whether this affects Npas4 expression.

Finally, the functional role of Npas4 during neural differentiation of mES cells is another area which needs to be investigated further. This study demonstrated for the first time that knock-down of Npas4 expression in mES cells affected several aspects of neural differentiation including expression of Sox1 and neurite sprouting, but it is possible that Npas4 may also be involved in other aspects of neurogenesis. For example, it would be of interest to know whether knock-down of Npas4 also affects neuronal survival, neuronal subtype specification, synapse formation or gliogenesis.

All of these questions could be answered relatively easily by comparing Empty vector control cultures to Npas4 KD cultures in their ability to undergo neural differentiation. Immunofluorescence techniques could be used to assess expression of various markers after 15-20 days of differentiation to answer specific questions. For example, the ratio of neurons:glia produced could be determined by comparing the ratio of Fox-3^{+}:GFAP^{+} cells, while TUNEL staining could be used to determine whether neuronal apoptosis of Fox-3^{+} cells is increased in Npas4 KD cultures. The effect of Npas4 on synapse formation could be assessed by comparing the expression of Synapsin I, a marker of presynaptic vesicles, while expression of markers specific to excitatory (i.e. vesicular glutamate transporter) or inhibitory neurons (i.e. GAD65) would indicate whether knock-down of Npas4 alters the outcome of neuronal subtype specification.

6.4 Concluding remarks

The work described in this thesis provides evidence that *Npas4* is expressed in the embryo during mammalian development. Furthermore, the data presented here suggest that *Npas4* may also play a role in embryonic development that is distinct to its many functions in neurons of the adult brain. Based on these findings, it is possible to propose the hypothesis that *Npas4* has dual modality. In the mature brain it is up-regulated in neurons in response to neural activity where it regulates a specific set of target genes (including *Bdnf* and *Drebrin*) and is involved in neuroprotection, synaptic plasticity and inhibitory synapse formation. In the embryo, it is expressed in an early progenitor cell type in response to Noggin-induced Ca^{2+} signalling where it regulates a distinct set of target genes (which may include indirect regulation of *Sox1*) and participates in cellular processes which may include NPC maintenance and/or neuronal maturation (see Figure 6.1).

7 – References

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8 – Appendices

8.1 Appendix 1

Accession numbers for sequences used to construct bHLH PAS phylogenetic trees

Protein	Species	Accession number
aha-1	*Caenorhabditis elegans*	C25A1.11a
ahr-1	*Caenorhabditis elegans*	C41G7.5a
C15C8.2	*Caenorhabditis elegans*	C15C8.2b
hif-1	*Caenorhabditis elegans*	F38A6.3b
T01D3.2	*Caenorhabditis elegans*	T01D3.2
Clock	*Drosophila melanogaster*	FBpp0099478
Cyc	*Drosophila melanogaster*	FBpp0074693
Dys	*Drosophila melanogaster*	FBpp0289845
Met	*Drosophila melanogaster*	FBpp0073368
Sima	*Drosophila melanogaster*	FBpp0084931
Sim	*Drosophila melanogaster*	FBpp0082178
Ss	*Drosophila melanogaster*	FBpp0084931
Tai	*Drosophila melanogaster*	FBpp0079394
Tgo	*Drosophila melanogaster*	FBpp0081483
Trh	*Drosophila melanogaster*	FBpp0072466
Actin	*Mus musculus*	ENSMUSP00000031564
Ahr	*Mus musculus*	ENSMUSP00000112137
Ahrr	*Mus musculus*	ENSMUSP00000022059
Arnt	*Mus musculus*	ENSMUSP00000099810
Arnt2	*Mus musculus*	ENSMUSP00000082154
Bmal1	*Mus musculus*	ENSMUSP00000046235
Bmal2	*Mus musculus*	ENSMUSP00000107266
Clock	*Mus musculus*	ENSMUSP00000031148
Hif1a	*Mus musculus*	ENSMUSP00000021530
Hif2a	*Mus musculus*	ENSMUSP00000024954
Hif3a	*Mus musculus*	ENSMUSP00000048248
Ncoa1	*Mus musculus*	ENSMUSP00000072285
Ncoa2	*Mus musculus*	ENSMUSP00000006037
Ncoa3	*Mus musculus*	ENSMUSP00000085416
Npas1	*Mus musculus*	ENSMUSP00000002053
Npas2	*Mus musculus*	ENSMUSP00000054719
Npas3	*Mus musculus*	ENSMUSP00000098975
Npas4	*Mus musculus*	ENSMUSP00000062992
SIm1	*Mus musculus*	ENSMUSP00000020071
Sim2	*Mus musculus*	ENSMUSP00000072043
AHR	*Homo sapiens*	ENSP00000242057

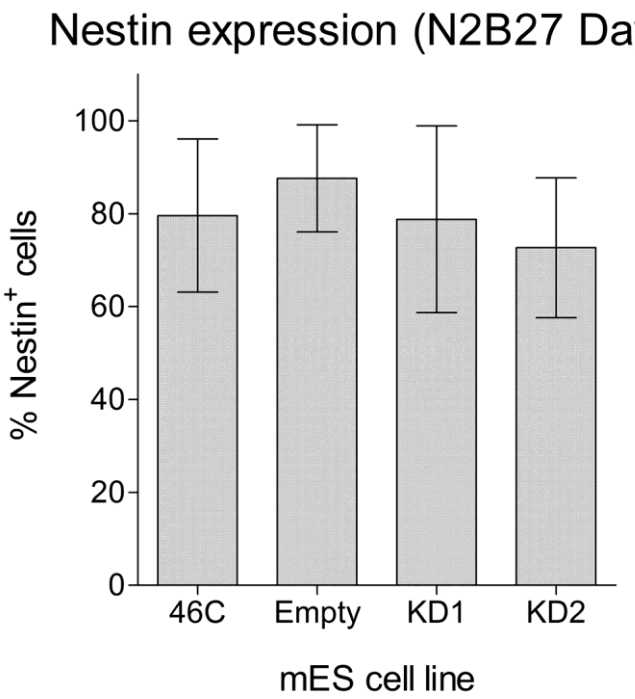
AHRR	*Homo sapiens*	ENSP00000323816
ARNT	*Homo sapiens*	ENSP00000351407
ARNT2	*Homo sapiens*	ENSP00000307479
BMAL1	*Homo sapiens*	ENSP00000374357
BMAL2	*Homo sapiens*	ENSP00000266503
CLOCK	*Homo sapiens*	ENSP00000426983
HIF1a	*Homo sapiens*	ENSP00000338018
HIF2a	*Homo sapiens*	ENSP00000263734
HIF3a	*Homo sapiens*	ENSP00000300862
NCOA1	*Homo sapiens*	ENSP00000385216
NCOA2	*Homo sapiens*	ENSP00000399968
NCOA3	*Homo sapiens*	ENSP00000361073
NPAS1	*Homo sapiens*	ENSP00000405290
NPAS2	*Homo sapiens*	ENSP00000338283
NPAS3	*Homo sapiens*	ENSP00000348460
NPAS4	*Homo sapiens*	ENSP00000311196
SIM1	*Homo sapiens*	ENSP00000358210
SIM2	*Homo sapiens*	ENSP00000290399

8.2 Appendix 2

Key for mouse embryonic stem (mES) cell lines generated in this study. The small interfering RNA (siRNA) vectors used to make each cell line are listed as well as the name of the clone from which the cell line was derived.

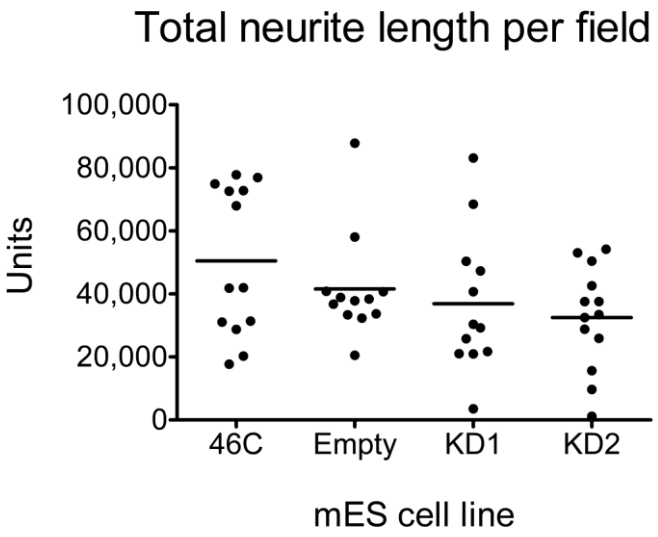
mES cell line	siRNA used	Clone
Empty vector control	Empty vector control	Empty 5A
Npas4 KD1	Npas4 siRNA #3	#3 10A
Npas4 KD2	Npas4 siRNA #2	#2 2D

8.3 Appendix 3



Appendix 3 - Nestin expression after four days of differentiation in N2B27 medium
After four days of differentiation in N2B27 medium, cultures were fixed and expression of Nestin was analysed in each of the mES cell lines using immunocytochemistry (see Figure 5.1D). The percentage of cells expressing Nestin after four days of differentiation in N2B27 medium was determined by manually couting the number of Nestin$^+$ cells. When analysed using an unpaired t test, there was no statistically significant difference in the mean number of Nestin$^+$ cells between; 46C and Empty vector control cultures (P = 0.3995), Empty vector control and KD1 cultures (P = 0.4203) or Empty vector control and KD2 cultures (P = 0.1614).

8.4 Appendix 4



Appendix 4 - Total neurite length after 10 days of differentiation in N2B27 medium
After 10 days of differentiation in N2B27 medium, cultures were fixed and neurite sprouting was assessed in each of the mES cell lines using immunocytochemistry for the neuronal marker βIII tubulin (see Figure 5.1E). The overall extent of neurite sprouting was determined by using automated neurite tracing software to measure the total neurite length in each field (see Section 2.2.2). When analysed using an unpaired t test, there was no statistically significant difference in the mean total neurite length per field between; 46C and Empty vector control cultures (P = 0.2957), Empty vector control and KD1 cultures (P = 0.5655) or Empty vector control and KD2 cultures (P = 0.1837).