The De-ubiquitylating Enzyme USP9X
is Essential for Normal Neural Development in Mouse

Shane Stegeman
B.Sc. (Hons)

School of Biomolecular and Physical Sciences
Science, Environment, Engineering and Technology
Griffith University

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Abstract

The ubiquitin system is involved in almost all aspects of protein function and cell fate and is thought to play an important role during neural development. Defects within the ubiquitin system have been linked to a range of neuro-degenerative diseases including Parkinson’s and Alzheimer’s disease. De-ubiquitylating enzymes function downstream in the ubiquitin pathway having potential to act as the final arbiters of substrate fate and function. Several studies have shown that de-ubiquitylating enzymes play important roles in the growth, function and maintenance of neurons.

The substrate-specific de-ubiquitylating enzyme USP9X is highly expressed in the developing central nervous system. *In vitro* analyses have shown that USP9X is highly expressed in neural stem cells while expression remains at lower levels in more differentiated neural cell types. As the human and mouse USP9X genes share a 97% nucleotide identity within the coding sequence, this study uses the mouse as a model to analyse the role of USP9X during mammalian neural development. Previous studies have shown that loss of USP9X in the pre-implantation mouse embryo results in pre-implantation lethality. To circumvent this early developmental stage and analyse USP9X function in the developing brain we utilized a conditional knockout strategy involving the Cre/loxP recombination system. Using the Cre/loxP recombination system we permanently disable USP9X via exon removal specifically in the CNS during embryonic development. Two models were utilised, the first disabling USP9X in the whole CNS using Nestin-Cre mediated deletion and the second disabling USP9X in the dorsal telencephalon (dorsal forebrain) using Emx1-Cre. Using these models, USP9X is targeted in neural stem cells / neural progenitor cells prior to differentiation. This approach presents the ability to examine the effects of loss of USP9X on neural progenitor cells as well as neuronal and glial cells that derive from these progenitors.

Loss of USP9X throughout the whole CNS results in early postnatal lethality confirming an essential role for USP9X in brain development. In the late embryo and newborn a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus was observed. An *in vitro* analysis of cortical-derived neuronal cultures examining early neuronal development identified deficiencies in early axonal specification. A similar analysis of hippocampal-derived neuronal cultures, examining late stage neuronal development, showed that loss of
USP9X resulted in reduced elongation of the primary axonal process and a reduction in the number of axonal neurite termini. Loss of USP9X restricted to the dorsal forebrain during development allowed mice to survive into adulthood. In the late embryo these mice also displayed a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus. In the adult forebrain a significant reduction in the size of both the corpus callosum and the hippocampus was observed. The reduction in the corpus callosum suggests that USP9X may be required for normal axonal development as the *in vitro* analysis suggests. Loss of USP9X also resulted in the presence of aberrant neuronal processes in the adult cerebral cortex.

USP9X has multiple substrates many of which play important roles during neural development. These substrates are known to regulate a range cellular processes including mitosis, apoptosis, protein trafficking, cell signalling, transcriptional regulation, migration, cellular polarity and cell-to-cell adhesion, phosphorylation and ubiquitylation. It is hence likely that USP9X regulates neural development via multiple pathways.
Signed statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Shane Stegeman
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Dr Stephen Wood  
National Centre for Adult Stem Cell Research  
Eskitis Institute  
Griffith University

Professor Alan Mackay-Sim  
National Centre for Adult Stem Cell Research  
Eskitis Institute  
Griffith University

Professor Linda Richards  
Queensland Brain Institute  
University of Queensland

Dr Lachlan Jolly  
Neurogenetics Research Program  
SA Pathology  
University of Adelaide

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An updated version of the poster is presented in Appendix 1

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<tr>
<td>AF-6</td>
<td>Acute lymphoblastic leukemia-1 fusion partner from chromosome 6</td>
</tr>
<tr>
<td>AGS3</td>
<td>Activator of G protein Signalling 3</td>
</tr>
<tr>
<td>CA</td>
<td>cornu ammonis</td>
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<tr>
<td>cKO</td>
<td>conditional knockout</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>Cre</td>
<td>cre-recombinase</td>
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<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>DUB</td>
<td>de-ubiquitylating enzyme</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic Day (e.g. E15.5)</td>
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<tr>
<td>EFA6</td>
<td>Exchange factor for ARF6</td>
</tr>
<tr>
<td>faf</td>
<td>fat facets (<em>Drosophila</em> homologue of USP9X)</td>
</tr>
<tr>
<td>fam</td>
<td>fat facets in mouse (official name: USP9X)</td>
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<td>MARCH7</td>
<td>Membrane-Associated RING-CH 7</td>
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<td>MARK4</td>
<td>Microtubule-affinity-regulating kinase 4</td>
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<td>MCL1</td>
<td>Myeloid cell leukemia 1</td>
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<td>NF160</td>
<td>Neurofilament 160</td>
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<td>NP</td>
<td>neural progenitor</td>
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<td>NSC</td>
<td>neural stem cell</td>
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<td>P</td>
<td>postnatal day (e.g. P1)</td>
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<td>RT-PCR</td>
<td>reverse transcription - polymerase chain reaction</td>
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<td>SVZ</td>
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<td>Ub</td>
<td>ubiquitin</td>
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<td>UCH</td>
<td>ubiquitin C-terminal hydrolase</td>
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<td>USP</td>
<td>ubiquitin-specific protease</td>
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Chapter 1

Introduction

1.1 USP9X

USP9X is a substrate specific de-ubiquitylating enzyme. This introduction begins with an overview of the ubiquitin system and the role of de-ubiquitylating enzymes followed by a specific discussion of USP9X (starting in section 1.1.3).

1.1.1 The ubiquitin system

The ubiquitin system is involved in almost all aspects of protein function and cell fate (Andersen et al., 2005, Hicke et al., 2005, Strieter and Korasick, 2012). Ubiquitin (Ub) is a 76 amino acid protein highly conserved in eukaryotes (Ciechanover et al., 1980, Pickart and Eddins, 2004). The enzyme mediated attachment of Ub to a target protein (referred to as ubiquitylation or ubiquitination) plays various roles including the regulation of protein degradation (normal & misfolded proteins), cell signalling, cell cycle regulation, transcription, protein trafficking and endocytosis (Dhananjayan et al., 2005, Gill, 2004, Hershko and Ciechanover, 1998, Spence et al., 1995, Varshavsky, 2005, Weissman, 2001, Fredrickson and Gardner, 2012, Wright et al., 2012). A series of sequential steps involving three enzymes is required to covalently attach Ub to its target protein via an isopeptide bond between a lysine residue of the target protein and the carboxy-terminal glycine residue of the Ub molecule. In the first step an ubiquitin-activating enzyme (E1) activates the carboxy-terminal glycine residue of the Ub molecule. In this step the Ub molecule is bound to a cysteine residue on the E1 enzyme. The activated Ub molecule is then transferred to a cysteine residue on an ubiquitin-carrier protein (E2) (also called a ubiquitin-conjugating enzyme). In the final step an ubiquitin-ligase (E3) catalyses the carboxy-terminal linkage of the Ub molecule to a lysine residue on the target protein (Hershko and Ciechanover, 1998). A large number of highly conserved E2 and E3 proteins have been identified in a variety of eukaryotes including yeasts, fruit flies and mammals (Cenci et al., 1997, Jentsch, 1992, Tong et al.,
Though E2 enzymes show specificity for recognising particular E3 ligases, it is predominantly the E3 ligases which are responsible for substrate recognition (French et al., 2005, Hicke et al., 2005). The two major classes of E3 ligases characterised by their ubiquitin-associated domains include the HECT (Homology E6 C-terminus) domain family and the RING-type (Really Interesting New Gene) family (Andersen et al., 2005, Petroski, 2008). Proteins can be ubiquitylated in a variety of ways including mono-ubiquitylation with a single Ub molecule, multi-ubiquitylation with single Ub molecules bound to multiple lysine residues within the substrate and poly-ubiquitylation consisting of a chain of Ub molecules bound from a single lysine residue on the target protein (Lucero et al., 2000, Peng et al., 2003, Xu and Peng, 2006). Poly-ubiquitylation is achieved by linking the carboxy-terminal of the incoming Ub molecule to a lysine residue on the existing Ub molecule (Pickart, 2001). All lysine residues within the Ub molecule are able to participate in chain formation however residues K48 and K63 are the most common linkage sites (Pickart and Eddins, 2004). The mode of ubiquitylation is a major determent of protein fate (Shih et al., 2000, Terrell et al., 1998, Thrower et al., 2000). Mono-ubiquitylation often serves as an endocytosis signal (Chen and Fischer, 2000). Poly-ubiquitylation usually targets a protein for degradation in the proteasome. The majority of proteins are targeted for degradation via this pathway (Ciechanover et al., 2000, Petroski and Deshaies, 2003). Following degradation Ub chains are recycled into free re-usable Ub units by the action of de-ubiquitylating enzymes (Wilkinson, 2000). De-ubiquitylating enzymes are discussed in section 1.1.2. The ubiquitin system is thought to play an important role during neurogenesis (Tai and Schuman, 2008, Tuoc and Stoykova, 2010, Chen et al., 2011). Defects within the ubiquitin system have been linked to a variety of neuro-degenerative diseases including Parkinson’s and Alzheimer’s disease (Imai et al., 2000, Lim and Tan, 2007, Oddo, 2008, Pasinetti, 2001, Rogers et al., 2010). A common thread associated with a range of neuro-degenerative diseases is the accumulation of abnormal ubiquitylated protein aggregates. These deposits are thought to contribute to the loss of neuronal function in specific regions of the brain. Excess build up of Ub has been detected in Alzheimer’s, Parkinson’s, Huntington’s and motor neuron disease (DiFiglia et al., 1997, Lowe et al., 1988a, Probst et al., 2001, Riederer et al., 2011). Ub inclusions have also been identified in tissues beyond the brain and are known to contribute to other disorders such as
alcoholic liver disease (Lowe et al., 1988b). Defects within the ubiquitin system have also been implicated in a range of cancers (Brummelkamp et al., 2003, Pagano and Benmaamar, 2003). Disruptions to E3 ligase function are often seen in many of these diseases (Ardley and Robinson, 2004, Michael and Oren, 2002, Scheffner and Staub, 2007).

1.1.2 De-ubiquitylating enzymes

Ubiquitylation is a reversible process. Ubiquitin-specific proteases also called de-ubiquitylating enzymes (DUB) function downstream in the ubiquitin pathway having potential to act as the final arbiters of substrate fate and function. The removal of ubiquitin (Ub) by DUBs not only plays a recycling role following proteasomal degradation but also a regulatory role. DUBs regulate a wide range of cellular activities including protein stability, localisation, trafficking and signalling (Burrows and Johnston, 2012, Fischer, 2003, Hershko and Ciechanover, 1998, Millard and Wood, 2006, Wilkinson, 2000). Most early work on DUBs focussed on the yeast *S.cerevisiae*. The catalytic domains identified in yeast DUBs are highly conserved across a range of species including nematodes, fruit flies, mice and humans (Baker et al., 1992, Isaksson et al., 1996, Jentsch, 1992, Tobias and Varshavsky, 1991, Wilkinson and Hochstrasser, 1998, Wilkinson et al., 1989). It is estimated that the human genome encodes for at least 98 DUBs which are divided into five classes based on differences within their catalytic domain structure. Evidence has recently emerged suggesting a sixth class. The five original classes include ubiquitin-specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), Machado-Joseph disease proteases, Ovarian tumour proteases and Metallo-protease-JAMM-motif containing proteases (Baek, 2003, Fraile et al., 2011).

The two largest classes of DUBs consist of the USPs and the UCHs (Baek, 2003). The highly conserved UCHs are smaller (20–30 kDa) proteasome associated enzymes that primarily function in the removal and recycling of single Ub molecules from poly-ubiquitin chains following protein degradation (Baek, 2003, Hochstrasser, 1995, Johnston et al., 1999). The larger USPs (50-300 kDa) for which there are more than fifty in humans are substrate specific and are identified primarily by their conserved cysteine / histidine catalytic domains (Baek, 2003, Nijman et al., 2005). One of the roles
of USPs is to remove Ub from their substrates prior to proteasomal entry, rescuing their substrates from degradation (See Figure 1.1). Other roles include the induction of substrate endocytosis, signal transduction, trafficking and activation of substrates to initiate involvement in a diverse range of processes including transcriptional regulation, mitosis and apoptosis (Baek et al., 2001, Cadavid et al., 2000, Hanna et al., 2006, Katz et al., 2010, Lucero and Lagunas, 1997, Park et al., 2002, Reyes-Turcu et al., 2009, Verma et al., 2002, Wilkinson, 1997). Identifying specific substrates for individual DUBs is difficult due to the often weak and transient interactions between DUBs and their target substrates (Nijman et al., 2005, Theard et al., 2010).

DUBs have been implicated in a range of diseases with most prominent studies focusing on their roles in cancer (Brichory et al., 2001, Fraile et al., 2011, Rolen et al., 2006, Yang, 2007). Several studies have also shown that DUBs play important roles in the growth, function and maintenance of neurons (DiAntonio et al., 2001, Staropoli and Abeliovich, 2005). This present study focuses on the substrate-specific de-ubiquitylating enzyme USP9X and its role during neural development.
Figure 1.1 The ubiquitin system
The attachment of ubiquitin (Ub) is carried out sequentially by the action of three enzymes respectively: ubiquitin-activating enzymes (E1), ubiquitin-carrier proteins (E2) and ubiquitin-ligases (E3) that consist of two major classes: the HECT (Homology E6 C-terminus) domain family and the RING-type (Really Interesting New Gene) family. The two largest classes of de-ubiquitylating enzymes (DUB) include: The ubiquitin-specific proteases (USP) which perform several roles including the removal of Ub from their substrates prior to proteasomal entry rescuing their substrates from degradation. And the ubiquitin C-terminal hydrolases (UCH) which primarily function in the removal and recycling of single Ub molecules from poly-ubiquitin chains following protein degradation. Image modified from (Devoy et al., 2005)
1.1.3 USP9X

**fat**

In a search for mutations affecting eye development in *Drosophila*, the Fischer-Vize laboratory discovered *fat facets* (*faf*). In *Drosophila* faf is essential for normal eye and oocyte development but is also expressed in many other tissues where it is non-essential. (*faf* mutants develop eye facets with additional photoreceptors hence the name *fat facets*). In the oocyte faf localises to the posterior pole (Fischer-Vize et al., 1992). The establishment of anterior-posterior polarity and the localisation of various proteins to opposite poles plays an important role during development (St Johnston and Nusslein-Volhard, 1992).

The Fischer-Vize laboratory later identified faf as a de-ubiquitylating enzyme (DUB) that functions upstream of the proteasome (Huang et al., 1995). In this role faf is thought to de-ubiquitylate substrates prior to proteasomal entry saving them from degradation. Interestingly it was later shown that faf is only essential early in eye development clearly showing that the role of faf during eye development is stage-specific (Huang and Fischer-Vize, 1996).

faf displays little sequence similarity outside the conserved cysteine / histidine catalytic domain compared to all other known members of the ubiquitin-specific protease class in *Drosophila* (Chen and Fischer, 2000, Rubin et al., 2000). Whilst the cysteine / histidine catalytic domain is responsible for de-ubiquitylase activity sequences outside the catalytic domain are thought to be responsible for substrate recognition suggesting that faf may have a substrate-specific role distinct from other DUBs (Wilkinson and Hochstrasser, 1998).

**DFFRX**

Genes with sequence homology to the *Drosophila faf* gene have been identified on the human sex chromosomes. These X and Y-linked homologues were originally named *DFFRX* and *DFFRY* standing for *Drosophila fat facets* related on the X and Y chromosomes. The X-linked protein displays 44% identity and 88% similarity to its *Drosophila* homologue (Jones et al., 1996). In humans *DFFRX* escapes random X-inactivation (Carrel et al., 1999, Jones et al., 1996). DFFRX expression is found in a
range of embryonic tissues including the brain, eye, heart, kidney, liver and lung, suggesting a developmental role in humans (Jones et al., 1996).

**Fam**

In a gene-trap screen for genes expressed during embryonic development in mouse Wood et al identified a gene with high sequence similarity to the *Drosophila* *faf* gene. This gene was originally named *Fam* short for *fat facets in mouse* (Wood et al., 1997). Fam expression was detected in the developing central nervous system as well as other developing organs including the liver and lung (Wood et al., 1997). Like the human homologue *DFFRX*, *Fam* was also found on the X chromosome (Wood et al., 1997). The Fam protein displays 50% identity and 70% similarity to *faf* and in *Drosophila* Fam is able to substitute for loss of *faf* (Chen et al., 2000, Wood et al., 1997). The X-linked human and mouse genes share 97% nucleotide identity within their coding sequences (Brown et al., 1998). Mice have far fewer genes that escape random X-inactivation compared to humans. In mouse it is thought that Fam is subject to X-inactivation (Yang et al., 2010).

**USP9X**

These *faf* homologues found in human and mouse originally called *DFFRX* and *Fam* were later renamed *USP9X* to reflect the USP class of DUBs they belong to, number 9 within that class and the X chromosomal location in both human and mouse. For the remainder of this thesis *DFFRX* and *Fam* are referred to as *USP9X*. The Y chromosome gene first identified in humans has also been identified in mouse and is now referred to as *USP9Y* in both human and mouse. *USP9Y* is discussed in section 1.1.4.

Besides human and mouse, USP9X expression has also been investigated in rat where it is expressed in the brain, thymus, lung, heart, ovaries, pancreas and spleen (Mouchantaf et al., 2006). USP9X has been shown to interact with a growing list of substrates and binding partners in a range of cells and tissues. A substrate is defined as a protein for which USP9X removes ubiquitin whilst a binding partner though interacting with USP9X is not subject to USP9X de-ubiquitylase activity. USP9X de-ubiquitylase activity / substrate identification may be assessed using a range of *in vitro* approaches. One approach utilises exogenous expression of functional USP9X versus mutant (catalytically inactive) USP9X expression followed by immuno-precipitation of
exogenously expressed tagged targets (potential substrates). The ubiquitylation levels of these immuno-precipitated targets are then assessed (Theard et al., 2010).

USP9X substrates (and binding partners) are known to regulate a range of cellular processes including mitosis, apoptosis, protein trafficking, cell signalling, transcriptional regulation, migration, cellular polarity and cell-cell adhesion, and phosphorylation. Several of these substrates themselves are components of the ubiquitin system (Table 1.1). Though USP9X substrates Itch, MARK4 and NUAK1 are ubiquitylated at lys29, Survivin is ubiquitylated at Lys63 and MCL1 at Lys48 suggesting that USP9X does not have any specific linkage preference for substrate recognition (Al-Hakim et al., 2008, Schwickart et al., 2009). Nor is there any strict role for the function of USP9X in regard to its substrates. USP9X is proposed to stabilise substrates like AF-6 and β-catenin while regulating the activation of substrates like MARK4 and NUAK1 rather then their stability (Al-Hakim et al., 2008). USP9X-substrate interactions are discussed further in latter sections.

Human USP9X is a large 2547 amino acid protein encoded by a 45 exon gene (Brown et al., 1998; Jones et al., 1996) (Genbank entry: NM_001039590). The mouse USP9X gene also transcribes into a 45 exon mRNA product that produces a 2554 amino acid protein (Wood et al., 1997) (Genbank entry: NM_009481). In Drosophila faf is the largest de-ubiquitylating enzyme, more then twice the size of the next largest (Chen and Fischer, 2000). The large size of USP9X and faf may reflect its capacity to interact with multiple substrates.
### USP9X substrates & binding partners

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Major Role</th>
<th>Primary Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-6</td>
<td>Cell adhesion &amp; polarity</td>
<td>(Taya et al., 1998)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Cell adhesion &amp; polarity. Signalling</td>
<td>(Taya et al., 1999)</td>
</tr>
<tr>
<td>Epsin</td>
<td>Signalling</td>
<td>(Chen et al., 2003, Chen et al., 2002)</td>
</tr>
<tr>
<td>Lqf *</td>
<td>Transcriptional regulation</td>
<td>(Liu et al., 2003)</td>
</tr>
<tr>
<td>Vasa *</td>
<td>Neuronal migration. Trafficking</td>
<td>(Friocourt et al., 2005)</td>
</tr>
<tr>
<td>Survivin</td>
<td>Mitosis. Apoptosis</td>
<td>(Vong et al., 2005)</td>
</tr>
<tr>
<td>Itch</td>
<td>Signalling. Ubiquitin system</td>
<td>(Mouchantaf et al., 2006)</td>
</tr>
<tr>
<td>Mindbomb1</td>
<td>Signalling. Ubiquitin system</td>
<td>(Choe et al., 2007)</td>
</tr>
<tr>
<td>Huntingtin *</td>
<td>Trafficking. Transcription</td>
<td>(Kaltenbach et al., 2007)</td>
</tr>
<tr>
<td>MARK4</td>
<td>Cell adhesion &amp; polarity</td>
<td>(Al-Hakim et al., 2008)</td>
</tr>
<tr>
<td>MARCH7</td>
<td>Ubiquitin system</td>
<td>(Nathan et al., 2008)</td>
</tr>
<tr>
<td>NUAK1</td>
<td>Phosphorylation (Kinase)</td>
<td>(Al-Hakim et al., 2008)</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis</td>
<td>(Nagai, 2009)</td>
</tr>
<tr>
<td>MCL1</td>
<td>Apoptosis</td>
<td>(Schwickart et al., 2009)</td>
</tr>
<tr>
<td>Smad4</td>
<td>Signalling</td>
<td>(Dupont et al., 2009)</td>
</tr>
<tr>
<td>AGS3</td>
<td>Cell adhesion &amp; polarity</td>
<td>(Xu et al., 2010)</td>
</tr>
<tr>
<td>EFA6</td>
<td>Cell adhesion &amp; polarity</td>
<td>(Theard et al., 2010)</td>
</tr>
</tbody>
</table>

**Table 1.1** Single asterisks (*) indicate proteins that have been identified as substrates in *Drosophila*. Double asterisks (**) indicate proteins (referred to as binding partners) that bind with USP9X but are not substrates de-ubiquitylated by USP9X. Triple asterisks (***) indicate proteins for which the nature of the USP9X interaction is not yet known.
1.1.4 USP9Y

Both USP9X and USP9Y transcribe an 8-9 kb mRNA product. The human USP9Y gene contains a coding sequence with 89% nucleotide identity and 98% nucleotide similarity to USP9X (Brown et al., 1998; Jones et al., 1996). In mouse USP9Y contains an 83% nucleotide identity within the coding sequence and an 82% amino acid identity to USP9X (Hall et al., 2003). USP9Y in both humans and mouse contains the trademark catalytic cysteine / histidine domain found in USP9X suggesting that it too may function as a de-ubiquitylating enzyme (DUB). Though USP9X and USP9Y may both function as DUBs it remains to be tested whether they are interchangeable in their function. As homologous genes located on mammalian X and Y chromosomes no longer recombine, though similar in structure they are hence able to evolve over time into distinctive forms with differing functions (Graves, 1995). As sequences outside the catalytic domain are thought to be responsible for substrate recognition the small portion of amino acid differences found outside this region may result in different substrate specificities (Brown et al., 1998; Wilkinson and Hochstrasser, 1998).

Though USP9Y is ubiquitously expressed in humans, as a Y-linked gene much of the interest has focused on its potential role in testis development (Brown et al., 1998, Sargent et al., 1999, Wilkinson and Hochstrasser, 1998). In human testes though not specifically required for fertility, USP9Y is thought to have a developmental role (Krausz et al., 2006, Luddi et al., 2009, Sun et al., 1999). Though USP9Y is ubiquitously expressed in humans many other Y-linked genes show testis-specific expression patterns (Blanco et al., 2000, Lahn et al., 2001). Studies of USP9Y mRNA expression in mouse do suggest a testis-specific pattern. In mouse testes USP9Y expression first appears between day seven and ten after birth at a time when spermatogenesis begins and continues into adulthood suggesting a possible role in spermatogenesis (Brown et al., 1998).

Though the mouse USP9Y gene has a coding sequence homologous to USP9X it has no significant homology outside the coding region. Mouse USP9Y contains a promoter sequence characteristic of other testis specific genes (Hall et al., 2003). As USP9Y expression is restricted to the testes in mouse, the mouse presents an ideal model for specific analysis of USP9X function in all other tissues.
1.1.5 USP9X and stem cells

Embryonic stem cells have the capacity to differentiate into all the cell types that make up the adult organism whereas adult stem cells in their natural environment (in vivo) are more lineage restricted (Keller, 2005). In mature mammals, adult stem cells have been found in a variety of tissues including bone marrow, epidermis, kidney, liver, skeletal muscle and in distinct regions of the CNS (Bailey et al., 2001, Lledo et al., 2006, Slack and Tosh, 2001, Wagers and Weissman, 2004). Stem cells offer great hope for regenerative medicine. Cell transplantation therapies using patient-derived adult stem cells may be of great advantage due to their acceptance by the patient’s immune system and their accessibility, avoiding the ethical issues associated with embryonic stem cells (Mackay-Sim and St John, 2011, Wetzig et al., 2011). To date much progress has been made. For example, inner ear stem cells have been differentiated in vitro to produce functional sensory neurons (Martinez-Monedero et al., 2008). Human mesenchymal stem cells have been induced into dopamine producing cells (Barzilay et al., 2008). And olfactory mucosa derived neural progenitors have been identified as a potential source for autologous stem cell therapy to treat Parkinson’s disease and a range of other neurodegenerative diseases (Murrell et al., 2008). For this reason it is essential to identify factors that regulate both embryonic and adult stem cell function and fate. The expression levels of various genes involved in the ubiquitin system are enriched in both embryonic and adult stem cells (Ramalho-Santos et al., 2002).

In a microarray transcriptional profiling screen of approximately 12000 genes USP9X (previously identified in the gastrulating and neurulating embryo) was identified as one of 216 genes up-regulated in mouse in all three stem cell populations examined: embryonic (ESC), bone marrow derived hematopoietic (HSC) and lateral ventricle derived neural (NSC) stem cells (Ramalho-Santos et al., 2002; Wood et al., 1997). A similar study found that USP9X is highly expressed in mouse HSCs of both foetal and adult origin and that USP9X is also highly expressed in human HSCs (Ivanova et al., 2002). Only 14% of the genes expressed at higher levels in ESCs, HSCs and NSCs were also enriched in epidermal bulge stem cells from hair follicles within the adult skin. USP9X was among this group of genes enriched throughout these four stem cell populations (Blanpain et al., 2004). A proteomic analysis complementing the ESC microarray studies confirmed USP9X as one of 191 proteins highly expressed in both
human and mouse ESCs (Van Hoof et al., 2006). A recent study has also shown that USP9X is highly expressed at the protein level in both ESCs and NSCs (Jolly, 2009).

These findings suggest that USP9X may play a distinct role in the regulation of stem cell function and fate choices. For example USP9X / faf has been shown to interact with Lqf (Epsin in mammals), a positive regulator of the Notch signalling pathway as well as Itch, a negative regulator of Notch giving USP9X / faf the potential to act as both a regulator of proliferation and differentiation in stem cells (Chen et al., 2002; Mouchantaf et al., 2006). Though at lower levels, the expression of USP9X in more differentiated cell types, clearly suggests that the role of USP9X goes beyond that of stem cell regulation. In neurons for example USP9X interacts with DCX, a microtubule-associated protein involved in migration, protein sorting and vesicle trafficking (Friocourt et al., 2001, Friocourt et al., 2005). The role of USP9X in neural development is discussed in the following section.

1.1.6 USP9X and neural development

USP9X is highly expressed throughout the developing CNS in both humans and mouse. Though expressed at lower levels compared to the embryo and newborn, USP9X is also found throughout the adult mouse brain. In the adult mouse brain expression remains high in a few distinct regions such as the hippocampus, one of the limited regions of the adult brain where neurogenesis takes place (Friocourt et al., 2005; Wood et al., 1997) (Figures 1.2 and 1.3). USP9X expression at the mRNA level in the adult mouse brain can also be compared in the Allen Brain Atlas at http://mouse.brain-map.org. The in vitro findings identifying high USP9X expression in neural stem cells and reduced expression in more differentiated neural cell types are compatible with these in vivo observations (Blanpain et al., 2004; Ramalho-Santos et al., 2002). A recent study has shown that in vitro over-expression of USP9X in neural progenitors results in an increase in the number of neural progenitors as well as an increase in neuronal differentiation strongly suggesting a functional role for USP9X during neural development (Jolly, 2009).
Most of USP9X’s known substrates are expressed in the CNS. Many of these are known to play important roles during neural development. However as the majority of these substrates are also co-expressed with USP9X in other tissues it remains to be determined which of these substrates require an interaction with USP9X to regulate their normal activity in the CNS. The interaction between USP9X and these substrates is discussed in the following sections.

![Figure 1.2 USP9X expression in the developing embryonic CNS in mouse](image)

*In situ* hybridization of sagittal sections showing USP9X expression in the developing CNS in mouse at day E16.5 in embryonic development (A). Negative control (B). Image from (Friocourt et al., 2005).

![Figure 1.3 USP9X expression in the newborn and adult mouse brain](image)

*In situ* hybridization of coronal sections showing USP9X mRNA expression in the newborn mouse brain (A) and the adult mouse brain (C). Notice the high expression in the adult hippocampus (arrow). Negative controls (B, D). Image modified from (Friocourt et al., 2005).
1.1.7 Protein trafficking and neuronal migration

One of the mechanisms by which neurons migrate involves somal translocation. The newborn neuron experiences a continuous series of attachments and detachments from its surroundings before settling into its final position in the CNS (Shieh et al., 2011). To facilitate these events the neuron must traffic an enormous number of proteins up and down the leading and lagging processes and around the continually changing somal body. USP9X is known to associate with DCX a key regulator involved in protein trafficking and neuronal migration (Horesh et al., 1999, Koizumi et al., 2006a, Koizumi et al., 2006b).

DCX

Doublecortin (DCX) is a microtubule binding protein expressed in neuronal cell bodies, processes and axons (Francis et al., 1999). DCX is highly expressed in the embryonic and newborn CNS but expression is limited in the adult. In the adult DCX is primarily found in neurons, which migrate from the sub-ventricular zone to the olfactory bulb via the rostral migratory stream (Friocourt et al., 2005). Loss of DCX results in premature termination of neuronal migration (Bai et al., 2003). DCX is an X-linked gene. Lissencephaly, a disorder more common in males is caused by defective neuronal migration during the 12th to 24th week of gestation resulting in deficient development of gyri (folds) and sulci (grooves) of the brain. Affected children usually have severe neurological impairment and often die within several months after birth. Lissencephaly may be caused by viral infections, insufficient blood supply or a number of genetic factors. Genetic causes in some cases include defects within the DCX gene (des Portes et al., 1998, Dobyns et al., 1996, Gleeson et al., 1998).

DCX possibly regulates the localisation of USP9X in neurons. DCX is not ubiquitylated, nor does it interact with the catalytic domain of USP9X. Hence DCX is not a substrate de-ubiquitylated by USP9X (Friocourt et al., 2005). Besides a role in neuronal migration DCX is also thought to play a role in protein sorting and vesicle trafficking in the CNS (Friocourt et al., 2001). DCX may recruit USP9X to points of protein sorting and vesicle trafficking facilitating the interactions between USP9X and key neuronally expressed substrates. In epithelial cells USP9X localises to the Golgi apparatus and other cytoplasmic vesicles responsible for protein processing and trafficking (Murray et al., 2004). The recruitment of USP9X to particular sites in
various cell types may be crucial as USP9X has been shown to function in a concentration-dependent manner (Al-Hakim et al., 2008). This may be of greatest importance in neurons considering the relatively long distances proteins need to be transported within axons and other neuronal processes. High expression of USP9X has previously been observed in synapses and USP9X has been shown to affect the number of synapses as well as synaptic function (Chen et al., 2003; DiAntonio et al., 2001). Figure 1.4 shows co-localisation of USP9X and DCX in axons and neurites (Friocourt et al., 2005). The following sections focus on specific USP9X substrates and the roles they are known to play during neural development.

![Figure 1.4 USP9X and DCX co-localise in axons and neurites](image)

**Figure 1.4 USP9X and DCX co-localise in axons and neurites**

Neuronal cultures derived from the E18 foetal rat brain show co-expression of USP9X and DCX in axons and neurites. Image modified from (Friocourt et al., 2005)

### 1.1.8 Cellular polarity in the CNS

Neural progenitors (NP) in the developing CNS are highly polarized. Central to the maintenance of cell polarity in NPs is cell-cell adhesion. Cell-cell adhesion in NPs is facilitated primarily by adherens junctions that reside at the apical end of the plasma membrane (Zhadanov et al., 1999). Cell division is able to occur symmetrically along the apical-basal axis to create two identical daughter cells which retain equal apical cellular constituents or asymmetrically resulting in each daughter cell inheriting different apical and basal membrane constituents (Chenn and McConnell, 1995, Hitoshi et al., 2004, Mione et al., 1996). The apical plasma membrane represents a very small percentage of the whole plasma membrane (approximately 1 – 2%). A variety of important trans-membrane proteins are found specifically in this apical region (Kosodo
et al., 2004, Weigmann et al., 1997). It has been proposed that the inheritance of specific apical or basal membrane constituents plays a role in initiating cell fate choices. Asymmetrical cell division may facilitate the signalling of one daughter cell to remain a NP while the other daughter cell differentiates towards a neuronal or glial fate (Gotz and Huttner, 2005, Huttner and Kosodo, 2005, Kosodo et al., 2004). This is thought to allow for differentiation while at the same time expanding the progenitor pool (Haubensak et al., 2004). However neurons can also arise through symmetric cell division clearly showing there are additional factors that are able to facilitate cell fate choices. Little is known as to what factors initially trigger choices between symmetric and asymmetric cell division in progenitors. The most likely candidates are factors associated with spindle orientation.

Post-mitotic neurons do not contain adherens junctions however other factors associated with the actin / microtubule cytoskeleton play important roles in maintaining neuronal polarity. Proper coordination of cytoskeletal shape changes is essential for normal neuronal migration, axon guidance and growth cone motility (Dent and Gertler, 2003, Kosodo et al., 2004, Sakagami, 2008). USP9X is known to interact with key regulators of cell polarity found in both NPs and neurons. A recent study has shown that over-expression of USP9X in NPs results in a significant increase in polarised cellular architecture (Jolly, 2009).

**AF-6**
Acute lymphoblastic leukemia-1 fusion partner from chromosome 6 (AF-6) and β-catenin are two adherens junction proteins found in NPs that have been identified as substrates of USP9X in other polarised epithelial cells (Taya et al., 1999; Taya et al., 1998). Besides a role in cell-cell adhesion AF-6 has also been identified as a Ras target (Kuriyama et al., 1996). Knockdown of USP9X using antisense oligonucleotides in the pre-implantation mouse embryo resulted in a reduction of AF-6 and β-catenin, an inhibition of cell-cell adhesion and a decrease in cell cleavage events (Pantaleon et al., 2001). In epithelial cells USP9X co-localises with AF-6 at the points of cell-cell contact and inhibits the ubiquitylation of AF-6 (Murray et al., 2004; Taya et al., 1998).

AF-6 null embryos die around E10. At earlier stages the ectoderm (the primary germ layer that eventually gives rise to the nervous system) is disorganised and the neuroepithelium, though able to arise prior to lethality, is also poorly developed (Ikeda
et al., 1999, Zhadanov et al., 1999). Besides NPs, AF-6 is also found in neurons where it co-ordinates with other adhesion partners including N-cadherin to modulate dendritic spine morphology and synaptic remodelling (Xie et al., 2008). USP9X has been shown to bind AF-6 in the bovine brain, however it remains to be determined if this interaction between USP9X and AF-6 is essential for normal neural development (Taya et al., 1998).

**β-catenin**

β-catenin is a multifunctional protein that plays an essential role in cell-cell adhesion in the CNS as well as in many other tissues. β-catenin is also a key effector of the Wnt signalling pathway (See section 1.1.10) (Behrens et al., 1996, Gottardi and Gumbiner, 2004). In NPs β-catenin binds with E-cadherin and α-catenin to form a larger complex with Vinculin and other proteins associated with the actin cytoskeleton (Adams and Nelson, 1998, Conacci-Sorrell et al., 2002). Loss of β-catenin in the developing CNS results in the disruption of cell-cell adhesion and the loss of neuroepithelium integrity (Junghans et al., 2005). Over-expression of β-catenin in the CNS during mouse development results in an increase in cerebral cortex tissue mass. These mice contain a greater portion of NPs suggesting that β-catenin enhances proliferation (Chenn and Walsh, 2002, Chenn and Walsh, 2003). Whilst up-regulation of β-catenin is thought to promote NP proliferation, down-regulation is thought to promote differentiation (Wrobel et al., 2007, Zechner et al., 2003).

In kidney epithelial cells (MDCK) and fibroblasts (mouse EL) β-catenin has been shown to immuno-precipitate with USP9X. Over-expression of USP9X in these cell lines results in an increase in β-catenin levels suggesting that USP9X stabilizes β-catenin (Taya et al., 1999). Further investigation is required to determine if this stabilization is achieved by USP9X mediated de-ubiquitylation. β-catenin and USP9X immuno-precipitate together in sub-confluent intestinal epithelial cells (T84) but do not in confluent cells (Murray et al., 2004). Similarly another study showed USP9X co-localised with its substrate EFA6 at tight junction contact points in sub-confluent kidney cells (MDCK) but USP9X was absent at confluence (Theard et al., 2010). These findings suggest that USP9X is only required to interact with β-catenin as cell-cell adhesion is being established. Interestingly a partial knockdown of USP9X in MDCK cells does not affect the levels of β-catenin (Theard et al., 2010). This suggests that the function of USP9X is also concentration dependent (Al-Hakim et al., 2008). As a
number of USP9X’s substrates are expressed in the same tissues and USP9X is possibly interacting with multiple substrates simultaneously, only certain substrates may require the expression of USP9X at a high level. As β-catenin and USP9X are co-expressed in a range of tissues besides the brain it remains to be determined if a specific interaction between USP9X and β-catenin plays a role in neural development.

In *Drosophila* USP9X’s homologue faf is essential for normal eye development however β-catenin (called armadillo) is not an essential substrate. This suggests that though co-expressed in any given tissue the interaction between USP9X and its substrates may not always occur or be critical (Chen et al., 2000). The interaction between USP9X and its substrates may be regulated in a cell-specific manner or even a stage-specific manner by the presence of any antagonists of USP9X activity, the presence of other positive and negative regulators of ubiquitylation or any other tissue-specific protein interactions these substrates may be involved in (DiAntonio et al., 2001).

**AGS3**  
Activator of G protein Signalling 3 (AGS3) is a receptor-independent G protein activator widely expressed in a range of tissues where it is thought to play a role in Golgi structure and function (Groves et al., 2007, Pattingre et al., 2003). In the CNS AGS3 is also involved in mitotic spindle orientation and asymmetric cell division in cortical progenitors (Sanada and Tsai, 2005).

AGS3 has recently been identified as a USP9X substrate. Knockdown of USP9X results in a moderate reduction in AGS3 levels while over-expression of USP9X leads to an increase in AGS3 suggesting that USP9X plays a stabilising role in AGS3 function (Xu et al., 2010). These interactions were identified in a range of cells including human embryonic kidney cells (HEK293), pheochromocytoma cells from the rat adrenal medulla (PC12), immortalised African Green monkey kidney cells (COS7) and immortalised cervical cancer cells (HeLa). USP9X was also shown to immunoprecipitate with AGS3 from the rat brain though the specific function remains unknown (Xu et al., 2010). USP9X and AGS3 not only interact to some degree in the CNS but are both thought to play a role in polarity. Both are also thought to associate with the Golgi apparatus (Murray et al., 2004). This places AGS3 as a potential candidate for USP9X regulation during neural development.
Exchange factor for ARF6 (EFA6) modulates tight junction formation. Tight junctions primarily function as a permeability barrier. Tight junction synthesis, re-organisation and de-synthesis dynamics play prominent roles in regulating both proliferation and differentiation (Tsukita et al., 2008). The EFA6 family members (EFA6A, EFA6B, EFA6C, EFA6D) are expressed in a range of tissues including the brain, intestines, kidney, liver, lung, spleen and thymus (Derrien et al., 2002, Sakagami et al., 2006). In epithelial cells USP9X de-ubiquitylates EFA6A and EFA6B protecting EFA6A/B from proteasomal degradation. Loss of either USP9X or EFA6B impairs tight junction synthesis whilst over-expression of EFA6A restores tight junction synthesis in USP9X deficient cells. Interestingly this study showed that the interaction between USP9X and EFA6A/B is transient most likely occurring at specific stages in the cell-cell adhesion cycle (Theard et al., 2010).

ARF6 is a GTPase that requires activation by guanine nucleotide exchange factors including members of the EFA6 family. In the CNS ARF6 mediates a range of neuronal functions including cytoskeletal modelling and membrane trafficking. A specific role for cytoskeletal reorganisation during axonal elongation and branching has been shown (Hernandez-Deviez et al., 2004). Three members of the EFA6 family (EFA6A, EFA6C and EFA6D) are highly expressed in the brain and EFA6C is thought to be neuron specific. In mouse EFA6A is predominantly expressed in forebrain regions including the olfactory bulb, cerebral cortex, hippocampus and striatum (Sakagami et al., 2004). EFA6D is also highly expressed in the olfactory bulb, cerebral cortex and hippocampus though the regional expression within the hippocampus various for EFA6A and EFA6D (Sakagami et al., 2006). Though EFA6 expression is generally high during early neural development, none of the EFA6 members are expressed in the proliferative zones and EFA6A expression in particular peaks in the cerebral cortex in the second and third weeks postnatal suggesting a role in late stage neuronal differentiation (Matsuya et al., 2005, Sakagami, 2008). Further investigation is required to ascertain if a relationship exists between USP9X and any of the EFA6 family members in the CNS.

MARK4
A recent study has identified Microtubule-affinity-regulating kinase 4 (MARK4) an AMPK-related kinase as a substrate of USP9X. MARK4 is thought to play a role in polarity and proliferation. De-ubiquitylation of MARK4 by USP9X in kidney cells
(HEK293) not only to controls MARK4 stability but also regulates its activation (Al-Hakim et al., 2008). A role for MARK4 has also been implicated in neuronal differentiation (Moroni et al., 2006). USP9X immuno-precipitates with MARK4 from rat brain yet further investigation is required to determine the functional significance of this relationship (Al-Hakim et al., 2008).

1.1.9 Regulation of cell survival in the CNS

Dying cells are often found in proliferating tissues. During normal CNS development programmed cell death (apoptosis) occurs in both NPs and mature neurons and glia. Normal NPs may undergo proliferative apoptosis as a mechanism for regulating cell specific numbers during development. For example, in the late embryonic and newborn rat cortex, apoptosis is most widespread in proliferating cells in the ventricular zone and sub-ventricular zone (Thomaidou et al., 1997).

Various factors induce apoptosis in the mature cells. Neurons in the embryo, newborn and adult may undergo apoptosis triggered by a lack of synaptic inputs or oxidative stress (Mattson, 2000, Raff et al., 1994). USP9X is known to stabilise and activate several proteins that regulate cell survival in the CNS.

MCL1

The anti-apoptotic protein Myeloid cell leukemia 1 (MCL1) is involved in immune system development and neural development and has also been identified as an oncogene (Arbour et al., 2008, Opferman et al., 2003, Schwickart et al., 2009). Both USP9X and MCL1 are up-regulated in a range of human cancers including those of the colon, lung and lymph nodes. A distinct interaction between USP9X and MCL1 has been experimentally shown in several proliferative and tumorogenic cell lines including human embryonic kidney cells (HEK293T), colon carcinoma cells (HCT116) and HeLa cells. In these cell lines USP9X was shown to de-ubiquitylate and stabilise MCL1 promoting cell survival. Knockdown of USP9X resulted in a reduction of MCL1 levels and an increase in cell death but did not affect the cells capacity to proliferate. Knockdown of USP9X in vivo using a xenograft tumour model (BxPC-3 pancreatic carcinoma) also results in a reduction of MCL1. The loss of USP9X and subsequently
MCL1 resulted in a small reduction in tumour growth most likely due to increased apoptosis as observed by cleaved caspase-3 reactivity (Schwickart et al., 2009). In another study WP1130 mediated inhibition of the ubiquitin cycle in chronic myelogeneous leukemia patient derived cells inhibited USP9X de-ubiquitylase activity resulting in down-regulation of MCL1 levels and an increase in cell death (Sun et al., 2011). These finding identify USP9X as a potential prognostic and therapeutic target in human tumours over-expressing MCL1.

In the CNS MCL1 regulates apoptosis during development. In the developing cortex MCL1 is highly expressed in neural progenitors and newborn neurons. Newborn neurons deficient in MCL1 undergo apoptosis as they migrate away from the ventricular zone showing that MCL1 is required for neuronal survival following differentiation (Arbour et al., 2008). Though a distinct relationship between USP9X and MCL1 has been identified during tumourogenesis it remains to be determined if USP9X regulates MCL1 stability during neural development.

ASK1

Apoptosis signal-regulating kinase 1 (ASK1) is thought to mediate cell death in response to oxidative stress and a range of other intra- and extracellular stresses. In oxidative stress-induced cell models (HEK293A embryonic kidney cells) USP9X stabilises active ASK1 preventing its ubiquitin dependent degradation. Loss of USP9X and subsequent loss of ASK1 results in a reduction in oxidative stress induced apoptosis (Nagai, 2009).

In neural systems, experimental animal models of brain inflammation show a close link between ASK1 activity and apoptotic neuro-inflammation (Guo et al., 2010). In particular endoplasmic reticulum (ER) stress has been identified as a significant factor contributing to the pathogenesis of motor neuron disease and a range of other neurodegenerative diseases (Sekine et al., 2006). ER stress is caused by an accumulation of aberrant proteins. ER stress activates an ASK1 dependent cascade that results in motor neuron death (Nishitoh et al., 2008). Further investigation is required to determine if ASK1 stability is regulated by USP9X in the CNS.
Survivin

During neural development Survivin functions as an inhibitor of apoptosis. Mice lacking Survivin in the CNS die shortly after birth. These mice display reduced brain sizes and an increase in apoptosis in the cerebrum, cerebellum, brainstem and spinal cord (Jiang et al., 2005). During mitosis Survivin operates in a complex to assist proper chromosomal segregation (Beardmore et al., 2004). The USP9X-Survivin interaction was first identified using a highly mitotic Xenopus egg model. This relationship was further characterized in human HeLa cells. In HeLa cells the ubiquitylation of Survivin by the ubiquitin ligase Ufd1 does not target Survivin for degradation but rather activates Survivin facilitating its association with the centromeres. USP9X de-ubiquitylation of Survivin is required for its final dissociation following chromosomal segregation. Down-regulation of either Survivin or USP9X results in an increase in chromosomal misalignment at metaphase and an inhibition of cell proliferation. This indicates that there is a delicate balance between the timing of ubiquitylation versus de-ubiquitylation, and also that this relationship is likely to be concentration dependent. Down-regulation of USP9X however does not affect Survivin’s actual expression levels suggesting that USP9X may only be involved in modulating Survivin’s activity but not its stability (Vong et al., 2005).

Survivin may be a multifunctional protein that plays a role in both mitosis and apoptosis through separate mechanisms. Alternatively the failure of Survivin to dissociate from the chromosome during mitosis (mediated via ubiquitylation / de-ubiquitylation) may be the trigger that induces apoptosis. Mitotic integrity is essential to all tissues during development. However as the positive and negative regulators of ubiquitin function including any antagonists of USP9X activity may be differentially expressed in a tissue specific manner further investigation is required to determine what relationship if any USP9X and Survivin have in the CNS.
1.1.10 Signalling pathways and neural development

Almost every aspect of cellular function is governed by signal transduction. In broad terms signal transduction is a process by which a signalling molecule triggers a cascade of molecular events to initiate a functional change within the cell. The biological advantage of signalling pathways is the ability to regulate a multitude of cellular functions via a single pathway simply through the modification of existing components within that pathway. Furthermore the capacity to differentially express individual components or regulators of those components in a tissue-specific manner gives an even greater dimension to the range of functions a single pathway can initiate.

An enormous number of signalling pathways have been identified in the CNS. The ubiquitin system is one mechanism via which many of these signalling pathways are regulated. USP9X interacts with several key components involved in some of the most significant neural pathways. USP9X has the potential to mediate many aspects of these signalling pathways including continuation, termination, duration, amplification, attenuation as well as influencing the specific end targets of these pathways.

The TGFβ and BMP signalling pathways - Smad4

The Transforming growth factor beta (TGFβ) and Bone morphogenetic protein (BMP) signalling pathways regulate a range of biological processes including cell proliferation, differentiation and apoptosis in both embryos and adults (Graham et al., 1994, Hogan, 1996, Iuzzi and Attisano, 2006, Lee et al., 1998). Not surprisingly, TGFβ / BMP signalling abnormalities have also been implicated in a range of cancers (Derynck et al., 2001). The various tissue type and stage-specific responses inducible by TGFβ / BMP signalling are dependent not only on the types of ligands activated but also the concentration of those ligands and the duration of exposure (Niehrs, 2004, Schmierer and Hill, 2007). Like many signalling pathways the TGFβ / BMP pathways are regulated by both phosphorylation and ubiquitylation (Lin et al., 2006, McCabe et al., 2004).

TGFβ / BMP signalling plays an important role in neural development. TGFβ signalling via the TGFβ receptor TBR2 is essential for normal axon formation (Dwyer and Winckler, 2010). BMP signalling is thought to regulate hippocampal development (Grove and Tole, 1999). Both the TGFβ and BMP pathways promote dendritic growth
Synaptic development in *Drosophila* is regulated by the BMP pathway which requires coordination between positive and negative regulators of ubiquitylation (DiAntonio et al., 2001; McCabe et al., 2004).

**Smad4**

Smad4 is an essential component of both the TGFβ and BMP signalling pathways (Dupont et al., 2009, Guo et al., 2001, Massague, 1998, Schmierer and Hill, 2007, Yi et al., 2010). Upon receptor activation (TGFβ signalling) a series of phosphorylation events activates the intracellular Smad proteins Smad2 and Smad3, which then associate with de-ubiquitylated (activated) Smad4 forming an active complex before translocating to the nucleus. In the nucleus the active Smad complex interacts with transcription factors that initiate a range of target gene responses (Dupont et al., 2009, Feng and Derynck, 2005, Whitman, 1998). Similarly receptor activation (BMP signalling) leads to phosphorylation of Smads 1, 5 and 8, which then associate with activated Smad4 before translocating to the nucleus to activate various transcriptional target gene responses.

A recent study has shown that Smad4 is activated via de-ubiquitylation by USP9X. A screen using siRNA to inhibit 75 known or predicted human de-ubiquitylating enzymes identified USP9X as most important for modulating TGFβ pathway responses. USP9X was required to induce multiple TGFβ target gene responses including PAI1, JunB, p21\textsuperscript{Waf1}, p15\textsuperscript{INK4B}, Smad7 and Smurf1. De-ubiquitylation by USP9X was shown to activate Smad4 rather than stabilise it as the loss of USP9X did not affect the levels of Smad4. Mono-ubiquitylation of Smad4 at lysine 519 by the ubiquitin ligase Ecto (shown in HaCaT keratinocytes *in vitro*) inhibits Smad4 interaction with activated Smad2/3 negatively regulating TGFβ pathway responses. It is thought that USP9X and Ecto act on Smad4 from opposite cellular compartments. USP9X is thought to de-ubiquitylate Smad4 in the cytoplasm assisting in Smad4/Smad2/3 formation and shuttling to the nucleus whilst Ecto serves as an antagonist, ubiquitylating Smad4 in the nucleus disrupting the Smad4/Smad2/3 complex (Dupont et al., 2009).

The E3 ubiquitin ligase Highwire (Hiw) negatively regulates synaptic growth in *Drosophila* via binding with the Smad homologue Med. The binding and subsequent ubiquitylation renders Med inactive. Loss of Hiw results in synaptic overgrowth. In contrast over-expression of faf (the USP9X homologue in *Drosophila*) also results in
synaptic overgrowth further highlighting the role of ubiquitylation / de-ubiquitylation in the regulation of this pathway. However loss faf does not result in synaptic reduction suggesting additional de-ubiquitylating enzymes may play a role activating this pathway in the absence of faf (DiAntonio et al., 2001; McCabe et al., 2004). Other studies have shown that Smad activation during BMP signalling is essential for normal dendritic growth (Guo et al., 2001).

Though faf mediated regulation of the TGFβ / BMP pathways has been shown to effect neural development in Drosophila and an interaction between USP9X and Smad4 has been identified in specific mammalian tissues it remains to be determined if a USP9X - TGFβ / BMP engagement plays a role in mammalian neural development.

The Notch signalling pathway - Epsin, Itch and Mindbomb1
Epsin, Itch and Mindbomb1 are regulators of the Notch signalling pathway, a pathway highly conserved in vertebrates (Chen et al., 2002, Choe et al., 2007, Lardelli et al., 1994, Mouchantaf et al., 2006, Yoon and Gaiano, 2005). During neural development the Notch signalling pathway plays an important role in cell fate choices (Lardelli et al., 1996). Notch1, a key component of this pathway is highly expressed in the developing brain in both humans and mouse and is expressed at lower levels in the adult brain (Berezovska et al., 1998, Berezovska et al., 1997, Higuchi et al., 1995, Williams et al., 1995). In the developing mouse brain highest Notch1 expression is observed in neural progenitors (NP) within the ventricular zone (Lindsell et al., 1996, Tokunaga et al., 2004). Notch signalling is thought to regulate the balance between NP proliferation and early neuronal differentiation where high Notch activation maintains NPs in a proliferative state (Artavanis-Tsakonas et al., 1999, Basak and Taylor, 2007, Breunig et al., 2007, Hitoshi et al., 2002, Lowell et al., 2006, Shimojo et al., 2008). Over-expression of Notch1 in the mouse embryo results in an increase in NP proliferation leading to an expansion of the ventricular zone and the collapse of the third and fourth ventricles whereas loss of Notch activity results in premature differentiation (Cai and Grabel, 2007, Lardelli et al., 1996, Ross et al., 2003, Yoon and Gaiano, 2005). It has also been suggested that Notch signalling plays a role in axon guidance (Giniger, 1998). Notch1 and its ligand Jagged1 are expressed in cell bodies as well as the processes of post-mitotic neurons however, over-expression of Notch1 inhibits neurite outgrowth suggesting that Notch function is concentration-dependent (Berezovska et al., 1999). Notch signalling is also thought to regulate gliogenesis (Gaiano and Fishell, 2002,
Considering the range of functions it is not surprising that aberrant Notch signalling has been implicated in a range of developmental disorders and neuro-degenerative diseases (Joutel and Tournier-Lasserve, 1998).

The Notch1 receptor consists of an extra cellular domain, a single transmembrane domain and an intracellular domain. In mammals members of the Delta-like and Jagged family of ligands activate the Notch receptors (Baron, 2003). Upon ligand binding the Notch intracellular domain (NotchIC) is cleaved and translocated to the nucleus where it associates with the CSL family of transcription factors. In the nucleus this association (in particular with CBF1, RBPj, several histone acetylases and the nuclear protein Mastermind) activates expression of target genes including Myc, p21 and the HES family of transcription factors which include Hes1 and Hes5 (Artavanis-Tsakonas et al., 1999, Baron, 2003, Fehon et al., 1990, Louvi and Artavanis-Tsakonas, 2006, Schroeter et al., 1998, Wu et al., 2000). Hes5 is highly expressed in all NPs throughout the developing CNS. In the absence of Hes5, NPs prematurely differentiate into neurons (Hatakeyama et al., 2004).

The Notch signalling pathway involves many enzymatic steps that are not yet completely understood. Ubiquitylation is one of the mechanisms by which this pathway is regulated. The E3 ubiquitin ligase Itch directly ubiquitylates Notch1, the E3 ubiquitin ligase Sel-10 negatively regulates Notch signalling by some mechanism not yet fully understood and both the E3 ubiquitin ligases Mind bomb-1 and Neuralized play a role in endocytosis of the transmembrane Notch receptor ligand Delta (Itoh et al., 2003, Oberg et al., 2001, Pavlopoulos et al., 2001, Qiu et al., 2000). USP9X is known to stabilise and activate several proteins that regulate Notch signalling.

Epsin
Epsin is a trafficking protein originally identified as an essential endocytosis component in vertebrates and yeast. Epsin has many domains capable of interacting with multiple proteins associated with endocytosis. Epsin is normally mono-ubiquitylated (Chen et al., 1998, Ford et al., 2002, Wendland et al., 1999). De-ubiquitylation by faf (the USP9X homologue in Drosophila) activates Lqf (the Epsin homologue in Drosophila) at the plasma membrane where it triggers endocytosis of Delta which then activates the Notch receptor as mentioned above (Cadavid et al., 2000, Chen et al., 2002, Eun et al., 2007, Overstreet et al., 2004). In Drosophila Lqf mediated endocytosis of Delta is essential
during eye development. Both rat and human Epsin are able to substitute for Lqf function in *Drosophila* suggesting a highly conserved role for this protein (Chen et al., 2000, Overstreet et al., 2003). USP9X and Epsin immuno-precipitate together in the rat brain where co-localization is also observed at the synapses (Chen et al., 2003).

As Notch1 plays a distinct role in NP proliferation but is also expressed in the processes of postmitotic neurons and Epsin is expressed in NPs as well as neuronal synapses the question is raised as to the limits for Epsin regulation of Notch activity. Besides playing a role in the Notch pathway Epsin is also involved in other protein trafficking events. This places USP9X with the potential through Epsin to regulate multiple aspect of neural function.

Itch
The E3 ubiquitin ligase Itch ubiquitylates Notch1 as well as its own auto-ubiquitylation (Qiu et al., 2000). Itch was originally identified as having a role in immune response and inflammation. Besides CNS development the Notch pathway is also involved in other developmental processes including immune system development (Laky and Fowlkes, 2008, Nobta et al., 2005, Perry et al., 1998). The Itch-Notch interaction may be specifically required for immune system development. A specific link for Itch-Notch regulation in the CNS is yet to be determined.

In kidney cells (HEK293T) USP9X de-ubiquitylates Itch playing a stabilising role. Both over-expression and down regulation of USP9X were shown to correlate with changes in Itch expression levels (Mouchantaf et al., 2006). In the rat brain an interaction between USP9X and Itch has been identified, however Itch is highly expressed in some tissues where USP9X is not detected suggesting that Itch de-ubiquitylation may be regulated by multiple de-ubiquitylating enzymes (Mouchantaf et al., 2006). Further investigation is required to determine the nature of the USP9X-Itch interaction in the CNS and if that interaction participates in the Notch signalling pathway.

Mind bomb-1
Recent studies have also identified the E3 ubiquitin ligase Mind bomb-1 (an essential component for Notch ligand Delta endocytosis) as a protein shown by affinity purification from rat brain lysates to associate with USP9X (Choe et al., 2007; Yoon et al., 2008). In neurons Mindbomb1 is enriched at the synaptic junction and over-
expression of Mindbomb1 in neurons results in a reduction in neurite length and branching (Choe et al., 2007).

Though it may seem contradictory for USP9X to interact with both positive (Epsin & Mindbomb1) and negative (Itch) regulators of Notch signalling the co-expression of USP9X with these proteins in both immature and differentiated cells presents the possibility that USP9X may interact with one substrate regulating the Notch role in progenitor proliferation whilst interacting with another substrate in post-mitotic cells to facilitate neuronal growth and function. Alternatively some of these USP9X-substrate interactions may not participate in the Notch signalling pathway as some of these substrates also participate in additional cellular functions.

The Wnt signalling pathway and β-catenin
Wnt signalling plays a role in the regulation of NP proliferation and neural fate choices. Wnt signalling is also thought to regulate hippocampal development whilst in the adult regulating neurogenesis in the dentate gyrus (Grove and Tole, 1999, Lie et al., 2005). In *C. elegans* Wnt proteins are also involved in directing both axonal and dendritic trajectories (Hilliard and Bargmann, 2006). Secreted Wnt5 in *Drosophila* and secreted Wnt4 in mouse act as axon guidance molecules that direct neuronal growth cones (Lyuksyutova et al., 2003, Salinas and Zou, 2008, Yoshikawa et al., 2003). Wnt signalling can also facilitate axon growth and guidance in the developing corpus callosum (Keeble and Cooper, 2006).

The ubiquitin system is an important regulator of Wnt signalling (Maniatis, 1999). When β-catenin is not active in the Wnt signalling pathway it is rapidly degraded from the cytoplasm via the ubiquitin-proteasome pathway (Conacci-Sorrell et al., 2002). Considering that β-catenin is a multifunctional protein capable of playing a role in cell-cell adhesion and Wnt signalling simultaneously in NPs, this places USP9X with the potential to play a regulatory role in both events. However as β-catenin and USP9X are co-expressed in a range of tissues besides the brain it remains to be determined what relationship if any exists between USP9X and β-catenin in the CNS.
1.1.11 The ubiquitin system and self regulation

The ubiquitin system is involved in almost all aspects of protein function and cell fate. Considering just the negative regulators of ubiquitylation it is estimated that there is more then 80 de-ubiquitylating (DUB) enzymes in humans. If one DUB USP9X has the potential to interact with multiple proteins in the CNS the potential functions of all the neurally expressed DUBs may be quite significant. Yet another dimension to ubiquitin activity is the systems ability to regulate itself. USP9X is known to directly interact with several ubiquitin ligases including Itch, Mindbomb1 and MARCH7.

MARCH7
Membrane-Associated RING-CH 7 (MARCH7), also known as Axotrophin is an E3 ubiquitin ligase of the RING-CH family highly expressed in a range of stem cells (Ramalho-Santos et al., 2002). Limited studies have raised the possibility of a role for MARCH7 in immune and neural development (Baker et al., 1997, Metcalfe, 2005). MARCH7 is capable of auto-ubiquitylation but may also be ubiquitylated by other ligases. MARCH7 is de-ubiquitylated by USP9X but is also de-ubiquitylated by another DUB enzyme USP7. USP9X de-ubiquitylates MARCH7 in the cytosol whilst USP7 de-ubiquitylates MARCH7 in the nucleus (Nathan et al., 2008). These findings add yet another dimension to USP9X activity suggesting that USP9X is not only able to function in a tissue specific, stage specific and cell-cycle specific manner but also a compartmental specific role. The interaction between USP9X and MARCH7 was identified using human glioblastoma cell lines (U87) and embryonic kidney cells (HEK-293T) (Nathan et al., 2008). Though an interaction between USP9X and MARCH7 is found in glial cells under cancerous conditions it remains to be determined if a specific interaction between USP9X and MARCH7 plays a role in normal neural development.
1.1.12 USP9X summary

USP9X interacts with numerous substrates in a range of tissues and cell lines. USP9X interacts with many of these substrates in a tissue, cell-type, developmental stage and compartmental-specific manner. These interactions may also be regulated in a concentration-dependent manner. De-ubiquitylation by USP9X modulates the activation status of some substrates while maintaining the stability of others, as well as intracellular localisation.

In the CNS USP9X is highly expressed during development while expression remains at lower levels in the adult brain. However in the adult brain USP9X expression remains high in niche neurogenic regions. A cell specific analysis in vitro shows that USP9X is highly expressed in neural stem cells while expression remains at lower levels in more differentiated neural cell types. Many of USP9X’s substrates are known to play important roles in the CNS. Considering USP9X’s expression pattern and its range of substrates makes it difficult to determine the precise roles that USP9X may have during neural development. As several of USP9X’s substrates are co-expressed with USP9X in multiple tissues it remains to be determined which of these substrates require an interaction with USP9X for their normal activity in the CNS. It is possible that USP9X regulates neural development via multiple pathways.
1.2 Neural development

Though USP9X is highly expressed throughout the developing brain this study focuses primarily on the forebrain as a model region to analyze the role of USP9X during neural development. This section begins with an overview of primary brain formation followed by a specific discussion of forebrain development including specific regions of the forebrain of focus in this study.

1.2.1 Neural induction and primary brain formation

During mammalian embryogenesis the single-layered blastula develops into three primary germ layers; the endoderm, mesoderm and ectoderm (a process termed gastrulation). These three germ layers eventually give rise to all the tissues and organs that make up the adult. The nervous system in mammals arises from the embryonic ectoderm which also gives rise to the epidermis (skin). The commencement of nervous system formation (neural induction) begins with a single cell layer of polarized neuroepithelial cells that first form a structure termed the neural plate (also called the neuroectoderm) which then develops into the neural tube (a process termed neurulation). As the neural tube forms, cells along the edge form the neural crest. The neural tube gives rise to the CNS whilst neural crest cells give rise to most of the peripheral nervous system (PNS) (Figure 1.5). These neuro-epithelial cells or neural stem cells (NSC) making up the neural tube rapidly proliferate, later differentiating to form all the neurons and supporting glial cells of the developed brain and spinal cord (Greene and Copp, 2009, Schoenwolf and Smith, 1990). In the mouse gastrulation usually commences around embryonic day E6.5 (day 15 – 16 in humans) and neural induction usually commences around E7.5 (day 17 – 18 in humans). Following neurulation the anterior portion of the neural tube expands and constricts in specific locations to mark off the three primary brain vesicles: the prosencephalon which eventually becomes the forebrain, the mesencephalon which becomes the midbrain and the rhombencephalon which becomes the hindbrain. The remainder of the neural tube becomes the spinal cord (Figure 1.6).
Nestin is widely used as a marker for NSCs as it is first expressed at the onset of neural induction (Lendahl et al., 1990, Lothian and Lendahl, 1997, Zimmerman et al., 1994). Nestin is an intermediate filament protein that constitutes a major component of the cytoskeleton. Transient expression has also been detected at lower levels in intermediate progenitors and other cell types within the CNS prior to terminal differentiation including immature astrocytes and developing neurons though this expression disappears upon differentiation into mature astrocytes and neurons (Clarke et al., 1994, Lendahl et al., 1990, McKay, 1997, Ramalho-Santos et al., 2002). Nestin is eventually replaced by glial fibrillary acidic protein in astrocytes and neurofilaments in neurons (Edwards et al., 1990, Hockfield and McKay, 1985, Lothian et al., 1999, Misson et al., 1988). In vitro the expression of Nestin is lost when cultures are differentiated towards neurons, astrocytes and oligodendrocytes (Johe et al., 1996, Reynolds.B.A, 1992). The Nestin enhancer has been used in numerous studies to drive nervous system specific transgene expression commencing in NSCs (Graus-Porta et al., 2001, Tronche et al., 1999). The use of the Nestin enhancer in this study is discussed in section 2.4.

![Figure 1.5 Neurulation](image)

Figure 1.5 Neurulation

The nervous system and epidermis arise from the embryonic ectoderm. A single cell layer of neuro-epithelial cells (the neural plate or neuro-ectoderm) develops into the neural tube and neural crest. The neural tube gives rise to the CNS whilst neural crest cells give rise to most of the peripheral nervous system. Image modified from McGill Molson Medical Informatics, 2008.
1.2.2 Forebrain development

During CNS development the most anterior of the three primary brain vesicles the prosencephalon further subdivides into the telencephalon and diencephalon which constitute the forebrain. The telencephalon evolves into the left and right cerebral hemispheres (Figure 1.6). The dorsal portion of the telencephalon forms the cerebral cortex while the ventral telencephalon becomes the basal ganglia.

During development newly generated neurons deriving from the ventricular and sub-ventricular zones that line the lateral ventricles migrate through the cortical wall eventually forming six distinct layers that make up the adult cerebral cortex (Francis et al., 1999, Gupta et al., 2002, Hatten, 1999, Haubensak et al., 2004, Huttner and Kosodo, 2005). The six layers form starting with the deepest first with each successive layer forming as new neurons migrate through existing layers (Hatten, 1999, Miller and Nowakowski, 1988). Layers are labelled 1 to 6 from superficial to deep with each layer distinguished by the neuronal subtypes and fibres they contain (Figure 1.7). Neurogenesis is discussed further in the next section.

Figure 1.6 Primary brain vesicles

During development, the neural tube expands and constricts in specific locations to mark off the three primary brain vesicles: the prosencephalon, the mesencephalon and the rhombencephalon. The remainder of the neural tube becomes the spinal cord. The prosencephalon further subdivides into the telencephalon and diencephalon which constitute the forebrain. The telencephalon evolves into the left and right cerebral hemispheres of the cerebrum.

Image modified from McGill Molson Medical Informatics, 2008.
1.2.3 Neurogenesis

Neurons are the core functional cells making up the CNS and the PNS. Most neurons possess a cell body (soma), multiple dendrites and an axon. Neurons transmit signals both electrically and chemically. Electrical signalling is transmitted within individual neuronal processes (axons and dendrites) whilst chemical signalling occurs via synapses which facilitate the transmission of signals between neurons or between neurons and glands or muscle cells.
The mammalian CNS contains a vast array of neuronal subtypes. Neurons may be classified based on their dendritic morphology as either bipolar (with a single dendrite) or multipolar (with multiple dendrites). Multipolar neurons constitute the majority of neurons found in the brain. Bipolar neurons are most commonly found in the retina (Donovan and Dyer, 2005). Neurons are also classified based on axonal morphology. Pyramidal neurons and Purkinje cell neurons have long-projecting axons whilst Granule cell neurons have shorter locally projecting axons. Neuronal subtypes can also be identified by their localisation within the nervous system. For example, Purkinje cell neurons are found in the cerebellum. Cajal-Retzius neurons are the major type found in the marginal zone. Cajal-Retzius neurons are also numerous in the hippocampus (Soriano et al., 1994). Pyramidal cell neurons are the major cortical output (excitatory) neurons representing ~80% of neocortical neurons. Granule cell neurons are the most numerous neuronal type throughout the whole brain. Many of these neuronal subtypes form complex interactions with one another. For example, granule cell neurons in the cerebellum send their axons to synapse with Purkinje cell dendrites.

In the mouse neurogenesis commences around day E10.5 in embryonic development and peaks around day E14.5 - E15.5 (Au and Fishell, 2006, Haubensak et al., 2004). The majority of neurons that make up the cerebral cortex and a large portion of the neurons that make up other regions of the brain such as the cerebellum originate from ventricular zone neural progenitors (Donovan and Dyer, 2005, Francis et al., 1999, Haubensak et al., 2004, Miller and Nowakowski, 1988). The majority of GABA-containing cortical inter-neurons however derive from the striatum (Anderson et al., 2002).

Radial glia are the most common intermediate progenitor found in the developing cerebral cortex (Malatesta et al., 2003, Mori et al., 2005). Radial glia are defined by two dominant roles, firstly playing a supporting role during neuronal migration and secondly as precursor cells as neurogenesis continues and gliogenesis commences (Gaiano et al., 2000, Luskin et al., 1988, Rakic, 1990). In the cerebral cortex radial glia are characterised by their bipolar morphology and long radial processes. The cell bodies of radial glia reside in the ventricular zone whilst the major process extends through the neural wall to the pial surface in one direction and the minor process projects to the ventricular surface in the opposite direction (Gotz and Barde, 2005). Migrating neurons establish junctions with radial glia processes to assist in cell motility. 80 – 90% of
developing neurons migrate along these glial fibers (Anton et al., 1996). Late born neurons often migrate via somal translocation (Nadarajah et al., 2003). Radial glia cells also assist neuronal migration within the hippocampus (Barry et al., 2008). Radial glia may be identified by the expression of brain lipid binding protein (BLBP) which is highly expressed in their processes (Hunter and Hatten, 1995, Xu et al., 1996). Following the peak of neurogenesis, radial glia begin to differentiate into both neurons (commencing around E15 in mouse) as neurogenesis continues and into astrocytes as gliogenesis commences (Fox et al., 2004, Gaiano et al., 2000, Merkle et al., 2004, Noctor et al., 2001, Schmechel and Rakic, 1979, Wang and Barres, 2000). Gliogenesis is discussed in the following section.

1.2.4 Gliogenesis

Shortly after neurogenesis peaks gliogenesis commences. Glial cells are non-neuronal supporting cells that derive from both neural progenitor and intermediate progenitor cells such as radial glia (Gaiano et al., 2000). Little is known about the factors that stimulate gliogenesis. One line of thought is that neurons may induce glial proliferation and differentiation by secreting diffusible factors or by releasing membrane bound factors (Feng and Heintz, 1995, Gasser and Hatten, 1994, Wang and Barres, 2000).

Astrocytes

Astrocytes are the most abundant glial lineage in the CNS. Astrocytes are responsible for regulating the chemical environment. Astrocytes maintain the homeostatic balance by altering ion gradients and assisting in neurotransmitter transduction (Wang and Bordey, 2008). Being the homeostatic cells in the CNS it is not surprising that astrocytic changes are observed in many forms of neuropathology ranging from Alzheimer’s disease to cerebral ischemia (Giaume et al., 2007, Nedergaard and Dirnagl, 2005, Olabarria et al., 2010, Seifert et al., 2006). The most commonly used mature astrocyte marker GFAP (Glia Fibrillary Acidic Protein) is highly conserved between mouse and human (Barry et al., 2008, Messing and Brenner, 2003, Reeves et al., 1989, Sancho-Tello et al., 1995, Voigt, 1989). GFAP is an intermediate filament thought to be important for astrocyte motility and structural stability (Eng et al., 2000). In mouse
GFAP expressing astrocytes are first detected in the developing CNS around embryonic day E16.5 (Bovolenta et al., 1987, Brenner, 1994).

**Oligodendrocytes**

Oligodendrocytes secrete myelin to assist in axonal insulation and electrical signal transduction. A single oligodendrocyte is capable of extending its processes to numerous axons. The majority of cortical oligodendrocytes arise from precursor cells that migrate away from the sub-ventricular zone (Hardy and Reynolds, 1991). It is difficult to determine the precise stages that oligodendrogenesis commences. A range of markers have been identified for oligodendrocyte precursors (Spassky et al., 2001). Myelin basic protein (MBP) and O4, used as mature oligodendrocyte markers are predominantly found postnatally during mouse development (Eylar et al., 1971, Ke et al., 2007, Saxe et al., 1985, Schachner et al., 1981, Sommer and Schachner, 1981).

**Microglia**

Microglia function within the CNS as immuno-competent cells that respond to pathological conditions and also as phagocytic cells that remove cellular and foreign debris (Pivneva, 2008). Microglia also participate in the progression of neurological disorders including Alzheimer’s disease, Parkinson’s disease and multiple sclerosis where they release a range of cytotoxic molecules and pro-inflammatory cytokines (Dheen et al., 2007). For this reason it is thought that microglia have both beneficial and detrimental functions.

1.2.5 The corpus callosum

This study focuses primarily on the forebrain as a model region for analysis. The corpus callosum and hippocampus are two specific regions of the forebrain of focus in this study.

The corpus callosum (CC), also known as the colossal commissure, consists of an axonal tract that crosses the midline joining the two cerebral hemispheres (Figure 1.8). In mouse, CC development begins around embryonic day E15 continuing up to day 14 after birth. In mouse axons within the CC derive predominantly from neurons in layers
II, III and V of the cerebral cortex (Piper et al., 2009, Plachez and Richards, 2005, Rash and Richards, 2001, Yorke and Caviness, 1975). There is a significant delay between the time callosal neurons are first generated and the time their axons cross the midline. For example, in rat axons first cross the midline around embryonic day E17 though these axons derive from cortical neurons that were generated earlier around day E14 (Koester and O'Leary, 1994). In humans the failure to develop the CC occurs in 1 in 4000 individuals (Paul et al., 2007). Atrophy of the CC is associated with old age but is also observed in a range of neural disorders such as multiple sclerosis (Fling et al., 2011, Yaldizli et al., 2011). Two proteins that have been shown to interact with USP9X; β-catenin and DCX are known to play a role in CC development (Deuel et al., 2006, Koizumi et al., 2006a, Koizumi et al., 2006b, Machon et al., 2003).

Figure 1.8 The corpus callosum
Images left to right: Nissl stained coronal sections displaying the rostral, middle and caudal aspects of the corpus callosum (black arrows) in the adult mouse brain. Modified from (Paxinos and Franklin, 2001).
1.2.6 The hippocampus

The hippocampus develops from a fold in the telencephalon in a similar pattern in all mammals. Neural progenitors originating predominantly in the ammonic ventricular zone first migrate to form the subiculum and cornu ammonis (CA) regions of the developing hippocampus. These progenitors later differentiate into both neurons and glia (Barry et al., 2008, Bayer, 1980, Lavado and Oliver, 2007, Zhou et al., 2004). Neurogenesis in the hippocampus proceeds in a superficial to deep manner opposite to the cerebral cortex. As for the cerebral cortex radial glia also assist early neuronal migration within the hippocampus. In mouse hippocampal neurogenesis peaks around embryonic day E16 (Super et al., 1998). By E17 the majority of neurons in the CA1 and CA2 regions have already been produced (Smart, 1982). The dentate gyrus (first appearing around embryonic day E16.5) is the final hippocampal structure to develop. The dentate gyrus comprises mainly of granule neurons while the CA regions are comprised of mainly pyramidal neurons (Hagihara et al., 2009).

Signals are transmitted to the hippocampus from various regions of the brain. The entorhinal cortex is the main interface that forms a network between the hippocampus and the rest of the cortex. The major signalling pathway into the hippocampus (the perforant path) sends signals from neurons residing predominantly in cortical layers II and III with a lesser input from neurons in deeper layers V and VI. The deeper cortical layers however receive the most prominent output from the hippocampus (Hechler et al., 2006). In humans, neurons in cortical layer II project to the dentate gyrus and the CA3 region while neurons in layer III project primarily to the CA2 region and the subiculum but also to the CA1 region (Witter et al., 2000). In mice and rats, neurons from upper cortical layers terminate in the marginal zones of the hippocampus and in the outer molecular layer of the dentate gyrus (Amaral and Witter, 1995). The hippocampus also forms neuronal connections with the striatum (Anderson et al., 2002).

Mouse models have shown that when fully developed the hippocampus (Figures 1.9 and 1.10) plays a role in several important functions including memory and navigation (Goodman et al., 2010). In humans hippocampal atrophy is associated with old age and is also observed in a range of neuro-degenerative diseases such as Alzheimer’s disease and dementia (van de Pol et al., 2011, Zarow et al., 2011). Adult neurogenesis takes
place in very few places in the adult brain, the hippocampal dentate gyrus being one of these. Adult neurogenesis is discussed further in section 1.2.7.

Figure 1.9  The hippocampus
Images A to D: Nissl stained coronal sections displaying rostral, middle and caudal aspects of the hippocampus (black arrows) in the adult mouse brain. Modified from (Paxinos and Franklin, 2001).
1.2.7 Adult neurogenesis

Originally it was thought the adult nervous system was incapable of regeneration. However, adult neurogenesis has now been identified in birds, rodents, rabbits, primates and humans (Eriksson et al., 1998, Goldman and Nottebohm, 1983, Ponti et al., 2008, Rakic, 2002b, Rakic, 2002a). During development most NPs eventually differentiate into a neuronal or glial lineage with only a small portion remaining in an undifferentiated state. In the adult brain the small proportion of NPs that remain undifferentiated spend most of their time in a quiescent state, residing in distinct niches including the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the sub-ventricular zone (SVZ) lining the lateral ventricles (Alvarez-Buylla et al., 2001, Alvarez-Buylla and Lim, 2004, Lledo et al., 2006). NPs also reside in limited regions of the adult peripheral nervous system (PNS) such as the olfactory epithelium (Mackay-Sim, 2010).

NPs within the SGZ of the adult dentate gyrus are thought to mature locally into granule neurons that send out axonal projections to the CA3 region of the hippocampus and dendritic projections into the hippocampal molecular layer (Doetsch et al., 1999, Garcia et al., 2004, Laywell et al., 2000, Markakis and Gage, 1999, Stanfield and Trice, 1988).

NPs within the SVZ lining the lateral ventricles migrate along the rostral migratory stream maturing from neuroblasts into new inter-neurons within the human olfactory
bulb (Alvarez-Buylla and Garcia-Verdugo, 2002, Bedard and Parent, 2004, Bonaguidi et al., 2008, Lledo et al., 2006). One train of thought holds that astrocytes in the SVZ act as slow-dividing progenitors capable of producing neuroblasts (Doetsch et al., 1999; Garcia et al., 2004; Laywell et al., 2000).

The olfactory epithelium (located in the upper region of the nasal cavity in humans) is responsible for the direct detection of odours. Olfactory sensory neurons within the olfactory epithelium (also called olfactory receptor neurons) transmit signals via the olfactory nerve to the olfactory bulb within the CNS. NPs within the olfactory epithelium are able to give rise to new olfactory sensory neurons throughout adult life. As this region of the PNS has a degree of exposure to the external environment the need to maintain a regenerative capacity is important (Schwob, 2002). As the olfactory mucosa is easily biopsied, NPs and other cells from this region are of distinct interest for their potential use in cell transplantation therapies (Mackay-Sim and St John, 2011, Murrell et al., 2008, Wetzig et al., 2011).

Little is known about the factors that regulate adult neurogenesis. USP9X is ubiquitously expressed at low levels throughout the adult mouse brain however expression remains high in the hippocampus (Friocourt et al., 2005) (Figure 1.3). The highest expression is found in the CA3 region of the hippocampus (Xu et al., 2005) (Figure 1.11). USP9X expression at the mRNA level in the adult hippocampus in mouse can be compared in the Allen Brain Atlas at http://mouse.brain-map.org. Further study is needed to determine the role USP9X plays in the adult hippocampus and what role if any USP9X contributes towards adult neurogenesis.

![Figure 1.11 USP9X is highly expressed in the hippocampal CA3 region in the adult mouse brain](image)

USP9X protein expression is more abundant in the CA3 region than any other region of the adult hippocampus. Image modified from (Xu et al., 2005).
1.3 Aim

Diseases of the nervous system accounted for 4.1% of all registered deaths in Australia for the year 2008 (Australian Bureau of Statistics). According to the Brain Foundation of Australia 40 000 Australians annually suffer a stroke with a significant portion of these resulting in some permanent form of brain damage. For the year 2009 strokes were the second leading cause of deaths in Australia whilst Dementia and Alzheimer’s ranked third (Australian Bureau of Statistics). In 1999 the Australian Institute of Health and Welfare reported that approximately 1.9% of the Australian population was living with a disability related to an acquired brain injury. According to Brain Injury Australia this figure has only increased with over 500 000 Australians now suffering from an acquired brain injury, with three quarters of these being under 65 years of age and two thirds of these acquiring the injury prior to the age of 25. With only a limited capacity for neurogenesis in the adult CNS it is important to identify factors that regulate neural development. This study aims to investigate the role of USP9X during neural development using the developing CNS in mouse as a model.

USP9X is highly expressed during ovulation and implantation during normal mouse development. A previous study found knockdown of USP9X using antisense oligonucleotides in the pre-implantation mouse embryo resulted in pre-implantation lethality (Pantaleon et al., 2001). In contrast to this finding, mutations in $f_{a}f$ ($USP9X$ homologue) prevented the fertilised egg from undergoing normal embryogenesis in $Drosophila$ (Fischer-Vize et al., 1992). To circumvent the early developmental stage in mouse and analyse the role of USP9X in the developing brain we utilized a conditional knockout (cKO) strategy using the Cre/loxP recombination system (Orban et al., 1992, Sauer, 1998). Two cKO models were utilised: the first permanently disabling $USP9X$ in the whole CNS during development using $Nestin-Cre$ mediation and the second disabling $USP9X$ in the dorsal telencephalon (dorsal forebrain) during development using $Emx1-Cre$.

Considering that USP9X is known to interact with at least 13 substrates in a range of mammalian tissues and cell lines (Table 1.1) and all of these substrates are expressed in the mammalian CNS, it is reasonable to commence this study with a rather broad hypothesis proposing that USP9X is likely to play a significant role during mammalian neural development. As the role of a de-ubiquitylating enzyme is likely to be defined by
the role of its substrates and USP9X has numerous substrates, many of which are multifunctional, a broad hypothesis was acceptable prior to analysis. Hence this study initially focuses on characterising the phenotypical / morphological response to loss of USP9X with more refined hypotheses considered thereafter.

In this study USP9X is knocked out in NPs early in CNS development. Considering that NPs have the potential for use in a variety of applications including cell transplantation therapies it is important to investigate factors that regulate NP function. In cell transplantation medicine NPs may be used to treat a range of neurological disorders including Parkinson’s disease, Alzheimer’s disease, epilepsy, motor neuron disease, schizophrenia, multiple sclerosis, spinal cord injury, acquired brain injury and stroke. However considering the tumour associated risks of stem cell / progenitor cell transplantation approaches, researchers also focus attention on transplantation therapies using post-mitotic neural cell types (Arnhold et al., 2004, Ferrari et al., 2006). The knockout approach used in this study allows us to examine the effects of loss of USP9X on NPs as well as neurons and glial cells that derive from these progenitors.
Chapter 2

Materials and Methods

2.1 Animal ethics

Ethical clearance (AEC-ESK/01/09) (AEC-ESK/11/09) was granted for animal experimentation by the Griffith University Animal Ethics Committee (AEC) and research was conducted in accordance with the approved protocol at the Eskitis Institute for Cell and Molecular Therapies (ESK), Griffith University.

2.2 Conditional knockout of USP9X using the Cre/loxP recombination system

In this study we achieve conditional knockout (cKO) of USP9X in the developing CNS using the Cre/loxP recombination system (Bjorkman et al., 2002, Hebert and McConnell, 2000, Junghans et al., 2005, Kwan, 2002, Nagy, 2000, Savitt et al., 2005, Scacheri et al., 2004). The crossing of two separate mouse strains is required to achieve conditional gene knockout using this system: One mouse strain carrying a transgene expressing the P1 bacteriophage Cre-recombinase enzyme (Cre) under the regulatory control of a tissue/stage specific promoter is crossed with a second mouse strain carrying a loxP modified target gene in which an essential sequence of this gene is flanked by loxP sites (Nagy and Mar, 2001, Van Duyne, 2001). In this study we crossed Nestin-Cre mice and Emx1-Cre mice with USP9X^{loxP/loxP} mice (Figure 2.1). In cells expressing Cre the loxP flanked target sequence is removed and the gene is ligated together again recreating a continuous DNA strand. Loss of the loxP flanked sequence is designed to remove an essential part of the gene rendering the gene “knocked out”. The “knocked out” gene will only produce, if any, a dysfunctional mRNA or protein product. In the absence of Cre the loxP modified target gene functions as normal (Babinet, 2000, Kilby et al., 1993). The USP9X^{loxP} knock-out allele is discussed further in the following section.
Figure 2.1 Conditional knockout of USP9X in the CNS using the Cre/loxP system

Nestin-Cre or Emx1-Cre mice were mated with USP9XloxP/loxP mice. In CNS tissues expressing Cre the loxP flanked target sequence is removed and the gene is ligated together again recreating a continuous DNA strand. In the absence of Cre expression in all other tissues the loxP modified target gene functions as normal.

2.3 The USP9XloxP knock-out allele

USP9XloxP mice were generated by Ozgene Pty Ltd, Bentley, Australia following a strategy designed by Dr Stephen Wood (See acknowledgements). USP9X is a large 45 exon gene coding for a 2554 amino acid protein (Wood et al., 1997). The start codon is located in the second exon. loxP sites were incorporated into the second and third introns flanking exon three (Figure 2.2). The insertion of loxP sites into non-coding regions allows for normal transcriptional processing in the absence of Cre-recombinase (Cre). The presence of Cre facilitates the removal of the loxP flanked sequence including the whole of exon three (Figure 2.3). Loss of exon three generates a downstream frame shift. Should translation take place from the start codon located in exon two only the first 93 base pairs (31 codons) will be in frame. Should any translation take place downstream from an alternative start codon the removal of exon three renders the next six alternative start codons out of frame. To ascertain if
translation could take place from an in frame start codon further downstream from the first six out of frame alternative start codons a USP9X C-terminal antibody was used to determine the level if any that this may take place.

In this study we use two distinct Cre models, Nestin-Cre to knock out USP9X in the whole CNS and Emx1-Cre to knock out USP9X in the forebrain only (See sections 2.4 & 2.5). These models allow the retention of USP9X function in all other tissues. These models first induce Cre expression in neural stem cells / neural progenitor cells prior to the onset of differentiation (Chou et al., 2009). The complementary use of both whole brain and forebrain conditional models has been successfully used in other neural studies (Arbour et al., 2008).

![Figure 2.2 USP9X<sup>loxP</sup> gene modification](chart)

**Figure 2.2 USP9X<sup>loxP</sup> gene modification**

The start codon is located 93 base pairs from the end of the second exon in the USP9X gene. The USP9X gene was modified by incorporating loxP sites into the second and third introns flanking exon three.
Figure 2.3 Cre mediates removal of exon three from the USP9X<sup>loxP</sup> gene
Cre expression facilitates the removal of the loxP flanked sequence including the whole of exon three.

2.4 Nestin-Cre mediated conditional knockout of USP9X in the developing CNS

*Nestin-Cre* mice first developed by Tronche *et al* were kindly supplied by Professor Denis Crane, Griffith University. Expression of Cre in these mice is driven by a rat *Nestin* enhancer designed to drive NP specific expression throughout the CNS in both the brain and spinal cord (Arbour et al., 2008, Berube et al., 2005, Graus-Porta et al., 2001, Isaka et al., 1999, Ke et al., 2007, Tronche et al., 1999). The *Nestin* promoters ability to drive transgene expression in NPs is well characterised. A downstream enhancer element within the second intron of the rat *Nestin* gene is capable of driving Nestin expression in the CNS. This enhancer element (also referred to as the *Nestin* promoter) was shown to drive expression regardless of its orientation (upstream or downstream) in relation to the transcriptional start site. *Nestin-lacZ* reporter mice
display β-galactosidase expression in the forebrain, midbrain and hindbrain at early stages of development (Figure 2.4) starting around day E9.5 in embryonic development (Lothian and Lendahl, 1997; Zimmerman et al., 1994). The Nestin promoter has been used in numerous studies to drive expression of various genes within NPs (Josephson et al., 1998, Lardelli et al., 1996, Ringstedt et al., 1997, Ringstedt et al., 1998, Yaworsky and Kappen, 1999). The expression pattern of Nestin-Cre is shown in figure 2.5.

**Figure 2.4 Nestin- lacZ reporter mice**
Expression of the lacZ reporter gene under the transcriptional regulation of the Nestin enhancer from intron two of the rat Nestin gene. Nestin- lacZ transgenic mouse embryos expressing β-galactosidase at days E11.5 (A) and E13.5 (B). Images not to scale. Image from (Zimmerman et al., 1994).

**Figure 2.5 Nestin-Cre / lacZ<sup>loxP</sup> reporter mice**
Nestin-Cre induces β-galactosidase expression during embryonic development in lacZ<sup>loxP</sup> modified mice. In this case the presence of the loxP flanked insert prevents β-galactosidase expression. Removal of the insert by cre-recombinase under the control of the rat Nestin promoter activates the lacZ transgene. This reporter gene is used to show the stage and tissue specific expression patterns of Nestin-Cre. Image from (Graus-Porta et al., 2001).
2.5 *Emx1-Cre* mediated conditional knockout of USP9X in the developing forebrain

*Emx1-Cre* mice were kindly supplied by Shigeyoshi Itohara, RIKEN Brain Institute, Japan. *Emx1-Cre* expression commences in NPs within the developing embryonic dorsal telencephalon / dorsal forebrain (Gorski et al., 2002, Iwasato et al., 2000, Iwasato et al., 2004). Though *Nestin-Cre* expression commences in NPs throughout the whole CNS, the expression patterns of *Emx1-Cre* and *Nestin-Cre* are comparable in the forebrain (Graus-Porta et al., 2001; Tronche et al., 1999). The complementary use of both *Emx1-Cre* and *Nestin-Cre* has been used in previous neural studies (Chou et al., 2009).

*Emx1* is a homeobox gene expressed in NPs in the dorsal telencephalon from embryonic stages E9.5 up to adulthood (Briata et al., 1996, Gulisano et al., 1996, Simeone et al., 1992). The *Emx1* promoter drives gene expression in the future neocortex, the hippocampus and the olfactory bulb (Iwasato et al., 2004). Radial glia, Cajal-Retzius neurons, glutamatergic neurons, astrocytes and oligodendrocytes derive from *Emx1* expressing progenitors (Gorski et al., 2002). Figure 2.6 shows *Emx1* expressing regions in the adult dorsal forebrain of mouse using *Emx1-Cre / lacZloxP* reporter mice.

![Image](Image)

**Figure 2.6** *Emx1-Cre / lacZloxP* reporter mice

*Emx1-Cre* induces β-galactosidase expression in *lacZloxP* modified mice. In this case the presence of the *loxP* flanked insert prevents β-galactosidase expression. Removal of the insert by cre-recombinase activates the *lacZ* transgene during embryonic development. The *lacZ* transgene remains active, shown here in the adult. Image from (Iwasato et al., 2004).
2.6 *Cre*/USP9X\textsuperscript{ΔloxP} mouse mating plan

Considering *USP9X* is an x-linked gene (females *USP9X*/*USP9X*, males *USP9X*/*Y*) we mated homozygous *USP9X\textsuperscript{loxP}/USP9X\textsuperscript{loxP}* female mice with heterozygous *Nestin-Cre* or *Emx1-Cre* (wild type *USP9X*/*Y*) male mice. Male offspring inheriting *Nestin-Cre* or *Emx1-Cre* were used for analysis (*USP9X* knockout males: *USP9X\textsuperscript{cKO}/Y*) and male offspring negative for *Nestin-Cre* or *Emx1-Cre* as littermate controls (Figure 2.7). Though female offspring inherit a *USP9X\textsuperscript{ΔloxP}* gene, all female offspring also retain a paternally inherited wild type *USP9X* gene (*USP9X\textsuperscript{WT}/USP9X\textsuperscript{loxP})*. Hence in the presence of Cre these females will only be heterozygous for the “knockout” of *USP9X* at the genomic level (*USP9X\textsuperscript{WT}/USP9X\textsuperscript{cKO})*. In mouse it is thought that USP9X is subject to X-inactivation, hence females would be chimeric for USP9X expression.

![Figure 2.7 Cre / USP9X\textsuperscript{ΔloxP} mouse mating plan for producing USP9X cKO male mice](image)

*USP9X\textsuperscript{loxP}/USP9X\textsuperscript{loxP}* female mice were mated with heterozygous *Nestin-Cre* or *Emx1-Cre* male mice. The presence of Cre mediates the removal of the *loxP* flanked sequence containing exon 3 \(\rightarrow\) cKO. Male offspring inheriting Cre were used for analysis.
2.7 General analysis and statistics

For all analyses a minimum of three USP9X knockout mice versus three or more littermate controls were used. Differences between knockout mice and littermate controls were assessed statistically using a Student’s $t$ test. Though biological samples may be expected to display a normal distribution as the sample numbers are generally three versus three a normal distribution can only be inferred. For samples with a large variability the Mann-Whitney U test was also used as a non-parametric significance test in comparison with the Student’s $t$ test.

2.8 DNA extraction, genotyping and PCR analysis

DNA was extracted from tail tip tissue. Tail tip DNA samples allow genotyping to determine the sex of embryos and newborns. For embryos, newborns and adults tail tip DNA also allows genotyping to determine the inheritance of transgenes (e.g. Cre-recombinase). To confirm Cre-recombinase (Cre) activity targeting the $USP9X^{loxP}$ gene in vivo, tail tip DNA from Nestin-Cre matings was used. As Nestin-Cre is expressed in all neural stem cells / progenitor cells which reside in both the brain and spinal cord of the developing CNS, tail tip DNA allows confirmation of Cre targeting of the $USP9X^{loxP}$ gene in the CNS via spinal cord genomic DNA.

DNA was extracted using DNA extraction buffer containing 0.5M Tris, 0.2% SDS, 5mM EDTA, 0.2M NaCl and proteinase K (Thermo Scientific). Proteinase K was supplemented according to manufacturer’s recommendations. Tissues were mechanically homogenised then incubated in DNA extraction buffer overnight at 55°C. Samples were then centrifuged at 14000 rpm for 5 min to pellet cellular debris. The DNA containing aqueous phase was transferred to a new tube and centrifuged at 14000 rpm with an equal volume of isopropanol for 20 min to precipitate the DNA. The DNA pellet was washed with 75% ethanol and centrifuged again at 14000 rpm for 5 min to a pellet, air dried and finally resuspended in 100μl of autoclaved MilliQ water. DNA concentration was determined on a Nanodrop 1000 spectrophotometer prior to PCR analysis.
25μl PCR reactions were performed using between 20 and 200ng of DNA as a template. PCR was performed on a BioRad thermal cycler using Taq DNA polymerase (Invitrogen) according to the manufacturer’s instructions. See table 2.1 for primer sequences, annealing temperatures and product lengths. Cycling conditions varied depending on primer melting temperatures and product length but generally were as follows for 30 cycles:

94 °C for 30 seconds (denature DNA template)
X °C for 30 seconds (primer annealing)
72 °C for X seconds (extension)

Products were run at 100 volts for 30 - 45 minutes in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) on a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Gels were then photographed under UV light.

### Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Orientation</th>
<th>Sequence 5’ → 3’</th>
<th>Anneal Temp. (°C)</th>
<th>Product Length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ß-actin</td>
<td>Forward</td>
<td>cta agg cca acc gtg aaa ag</td>
<td>68</td>
<td>558 DNA 104 mRNA/cDNA</td>
</tr>
<tr>
<td>ß-actin</td>
<td>Reverse</td>
<td>acc aga ggc ata cag gga ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cre</td>
<td>Forward</td>
<td>tga tga ggt tgc caa gaa cc</td>
<td>65</td>
<td>389 DNA</td>
</tr>
<tr>
<td>Cre</td>
<td>Reverse</td>
<td>cca tga gtg aac gaa cct gg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRY</td>
<td>Forward</td>
<td>gag gca caa gtt ggc cca gca g</td>
<td>70</td>
<td>266 DNA</td>
</tr>
<tr>
<td>SRY</td>
<td>Reverse</td>
<td>ggt tcc tgt ccc act gca gaa g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP9X</td>
<td>Forward</td>
<td>gct cac cat tag gtt gtt ag</td>
<td>61</td>
<td>505 DNA 207 DNA (minus exon 3)</td>
</tr>
<tr>
<td>USP9X</td>
<td>Reverse</td>
<td>tag acc cat cat gaa cca tg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP9Y</td>
<td>Forward</td>
<td>atg gca ggt tgc aca ttc ac</td>
<td>60</td>
<td>272 mRNA/cDNA</td>
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<tr>
<td>USP9Y</td>
<td>Reverse</td>
<td>gtc ttc att acc ctc caa gat c</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2.1 Primers were synthesized by Sigma. Except for Cre all primers are for mouse.
β-actin primers were used to confirm the DNA integrity of each sample. Male embryos were identified using primers for the SRY region of the Y chromosome. Knockout mice were identified by the presence of the Cre-recombinase (Cre) transgene and further confirmed using USP9X primers designed to detect a 505 base pair (bp) product representing wild type USP9X (Figure 2.8 A) or a 207 bp product representing $USP9X^{loxP}$ with the exon three containing $loxp$ flanked sequence removed (Figure 2.8 C). The $USP9X^{loxP}$ gene contains extra exogenous sequences (associated with the Cre/loxp system) incorporated into intron 3 labelled ‘gene modification cassette’ (Figure 2.8 B) making intron 3 longer in the $USP9X^{loxP}$ gene in comparison to the wild type USP9X gene. As this modification is localised to a non-coding region the $USP9X^{loxP}$ gene (in the absence of Cre) functions as for the wild type USP9X gene.

Figure 2.8 USP9X primers detect a 207 bp product following removal of exon 3
USP9X primers detect a 505 base pair (bp) product representing wild type USP9X (A), or a 207 bp product representing $USP9X^{loxP}$ with the exon three containing $loxp$ flanked sequence removed (C).
2.9 RNA extraction and reverse transcription

Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total lysate was centrifuged with chloroform at 12000g at 4°C for 15 minutes. The upper aqueous phase containing total RNA was transferred to a new eppendorf tube, centrifuged with an equal volume of isopropanol to precipitate the RNA, resuspended with 75% ethanol to wash, re-centrifuged to a pellet again, air dried and resuspended in 20μl of sterile diethyl pyrocarbonate (DEPC) treated water (0.01% v/v). The total RNA concentration was analyzed using a Nanodrop 1000 and a portion was run on a 1% agarose gel to ensure a non-degraded high quality sample defined by intact ribosomal RNA. Total RNA was then subjected to a mRNA purification step using a MicroPoly(A) Purist mRNA purification kit (Ambion) according to the manufacturer’s instructions. Total mRNA was then treated with DNase I (Invitrogen) as a second precautionary step to ensure a pure mRNA sample. The total mRNA concentration was analyzed using a Nanodrop 1000 spectrophotometer. First-strand cDNA synthesis was performed on a BioRad thermal cycler using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions. A 20μl reaction used 40ng of mRNA template, primed with oligo(dT) (Invitrogen). Following 1st strand synthesis PCR was performed using 10% of the reverse transcription reaction as a template. PCR conditions were performed as described in section 2.8. β-actin primers were used to confirm the cDNA integrity of each sample. Samples from brain tissues and testes were screened with primers (Table 2.1) for the y-linked USP9X homologue USP9Y.

2.10 Tissue processing for analysis of brain sections

Heads from embryos and early postnatal mice were drop-fixed in 4% paraformaldehyde (PFA). Adults were anesthetized with ketamine-xylazene and perfused transcardially with 4% PFA before heads were drop-fixed in 4% PFA. Brains were removed from the skull for ages E17.5 and older. Samples were cryo-protected in 30% sucrose then frozen in Tissue-Tek O.C.T. compound (Sakura) then sectioned at 10μm on a Leica CM3050 S cryostat.
2.11 Histology, immuno-fluorescence and image acquisition for brain sections

For coronal analysis sections from a comparable position along the rostral-caudal axis were used (Piper et al., 2010). Sections were matched by counting the number of coronal sections starting at the rostral-most edge of the brain and confirmed by closely matching any unchanged anatomical landmarks (Pramparo, 2010). Histological analyses were performed using standard Cresyl Violet staining (also called Nissl staining) and imaged on an Olympus BX50 microscope with a SPOT camera and SPOT software (Diagnostic Instruments Inc).

For immuno-fluorescence brain sections were washed in PBS with 0.1% Triton X-100, blocked with 2% normal donkey serum (Invitrogen), incubated overnight at 4 °C with primary antibodies, then incubated for 3h at room temperature with fluorescent secondary antibodies and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Images were photographed on a Zeiss AxioImager Z1 microscope.

2.12 Mitotic and apoptotic analysis

Mitotic and apoptotic analyses were performed on Nestin-Cre mediated USP9X cKO mice. For mitotic analyses antibodies that recognize only the phosphorylated form of Histone H3 were used to distinguish mitotic nuclei (Cheung et al., 2007, Veras et al., 2009). For apoptotic analyses antibodies for Cleaved caspase-3 (commonly used in the analysis of apoptosis in the developing CNS) were used (Barry et al., 2008, Piper et al., 2010). At each stage of development at least three USP9X cKO mice were compared with three littermate controls. Counts were performed blind to the genotype of the sample. For each mouse a minimum of three coronal brain sections were analysed. Coronal sections from each mouse were taken from the middle section of the hippocampus along the rostral-caudal axis and were serial / within 10µm of one another. For each brain section counts were made separately from both cerebral hemispheres totalling at least six counts per mouse. The mean value of six counts was calculated for each mouse. The means from all individual cKO mice were combined to calculate an overall mean value for cKO mice versus controls. Specific brain regions such as
hippocampi were defined using Dapi nuclear staining. Considering that markers stain the nuclei and that cellular edges are not discernable cells were counted in the ventricular zone if they resided within 50\(\mu\)m of the lateral ventricle.

2.13 Hippocampal area analysis

Adult hippocampi were defined (Figure 2.9) and hippocampal area was calculated using SPOT software (Diagnostic Instruments Inc). The mean hippocampal area of controls was designated 100. The hippocampal areas of cKO mice were converted to ratios compared to the mean hippocampal area of controls.

![Figure 2.9 Hippocampal area](image)

Adult hippocampal area was defined using SPOT software (Diagnostic Instruments Inc).

2.14 Cell culture

The in vitro analyses in this study were performed by Shane Stegeman and Dr Lachlan Jolly (see acknowledgements).

Isolation of neural cells for in vitro culture by Dr Lachlan Jolly:
Mice were time mated by Shane Stegeman at Griffith University and sent to Dr Lachlan Jolly at the University of Adelaide. For hippocampal derived neuronal cultures, embryos were harvested at E18.5 and hippocampal neurons were cultured as previously described (Kaech and Banker, 2006).
Isolation of neural cells for in vitro culture by Shane Stegeman:
For cortical derived neuronal cultures embryos were harvested at E18.5 and brains were isolated in Hanks’ Balanced Salt Solution (Invitrogen 14175-095) to maintain cells in a viable state at physiological pH. Meninges were removed and cortices were isolated under sterile conditions and placed into TrypLE Express (Invitrogen 12604-013) trypsin replacement, dissociated by pipette trituration then incubated for 15 minutes at 37°C, then an equal volume of serum free Neurobasal media (Invitrogen 21103-049) was added, solution was centrifuged at 200g – 300g to pellet cells, media was removed then cells were resuspended in serum free Neurobasal media containing 2% (vol/vol) B-27 (Invitrogen 17504-044). To determine viable cell concentration a portion was diluted 1:5 in medium, mixed 1:1 with trypan blue and counted on a haemocytometer. Cells were plated on Lab-Tek Permanox™ coated 8 well chamber slides (Nunc 177445). 20 000 cells were plated per well in 300ul of serum free Neurobasal / B-27 media and incubated in a humidified incubator at 37°C with 5% CO₂. Media was changed after the first day and neurons were analysed via immuno-fluorescence on the 4th day.

2.15 Analyses and immuno-fluorescence on cell cultures

Analyses by Dr Lachlan Jolly:
For hippocampal derived neuronal cultures axons and neurites were identified using Tau1 immuno-reactivity and measured using ImageJ software (National Institute of Health). Only neurites of >10µm were included. Cultures from five USP9X cKO embryos were grown in duplicate and compared with littermate control cultures from four different embryos. Twenty neurons were scored per culture.

Analyses by Shane Stegeman:
For cortical derived neuronal cultures cells were fixed with 4% PFA, washed with PBS, permeabilised with 0.1% Triton X-100 in PBS, blocked with 2% normal donkey serum (Invitrogen), incubated for 1 - 2 hours at room temperature with primary antibodies, then incubated for 0.5 - 1 hour at room temperature with fluorescent secondary antibodies and mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Images were photographed on a Zeiss AxioImager Z1 microscope.
2.16 Protein extraction from brain tissues

Protein was extracted from brain tissue samples using SDS-PAGE general lysis buffer containing 10mM Tris pH 7.4, 1% SDS, 10% glycerol and Sigmaplast Protease Inhibitor Tablets (Sigma-Aldrich) according to manufacturer’s recommendations. Tissue was washed with cold PBS, homogenised in SDS-PAGE general lysis buffer, DNA was sheared with a 21 – 27 gauge needle, centrifuged at 12000g at 4ºC for 5 - 20 min to pellet cellular debris and supernatant was transfered to a new tube. Protein concentration was estimated using a Bicinchoninic acid (BCA) kit (Thermo Scientific - Pierce) according to manufacturer’s instructions.

2.17 Western blotting

25 ug of protein was run per well on a 4-12% NuPAGE Novex gradient gel (Invitrogen). A pre-stained protein ladder (PageRuler Plus Prestained Protein Ladder – Fermentas) was used to determine position of target proteins and when to stop running the gel. Protein was transferred from the gel to a nitrocellulose membrane (Amersham Biosciences) using a BioRad transfer apparatus (BioRad). The membrane was then blocked with 3% skim milk powder in PBS 0.1% Tween, incubated with primary antibodies for two hours at room temperature, then incubated with horse radish peroxidase secondary antibodies (Millipore) for one hour at room temperature and developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) according to manufacturer’s instructions before imaging on a VersaDoc 4000 MP Imaging System (BioRad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading normalization control.

2.18 Primary and secondary antibodies

A vast array of markers has been identified for the study of neurons. Listed below is a selection of neuronal markers utilised in this study:
**NF160**

Neurofilaments are intermediate filaments that constitute a major component of the cytoskeletal network in neurons. Neurofilament medium (NF160) is a common marker for mature neurons. NF160 is expressed in the cell body, dendritic processes and axons (Rao et al., 2003).

**Beta-III-tubulin**

Beta-III-tubulin is a microtubule protein found in neurons. It is thought to play a role in axon guidance and maintenance (Katsetos et al., 2003).

**MAP2**

MAP2 (Microtubule-associated protein 2) is the major microtubule associated protein expressed in neurons in the CNS. MAP2 is highly expressed in dendrites where it is involved in microtubule stabilization (Harada et al., 2002, Ke et al., 2007, Matus et al., 1990).

**Tau-1**

There are six Tau proteins (also called MAPT proteins) found in the brain (Goedert et al., 1989). Tau is a neuronal microtubule-associated protein (MAP) that associates with tubulin to stabilise microtubules. Tau-1 is highly expressed in axons.

**NeuN**

NeuN is a DNA binding neuron-specific protein primarily restricted to neuronal nuclei. NeuN expression first appears when early neurons withdrawal from the cell cycle. Expression is found throughout the developing CNS in mouse (Mullen et al., 1992).

**Synaptophysin**

Synaptophysin is a membrane bound pre-synaptic vesicle glycoprotein found in almost all neurons of the brain and spinal cord (McMahon et al., 1996).

The full range of primary antibodies utilised in this study are listed in Table 2.2.
### Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Raised in</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS3</td>
<td>rabbit</td>
<td>(Groves et al., 2010)</td>
</tr>
<tr>
<td>Beta-III-tubulin</td>
<td>mouse</td>
<td>Millipore MAB1637</td>
</tr>
<tr>
<td>BLBP</td>
<td>rabbit</td>
<td>Millipore AB9558</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>rabbit</td>
<td>Cell Signaling Technology 9661</td>
</tr>
<tr>
<td>DCX</td>
<td>rabbit</td>
<td>Abcam ab18723</td>
</tr>
<tr>
<td>FOXP1</td>
<td>rabbit</td>
<td>Abcam ab16645</td>
</tr>
<tr>
<td>FOXP2</td>
<td>rabbit</td>
<td>Abcam ab16046</td>
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<tr>
<td>GapDH</td>
<td>rabbit</td>
<td>R&amp;D Systems 2275-PC-100</td>
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<tr>
<td>GFAP</td>
<td>rabbit</td>
<td>DakoCytomation Z0334</td>
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<tr>
<td>MAP2</td>
<td>chicken</td>
<td>Millipore AB15452</td>
</tr>
<tr>
<td>MAP2</td>
<td>mouse</td>
<td>Sigma-Aldrich M 1406</td>
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<tr>
<td>MBP</td>
<td>rabbit</td>
<td>Millipore AB980</td>
</tr>
<tr>
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<td>mouse</td>
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</tr>
<tr>
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<td>mouse</td>
<td>Millipore MAB377</td>
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<tr>
<td>NF160</td>
<td>rabbit</td>
<td>Abcam ab9034</td>
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<tr>
<td>O4</td>
<td>mouse</td>
<td>Millipore MAB345</td>
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<tr>
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<td>Abcam ab5176</td>
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<tr>
<td>Synaptophysin</td>
<td>mouse</td>
<td>Millipore MAB5258</td>
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<td>Tau1</td>
<td>mouse</td>
<td>Millipore AB1512</td>
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<td>rabbit</td>
<td>Bethyl Laboratories A301-351A</td>
</tr>
<tr>
<td>USP9X-N-terminal</td>
<td>rabbit</td>
<td>(Murray et al., 2004)</td>
</tr>
</tbody>
</table>

Table 2.2 Primary antibodies

### Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody Name</th>
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<th>Source</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-rabbit Alexa-Fluor 594</td>
<td>donkey</td>
<td>Invitrogen</td>
<td>IF</td>
</tr>
<tr>
<td>anti-mouse Alexa-Fluor 488</td>
<td>donkey</td>
<td>Invitrogen</td>
<td>IF</td>
</tr>
<tr>
<td>anti-chicken Cy3</td>
<td>donkey</td>
<td>Jackson Laboratories</td>
<td>IF</td>
</tr>
<tr>
<td>anti-mouse Alexa-Fluor 647</td>
<td>donkey</td>
<td>Invitrogen</td>
<td>IF</td>
</tr>
<tr>
<td>anti-rabbit HRP</td>
<td>goat</td>
<td>Millipore</td>
<td>WB</td>
</tr>
</tbody>
</table>

Table 2.3 Secondary antibodies

IF = immuno-fluorescence  
WB = Western blotting
Chapter 3 - Results and Discussion

Loss of USP9X in the developing embryonic and newborn brain

3.1 Introduction

USP9X is highly expressed throughout the developing embryonic CNS in mouse (Wood et al., 1997). The developing mouse brain at E10.5 – E14.5 consists largely of neural stem cells / neural progenitor cells along with intermediate progenitors and early neuronal subtypes. An *in vitro* analysis shows that USP9X is highly expressed in neural stem cells and at lower levels in more differentiated neural cell types (Ramalho-Santos et al., 2002). In the developing mouse brain USP9X expression is found in mitotic cells as well as post-mitotic neurons (Friocourt et al., 2005; Wood et al., 1997). The expression of USP9X in both neural progenitors and in more mature neural cell types suggests that USP9X may play multiple roles during neural development including a role in neural progenitor maintenance as well as the differentiation to mature cell types. In this study we use the mouse as a model to analyse the role of USP9X during mammalian neural development by knocking out USP9X in neural stem cells / neural progenitor cells in the developing CNS prior to differentiation. Though USP9X is highly expressed throughout the developing brain (and hence we disable *USP9X* throughout the whole CNS) this study focuses primarily on the forebrain (the cerebral cortex and hippocampus) to analyse cellular / morphological changes in response to loss of USP9X.

3.2 USP9X is expressed at high levels in the developing CNS in mouse

USP9X localisation in the CNS at the mRNA and protein levels has been described previously (Friocourt et al., 2005). Prior to analysing the effects of USP9X loss, the localisation during early embryonic brain development was examined to confirm and expand on previous results. A well characterised rabbit polyclonal USP9X antibody
targeting the NH₂-terminal of mouse USP9X was used (Kanai-Azuma et al., 2000, Murray et al., 2004, Pantaleon et al., 2001). USP9X protein was highly expressed at E10.5 and E12.5 in the developing brain along with the neural progenitor cell marker Nestin (Lendahl et al., 1990) (Figure 3.1 A and B). USP9X expression remains high throughout the developing brain at E14.5 (Figure 3.1 C) (Appendix 2 - Supplementary figure 1). These results confirm the high levels of USP9X mRNA at E12.5 and E14.5.

The expression pattern of USP9X protein in the developing brain at E16.5 has been published previously and is consistent with our results (Friocourt et al., 2005). These results confirm high USP9X expression throughout the cytoplasm in neural cells as reported previously (Jolly, 2009) (Xu et al., 2005) and is consistent with the expression pattern reported in other cell types outside the CNS (Theard et al., 2010). The cellular localisation observed in vitro in intestinal epithelial cells shows expression levels of USP9X highest in the Golgi apparatus and other cytoplasmic vesicles responsible for protein processing and trafficking (Murray et al., 2004).
Figure 3.1 USP9X is highly expressed in the developing embryonic brain

Wild-type mouse embryos were stained with antibodies for USP9X (red) and Nestin (green).

A. E10.5 forebrain / telencephalic vesicle (T). B. E12.5 roof of the hindbrain
C. E14.5 hindbrain (left), forebrain (right). 10µm sagittal cryo-sections. Scale bars 20µm.
3.3 *Nestin-Cre* mediates knockout of USP9X in the developing CNS

Knockdown of USP9X using RNA interference in the pre-implantation mouse embryo results in pre-implantation lethality (Pantaleon et al., 2001). Therefore we chose a conditional knockout (cKO) strategy using the Cre/loxP recombination system to knock out USP9X specifically in the CNS during embryonic development. In this study we use *Nestin-Cre* mediation to permanently disable USP9X throughout the whole CNS via exon removal. Considering that *USP9X* is an X-linked gene, matings were designed to produce USP9X cKO male offspring as the primary models for analysis (See figure 2.7). Confirmation of deletion of exon three in genomic DNA was confirmed by PCR (Figure 3.2). (For methods see section 2.8). A 207 base pair product is detected following removal of exon three from the *USP9X* \(^{\text{loxP}}\) gene. The same primer set detects a 505 base pair product representing full length wild type *USP9X* (Also see figure 2.8).

**Figure 3.2 Cre mediates knockout of USP9X at the genomic level**

Genomic DNA was extracted from the CNS of E18.5 embryos. PCR was performed generating a 505 base pair product representing full length wild type *USP9X* from a *Nestin-Cre* negative control embryo and a 207 base pair product representing removal of exon three from the *USP9X* \(^{\text{loxP}}\) gene from a *Nestin-Cre* positive USP9X cKO embryo.

Nestin expression is first detected in the earliest stages of neural plate formation around day E7.5 hence the *Nestin-Cre* transgene may be first induced around this time point. Previous studies have confirmed initial *Nestin-Cre* activity around day E9.5 - E10.5 which is most likely a reflection of detection sensitivity (Graus-Porta et al., 2001; Zimmerman et al., 1994). Following removal of exon three any remaining mRNA and protein needs to be degraded before USP9X is completely depleted from the cell at the protein level. Loss of USP9X protein was confirmed at E18.5 via immuno-blot using a
USP9X C-terminal antibody (Figure 3.3 & Appendix 2 - Supplementary figure 2) and at E16.5 by immuno-fluorescence (Figure 3.4). In mouse, USP9X is also expressed in other tissues including the heart, lungs, ovaries, pancreas, spleen and thymus (Mouchantaf et al., 2006). The specificity of USP9X detection and of the cKO strategy is confirmed by normal USP9X expression outside the CNS while USP9X is lost in CNS tissue (Figure 3.5). The residual protein expression detected in the whole brain cKO immuno-blot (Figure 3.3) is likely due to blood vessels and meninges of the brain (Isaka et al., 1999, Ke et al., 2007, Tronche et al., 1999).

![Figure 3.3 Nestin-Cre mediates knockout of USP9X at the protein level](image)

**Figure 3.3 Nestin-Cre mediates knockout of USP9X at the protein level**

Western blot analyses of whole brain protein extracts from E18.5 embryos. Image = 1 control vs 1 cKO. USP9X = C-terminal antibody. (Also see Appendix 2 - Supplementary figure 2 for two controls vs six cKO mice at E18.5).

![Figure 3.4 Nestin-Cre mediates knockout of USP9X at the protein level in the developing brain](image)

**Figure 3.4 Nestin-Cre mediates knockout of USP9X at the protein level in the developing brain**

USP9X (red) is depleted whilst Nestin expression (green) is retained shown here in the wall of the midbrain in the E16.5 mouse embryo. USP9X control (top panels), USP9X cKO (bottom panels). Dapi nuclear marker - blue, merge - right panels. 10µm sagittal cryo-sections. Scale bar 20µm.
3.4 *Nestin-Cre* mediated loss of USP9X results in early postnatal lethality

*Nestin-Cre* mediated knockout of USP9X throughout the whole CNS during development results in early postnatal lethality. USP9X cKO males (*USP9X<sup>cKO</sup>/Y*) die within approximately 24 hours after birth confirming an essential role for USP9X in the developing CNS. No USP9X cKO males survive beyond postnatal day one. All surviving males genotyped at three weeks of age were negative for the *Nestin-Cre* transgene (Tables 3.1 and 3.2). Dead USP9X cKO males were found in non-Mendelian ratios most likely due to postnatal cannibalization (Stumpo et al., 1995). Several litters were born during the night allowing mothers to eat defective or dead pups before they were collected for analysis. Surviving *Nestin-Cre* negative males (littermate controls) were found in normal Mendelian ratios. Female offspring heterozygous for the knockout of *USP9X* at the genomic level (*USP9X<sup>WT</sup>/USP9X<sup>cKO</sup>*) appeared normal at birth and survived into adulthood. *USP9X* cKO heterozygous females are discussed in section 4.8. Two females and two *Nestin-Cre* negative males were also found dead shortly after birth, but these small numbers most likely represent normal postnatal lethality and are not significant compared to the total number (154) of mice born. A genotyping example using PCR is shown in figure 3.6.
At birth USP9X cKO males were observed moving all four limbs comparable to littermate controls suggesting some degree of neuronal connectivity. However USP9X cKO males did not suckle as evidenced by lack of milk in their stomachs at the time of death. The exact mechanism of lethality could be attributed to a range of reasons (Lu et al., 2007, Planells-Cases et al., 2000, Young et al., 1998, Yu et al., 2004). Defects in CNS development leading to deficiencies in sensory and spatial awareness may result in failure to suckle. Though a degree of neuronal connectivity is evident in USP9X cKO mice an immuno-histochemical analysis shows that USP9X cKO mice have abnormalities in neuronal development (Discussed in section 3.7).

Table 3.1 Nestin-Cre mediated knockout of USP9X results in male postnatal lethality

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>No. of litters</td>
<td>18</td>
</tr>
<tr>
<td>Total no. of mice born (or found)</td>
<td>154</td>
</tr>
<tr>
<td>No. of mice (males and females) found dead by P1</td>
<td>23</td>
</tr>
<tr>
<td>No. of male mice found dead by P1</td>
<td>21</td>
</tr>
<tr>
<td>No. of male mice found dead by P1 genotyped +ve for Nestin-Cre</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 3.2 No USP9X knockout males survive the early postnatal stage

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of litters examined</td>
<td>15</td>
</tr>
<tr>
<td>Total no. of mice born</td>
<td>125</td>
</tr>
<tr>
<td>No. of surviving males at 3 weeks of age</td>
<td>32</td>
</tr>
<tr>
<td>No. of surviving males at 3 weeks of age genotyped +ve for Nestin-Cre</td>
<td>0</td>
</tr>
</tbody>
</table>
Genotyping was performed via PCR using tail tip DNA. Samples 1 – 5 represent five dead pups collected around postnatal day one. All five samples were screened for the endogenous β-actin gene to ensure DNA sample integrity (row A). Male pups were detected (samples 1, 2 and 5) using male specific primers for the SRY region of the Y chromosome (row B). All three male pups and one female pup (sample 3) screened positive for the Nestin-Cre transgene (row C). All three dead male pups that inherited the Nestin-Cre transgene display a 207 base pair product representing the disrupted USP9XloxP gene (exon three removed) in the spinal cord (row D). Female pups are heterozygous USP9XWT/USP9XloxP containing both a wild type USP9X gene and a modified USP9XloxP gene susceptible to Cre targeting (See section 2.6). The Nestin-Cre transgene negative female (sample 4) displays only a 505 base pair product representing full length (active) USP9X while the Nestin-Cre transgene positive female (sample 3) displays both a 505 base pair product and a 207 base pair product (row D). (Red box: band pattern for identifying USP9X cKO males).

3.5 USP9Y is not expressed in the brain in response to loss of USP9X

Knockout of USP9X in males allows us to investigate if the y-linked homologue USP9Y (discussed in section 1.1.4) is able to provide any compensatory response to loss of USP9X. Considering that USP9X cKO mice die shortly after birth it is unlikely that USP9Y which normally displays a testis-specific expression pattern is induced in the CNS (Brown et al., 1998). In human testes though not specifically required for fertility, USP9Y is thought to have a developmental role (Krausz et al., 2006, Luddi et al., 2009, Sun et al., 1999). Though USP9Y in mouse shows an 83% nucleotide identity to USP9X within the coding sequence, USP9Y contains a promoter sequence characteristic of other testis specific genes (Hall et al., 2003). Considering USP9Y normally displays a testis-specific expression pattern in mouse and USP9X cKO mice die shortly after birth it is unlikely that the USP9Y gene is able to provide any compensatory response in the CNS.
In consideration of these points we chose to perform a limited screen at postnatal day P0, just prior to death to address this question.

Reverse transcription - polymerase chain reaction (RT-PCR) failed to detect any USP9Y mRNA from whole brain mRNA extracts indicating that USP9Y is not induced in response to loss of USP9X, at least at P0 (Figure 3.7). We confirmed in the wild type male that USP9Y is expressed in the testes but not in the brain (Brown et al., 1998). This expression analysis combined with other analysis (including male postnatal lethality and abnormal neuronal development discussed in the next section) shows that USP9Y does not compensate in response to loss of USP9X. The lack of homology with USP9X within the promoter region or any other area outside the coding region suggests that USP9Y may play a different role to USP9X which may be restricted in a tissue / testis specific manner.

Figure 3.7 USP9Y is not expressed at P0 in the male wild type or USP9X cKO mouse brain
Reverse Transcription-PCR analysis: USP9Y expression was found in the testis sample (T) taken from a wild type adult mouse. No USP9Y expression was found in the developing brain at P0 in the wild type male mouse (WT) confirming previous published results (Brown et al., 1998) or in the developing brain at P0 in USP9X cKO male mice (cKO). F = female P0 mouse brain - negative control. All samples were screened for β-actin mRNA (cDNA) to ensure sample integrity.
3.6 *Nestin-Cre* mediated loss of USP9X results in a diffuse ventricular architecture

Considering that loss of USP9X results in early postnatal lethality a histological and neuronal analysis was performed on the late embryonic and newborn brain. A preliminary analysis suggested that loss of USP9X results in a diffuse ventricular architecture (Figures 3.8 and 3.9). A litter at E16.5 consisting of two USP9X cKO embryos found a diffuse ventricular architecture in one USP9X cKO embryo whilst another presented a more similar morphology to littermate controls. A litter at E17.5 consisting of one USP9X cKO embryo also found a diffuse ventricular architecture complementing results observed at E16.5. Hence varied results at E16.5 may reflect differences in susceptibility to loss of USP9X due to natural genetic variation. A neuronal analysis (Section 3.7) at E18.5 and P0 revealed significant changes in response to loss of USP9X. Considering the significance of these neuronal findings a decision was made to devote resources to further investigate these changes. Further histological analysis is therefore required in future studies to statistically verify these diffuse ventricular findings.

During development the ventricular zone consists predominantly of neural progenitors (NP). NPs within the ventricular zone are highly polarized. NP polarity is maintained by a range of cell-cell adhesion proteins including adherens junction and tight junction proteins (Zhadanov et al., 1999). USP9X interacts with several regulators of cell-cell adhesion in a range of tissues and cell lines including the adherens junction proteins AF-6 and β-catenin as well as EFA6, a regulator of tight junction formation (Taya et al., 1999, Taya et al., 1998, Theard et al., 2010). Besides regulators of cell-cell adhesion USP9X also interacts with other enhancers of cellular polarity including MARK4, a microtubule-affinity-regulating kinase and AGS3 a regulator of mitotic spindle orientation (Al-Hakim et al., 2008, Xu et al., 2010). Previous studies have shown that USP9X immuno-precipitates with AF-6, AGS3 and MARK4 in the mammalian CNS. However further analysis is required to access the functional relevance of these interactions. Less is known however about any interactions USP9X may have with β-catenin and EFA6 in the CNS. In this study we specifically indentified a reduction in AGS3 cortical expression at E17.5 in response to loss of USP9X (Section 3.12).
Figure 3.8 Loss of USP9X results in a diffuse ventricular architecture at E16.5

One USP9X cKO embryo displayed a diffuse ventricular architecture (B) as shown by the red arrow whilst another (C) presented a more similar morphology to littermate controls (black arrow). Nissl stain. USP9X control (A). USP9X cKO (centre and bottom). 10µm sagittal cryo-sections.
Figure 3.9 Loss of USP9X results in a diffuse ventricular architecture at E17.5

A limited screen at E17.5 of a litter consisting of one USP9X cKO embryo identified a diffuse ventricular architecture (black arrow) comparable to results observed at E16.5.

Nissl stain. USP9X control (top). USP9X cKO (bottom). 10µm sagittal cryo-sections.
3.7 *Nestin-Cre* mediated loss of USP9X results in reduced neuronal processes

*Nestin-Cre* mediated loss of USP9X throughout the whole CNS results in a reduction in neuronal processes showing that USP9X is required for normal neuronal development. The reduction in neuronal processes was observed at day E18.5 in all five USP9X cKO embryos analysed and on postnatal day P0 in all four USP9X cKO newborn mice analysed. (E18.5 is the final day in embryonic development prior to birth). In control mice neuronal processes were seen projecting between the anterior cingulate cortex and the subiculum of the hippocampus while absent or reduced in USP9X cKO mice (Figures 3.10 and 3.11). In Purkinje cell neurons of the cerebellum USP9X expression is found in both the soma and in the axonal processes suggesting a possible role for USP9X in axons (Friocourt et al., 2005).

Following neuronal analysis a glial analysis was performed. Expression of the radial glia marker brain lipid binding protein (BLBP) was localised to the ventricular / sub-ventricular zone of the cerebral cortex at E18.5 in both controls and USP9X cKO mice (Figure 3.12). No obvious differences were seen in the embryonic expression pattern of the the astrocytic marker Glial Fibrillary Acidic Protein (GFAP). At day E18.5 the fimbrial glial bundle in the hippocampus displayed a GFAP expression pattern comparable in both USP9X cKO mice and controls (Figure 3.12) (Barry et al., 2008).
**Figure 3.10 Nestin-Cre mediated knockout of USP9X results in reduced neuronal processes**

A reduction in neuronal processes (white arrow) was observed projecting between the anterior cingulate cortex (C) and the subiculum of the hippocampus (H) in USP9X cKO mice at day E18.5 in embryonic development. USP9X control (top). USP9X cKO (bottom). n = 5. Neuronal marker NF160 - red. 10μm coronal paraffin sections. Scale bar 20μm. The red box in the Nissl stained image above of the E18.5 forebrain defines the specific region from which the above NF160 stained images derive.
Figure 3.11 *Nestin-Cre* mediated knockout of USP9X results in reduced neuronal processes

Loss of USP9X results in a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus. Neuronal processes (white arrows) are clearly observed on sections both rostral (Control 1) and caudal (Control 2) to the mid rostral-caudal hippocampal aspect in controls while greatly reduced in USP9X cKO mice. C = cerebral cortex. H = hippocampus.

P0 mouse brain. USP9X controls (left panels). USP9X cKO (right panels). n = 4.

Neuronal marker NF160 - red. 10µm coronal cryo-sections. Scale bar 100µm.

The red box in the Nissl stained image above of the E18.5 forebrain defines the region from which the above P0 images derive. The black arrow specifies the dominant region from which neuronal processes are lost in USP9X cKO mice at both E18.5 and P0.
Figure 3.12 Nestin-Cre mediated knockout of USP9X – BLBP & GFAP expression at E18.5

A. BLBP expression was localised to the ventricular / subventricular zone of the cerebral cortex at E18.5 in both controls and USP9X cKO mice. USP9X control (left). USP9X cKO (right). n = 3. BLBP - red. 10µm coronal cryo-sections. Scale bar 100µm. The green box highlighted in the Nissl stained image above of the E18.5 embryonic forebrain defines the region from which the above BLBP stained images derive. **B.** No obvious differences were seen in GFAP expression in the fimbrial glial bundle in the hippocampus at E18.5. USP9X control (left). USP9X cKO (right). n = 3. GFAP - red. 10µm coronal paraffin sections. Scale bar 100µm. The red box highlighted in the Nissl stained image above of the E18.5 embryonic forebrain defines the region from which the above GFAP stained images derive.
3.8 Nestin-Cre mediated loss of USP9X results in axonal deficiencies in vitro

The in vitro analyses presented here were performed by Dr Lachlan Jolly (see acknowledgements) and Shane Stegeman.

Analyses by Dr Lachlan Jolly:
Given that the most common model for studying neuronal growth in vitro uses neurons derived from the rodent hippocampus we chose to start our in vitro analyses from this point (Dwyer and Winckler, 2010). Samples were grown in duplicate and twenty neurons were analyzed per culture. Loss of USP9X resulted in a reduction in elongation of the primary axonal process (Figures 3.13 and 3.14). Besides a reduction in primary axonal length, loss of USP9X also resulted in a reduction in the number of axonal neurite termini (Figures 3.13 and 3.15). Differences in dendritic morphology were observed in vitro but were not statistically significant. These findings suggest the reduction in neuronal processes observed in vivo may stem from abnormalities in axonal and neurite development rather then the loss of entire neurons.

Analyses by Shane Stegeman:
Given the reduction in neuronal processes at E18.5 and P0 was observed in the cerebral cortex, cortical derived neuronal cultures from Nestin-Cre mediated USP9X cKO embryos at E18.5 were also analysed. Considering our hippocampal derived neuronal analyses identified a reduction in primary axonal length, cortical derived neuronal cultures were grown for only four days to access the capacity for early axonal specification. Four USP9X cKO cultures were compared to six control cultures. By day four of culture five out of the six control cultures (~83%) displayed neurons that had specified axonal processes longer than 100µm whilst only one out of the four USP9X cKO cultures (25%) had specified axonal processes longer than 100µm. Representative images from these cultures are presented in figure 3.16. These findings suggest that USP9X is required for both early and late stage axonal development.
Figure 3.13 Nestin-Cre mediated knockout of USP9X results in reduced primary axonal length and a reduction in the number of axonal neurite termini

Hippocampal derived neuronal cultures were grown from E18.5 embryos. Loss of USP9X results in reduced primary axonal length (red arrow) and a reduction in the number of axonal neurite termini (white arrows). n = 5. MAP2 - red. Tau1 - blue. 200x magnification. (Analyses by Dr Lachlan Jolly).

Figure 3.14 Nestin-Cre mediated knockout of USP9X results in reduced primary axonal length

Hippocampal derived neuronal cultures were grown from E18.5 embryos. Primary axons were measured and analysed via a Student’s t test. Loss of USP9X results in reduced primary axonal length. cKO = USP9X<sup>KO</sup>/Y. * p = <0.05 n = 5 embryos. Error bars = standard deviation of the mean. Analyses by Dr Lachlan Jolly.
Figure 3.15 Nestin-Cre mediated loss of USP9X results in reduced axonal neurite termini
Hippocampal derived neuronal cultures were grown from E18.5 embryos. Axonal neurites and dendrites were measured and analysed via a Student’s t test. No significant difference was observed in the number of dendritic processes. Loss of USP9X results in a reduction in the number of axonal neurite termini. cKO = USP9X\textsuperscript{cKO}/Y. * p = <0.05 n = 5 embryos. Error bars = standard deviation of the mean. Analyses by Dr Lachlan Jolly.

Figure 3.16 Nestin-Cre mediated knockout of USP9X results in deficient axonal elongation
E18.5 cortical derived neuronal cultures were grown for four days. Nestin-Cre mediated knockout of USP9X results in deficient axonal initiation / elongation. Note the axonal length in the USP9X cKO sample (white arrow) compared to the controls (top panels). Neuronal marker: Beta-III-tubulin - green. Scale bar 10µm. Analyses by Shane Stegeman
3.9 Loss of USP9X - a mitotic analysis

Histone H3 is one of the five histone proteins involved in chromatin packaging during mitosis. Histone H3 is phosphorylated during prophase but becomes de-phosphorylated again once the cell enters anaphase. Antibodies that recognize only the phosphorylated form of Histone H3 allow mitotic nuclei to be distinguished (Cheung et al., 2007, Veras et al., 2009). Nestin-Cre mediated USP9X cKO mice were screened with markers for phosphorylated Histone H3. Counts were performed blind to the genotype of the sample. (See methods - section 2.12). No significant mitotic change (at prophase) was observed in the ventricular zone (the most proliferative region of the cortex) or any other part of the cerebral cortex at E15.5, E17.5, E18.5 or P0. Representative images at E15.5, E17.5 and P0 are presented in figure 3.17. Nor were any significant changes observed within the developing hippocampus (including the ammonic ventricular zone adjacent to the hippocampus) at E18.5 or P0. Graphed data is presented in figure 3.18. Samples were statistically analysed using a Student’s t test. Considering the variability between individual control mice and between individual cKO mice, samples were also analysed using the non-parametric Mann-Whitney U test. (See methods - section 2.7). At these stages of development no obvious differences were observed in the size of the brain and all major anatomical structures were present.
Figure 3.17 Loss of USP9X - proliferation in the ventricular zone and cerebral cortex
Mitotic cells were labelled (red) at prophase with antibodies for Histone H3 (phosphorylated). No significant mitotic change (at prophase) was observed in the ventricular zone (white arrows) or any other region of the cerebral cortex at E15.5 (A). Nor were any significant mitotic changes observed in the ventricular zone (white arrows) at E17.5 (B) or P0 (C). USP9X controls (top panels). USP9X cKO (bottom panels). n = 3 different mice at each stage (E15.5, E17.5 & P0). 10µm coronal cryo-sections. Scale bar 50µm.
Figure 3.18 Loss of USP9X - proliferative analysis in the ventricular zone and hippocampus

Mitotic cells were labelled at prophase with antibodies for Histone H3 (phosphorylated) and positive cells per 10µm thick section were counted blind to the genotype and statistically analysed via a Student’s t test with p values highlighted in bold. For comparison Mann-Whitney U test p values are shown in brackets adjacent to the bold type Student’s t test p values. No significant mitotic change (at prophase) was observed in the ventricular zone (A) or any other region of the cerebral cortex (B) at E15.5. No significant mitotic change (at prophase) was observed in the ventricular zone at E17.5 (C), E18.5 (D) or P0 (F). Nor was any significant mitotic change observed in the hippocampus at E18.5 (E) or P0 (G). n = 3 control mice versus 3 cKO mice at each stage.

Error bars = standard error of the mean.

3.10 Loss of USP9X - an apoptotic analysis

During normal CNS development programmed cell death occurs in neural progenitors as well as neurons and glia. Caspase-3 is a central regulator of apoptosis in mammalian cells. Caspase-3 normally exists in an inactive form. Upon proteolytic cleavage two subunits are produced which then dimerize to form an active enzyme. The active enzyme (Cleaved caspase-3) initiates activation of other caspases in a sequence of events leading to apoptosis. Loss of caspase-3 activity during CNS development results in abnormal enlargement of the neocortex (Kuida et al., 1996). The use of antibodies for Cleaved caspase-3 is commonly used in the analysis of apoptosis in the developing CNS (Barry et al., 2008, Piper et al., 2010). In this study we use Cleaved caspase-3 to analyse
changes in the levels of apoptosis in response to loss of USP9X. (See methods - section 2.12).

No statistically significant increase in apoptosis was observed in the hippocampus or the medial cortex of the newborn on postnatal day zero (Figures 3.19, 3.20, 3.21 and 3.22) or in the medial cortex on day E18.5 in embryonic development (Figures 3.23 and 3.24). Though cortical apoptosis was not statistically significant mean values for cKO mice were close to double that of controls. It is possible that the use of a larger sample size in future studies may identify significant differences in apoptotic levels. At P0 the mean value for hippocampal apoptosis for cKO samples was seven fold greater than the control value. Considering that high variability in the cKO sample may affect the statistical outcome the use of a larger sample size in future studies may provide greater clarity. Besides statistical analyses via Student’s t tests, samples were also analysed using the non-parametric Mann-Whitney U test.

In the late embryonic and newborn rodent cortex, proliferative apoptosis is most widespread in cells within the ventricular and sub-ventricular zones (Thomaidou et al., 1997). Normal neural progenitors may undergo proliferative apoptosis as a mechanism for regulating development, however no significant change in apoptosis was observed in USP9X cKO mice in any part of the forebrain at earlier more proliferative stages of development analysed including E17.5 and E15.5 (data not shown). Neurons in the embryo, newborn and adult may undergo apoptosis triggered by a lack of synaptic inputs (Raff et al., 1994). The reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus (Section 3.7) may correlate with increased apoptotic changes in these regions. Future studies utilising larger sample sizes are required to clarify these possibilities. Alternatively any apoptotic changes observed in USP9X cKO mice may be directly induced by an interruption in some USP9X dependent apoptotic pathway. USP9X has been shown to interact with several regulators of apoptosis that are also known to play roles during neural development. These include ASK1, MCL1 and Survivin (Nagai, 2009, Schwickart et al., 2009, Vong et al., 2005) (See section 1.1.9).
Figure 3.19 Loss of USP9X - apoptosis in the hippocampal ammonic ventricular zone at P0
Apoptotic cells were labelled (red) with antibodies for Cleaved caspase-3 (white arrows). No significant increase in apoptosis was observed in the hippocampus of Nestin-Cre mediated USP9X cKO mice at P0. H = hippocampus, VZ = ventricular zone. Control (left), USP9X cKO (right). n = 4. DAPI nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.

Figure 3.20 Loss of USP9X - apoptosis in the hippocampus at P0
Apoptotic cells were labelled with antibodies for Cleaved caspase-3 and positive cells per 10µm thick section were counted blind to the genotype. A Student’s t test revealed no significant increase in apoptosis in the hippocampus of Nestin-Cre mediated USP9X cKO mice at P0 - p = 0.09 (n = 4 control mice and 4 cKO mice). Error bars = standard error of the mean. Considering the variability in cKO samples a Mann-Whitney U test was also performed - p = 0.021.
Figure 3.21 Loss of USP9X - apoptosis in the anterior cingulate cortex at P0

Apoptotic cells were labelled (red) with antibodies for Cleaved caspase-3 (white arrows). No significant increase in apoptosis was observed in the anterior cingulated cortex of Nestin-Cre mediated USP9X cKO mice at P0. USP9X control (top panels), USP9X cKO (bottom panels). n = 4.

DAPI nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.
Apoptotic cells were labelled with antibodies for Cleaved caspase-3 and positive cells per 10µm thick section were counted blind to the genotype. A Student’s t test revealed no significant increase in apoptosis in the medial cortex of Nestin-Cre mediated USP9X cKO mice at P0 - $p = 0.34$ (n = 4 control mice and 4 cKO mice). Error bars = standard error of the mean. Areas of the medial cortex analysed included the retrosplial cortex, anterior cingulate cortex and subiculum. Given the variability a Mann-Whitney U test was also performed - $p = 0.386$. 

**Figure 3.22 Loss of USP9X - apoptosis in the medial cortex at P0**

Apoptotic cells were labelled with antibodies for Cleaved caspase-3 and positive cells per 10µm thick section were counted blind to the genotype. A Student’s t test revealed no significant increase in apoptosis in the medial cortex of Nestin-Cre mediated USP9X cKO mice at P0 - $p = 0.34$ (n = 4 control mice and 4 cKO mice). Error bars = standard error of the mean. Areas of the medial cortex analysed included the retrosplenial cortex, anterior cingulate cortex and subiculum. Given the variability a Mann-Whitney U test was also performed - $p = 0.386$. 


Figure 3.23 Loss of USP9X - apoptosis in the anterior cingulate cortex at E18.5

Apoptotic cells were labelled (red) with antibodies for Cleaved caspase-3. No significant increase in apoptosis was observed in the anterior cingulate cortex of Nestin-Cre mediated USP9X cKO mice at E18.5. Control (top panels), cKO (bottom panels). n = 5. DAPI - blue.

10µm coronal cryo-sections. Scale bar 20µm.
Loss of USP9X - apoptosis in the medial cortex at E18.5

Apoptotic cells were labelled with antibodies for Cleaved caspase-3 and positive cells per 10µm thick section were counted blind to the genotype. A Student’s t test revealed no significant increase in apoptosis in the medial cortex of Nestin-Cre mediated USP9X cKO mice at E18.5 - \( p = 0.19 \) (n = 5 control mice and 5 cKO mice). Error bars = standard error of the mean. Areas of the medial cortex analysed included the retrosplenial cortex, anterior cingulate cortex and subiculum. Given the variability a Mann-Whitney U test was also performed - \( p = 0.391 \).

3.11 Loss of USP9X results in a reduction in AGS3 in the cerebral cortex

Activator of G protein Signalling 3 (AGS3) has recently been identified as a substrate de-ubiquitylated by USP9X in a range of cells including human embryonic kidney cells (HEK293), pheochromocytoma cells from the rat adrenal medulla (PC12), immortalised African Green monkey kidney cells (COS7) and immortalised cervical cancer cells (HeLa). Knockdown of USP9X resulted in a moderate reduction in AGS3 levels while over-expression of USP9X lead to an increase in AGS3, suggesting that USP9X plays a stabilising role in AGS3 function. USP9X was also shown to immuno-precipitate with AGS3 from the rat brain (Xu et al., 2010). In light of these findings we performed a limited screen identifying a decline in AGS3 expression in the cerebral cortex of USP9X cKO mice at E17.5 (Figure 3.25). These findings were confirmed in three USP9X cKO mice at E17.5. Further analysis is required to determine the functional nature of this USP9X-AGS3 relationship.
In the CNS AGS3 is involved in mitotic spindle orientation and asymmetric cell division in cortical progenitors as well as synaptic plasticity in developing neurons (Blumer et al., 2008, Sanada and Tsai, 2005). However conditional AGS3 knockout mice display no obvious changes in brain morphology, behaviour or motor skills (Blumer et al., 2008). Considering that AGS3 knockout mice are not postnatal lethal as whole CNS USP9X knockout mice are, it is unlikely that disruptions to USP9X-AGS3 interactions can completely account for the phenotype observed in USP9X cKO mice. It is clear that additional USP9X-substrate interactions must play more significant roles during neural development. Hence, it is likely that the USP9X cKO phenotype is a compound phenotype caused by multiple factors and that USP9X regulates neural development via multiple pathways.

Figure 3.25 Loss of USP9X results in down-regulation of AGS3 in the cerebral cortex at E17.5

*Nestin-Cre* mediated loss of USP9X results in down-regulation of AGS3 (red) in the cerebral cortex at E17.5 with loss of AGS3 most evident in the ventricular / sub-ventricular zone lining the lateral ventricles. L = lateral ventricle in the cerebral cortex. USP9X control (top), cKO (bottom). n = 3. 10µm coronal cryo-sections. Scale bar 50µm.
3.12 Loss of USP9X - DCX expression

Doublecortin (DCX) is the only protein that interacts with USP9X exclusively in the CNS. Considering that DCX expression is restricted almost entirely to immature neurons the interaction between USP9X and DCX can only have a neuronal-specific function. As DCX is not a substrate stabilised by USP9X but rather a protein that binds with USP9X no change in DCX expression levels were expected. An analysis was performed however to determine if any changes were evident in the localisation of DCX. No obvious change was observed in the expression pattern of DCX at E15.5 or E18.5 in the developing embryo or on postnatal day eight after birth following loss of USP9X. A representative image of DCX expression in the cerebral cortex at E15.5 (around the peak of neurogenesis) is shown in figure 3.26. As DCX is thought to facilitate trafficking of USP9X in neurons and not the other way around it is not surprising that no change in DCX cellular localisation was observed (Friocourt et al., 2005).

In neurons USP9X expression is not only found in the cell bodies but also at the extremities in the synapses (Chen et al., 2003). Previous studies have shown that USP9X functions in a concentration-dependent manner and in some circumstances also a compartment-specific manner (Al-Hakim et al., 2008, Nathan et al., 2008). Loss of USP9X results in a reduction in primary axonal length and in the number of axonal neurite termini (Section 3.9). It is possible that DCX normally traffics USP9X within axons and other neuronal processes (even up to the synapses) to facilitate USP9X interactions with other substrates. Substrates like AF-6 and EFA6 for example are also highly expressed at neuronal synapses (Sakagami, 2008, Xie et al., 2008). Loss of USP9X results in a reduction in neuronal processes projecting towards the hippocampus. Interestingly DCX knockout mice display hippocampal disorganisation at late embryonic stages (Pramparo, 2010). Though DCX may play a role in USP9X trafficking within neurons it remains unknown which other USP9X-substrate interactions are normally facilitated by such trafficking. It may be the interaction with these other substrates that is specifically required for normal neuronal development.
Figure 3.26 DCX expression patterns are retained in the forebrain of USP9X cKO mice at E15.5
No obvious differences were seen in the expression pattern of the immature neuronal marker DCX (red) at E15.5 following Nestin-Cre mediated loss of USP9X shown here in the cerebral cortex (C) and striatum (S). USP9X control (top), USP9X cKO (bottom). n = 3. 10µm coronal cryo-sections. Scale bar 100µm.
3.13 Summary

USP9X is highly expressed in the developing CNS. Loss of USP9X throughout the whole CNS results in early postnatal lethality. Newborn mice failed to suckle. In the late embryo and newborn a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus was observed. *In vitro* loss of USP9X results in defects in axonal development including deficiencies in early axonal specification and a reduction in elongation of the primary axonal process and a reduction in the number of axonal neurite termini. An increase in hippocampal apoptosis in the newborn was observed. This increase in apoptosis may be directly induced by an interruption in some USP9X dependent apoptotic pathway. USP9X has been shown to interact with several regulators of apoptosis that are also known to play roles during neural development. These include ASK1, MCL1 and Survivin. Alternatively hippocampal apoptosis may be induced by other changes that may include a loss of synaptic inputs to the hippocampus. Loss of USP9X resulted in a reduction in AGS3 expression in the cerebral cortex in the late embryo. AGS3 is involved in mitotic spindle orientation in cortical progenitors as well as synaptic plasticity in developing neurons. However considering that conditional AGS3 knockout mice are not postnatal lethal it is unlikely that disruptions to USP9X-AGS3 interactions account for the phenotype observed in USP9X cKO mice. It is clear that additional USP9X-substrate or binding partner interactions must play more essential roles in neural development. It is hence likely that USP9X regulates neural development via multiple pathways. These pathways are likely modulated via USP9X’s role as a de-ubiquitylating enzyme but also possibly through its interaction with other binding partners via additional mechanisms.

In-depth conclusions are presented in chapter 5 following further analyses presented in chapter 4.
Chapter 4  -  Results and Discussion

Loss of USP9X in the developing and the adult forebrain

4.1 Introduction

As Nestin-Cre mediated loss of USP9X throughout the whole CNS results in early postnatal lethality preventing an analysis of the brain beyond postnatal day one we then used Emx1-Cre mediation to knockout USP9X in the developing dorsal forebrain only (Iwasato et al., 2004). Emx1-Cre mediated loss of USP9X in the dorsal forebrain during development allowed male cKO mice (USP9X\(^{kO}/Y\)) to survive into adulthood. This enabled us to examine the effects on adult forebrain development caused by loss of USP9X during embryonic development.

In the normal adult brain USP9X is expressed at lower levels compared to the embryo and newborn. Expression however remains high in a few distinct regions such as the hippocampus, one of the limited regions of the adult brain where neurogenesis takes place (Friocourt et al., 2005, Wood et al., 1997). Hence any phenotypical changes in the adult forebrain and specifically the hippocampus may be caused by a combination of loss of USP9X during development as well as the retained loss of USP9X which may be required in the adult forebrain.

4.2 Emx1-Cre mediated loss of USP9X results in reduced neuronal processes

In the developing embryonic dorsal telencephalon the Emx1-Cre transgene is expressed in neural progenitor cells starting around E10.5 in a similar cell-type and stage-specific manner to Nestin-Cre (Chou et al., 2009, Gorski et al., 2002, Graus-Porta et al., 2001, Tronche et al., 1999). Prior to analysing the adult forebrain we examined the Emx1-Cre mediated USP9X cKO embryonic forebrain for comparison with the Nestin-Cre mediated cKO embryonic brain. A reduction in neuronal processes projecting between
the anterior cingulate cortex and the subiculum of the hippocampus was also observed at E18.5 in Emx1-Cre mediated cKO mice (Figure 4.1) as was observed in Nestin-Cre mediated mice (See section 3.7).

Figure 4.1  
Emx1-Cre mediated knockout of USP9X results in reduced neuronal processes

A reduction in neuronal processes (arrows) was observed projecting between the anterior cingulate cortex and the subiculum of the hippocampus at E18.5 in embryonic development.

USP9X control (top). USP9X cKO (bottom). Neuronal marker NF160 - red.
10µm coronal cryo-sections. Scale bar 20µm.
4.3 *Emx1-Cre* mediated loss of *USP9X* in the dorsal forebrain allowed mice to survive into adulthood

*Nestin-Cre* mediated loss of *USP9X* throughout the whole CNS results in early postnatal lethality whilst *Emx1-Cre* mediated loss of *USP9X* restricted to the dorsal forebrain allowed mice to survive into adulthood (Figure 4.2). The residual protein expression detected in the forebrain immuno-blot is likely due to blood vessels and meninges of the brain (Isaka et al., 1999, Ke et al., 2007, Tronche et al., 1999). The first three litters analysed contained a total of 24 mice born for which 12 (50%) were male. Of these 12 male mice 5 (~42%) were *USP9X* cKO males positive for the Cre-recombinase transgene whilst the other 7 being male littermate controls. Mendianian ratios would expect *USP9X* cKO males to represent 50%. Given that 6 *USP9X* cKO males would represent Mendelian ratios these litters suggest that *USP9X* cKO mice are born in numbers close to Mendelian ratios.
Figure 4.2 *Emx1-Cre* mediates knockout of USP9X at the protein level in the dorsal forebrain

(A) USP9X (red) is depleted in the dorsal forebrain / cerebral cortex in the cKO (top right panel) whilst expression is maintained in the rest of the cKO brain as shown here in the striatum (bottom right panel). 7 week adult brain. USP9X control (left panels). Dapi nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.

(B) Western blot analyses of 7 week adult cerebral cortex extracts show USP9X depletion in the dorsal forebrain (forebrain). USP9X = C-terminal antibody.

4.4 *Emx1-Cre* mediated loss of USP9X results in adult corpus callosum reduction

Loss of USP9X in the dorsal forebrain during development resulted in a distinct reduction in the size of the corpus callosum in the adult forebrain (Figure 4.3). The portion of the forebrain containing the corpus callosum was sectioned coronally at a thickness of 10µm from the most rostral to caudal aspect and four USP9X cKO mice were examined under light microscopy against four littermate controls. A distinct reduction was observed in all four USP9X cKO mice with two out of the four having an almost undetectable corpus callosum. The reduction in the corpus callosum (an axonal tract connecting the two cerebral hemispheres) confirms the requirement of USP9X for normal neuronal development and suggests that USP9X may be specifically required for normal axonal development as the *in vitro* analysis suggests (See section 3.8). The corpus callosum is discussed further in sections 4.5 and 4.6.
4.5 *Emx1-Cre* mediated loss of USP9X results in adult hippocampal reduction

Brains were sectioned coronally through the hippocampus from its most rostral to caudal aspects and examined under light microscopy. Loss of USP9X resulted in a dramatic reduction in the size of the adult hippocampus in all four USP9X cKO mice analysed (Figure 4.4). The hippocampus in USP9X cKO mice did not project rostrally or caudally beyond the normal anatomical position observed in control mice showing that loss of USP9X resulted in a reduction in absolute hippocampal volume. In humans, hippocampal volume is normally analysed via magnetic resonance imaging (MRI) (Campbell et al., 2004, Videbech and Ravnkilde, 2004). In the absence of an MRI capacity we used a hippocampal area analysis to access the degree of hippocampal reduction in response to loss of USP9X. Had the hippocampal reduction been less obvious under visual inspection (requiring additional confirmation) an in-depth
volumetric estimation using the Cavilieri method would have been utilized. Considering that the reduction was obvious and that an in-depth volumetric analysis would not provide any additional insight into the role of USP9X, an area analysis was performed to provide some numerical scale for comparison. If required the Cavilieri method may be utilized in future studies (Rosen and Harry, 1990). For area analysis coronal sections from each mouse (three control mice and three USP9X mice) were taken from the middle section of the hippocampus along the rostral-caudal axis. (See methods - section 2.13). USP9X cKO mice had a mean area difference of approximately 74% less then littermate controls (Figure 4.5). Additional measurements showed that adult USP9X cKO mice had a mean difference in coronal hippocampal length of approximately 62% less and a mean difference in coronal dentate gyrus length of approximately 65% less then littermate controls (Figures 4.6 and 4.7). All hippocampal regions appeared to be intact suggesting the hippocampal reduction in the adult consists of an overall reduction rather than a region specific reduction. No obvious reduction in hippocampal size was observed in the embryo or the newborn (Emx1-Cre or Nestin-Cre mediated) suggesting the bulk of the hippocampal change occurs in the late postnatal or early adult (Figure 4.8). A preliminary analysis on postnatal day eight (2 controls versus 1 USP9X cKO mouse) found no major differences in overall hippocampal size, however deficient dentate gyrus development was observed at this stage (Figure 4.9). Further histological analysis is required in future studies to verify these postnatal findings.

Reduced adult hippocampi retained a similar expression pattern compared to controls for the major neuronal markers; Neurofilament medium (NF160), the neuronal nuclei marker NeuN, the neuronal / dendritic marker MAP2 and the pre-synaptic marker Synaptophysin (Figures 4.10, 4.11, 4.12 and 4.13). The expression of these markers was not surprising as the in vitro analyses showed that neuronal aberrancies stem not from the inability to specify neurons but in deficiencies in their morphologies. Disruptions in neuronal circuitry caused by aberrant morphologies may not be obvious from hippocampal analyses on brain sections (Blumer et al., 2008, Yao et al., 2006). In the late embryo and newborn a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus was observed. In mice neurons found predominantly in superficial cortical layers II and III with lesser input deriving from neurons in deeper layers V and VI normally transmit signals to the hippocampus. Neurons from superficial cortical layers normally terminate in the marginal zones of the hippocampus and in the molecular layer of the dentate gyrus.
The deeper cortical layers also receive the most prominent output from neurons within the hippocampus (Hechler et al., 2006). In humans, neurons in cortical layer II project to the dentate gyrus and the CA3 region while neurons in layer III project primarily to the CA2 region and the subiculum but also the CA1 region of the hippocampus (Witter et al., 2000). The actual number of axons that reach the hippocampus may be significantly reduced as may the number of termini branches stemming from those axons. The majority of neurons that form the hippocampus arise from neural progenitors in the ventricular zone. The secretion of signalling molecules from the hippocampus is required to attract and guide migration and final positioning in the hippocampus (Borrell et al., 1999, Chou et al., 2009, Del Rio et al., 1997, Hechler et al., 2010). A loss of synaptic inputs to the hippocampus may result in down-regulation of these signalling cues. This may result in localised migrational deficiencies to the hippocampus. Furthermore, any reduction in synaptic connections may also induce dendritic retraction or may inhibit initial dendritic expansion. Several studies have shown that growth and retraction of dendritic filipodia is associated with synaptic plasticity (Engert and Bonhoeffer, 1999, Yuste and Bonhoeffer, 2001).

Hippocampal reduction has been identified in a number of neurological diseases in humans including epilepsy, schizophrenia, dementia, Alzheimer’s, Parkinson’s and Huntington’s disease (Czeh and Lucassen, 2007). Neuronal cell loss in the hippocampus (hippocampal sclerosis) is found in a high number of patients suffering epilepsy (Kuruba et al., 2009, Sloviter, 2005). Defects in synaptic connectivity within the hippocampus are often found in schizophrenia patients (Harrison, 2004) (Boyer et al., 2007). In most neurological disorders the exact cellular and molecular mechanisms causing hippocampal reduction are not found. In Alzheimer’s disease the hippocampus is one of the first regions of the brain to suffer damage (Hampel et al., 2008, Olabarria et al., 2010, Zhang et al., 2010). Reduced hippocampi have been observed in transgenic mouse models for Alzheimer’s disease. One of the features of these reduced hippocampi is astroglial atrophy consisting of a reduction in astrocytic volume as well as a reduction in branching (Olabarria et al., 2010). In the adult, glial cells outnumber neurons in the hippocampus (and throughout the nervous system) and hence constitute a significant proportion of hippocampal volume (Joelving et al., 2006). A screen of adult hippocampi in USP9X cKO mice however found no obvious differences in the localisation or morphology of cells expressing the astrocytic marker GFAP (Figure 4.14). Nor were
there any obvious changes in the expression pattern of the oligodendrocyte markers Myelin Basic Protein (MBP) and O4 (n = 3 data not shown).

In knockout mouse studies a diverse range of hippocampal phenotypes have been reported. Some studies have reported the complete loss of the hippocampus while other studies observe region specific changes only. For example, loss of transcription factors Lef1, Tcf or Lhx2/Lhx5 results in a loss of the entire hippocampal complex while loss of NFIB, Emx2 or NeuroD results in the complete loss of the dentate gyrus (Barry et al., 2008, Galceran et al., 2000, Miyata et al., 1999, Pellegrini et al., 1996, Porter et al., 1997, Zhao et al., 1999). The Gli3 mutant develops a poorly characterised hippocampus while loss of of the CXCR4 receptor or NFIA results in a poorly defined dentate gyrus (Grove et al., 1998, Lu et al., 2002, Piper et al., 2010, Shu et al., 2003). Considering the diversity of these results no obvious conclusions can be made based on phenotype alone as to the potential mechanisms by which loss of USP9X induces its unique hippocampal response.

To access the cellular response, mitotic and apoptotic analyses were performed. No significant mitotic changes at prophase were observed within the developing hippocampus at E18.5 or P0 (Section 3.9). Normal apoptosis occurs in the healthy adult hippocampus and other regions of the CNS though at very low levels (Dayer et al., 2003). No significant change in apoptosis was observed in the adult hippocampus at seven weeks of age (data not shown). Loss of USP9X may result in an increase in apoptosis in the newborn hippocampus at postnatal day zero (Section 3.10). Any increase in apoptosis may result from an interruption in some USP9X dependent apoptotic pathway. Alternatively, factors such as a loss of synaptic inputs are also known to stimulate apoptosis (Raff et al., 1994). Smaller hippocampi have been observed in mice deficient in the chromatin-remodeling protein ATRX. These ATRX deficient mice are postnatal lethal displaying a reduction in the number of CA1 and CA3 pyramidal neurons in the hippocampus with fewer cells migrating to the dentate hilus. These deficiencies resulted from an increase in apoptosis in neural progenitors as well as post mitotic cells upon differentiation (Berube et al., 2005). Brca1 deficient mice also present with reduced hippocampi. Higher levels of apoptosis were found in these mice though cell death resulted in a more dramatic phenotype which included failure of the cerebral cortex to reach its normal size (Pulvers and Huttner, 2009).
The hippocampus plays a role in memory, learning and spatial navigation though the precise role is poorly understood (Moser et al., 2008, Vago and Kesner, 2008). The hippocampus may also play a minor role in the awareness and memory of odours (Vanderwolf, 2001). Defects in hippocampal development may have contributed to the observed failure to suckle in the newborn (Section 3.4). In comparison to the embryonic and newborn brain, USP9X is expressed at significantly lower levels in the adult. Expression however remains high in the dentate gyrus and CA regions of the adult hippocampus (Friocourt et al., 2005, Xu et al., 2005). Besides a role during hippocampal development USP9X may also play an important role in hippocampal maintenance in the adult.
Figure 4.4 Emx1-Cre mediated knockout of USP9X results in hippocampal reduction in the adult
Also note the severe reduction in the corpus callosum (arrows) shown here in the 7 wk old mouse brain.
USP9X controls (top 3 panels). USP9X cKO (bottom 3 panels). n = 4.
Nissl stain. 10µm coronal cryo-sections. Scale bar 100µm.
Figure 4.5  Hippocampal area analysis - USP9X cKO mice at seven weeks of age
USP9X cKO mice had a mean area difference in the mid rostral-caudal hippocampal aspect of ~74% less then littermate controls. The mean hippocampal area of controls was designated 100. The hippocampal areas of cKO mice were converted to ratios compared to the mean hippocampal area of controls.
cKO = USP9X<sup>KO/Y</sup>. Student’s <i>t</i> test <i>p</i> = 0.003 <i>n</i> = 4. Error bars = standard error of the mean.

Figure 4.6  Hippocampal length of Emx1-Cre mediated USP9X cKO mice at 7 weeks of age
USP9X cKO mice had a mean difference in hippocampal length of ~62% less then littermate controls.
cKO = USP9X<sup>KO/Y</sup>  Student’s <i>t</i> test <i>p</i> = 0.0001 <i>n</i> = 4. Error bars = standard error of the mean.
Figure 4.7 Dentate gyrus length in the hippocampus of USP9X cKO mice at 7 weeks of age
USP9X cKO mice had a mean difference in dentate gyrus length of ~65% less than littermate controls. cKO = USP9X<sup>cKO</sup>/Y  Student’s t test p = 0.0001 n = 4. Error bars = standard error of the mean.

Figure 4.8 Loss of USP9X - Hippocampal development in the late embryo
At E18.5 no obvious reduction in hippocampal size was observed and all hippocampal regions appeared to be intact. Lateral ventricle (black arrow). Hippocampus (white arrow). Nissl stain. USP9X control (left). USP9X cKO (right). 10µm coronal cryo-sections.
Figure 4.9 Loss of USP9X results in deficient dentate gyrus development at P8
A preliminary analysis of the hippocampal dentate gyrus found deficiencies in development on postnatal day eight. USP9X controls (top panels). USP9X cKO (bottom panel).
Nissl stain. n = 1 cKO. 10µm coronal cryo-sections.

Figure 4.10 Loss of USP9X - hippocampal reduction in the adult and NF160 expression
No obvious differences were seen in the hippocampal expression pattern of the neuronal marker NF160 (red) shown here in the 7 week adult mouse brain. n = 3. USP9X control (top panels), USP9X cKO (bottom panels). Dapi nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 100µm.
Figure 4.11 Loss of USP9X - hippocampal reduction in the adult and NeuN expression
No obvious differences were seen in the hippocampal expression pattern of the neuronal marker NeuN (green) shown here in the 7 week adult mouse brain. n = 3. USP9X control (left). USP9X cKO (right). 10µm coronal cryo-sections. Scale bar 100µm.

Figure 4.12 Loss of USP9X - hippocampal reduction in the adult and MAP2 expression
No obvious differences were seen in the hippocampal expression pattern of the neuronal / dendritic marker MAP2 (green) shown here in the 7 week adult mouse brain. n = 3. USP9X control (left). USP9X cKO (right). 10µm coronal cryo-sections. Scale bar 100µm.
Figure 4.13 Loss of USP9X - hippocampal reduction in the adult and Synaptophysin expression

No obvious differences were seen in the hippocampal expression pattern of the pre-synaptic marker Synaptophysin (green) shown here in the 7 week adult mouse brains. n = 3

USP9X control (left panels). USP9X cKO (right panels). Dentate gyrus (bottom panels).

10µm coronal cryo-sections. Scale bar 100µm (top panels), 20µm (bottom panels).
4.6 Loss of USP9X results in aberrant neuronal processes in the adult cerebral cortex

*Emx1-Cre* mediated knockout of USP9X results in the presence of aberrant neuronal processes in the adult cerebral cortex (Figure 4.15). Aberrant neuronal processes were observed in two USP9X cKO mice whilst another presented a more similar morphology to littermate controls. These variations may reflect differences in response to loss of USP9X due to natural genetic variation. The normal cerebral cortex contains neurons with both descending and ascending axons. The cerebral cortex also contains pyramidal neurons that consist of descending axons whilst presenting both apical and basal
projecting dendrites. The apical dendrite normally extends from the apex of the pyramidal shaped cell body often for several hundred microns before branching. Though the processes in USP9X cKO mice may represent apical dendrites of large pyramidal neurons no processes of this intensity were observed in any controls (n = 3). Further analysis is required to statistically verify these findings and clarify the nature of these processes.

No obvious change in the size of the cerebral cortex was observed and all six cortical layers were detected. A limited screen with the layer specific neuronal markers FOXP1 and FOXP2 was performed though no obvious differences were observed. FOXP2 expression was found in deeper layers where normally expressed (Figure 4.16). A screen was also performed for glial markers in the adult cortex. No obvious difference in the expression pattern of the GFAP, MBP or O4 was observed (n = 3 data not shown).

Neurons with aberrant processes were found predominantly in cortical layers II - V. In mouse neurons found predominantly in cortical layers II and III normally project axons to the hippocampus and neurons found in cortical layers II, III and V normally project axons across the corpus callosum (Amaral and Witter, 1995, Hechler et al., 2006, Piper et al., 2009, Placzek and Richards, 2005). The aberrant neurons found in USP9X cKO mice displayed what appeared to be a single thick process projecting from the cell body outwardly towards the cortical plate (Figure 4.17). During migration and maturation cortical neurons display bipolar morphologies with thick leading and thin trailing processes (O'Rourke et al., 1992). After migration is complete the trailing process (the axon) elongates to find its final destination and the leading process fans out forming multiple dendrites (Dwyer and Winckler, 2010, Noctor et al., 2004). Considering the axonal abnormalities observed in vitro, these neurons may fail to project axons to find their normal destinations. Neurons projecting axons across relatively short distances from neuron to neuron within the cerebral cortex may have less difficulty making connections compared to neurons projecting axons across larger distances from the cerebral cortex to the hippocampus or across the corpus callosum. Others studies have also shown a close link between the integrity of the hippocampus and the corpus callosum (Piper et al., 2010, Shu et al., 2003). Interestingly no dramatic changes were observed in the morphology of neuronal processes within the marginal zone a region abundant in relatively short projecting neurons (Figure 4.18). The failure of long
projecting neurons to make appropriate connections may induce feedback signalling which prevents the leading process from branching out into a normal dendritic tree. Further analysis is required to confirm either a dendritic or axonal nature for these aberrant processes.

Figure 4.15 Loss of USP9X results in aberrant neuronal processes in the adult cerebral cortex
No processes of this intensity were observed in any controls (n = 3 control mice & 2 cKO mice).
7 week adult cerebral cortex. USP9X control (left), USP9X cKO (right). Neuronal marker NF160 - red.
Dapi nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.

Figure 4.16 Loss of USP9X - FOXP2 expression in the adult cerebral cortex
FOXP2 expression was found in cortical layers V – VI in both USP9X cKO mice and controls. FOXP2 expression is shown here in layer VI adjacent to the lateral ventricle. (7 week adult mouse brain).
USP9X control (left), USP9X cKO (right). C = cerebral cortex. L = lateral ventricle. H = hippocampus.
FOXP2 - red. Dapi nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.
Figure 4.17 Aberrant neurons project thick processes towards the cortical plate
Aberrant neurons displayed what appeared to be a single thick process projecting from the cell body
towards the cortical plate shown here in the 7 week adult cerebral cortex of USP9X cKO at lower
magnification top and higher magnification bottom. Neuronal marker NF160 - red. Dapi nuclear marker -
blue. 10µm coronal cryo-sections. Scale bars 20µm.
Figure 4.18 Loss of USP9X - NF160 expression in the marginal zone
No dramatic changes were observed in the morphology of neuronal processes within the marginal zone of the cerebral cortex in the 7 week adult. USP9X control (left), USP9X cKO (right). n = 3. Neuronal marker NF160 - red. Dapi nuclear marker - blue. 10µm coronal cryo-sections. Scale bar 20µm.

4.7 Loss of USP9X in the dorsal forebrain results in a failure to mate

A total of four adult USP9X cKO male mice (germ line USP9X\textsuperscript{loxP}/Y & Emx1-Cre) were mated with eight female mice (two females per male). Each male was mated with a USP9X\textsuperscript{loxP}/USP9X\textsuperscript{loxP} female in attempt to generate homozygous cKO females (USP9X\textsuperscript{cKO}/USP9X\textsuperscript{cKO}) for analysis (Figure 4.19) as well as a wild type female to verify if these cKO males were able to mate. Mice were mated over a period of approximately four months starting at the onset of sexual maturity. No litters were produced from these matings though female mice used in these experiments were able to produce litters with non-cKO male mice. USP9X cKO males were not seen attempting to mate with females though such mating behaviour was observable in mice only carrying the Nestin-Cre or Emx1-Cre transgenes and in wild type male mice. As Cre is not expressed in the testes it is most likely that the failure to mate stems from neurological deficiencies rather than fertility deficiencies. This preliminary evidence suggests that changes in forebrain development caused by the loss of USP9X result in a behavioural response which includes failure to mate. Though no obvious difference was observed in the size of the olfactory bulbs the reduced hippocampus may play a role in this behaviour as this region of the brain is thought to play a role in the awareness of odours (Vanderwolf,
2001). Loss of USP9X also resulted in a behavioural response in the newborn which included failure to suckle (Section 3.4).

Figure 4.19 Mating plan for generating homozygous USP9X cKO female mice

USP9X<sup>loxP/loxP</sup> female mice were mated with USP9X cKO male mice with the goal of generating homozygous USP9X cKO female mice (highlighted red). USP9X cKO male mice failed to mate.

4.8 Nestin-Cre mediated heterozygous knockout of USP9X in the adult brain

Though Nestin-Cre mediated knockout of USP9X throughout the whole CNS results in early male postnatal lethality, female offspring heterozygous for the knockout of USP9X at the genomic level (USP9X<sup>WT</sup>/USP9X<sup>cKO</sup>) appeared normal at birth and survived into adulthood (Sections 2.6 and 3.4). In mouse it is thought that USP9X is subject to X-inactivation. If so USP9X<sup>WT</sup>/USP9X<sup>cKO</sup> females would be chimeric for USP9X expression (Yang et al., 2010).
After surviving for 20 months female mice were weighed and sacrificed for brain analysis. At 20 months of age heterozygous *USP9X* cKO female mice had a mean difference in weight of approximately 16% less then littermate (*Nestin-Cre* negative) female controls though both cKO females and controls were housed together with unlimited food (Figure 4.20). These mice also displayed a reduction in hippocampal size (n = 4) though this reduction was less dramatic in comparison to the hippocampal reduction observed in *Emx1-Cre* mediated adult cKO male mice (Figure 4.21). These mice displayed a mean difference in hippocampal area of approximately 25% less then littermate controls (Figure 4.22). A small number of aberrant neuronal processes were observed in the cerebral cortex of heterozygous *USP9X* cKO females similar though subtle in comparison to those seen in complete *USP9X* cKO males (Figure 4.23).

**Figure 4.20** Weight of heterozygous female *USP9X* cKO mice at 20 months of age

*Nestin-Cre* mediated heterozygous *USP9X* cKO female mice had a mean difference in body weight of approximately 16% less then littermate (*Nestin-Cre* negative) female controls. (Heterozygous cKO = *USP9X*<sup>WT</sup>/USP9X<sup>cKO</sup>). Student’s *t* test *p* = 0.1 n = 6. Error bars = standard error of the mean.
Figure 4.21 Adult female heterozygous USP9X cKO mice have reduced hippocampi
At 20 months of age female heterozygous USP9X cKO mice brains were sectioned and Nissl stained revealing a size reduction in the hippocampus (n = 4). USP9X female control (left), USP9X heterozygous female cKO (right). 10µm coronal cryo-sections. Scale bar 100µm.

Figure 4.22 Hippocampal area analysis - adult heterozygous USP9X cKO female mice
Heterozygous USP9X cKO female mice had a mean area difference in the mid rostral-caudal hippocampal aspect of ~25% less then littermate controls. The hippocampal area of the first control was designated 100. The hippocampal areas of all other controls and cKO mice were converted to ratios compared to the first control. Heterozygous cKO = USP9X\textsuperscript{WT}/USP9X\textsuperscript{cKO}
Student’s 𝑡 test 𝑝 = 0.06 n = 4. Error bars = standard error of the mean.
Figure 4.23 Adult female heterozygous USP9X cKO mice have aberrant neuronal processes in the cerebral cortex

At 20 months of age female heterozygous USP9X cKO mice (bottom panel) displayed numerous aberrant neuronal processes in the cerebral cortex. Neuronal processes observed in the cerebral cortex of control mice (top panel) were lesser in both number and size. Neuronal marker NF160 - red. 10µm coronal cryo-sections. Scale bar 50µm.
4.9 Summary

Loss of USP9X restricted to the dorsal forebrain during development allows mice to survive into adulthood. In the adult, loss of USP9X resulted in a significant reduction in the size of the corpus callosum and the hippocampus. The reduction in the corpus callosum, an axonal tract connecting the two cerebral hemispheres, suggests that USP9X may be required for normal axonal development as the in vitro analysis suggests. Loss of USP9X also resulted in the presence of aberrant neuronal processes in the cerebral cortex. Neurons with aberrant processes were found predominantly in cortical layers II - V. In mouse neurons found in cortical layers II and III normally project axons to the hippocampus and neurons found in cortical layers II, III and V normally project axons across the corpus callosum. Considering the axonal abnormalities in USP9X deficient mice the greatest deficiencies may be found in neurons projecting axons across relatively long distances such as from the cerebral cortex to the hippocampus or across the corpus callosum. Loss of USP9X also results in an adult behavioural response, which includes failure to mate. Adult female mice heterozygous for the knockout of USP9X at the genomic level also displayed reduced hippocampi and aberrant neuronal processes in the cerebral cortex similar to those seen in complete USP9X cKO males though subtle in comparison.

In-depth conclusions are presented in chapter 5 combining the analyses presented in chapters 3 and 4.
Chapter 5

Conclusions and Future directions

5.1 USP9X regulates multiple stages of neural development

In the CNS USP9X is highly expressed during development while expression remains at lower levels in the adult brain. However in the adult brain USP9X expression remains high in niche neurogenic regions such as the hippocampus (Friocourt et al., 2005). A cell-specific analysis in vitro shows that USP9X is highly expressed in neural stem cells while expression remains at lower levels in more differentiated neural cell types (Ramalho-Santos et al., 2002). This expression pattern suggests that USP9X has the potential to regulate multiple stages of neural development as well as retaining a maintenance role in the adult CNS. This study found abnormalities in neuronal development from late embryonic stages into adulthood. This study clearly identified a requirement for normal axonal development in vitro for which the in vivo evidence suggests is essential in both embryonic development as well as postnatal development towards adulthood.

5.2 USP9X regulates neural development via multiple pathways

As the primary role of a de-ubiquitylating enzyme is to remove ubiquitin from its substrates, the role of USP9X during neural development may likely be defined by the role of its substrates. However as human USP9X is a very large protein (2547 amino acids) capable also of interacting with binding partners that are not subject to its de-ubiquitylase activity these interactions may also define the strong role USP9X displays during neural development (Brown et al., 1998, Jones et al., 1996). USP9X has been shown to interact with at least 14 substrates and binding partners in a range of mammalian tissues and cell lines (refer Table 1.1). Though all 14 of these proteins are expressed in the mammalian CNS to date only seven of these proteins (AF-6, AGS3,
DCX, Epsin, Itch, MARK4 and Mind Bomb-1) have been shown (primarily via co-immunoprecipitation) to interact with USP9X in the CNS. In the CNS these proteins are known to regulate a range of important functions including protein trafficking, neuronal migration, cell signalling, cellular polarity and cell-cell adhesion. AF-6, AGS3, Epsin, Itch, and MARK4 have been identified as USP9X substrates in other tissues. DCX has been identified as a binding partner that does not interact with the catalytic domain of USP9X whilst the specific nature of the biochemical relationship between USP9X and Mind Bomb-1 is currently unknown (Al-Hakim et al., 2008, Chen et al., 2003, Choe et al., 2007, Friocourt et al., 2005, Mouchantaf et al., 2006, Taya et al., 1998, Xu et al., 2010).

**AF6 and MARK4**
AF6 and MARK4 are regulators of cellular polarity (See section 1.1.8). Given the diffuse ventricular architecture in USP9X cKO mice (discussed in section 3.6) AF6 and MARK4 are recommended in section 5.4 for further analysis in future studies.

**AGS3**
Previous studies confirmed a stabilizing role of USP9X for AGS3 levels (Xu et al., 2010). In this current study we found that loss of USP9X resulted in a reduction in AGS3 expression in the cerebral cortex in the late embryo. In the CNS AGS3 is normally involved in mitotic spindle orientation in cortical progenitors as well as synaptic plasticity in developing neurons. Further analysis is required to determine the functional significance of USP9X-dependent depletion of AGS3. Considering that conditional AGS3 knockout mice are not postnatal lethal as are USP9X knockout mice and no dramatic abnormalities are seen in the AGS3 knockout adult brain (unlike the USP9X forebrain knockout adult brain) it is unlikely that USP9X-dependent depletion of AGS3 accounts for the entire phenotype observed in USP9X cKO mice (Blumer et al., 2008). It is clear that other USP9X-substrate / binding partner interactions must play more essential roles during neural development.

**Epsin and Itch**
Epsin and Itch are regulators of Notch signaling, an important pathway that plays a role in cell fate choices during neural development (Lardelli et al., 1996). The Notch signaling pathway is recommended in section 5.6 for further analysis in future studies.
No direct interaction with USP9X has yet been observed in the CNS for ASK1, β-catenin, EFA6, MARCH7, MCL1, Smad4 and Survivin, however each of these proteins remain important candidates for USP9X mediated regulation of neural development. All of these proteins are substrates of USP9X and combined regulate a range of important cellular processes in the CNS (Dupont et al., 2009, Nagai, 2009, Nathan et al., 2008, Schwickart et al., 2009, Taya et al., 1999, Theard et al., 2010, Vong et al., 2005). In the CNS these proteins are known to play a role in cell cycle regulation, cell signaling, cellular polarity & cell-cell adhesion, apoptosis and ubiquitylation.

Previous studies have shown that USP9X interacts with its substrates (and binding partners) in a tissue, cell-type, developmental-stage, compartment-specific and concentration-dependent manner. The interaction between USP9X and its substrates may be regulated in a specific manner by the presence of any antagonists of USP9X activity, the presence of other positive and negative regulators of ubiquitylation or any other tissue-specific protein interactions these substrates may be involved in. The human genome encodes for at least 98 de-ubiquitylating enzymes (Fraile et al., 2011). The possibility exists for de-ubiquitylase redundancy. Any one of USP9X’s substrates may also be subject to de-ubiquitylase activity by another de-ubiquitylating enzyme(s). It remains to be determined which of these substrates require an interaction with USP9X to regulate their normal activity in the CNS.

Considering the large number of substrates, as well as many of these substrates such as β-catenin and Survivin having multifunctional roles, the use of microarray analyses in future studies may be a useful way to investigate a multitude of pathways simultaneously. In another study in which deficiencies in Nuclear factor IA (NFIA) contributed to aberrant corpus callosum and hippocampal development, microarray analyses were performed on brain tissue samples from E16 mouse embryos. These arrays revealed a differential expression pattern of over 3500 genes (Piper et al., 2010). Although such techniques often present overwhelming amounts of data, microarrays may be used preliminarily in future USP9X studies to quickly eliminate certain substrate associated pathways before moving on to more in-depth and conclusive proteomic approaches. As USP9X regulates proteins at the post-translational level a proteomic approach will also facilitate an analysis of the ubiquitylation status of any substrates under investigation. As USP9X in many cases regulates the levels of its
substrates a proteomic approach is also essential for comparing differences in mRNA versus protein degradation.

Considering the number of neurally expressed proteins USP9X is known to interact with and that many of these proteins play important roles during neural development it is hence likely that USP9X regulates neural development via multiple pathways and that multiple factors may contribute to a compound phenotype seen in USP9X cKO mice.

5.3 USP9X is required for normal axonal development

In the late embryo and newborn a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus was observed. In vitro analyses showed loss of USP9X results in defects in axonal development including deficiencies in early axonal specification, a reduction in elongation of the primary axonal process and a reduction in the number of axonal neurite termini. In the adult loss of USP9X resulted in a significant reduction in the size of the corpus callosum. The reduction in the corpus callosum, an axonal tract connecting the two cerebral hemispheres, suggests that USP9X may be specifically required for normal axonal development in vivo as the in vitro analysis suggests.

The thalamus is found between the cerebral cortex and the midbrain. The thalamus acts as a relay between the cerebral cortex and other sub-cortical areas (Steriade and Llinas, 1988). In future studies the thalamus may provide a model region in the adult brain for analysis of axonal projections deriving from the USP9X deficient cerebral cortex. In future studies the trigeminal ganglion (Section 5.5) may provide a model region for analysis of axonal development in the late embryo.

The TGFβ / BMP signalling pathways

Considering the phenotype in USP9X cKO mice, Smad4 may be an important candidate for regulation by USP9X during neural development (Dupont et al., 2009). Smad4 is an essential component of both the transforming growth factor beta (TGFβ) and bone morphogenetic protein (BMP) signalling pathways. TGFβ signalling is essential for normal axon formation (Dwyer and Winckler, 2010). Loss of TGFβ in vitro results in
failure of neurons to specify distinct axons (Yi et al., 2010). BMP signalling is thought to regulate hippocampal development (Grove and Tole, 1999). Smad4 null mouse embryos are smaller then littermate controls. By day E8.5 most Smad4 null embryos enter re-absorption or degeneration within the yolk sac. No Smad4 null embryos survive to birth (Sirard et al., 1998, Yang et al., 1998). When Smad4 is conditionally knocked out in the CNS (using the Cre/loxP system under the control of the Nestin promoter), post-implantation lethality also results similar to the observations made in Smad4 null mouse embryos (Yang et al., 2002, Zhou et al., 2003). However the lethality in Nestin-Cre mediated USP9X cKO mice is observed at a later stage compared to Smad4 cKO mice. Considering a possible USP9X-Smad4 requirement for normal neural development, the loss of USP9X may lead to a significant deficit in Smad4 modulated TGFß / BMP signalling but not the complete loss hence the more dramatic effect in Smad4 cKO mice where Smad4 is completely lost.

Loss of USP9X in the forebrain during development results in the presence of aberrant neuronal processes in the adult cerebral cortex. These aberrant neurons are characterised by the appearance of thick processes that project from the cell body towards the cortical plate. During migration and maturation cortical neurons display bipolar morphologies with thick leading processes (O'Rourke et al., 1992). Loss of TGFß signalling in vivo maintains neurons in a bipolar morphological state (Yi et al., 2010). These aberrant processes may be further characterised in future studies using 3-dimentional reconstruction combining a series (stack) of 2-dimentional brain slice images.

As de-ubiqitylation by USP9X activates Smad4 rather then stabilising its protein levels, gain or loss of USP9X is not expected to affect the levels of Smad4 (Dupont et al., 2009). An in depth analysis in future studies is required to access the potential role USP9X-Smad4 regulation may play for normal neuronal development. As Smad4 levels aren’t expected to change such investigations may commence with an analysis of neurally expressed downstream TGFß / Smad4 target gene responses. These may include JunB and PAI1 (Dupont et al., 2009, Rylski et al., 2009, Xin et al., 2010). Alternatively a Smad reporter such as the Cignal SMAD Reporter (luc) Kit: CCS-017L from SABiosciences Qiagen may be used.
Protein trafficking in neurons

DCX expression is restricted almost entirely to immature neurons (Francis et al., 1999). Hence DCX is the only protein that interacts with USP9X exclusively in the CNS. Though not a substrate de-ubiquitylated by USP9X, it is thought that DCX normally traffics USP9X within axons and other neuronal processes (Friocourt et al., 2005). This trafficking may facilitate interactions between USP9X and other substrates essential for axonal and neurite development. Besides the cell body and processes USP9X is expressed in synapses (Chen et al., 2003). Trafficking of USP9X may be of significant importance as USP9X has been shown to function in a concentration-dependent manner. Substrates like AF-6, EFA6 and Epsin are also highly expressed at the extremities of axons and in neuronal synapses (Chen et al., 2003, Sakagami, 2008, Xie et al., 2008). EFA6C like DCX is thought to be neuron-specific (Sakagami et al., 2004). Interestingly, when faf (the USP9X homologue in Drosophila) is over-expressed in neurons an increase in the number of synapses is observed (DiAntonio et al., 2001). Future studies may consider an analysis of these specific substrates.

5.4 Cellular polarity in the developing brain

USP9X interacts with several regulators of cellular polarity in a range of tissues and cell lines. These include AF-6, AGS3, β-catenin, EFA6 and MARK4 (Al-Hakim et al., 2008, Taya et al., 1999, Taya et al., 1998, Theard et al., 2010, Xu et al., 2010). Preliminary analysis suggests that loss of USP9X results in a diffuse ventricular architecture during embryonic development. Previous studies have shown that AGS3 immuno-precipitates with USP9X in the mammalian CNS whilst in this current study we show that loss of USP9X results in a reduction in AGS3 expression in the cerebral cortex at E17.5 (Xu et al., 2010). This loss of AGS3 was most evident around the ventricular zone. Interestingly the diffuse ventricular architecture was observed at E16.5 and E17.5. AF-6, β-catenin and EFA6A are highly expressed in several forebrain regions during normal development including the ventricular zone and the hippocampus (Buchert et al., 1999, Chenn and Walsh, 2002, Sakagami et al., 2004). Future studies should consider an analysis of AGS3 along with several of these other substrates. Whole brain USP9X cKO and control protein extracts from the late embryo have been prepared for use in future studies.
5.5 Loss of USP9X and the trigeminal ganglion

The trigeminal ganglion (TG) is a mass of neuronal cell bodies occupying a cavity found outside the brain at the base of the skull. High USP9X expression is evident in the developing TG at E14.5 (Friocourt et al., 2005) and E16.5 (Figure 5.1). For TG analysis a preliminary screen of 1 control versus 1 USP9X cKO mouse was performed. As the TG is found outside the brain whole embryonic heads at E18.5 were sectioned in the sagittal plane. Comparable sections were selected via matching TG sections with reference to other landmark features within as well as outside the brain. Landmark features within the brain including the forebrain, midbrain and hindbrain were used as reference points as were other features outside the brain including the brainstem, eye, nasal cavity and trachea. This screen found that loss of USP9X (Nestin-Cre mediated) results in a reduction in cell body volume of TG cells at day E18.5 in embryonic development (Figure 5.2). Trigeminal cells also appeared to display deficiencies in process specification though it is not possible to definitively characterise this from a single plane. Further analysis is required in future studies to confirm these deficiencies observed in the TG.

The TG is responsible for processing sensory aspects of the trigeminal nerve, receiving afferent impulses from multiple nerve branches. Neurons may respond detrimentally to a lack of synaptic inputs (Raff et al., 1994). As a requirement for USP9X for normal development of neuronal processes in the cerebral cortex has been established (Section 3.7) it is possible that similar deficiencies may also be present in the TG. In the TG deficiencies in specifying normal neuronal processes may generate significant effects as neuronal processes must project over relatively long and intricate pathways to reach their targets. Given the ease of accessing this region of the nervous system the trigeminal ganglion may provide a model region for analysis in future studies.
Figure 5.1 The Trigeminal ganglion

The trigeminal ganglion (TG) is a mass of neuronal cell bodies occupying a cavity found outside the brain at the base of the skull. USP9X (red) is highly expressed in the TG in the E16.5 mouse embryo. E = eye. F = forebrain. H = hindbrain. 10µm sagittal cryo-section. Scale bar 500µm.
Figure 5.2 Loss of USP9X results in reduced neuronal cell body volume in the trigeminal ganglion

Trigeminal neurons also appeared to display deficiencies in neuronal process specification (white arrows). Immature neuronal marker Beta-III-tubulin - green. USP9X control (top), USP9X cKO (bottom). n = 1. 10µm sagittal cryo-section. Scale bar 20µm.
5.6 Future analyses - the Notch signalling pathway

The transcription factor Nuclear factor IA (NFIA) acts downstream in the Notch signalling pathway. Loss of NFIA results in abnormal hippocampal development including dentate gyral reduction as well as defects in the development of the corpus callosum (Piper et al., 2010, Shu et al., 2003). Considering the phenotypical similarities between USP9X cKO mice and NFIA-Notch deficient mice and that three USP9X substrates (Epsin, Itch and Mindbomb1) are regulators of the Notch pathway, a Notch pathway analysis should be considered in any future studies (Overstreet et al., 2004, Qiu et al., 2000, Yoon et al., 2008).

5.7 Additional apoptotic and mitotic analyses

A mitotic analysis at prophase in the embryo at E15.5, E17.5 and E18.5 and newborn at P0 found no significant change in mitosis in the hippocampus, ventricular zone or any other part of the cerebral cortex (Section 3.9). Considering the dramatic reduction in hippocampal volume in adult USP9X cKO mice, a mitotic analysis focussing on the adult dentate gyrus (one of the distinct regions in the adult brain where neural progenitors reside) should be considered in future studies. The prophase marker phosphorylated Histone H3 may be used to label mitotic cells whilst the use of the thymidine analogue, ethynyl deoxyuridine (EdU) may be used to identify postmitotic cells that were undergoing mitosis at the time of EdU treatment (Chehrehasa et al., 2009).

An apoptotic analysis was performed in the embryo at E15.5, E17.5 and E18.5 and newborn at P0 (Section 3.10). The use of a larger sample size at E18.5 and P0 in future studies may identify significant differences in apoptotic levels. It is possible that a lack of synaptic inputs caused by a reduction in neuronal processes projecting between the anterior cingulate cortex and the subiculum of the hippocampus may induce an increase in apoptosis in these regions. Alternatively, any apoptotic changes identified in USP9X cKO mice may be directly induced by an interruption in some USP9X dependent apoptotic pathway as USP9X has been shown to interact with several regulators of apoptosis. These include ASK1, MCL1 and Survivin, all of which are known to play
roles during neural development (Nagai, 2009, Schwickart et al., 2009, Vong et al., 2005). Though no obvious change in hippocampal size was observed in the late embryo or newborn, preliminary analysis suggests distinct deficiencies in dentate gyrus development at postnatal day eight (Section 4.5). Considering that no significant change in apoptosis was observed in the adult hippocampus at seven weeks of age (Section 4.5), a staged apoptotic analysis from P0 to P8 should be considered in any future studies.

5.8 Future behavioural analyses

Loss of USP9X results in several behavioural responses which includes failure to suckle in the newborn and failure to mate in the adult. Adult female mice heterozygous for the knockout of USP9X at the genomic level were weighed at 20 months of age. These female mice had a mean difference in weight of approximately 16% less then littermate controls. Further behavioural analysis is required to determine if this weight difference results from a voluntary reduction in caloric intake or if there are any voluntary changes in physical activity, which may affect metabolism. Such analyses may also include motor skills testing as well as voluntary exercise (Blumer et al., 2008, Luong et al., 2011, Pietropaolo et al., 2006).

5.9 Future aged analyses

Loss of USP9X results in a significant reduction in the size of the adult hippocampus. Significant changes are often observed in the hippocampus as a result of normal aging. In rodents, aging results in a loss of synapses in specific regions of hippocampus, in particular the dentate gyrus and the CA1 region (Rosenzweig and Barnes, 2003). In the adult brain the small proportion of neural progenitors that remain undifferentiated spend most of their time in a quiescent state. These progenitors reside in distinct niches such as the subgranular zone (SGZ) of the dentate gyrus in the hippocampus one of the limited places in the adult brain where neurogenesis takes place (Alvarez-Buylla et al., 2001, Alvarez-Buylla and Lim, 2004, Lledo et al., 2006). It is thought that neural
progenitors within the SGZ mature locally into granule neurons that remain in the hippocampus (Markakis and Gage, 1999, Stanfield and Trice, 1988). Considering the abnormalities in neuronal development and the massive reduction in hippocampal volume in the young adult it is possible that loss of USP9X may also result in deficiencies in adult neurogenesis in older adults. Age related hippocampal degeneration may be accelerated in USP9X deficient mice. Further analysis is required to address these questions. As part of this project at least 30 $USP9X^{cKO}/Y$ mice were generated for this purpose and will shortly reach a suitable age to commence these analyses in future studies.
Appendix 1 - International conference presentations

Updated version of a poster presented at the Australian Neuroscience Society, 31st annual meeting, Auckland, New Zealand, Feb - 2011.
Appendix 2  - Supplementary figures

Figure 1  **USP9X is highly expressed in the developing CNS at E14.5**
Choroid plexus in the wild-type mouse embryo at day E14.5 in embryonic development. USP9X - red, Dapi nuclear marker - blue. 10µm sagittal cryo-sections. Scale bar 20µm.

![Image of choroid plexus](image1)

Figure 2  **Nestin-Cre mediates knockout of USP9X at the protein level**
Western blot analyses of whole brain protein extracts from E18.5 embryos. Image = 2 controls vs 6 cKO. USP9X = C-terminal antibody.

![Image of Western blot](image2)


DOBYS, W. B., ANDERMANN, E., ANDERMANN, F., CZAPANSKY-BEILMAN, D., DUBEAU, F., DULAC, O., GUERRINI, R., HIRSCH, B., LEDBETTER, D.


GOODMAN, T., TROUCHE, S., MASSOU, I., VERRET, L., ZERWAS, M., ROULLET, P. & RAMPON, C. 2010. Young hippocampal neurons are critical for recent and remote spatial memory in adult mice. Neuroscience, 171, 769-78.
GULISANO, M., BROCCOLI, V., PARDINI, C. & BONCINELLI, E. 1996. Emx1 and Emx2 show different patterns of expression during proliferation and


characterization of neuronal populations in the subplate and marginal zone. J Comp Neurol, 342, 571-95.


