Loss of Usp9x Disrupts Cortical Architecture, Hippocampal Development and TGFβ-Mediated Axonogenesis

Shane Stegeman1, Lachlan A. Jolly2,3, Susitha Premarathne1, Jozef Gecz2,3, Linda J. Richards4, Alan Mackay-Sim1, Stephen A. Wood1*

1 Eskitis Institute for Cell and Molecular Therapies, Griffith University, Brisbane, Queensland, Australia, 2Neurogenetics Research Program, South Australian Pathology at Women’s and Children’s Hospital, Adelaide, South Australia, Australia, 3 School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, Australia, 4 Queensland Brain Institute and The School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland, Australia

Abstract

The deubiquitylating enzyme Usp9x is highly expressed in the developing mouse brain, and increased Usp9x expression enhances the self-renewal of neural progenitors in vitro. USP9X is a candidate gene for human neurodevelopmental disorders, including lissencephaly, epilepsy and X-linked intellectual disability. To determine if Usp9x is critical to mammalian brain development we conditionally deleted the gene from neural progenitors, and their subsequent progeny. Mating Usp9x+/− Emx1-cre mice with mice expressing Cre recombinase from the Nestin promoter deleted Usp9x throughout the entire brain, and resulted in early postnatal lethality. Although the overall brain architecture was intact, loss of Usp9x disrupted the cellular organization of the ventricular and sub-ventricular zones, and cortical plate. Usp9x absence also led to dramatic reductions in axonal length, in vivo and in vitro, which could in part be explained by a failure in Tgf-β signaling. Deletion of Usp9x from the dorsal telencephalon only, by mating with Emx1-cre mice, was compatible with survival to adulthood but resulted in reduction or loss of the corpus callosum, a dramatic decrease in hippocampal size, and disorganization of the hippocampal CA3 region. This latter phenotypic aspect resembled that observed in Doublecortin knock-out mice, which is an Usp9x interacting protein. This study establishes that Usp9x is critical for several aspects of CNS development, and suggests that its regulation of Tgf-β signaling extends to neurons.

Citation: Stegeman S, Jolly LA, Premarathne S, Gecz J, Richards LJ, et al. (2013) Loss of Usp9x Disrupts Cortical Architecture, Hippocampal Development and TGFβ-Mediated Axonogenesis. PLoS ONE 8(7): e68287. doi:10.1371/journal.pone.0068287

Editor: Berta Alsina, Universitat Pompeu Fabra, Spain

Received February 24, 2013; Accepted May 28, 2013; Published July 5, 2013

Copyright: © 2013 Stegeman et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by a grant from the National Health and Medical Research Council of Australia APP1009248 to SAW, APP628952 and APP250340 to JG, a grant from the Women’s and Children’s Hospital Foundation to LAJ, and a grant to AMS from the Australian Government Department of Health and Ageing. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: s.wood@griffith.edu.au

† These authors contributed equally to this work.

Introduction

During embryonic development of the brain, neural cells need to respond rapidly to changing environmental cues. In the developing axon and dendrites, these decisions are made at a distance from the nucleus and so rely heavily on post-translational mechanisms. The ubiquitin system regulates protein stability, localisation and function in a rapid and quantitative manner and has been shown to regulate multiple aspects of neural development [1] [2] [3] [4]. Not surprisingly, given the precipitous consequences of protein ubiquitination, defects in the ubiquitin system have been linked to a range of neurodevelopmental diseases [5] [6] [7] [8] [9]. Specificity in the ubiquitin system is imparted by the hundreds of E3 ubiquitin ligases and deubiquitylating enzymes (DUBs), which add or remove ubiquitin, respectively. DUBs function downstream in the ubiquitin pathway, thus having the potential to act as final arbiters of protein substrate fate and function [10] [11] [12]. Several studies have shown that DUBs play important roles in the growth, function and maintenance of neurons and synapses [13] [14,15].

Ubiquitin specific protease 9, located in the X chromosome (Usp9x, also called FAM), is a substrate-specific DUB that is highly expressed in the developing CNS in both humans and mice [16–19]. Although Usp9x expression decreases in the mature CNS it remains strongly expressed in the neurogenic regions including, the sub-ventricular zone of the lateral ventricles and the sub-granular zone cells of the dentate gyrus [17,20]. Usp9x function has been implicated in several aspects of CNS development. Increased expression of Usp9x in embryonic stem cell-derived neural progenitors promotes their organisation into polarised clusters, and increases their self-renewal and cellular potency [17]. In Drosophila, Usp9x’s homologue, fat facets (faf) regulates photoreceptor fate as well as synaptic morphology and function [15,21]. In humans USP9X has been implicated in lissencephaly and epilepsy [16] and is an X-linked Intellectual Disability candidate gene [22].

Usp9x is a large DUB (2554 amino acids) and several of its substrates regulate aspects of neural development and/or homeostasis. These include components of neurodevelopmental signalling pathways such as Notch [23–25], Wnt [26] and TGF-β [27].
In the Notch pathway Usp9x regulates the trafficking accessory protein Epsin [29], as well as the ubiquitin ligase Mind Bomb1 [29] [30] in the signal sending cells. Usp9x also stabilises the Notch intracellular domain E3 ligase, Ictch [31], which functions in the signal receiving cell [23,25]. Usp9x directly binds and stabilises β-catenin, a component of cell-cell adhesion and a Wnt signalling pathway second messenger, in a range of the mammalian cells and tissues, including the CNS [26,32,33] where it is required for proper development [34–36]. Usp9x deubiquitylation of Smad4 is essential for signalling by members of the Tgfβ family [27,37].

Still other Usp9x substrates regulate neural progenitor adhesion and proliferation. Acute lymphoblastic leukemia-I fusion partner from chromosome 6 (AF-6) is essential for the establishment of adherens junctions and polarity in neural progenitor cells [38,39]. Usp9x regulates both the stability and localisation of AF-6 [40] [41]. Another Usp9x substrate, Activator of G protein Signalling 3 (AGS3) is involved in spindle orientation and asymmetric cell division in cortical progenitors [42] [43]. Finally, Usp9x binds the microtubule-assocated protein Doublecortin (DCX), which is involved in neuronal migration, protein sorting and vesicle trafficking [16] [44]. The interaction between Usp9x and DCX is clearly important for human CNS development as patients with point mutations in DCX that cannot bind Usp9x, develop lissencephaly [16].

The above evidence suggests that there are ample avenues through which Usp9x might regulate CNS development. However, there are two significant caveats to implying a role for Usp9x in neural development in vivo based simply on the observed substrate associations. These are, (i) most of the Usp9x-substrate interactions and regulation have been determined in cultured cells; (ii) whether or not Usp9x is the rate-limiting determinant of a substrate’s fate, and any subsequent developmental consequences, is largely cell-context-specific. Therefore, to assess the requirement for Usp9x during mammalian CNS development we conditionally deleted the Usp9x gene in neural progenitors using tissue-specific Cre recombinase gene expression. Loss of Usp9x in all neural progenitors resulted in early post-natal lethality, and was associated with disorganised cortical architecture and reduced corpus callosum and hippocampal volumes. More detailed analysis of neurons revealed neurite growth defects that can, in part, be explained by refractory responses to TGFβ stimulation. The results demonstrate novel roles for Usp9x in brain development.

Materials and Methods

Ethics Statement

All experiments were performed under ethical clearance from the Griffith University, and The Women’s and Children’s Health Ethics Committees. The research was conducted in accordance with the policy and guidelines of the National Health and Medical Research Council of Australia. Animals were monitored for signs of pain and distress, all euthanasia was performed using cervical dislocation, and all efforts were made to minimize suffering.

Generation of Usp9xloxP Mice

Usp9xloxP mice were generated by Ozgene Pty Ltd, Bentley, Australia, as described [45]. Briefly, loxP sites were incorporated into the second and third introns flanking exon three. Initiation of translation occurs in exon two, followed by 96bps of coding sequence. Deletion of exon three (146 bp), which contains an incomplete number of codons, would result in a frame shift with the next six alternative ATG start codons out of frame. To assess if any translation occurred from an in frame start codon downstream of exon three, an antibody raised against the Usp9X C-terminal was used.

Deletion of Usp9x in the Developing Mouse Brain

Usp9xloxP/loxP female mice were crossed with Nestin-Cre [46] or Emx1-Cre [47] males, to delete Usp9x from the whole brain (Nestin cre) or dorsal telencephalon (Emx1-cre), respectively. Using this breeding scheme Usp9x would be deleted from males, which inherited Cre recombinase and in this gender result in a Usp9x null genotype. Cre-negative male littersmates were used as negative controls in all experiments, except where noted. Cre-positive female offspring are heterozygous for Usp9x gene deletion.

For all analyses a minimum of three or more Usp9xloxP/Y mice versus three or more littermate controls were used. Results were assessed statistically using a Student’s t test unless otherwise specified.

Mouse Genotyping

DNA was extracted from neural tissues (brain or spinal cords) and PCR was performed using standard techniques. Primers were designed to detect Cre recombinase. for 5’-TATGAGGTGTCGGGAAGACC, rev 5’-CCATGAGTGAACCGACTGTT. Male embryos were identified using primers for the Sry region of the Y chromosome: for 5’-GAGCGCAAGTGGCCAGACCA, rev 5’-GTTTCCGTTCGCCACTGCAAGAG. Usp9x primers: for 5’-GGTACCACTTATTTGTTTAG, rev 5’-TAGACCATCATGACAGT.

Reverse Transcription-PCR Analysis

Total RNA was extracted from brains or testes using TRIzol reagent (Invitrogen). Total RNA was treated with DNase I (Invitrogen) then subjected to a mRNA purification step using a MicroPoly(A) Purist mRNA purification kit (Ambion). Reverse Transcription was performed using SuperScript III Reverse Transcriptase primed with oligo(dT) (Invitrogen). PCR was then performed on cDNA using standard techniques. Usp9x primers: F 5’-ATGGCGATTGACACGTATTAG, R 5’-GTCTTTCTCATCACCCCTCGATA. qPCR reactions on RNA isolated from hippocampal neurons were generated using the iTaq SYBR Green Supermix (Biorad), run on the StepOne Plus Real Time PCR System and analysed using StepOne Sortware V2.0 (Applied Biosystems). Primers included; Baf1 F: ACTGGCTGACACCTTTTGGAGC, R: GCCGTCTTTATGTTTCTCTTG; Has1 F: AATGACCTGTGAACGACCTGC R: GTCATGACACTGCTGAAGC; EphB2 F: GTTTGATCTCTAAGATGATGG R: GTCAACACCTCTACAGACTGG; Runx1 F: TCTGCGAGACTTTCCAGTCG R: GAAGTTGACCGCAGAGTAGG.

Western Blot Analysis of Brain Tissue

Protein was extracted from embryonic and adult brain tissues and Western blots were performed as described previously [33]. Signal was detected using horseradish peroxidase-conjugated secondary antibodies (Millipore) developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) then imaged on a VersaDoc 4000 MP Imaging System (BioRad).

Histology and Immuno-fluorescence on Brain Sections

For analysis of embryonic brains, samples were drop fixed in 4% paraformaldehyde. For adult brains animals were anesthetized, perfused trans-cardially with 4% PFA then heads were drop-fixed in 4% PFA. Following fixation brains were processed for
paraffin or cryo-sectioning using standard techniques and sectioned at 10 μm. Histological analyses were performed using standard cresyl violet (Nissl) staining. Hippocampal area was calculated using SPOT software (Diagnostic Instruments Inc). The hippocampal area of the first control was designated 100. The hippocampal areas of all other controls and Usp9x<sup>loxP/loxP</sup>/Y mice were converted to ratios compared to the first control. For immunofluorescence, brain sections were blocked with normal donkey serum (Invitrogen), incubated overnight at 4°C with primary antibodies, then incubated for 3 h at room temperature with secondary antibodies and mounted with Vectashield mounting medium (Vector Laboratories). Images were obtained on an AxioImager Z1 microscope (Carl Zeiss) or Olympus FV1000 confocal microscope. For corona
dal sections, sections from a comparable position along the rostral-caudal axis were used [48]. 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 [49].

**Neuronal Cell Assays**

For hippocampal-derived neuronal cultures, embryos were harvested at E18.5 and hippocampal neurons isolated and cultured as previously described [50]. For morphometric analysis, isolated cells were transfected with pMAX-EGFP using the mouse neuron nucleofector kit according to the manufactures instructions (Lonza). Cells were fixed with 4% PFA and immunofluorescence was performed as previously described [50]. Images were generated on an Axioplan2 microscope (Carl Zeiss). Axons and dendrites were identified using Tau1 and MAP2 immunoreactivity, respectively, and measured using ImageJ software (National Institute of Health). Only neurites 10 μm in length or longer were included. For neurite growth kinetic analysis Usp9x<sup>loxP/loxP</sup>/Y cultures were transfected with primary antibodies and cultured with Vectashield mounting medium with DAPI (Vector Laboratories). Images were obtained on a Axioliner Z1 microscope (Carl Zeiss) or Olympus FV1000 confocal microscope. For corona
dal sections, sections from a comparable position along the rostral-caudal axis were used [48]. 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 [49].

**Primary Antibodies**

Rabbit (Rb) anti-USP9X (Bethyl Laboratories A301-351A), Rb anti-GapDH (R&D Systems 2275-PC-100), Rb anti-NF160 (Abcam ab9034), Chicken (Ck) anti-MAP2 (Millipore AB15452), Mouse (Ms) anti-Tau1 (Millipore AB1512), Ms anti-MAP2 (Sigma-Aldrich M 1406), Rb anti-GFAP (DakoCytomation Z0334), Ck anti-MAP2 (Chemicon), Ms anti-04 (Millipore MAB345), Rb anti-Cleaved caspase-3 (Cell Signaling Technology 9661), Rb anti-DCX (Abcam ab18723), Rabbit anti-BLB (Millipore AB114).

**Secondary Antibodies**

Dk anti-rabbit Alexa-Fluor 594 (Invitrogen), Dk anti-mouse Alexa-Fluor 647 (Invitrogen), Dk anti-chicken Cy3 (Jackson Laboratories), Dk anti-mouse Alexa-Fluor 488 (Invitrogen), Gt anti-rabbit HRP (Millipore).

**Results**

**Deletion of Usp9x from the Developing CNS Results in Perinatal Lethality**

As Usp9x is required for pre-implantation mouse embryo development [41], in order to study Usp9x's role in brain development we generated Usp9x<sup>loxP/loxP</sup> females and bred them with heterozygous males expressing Cre recombinase from the Nestin promoter-enhancer, which is active in all CNS neural progenitors from E10.5 [52]. Using this strategy, males that inherited the Nestin-Cre transgene, would be potentially Usp9x null (hereafter referred to as Neo-Usp9x<sup>−/−</sup> mice). In preliminary studies using mouse ES cells in vitro, we had established that activation of Cre resulted in the loss of Usp9x exon3 and the Usp9x protein (data not shown). The efficiency of Usp9x exon3 and protein deletion in the brain in vivo was initially evaluated in E18.5 Usp9x<sup>−/−</sup> embryos (Figure S1A,B) by PCR and immunoblot using a C-terminally targeted Usp9x antibody (Figure S1B). Immunobots using an antibody against the N-terminal 20 amino acids of Usp9x showed a similar reduction in full-length (290kDa) Usp9x levels (data not shown). We observed some residual full-length Usp9x protein, but this was likely due to blood vessels and brain meninges that were present in the samples [53] [54] [52]. Immunofluorescence analyses detected very low, residual levels of Usp9x protein in neural cells in E12.5 embryos (n = 4) and confirmed complete loss of Usp9x protein in Neo-Usp9x<sup>−/−</sup> embryos by E14.5 (n = 4 embryos) (Figure S.1 C–F).

**Nestin-Cre** mediated loss of Usp9x throughout the brain resulted in early postnatal lethality. Of 154 pups analyzed, from 18 litters, all Neo-Usp9x<sup>−/−</sup> male mice died within 24 hours of birth. Although Neo-Usp9x<sup>−/−</sup> males could move at birth, were pink and appeared to breathe normally, they failed to suckle, as evidenced by lack of milk in their stomachs at the time of death. Female offspring heterozygous for the knockout of Neo-Usp9x (Usp9x<sup>−/−</sup>), appeared normal at birth and survived to adulthood at rates similar to wild-type females.

Usp9x has a homologue on the Y chromosome, Usp9y. Although Usp9y is reported to only be expressed during spermatogenesis in the mouse [55], we performed RT-PCR on P0 mouse brains to see if its expression is induced following the deletion of Usp9x. We failed to detect Usp9y expression in these brains indicating that it is not induced and potentially compensating for some Usp9x functions (Figure S2).

**Loss of Usp9x Results in Reduced Neuronal Processes**

Analysis of E16.5 to E18.5 brains failed to detect a significant decrease in the size of Neo-Usp9x<sup>−/−</sup> brains and low power
histological analysis revealed that all major CNS regions were present in the absence of Usp9x (not shown). However, Nissl staining revealed a general disorganization of the brain architecture. The most obvious loss was a clear demarcation between the neural progenitors in the ventricular and subventricular zones and the neuroblasts of the intermediate zone in E16.5 embryos (Fig. 1A–D) and E17.5 (not shown). These observations were confirmed in E18.5 embryos where the neural progenitor and radial glial markers Nestin and Brain Lipid Binding Protein (BLBP) were more diffusely localized and disorganized in Usp9x-/- embryos (Fig. 1E,H). The neurons of the cortical plate were also less densely packed in the absence of Usp9x (Fig. 1J).

We next sought to determine the affect of Usp9x loss on neurones and glia by staining for NF160 and GFAP, respectively. In the absence of Usp9x we detected a dramatic reduction in the number and length of NF160-positive neuronal processes. This was most evident in those projecting from the entorhinal cortex towards the hippocampus (Fig. 2A,B). GFAP staining was reduced in size in the hippocampus and did not extend as far medially in the cerebral cortex in E18.5 embryos (Fig. 2C,D). Within the dentate gyrus of the hippocampus GFAP stained projections were not as extensive in the absence of Usp9x (Fig. 2E,F).

As Nes-Usp9x-/- mice die at P0, to study the effects of Usp9x deletion on post-natal stages of brain development we mated Usp9x-loxP/loxP females to heterozygous Emx1-Cre males, where Cre expression is restricted to the neural progenitors of the dorsal telencephalon from E9.5 onwards [56]. Loss of almost all the dorsal telencephalon is compatible with survival [57] and indeed Emx1-Cre mediated Usp9x knockout mice (referred to as Emx1-Usp9x-/- hereafter) survived into adulthood. In an attempt to generate Emx1-Usp9x-/- females we paired four adult Emx1-Usp9x-/- males with eight Usp9x-loxP/loxP females over a period spanning four months, however, no litters were produced. Therefore, Emx1-Usp9x-/- mice fail to produce viable offspring.

In Emx1-Usp9x-/- mice, Emx1-Cre activity resulted in loss of Usp9x protein from the telencephalon (Figure S3). As observed in Nes-Usp9x-/- embryos, the neocortex of Emx1-Usp9x-/- mice had reduced neuronal processes projecting through the entorhinal cortex towards the hippocampus (not shown). In adult (7–8 wk old) Emx1-Usp9x-/- mice NF160 immunoreactivity detected aberrant neuronal processes (Fig. 3 A,B). In the absence of Usp9x, NF160-immunoreactive processes were much thicker and predominately projected toward the pial surface (top of image), rather than ventricle (Fig. 3A,B). Aberrant processes were found in all cortical layers. The localisation of the dentritic marker MAP2 was similar in the presence or absence of Usp9x. However while the number and length of NF160-positive neuronal processes. This suggests that Usp9x is required for the growth of axon tracts in the brain. Usp9x binds Doublecortin (Dcx) protein which is important for neuronal migration in the human cortex [50]. Loss of Usp9x however, in both Nes-Usp9x-/- (E12.5–E18.5) and Emx1-Usp9x-/- (P7) mice did not affect the overall localization or level of Dcx in the developing cerebral cortex (Figure S3).

Deletion of Usp9x Results in Reduced Hippocampal Size

Loss of Usp9x in the forebrain also dramatically affected the size of the hippocampus. Analysis of hippocampal area from matched sections detected a 74% reduction in hippocampal area in adult Emxl-Usp9x-/- mice (Fig. 5A–C). A similar degree of reduction in the absence of Usp9x was evident from the most rostral to caudal aspects of the hippocampus. We also analysed the hippocampi of adult Emx1-Usp9x-/- heterozygous females and detected a reduction of hippocampal area, though to a lesser extent (data not shown).

Despite the reduction in size, most regions of the adult hippocampus retained their relative organisation and cytoarchitecture. However, the cytoarchitecture of the CA3 region of the stratum pyramidale was particularly affected (Fig. 5 D–F). There was a significant thinning of the CA3 region in Emxl-Usp9x-/- mice (48.51+/−0.21 μm SEM (Usp9x+/+, n = 6); 21.95+/−6.43 μm SEM (Emxl-Usp9x-/-, n = 5, p = 0.011)). There were no significant alterations in the thickness of CA1 or CA2 areas, or within the granular cell layers of the dentate gyrus in Emxl-Usp9x-/- mice (data not shown). A less severe reduction in hippocampal size was already apparent in E17.5 and E18.5 embryos (data not shown).

A reduction in cell number may result from decreased proliferation, increased apoptosis or a reduction in the migration of neurons or glia within the hippocampus during development. Analysis of apoptosis by staining for cleaved caspase-3 [48] (Figure S4) revealed that loss of Usp9x increased apoptosis in the hippocampus and medial neocortex at E18.5 and P0 (Usp9x+/+ 1.1+/−1.1 cells (n = 9) versus Usp9x--/ 5.9+/−5.1 cells, n = 10; p = 0.013).

Deletion of Usp9x Results in Reduced Axonal Elongation and Tgf-β Response in vitro

To determine if reduced axon length was due to cell autonomous effects of Usp9x, we cultured hippocampal neurons in vitro and measured both neurite length and branching. [59]. Cultured Nes-Usp9x--/ hippocampal-derived neurons displayed both reductions in primary axonal length and in the number of axonal and dendritic termini, which reflects their degree of arborisation (Fig. 6). At day 3 of culture, Usp9x--/ axons were 30% shorter than control axons, whilst by day 7 of culture this difference had reached 44% (Fig. 6B). Likewise, the total number of neurite termini was reduced by 28–36% across all days of culture, and whilst the reduction in axonal termini number was the predominant contributor to this result (with reductions ranging between 43–53%), reductions in dendritic termini were also observed (Fig. 6C). Given the low cell density in these experiments the data strongly suggest that Usp9x function is required cell autonomously for the initiation and/or elongation of neurites, which supports our in vivo observation.

Usp9x is a major regulator of the Tgf-β family signalling pathway due to its deubiquitylation and hence activation of, the common Smad protein, Smad4 [27,37]. Recently, Tgf-β2 has been shown to be important for axon initiation and elongation [60], whilst it is well established that supplementing cultured neurons with TGFβ increases axonal growth [61]. Therefore we sought to determine if loss of Usp9x affected Tgf-β signalling in neurons (Fig. 7). To test the status of Tgf-β signalling in the absence of Usp9x we transfected hippocampal neurons with a Tgf-β-luciferase reporter construct [51]. We detected 2.6-fold higher basal levels of luciferase activity, normalised against renilla luciferase activity in the absence of Usp9x (Fig. 7A). However, while wild-type neurons could respond to increasing concentrations of Tgf-β with higher luciferase activity, there was no change in the
Figure 1. Loss of Usp9x disrupts the architecture of the embryonic neocortex. The Nestin-cre mediated deletion of Usp9x (B,D) results in loss of demarcation between the cells of the ventricular and sub-ventricular zones (VZ/SVZ), the more disperse cellular density of the intermediate zone (IZ) and the neurons of the cortical plate (CP) seen in control littermates (A,C). C and D are higher magnification images of A and B, respectively. Nestin (E,F) and BLBP (G,H) staining in E18.5 embryos indicated that neural progenitors were more loosely organized in the VZ/SVZ. Neurons of the
absence of Usp9x (Fig. 7B). This difference was greatest in the presence of 10 ng/ml TGFβ, resulting in a 3.3 fold elevation of luciferase activity relative to basal levels in control neurons, compared to only a 1.3 fold elevation in Usp9x<sup>-/-</sup> neurons. This suggested that Usp9x is required in neurons to respond to Tgf-β.

We next measured the mRNA levels of putative Tgf-β target genes in response to 1 ng/ml Tgf-β in the presence and absence of Usp9x. The TGF-β target gene *Bdnf* [62] showed no response to Tgf-β in the absence of Usp9x. *Bdnf* acts as a self-amplifying autocrine factor to promote axon formation and growth in hippocampal neurons [63]. Paradoxically, *Runx1* showed a higher response to Tgf-β in the absence of Usp9x. However as *Runx1* responds to TGF-β in a Smad4-independent manner [64] it might not be affected by the absence of Usp9x which deubiquitytes Smad4. The other putative target genes assayed, β-catenin, Ephb2 and *Hes1* did not respond to Tgf-β. Finally we measured the response of axon growth and neurite arborisation to 1 ng/ml Tgf-β exposure following 5 days of culture. Consistent with previous reports [61] we detected an 49% increase in axonal length in wild type neurons in response to Tgf-β. In comparison, only a negligible increase (14%) in axonal length was detected in Usp9x-null neurons (Fig. 7D). Likewise, whilst the number of neurite termini was increased by 40% in response to Tgf-β in control neurons, again predominately because of increases in axonal branching, no such response was observed in cells lacking Usp9x (Fig. 7C). These data establish that axon growth in...
hippocampal neurons requires Usp9x in order to respond to Tgf-β. Loss of Usp9x did not affect the viability of the cultured hippocampal neurons (Figure S6).

Discussion

Usp9x is highly expressed throughout the embryonic brain and in specific regions of the adult brain including the neurogenic zones [16,17]. To investigate whether Usp9x is required for neural development and maintenance we deleted Usp9x in neural progenitors of the developing brain using both a Nestin-Cre and Emx1-Cre deletion strategy. Here, we report that loss of Usp9x in the entire brain results in death within 24 hours of birth, possibly due to a failure to suckle. Although the overall architecture of the brain developed normally in the absence of Usp9x, its loss resulted in reduction of the corpus callosum and hippocampal size. At higher resolution, we revealed loss of Usp9x led to disorganisation of the developing neocortex architecture, observed early within the neural progenitor populations, and in the axonal projections of the neurons of the cortical plate.

Our data show that during neuronal development Usp9x is required for the correct development of CNS axons. The severe reduction of the corpus callosum in Emx1-Usp9x<sup>-/-</sup> mice is consistent with this observation. To examine the molecular mechanisms underlying the axonal defect we assessed Tgf-β signalling capacity in cultured hippocampal neurons. The rationale for this was based on two observations. First, Usp9x regulation of Tgf-β signalling has been demonstrated in other developmental systems, as diverse as Drosophila wing development and dorsal-ventral patterning, the gastrulating Xenopus embryo, and wound healing in human cells [27,37]. Usp9x regulation of these systems is due to its deubiquitylation of the common Smad protein, Smad4, which partners with receptor Smads to activate

Figure 3. Absence of Usp9x alters neuronal projections in the neocortex. NF160 staining of adult cerebral cortex (7–8 wk) Emx1-Usp9x<sup>+/Y</sup> (A,C,E) and Emx1-Usp9x<sup>-/-</sup> (B,D,F). NF160 stains thick, pial-oriented projections in Emx1-Usp9x<sup>-/-</sup> mice (B). NF160 (red) colocalises with the dendritic marker MAP2 (green) in the thick projections detected in Emx1-Usp9x<sup>-/-</sup> cortical neurons (arrowheads in D,F). There is little overlap in control littersates (C,E). Orientation is such that the pial surface (P) is to the top, and ventricle (V) to the bottom of each image. Scale bar = 30 μm (A), 40×μm (CE).

doi:10.1371/journal.pone.0068287.g003
Tgf-β family member target genes [65]. Cyclic ubiquitylation and deubiquitylation of Smad4 by the E3 ligase Ectodermin and Usp9x, respectively, regulates the nuclear/cytoplasmic shuttling of Smad4 which is required for continued Tgf-β signalling in the presence of ligand [27]. The second observation is that the Tgf-βRII receptor is required for axonogenesis in the mouse brain [60]. The axonal phenotype observed in these mice is similar to those in our Usp9x null mice. Our data show that axons from hippocampal neurons did not increase in length in response to exogenous Tgf-β in the absence of Usp9x. In addition, a Tgf-β reporter construct and the endogenous target gene Bdnf, did not respond to increasing TGF-β concentration in the absence of Usp9x (Fig. 7). These data strongly suggest there is little Tgf-β signalling in neurons in the absence of Usp9x explaining, at least in part, the failure or delay in axon growth in Usp9x deleted brains. However, Usp9x facilitation of Tgf-β signalling does not entirely explain our observed phenotype. Deletion of Smad4 in the brain results in a much milder phenotype than in the Usp9x−/− mice [66]. In addition, although Tgf-βRII is required for axon development it had no effect on dendrites, but dendritic development was also impaired in the absence of Usp9x, at least in vivo (Fig. 4) suggesting Usp9x regulates other critical substrates during CNS development.

In addition to its requirement during axonal development, loss of Usp9x also affected the localisation of the axonal protein NF160 in the forebrain of adult Emx1-Usp9x−/− mice. In a number of neurons NF160 co-localised with the dendritic marker MAP2 in thick processes projecting toward the pial surface (Fig. 4). This may reflect inappropriate trafficking of NF160 to dendrites, and Usp9x has been implicated in the trafficking of proteins in a polarised manner [33,67]. Alternatively, the mis-localisation of NF160 may reflect a more general loss of polarity in Usp9x null neurons. The earliest consequence of Usp9x loss was disorganised...
sation of neural progenitors in the VZ/SVZ of the neocortex observed in Nissl-stained sections and the tangled organisation of nestin and BLBP (Fig. 1). Usp9x regulates a number of cell adhesion and polarity proteins in polarised epithelial cells and increased expression of Usp9x enhances the polarised organisation of neural progenitors in vitro [17]. We have identified a number of

Figure 5. Usp9x is required for hippocampal development. The hippocampi of adult Emx1-Usp9x<sup>−/−</sup> mice (B) were reduced in area compared with Usp9x<sup>+/+</sup> littermates (A). Nissl stain of 7–8-week old mice. (C) Quantification of hippocampal area of Usp9x<sup>−/−</sup> adult males (Control, n = 4) compared with Usp9x<sup>−/−</sup> (cKO, n = 4) (** p < 0.01). (D–F) Higher magnification identifying disruption and reduction of the CA3 region in Usp9x<sup>−/−</sup> males. (D - Usp9x<sup>+/+</sup>; E,F independent Usp9x<sup>−/−</sup> males) Scale bar = 100 μm (A,B), 150 μm (D–F).
doi:10.1371/journal.pone.0068287.g005

Figure 6. Loss of Usp9x reduces neuronal outgrowth. Embryonic hippocampal neurons were isolated, transfected with a plasmid encoding Enhanced Green Fluorescent Protein, and grown in-vitro for 3, 5 or 7 days. (a) Example immunofluorescent images of wildtype (Nes-Usp9x<sup>+/+</sup>) and null (Nes-Usp9x<sup>−/−</sup>) neurons resolved using GFP expression (Green) and co-stained with the axonal and dendritic specific antibodies, TAU1 (cyan) and MAP2 (red) respectively. (b–c) Morphometric analysis was employed to record mean primary axonal length (b) and number of neurite termini (c). *p < 0.05 by student 2-tailed t-test.
doi:10.1371/journal.pone.0068287.g006
cell adhesion and polarity complex proteins mis-localised or mis-expressed in Usp9x null neural progenitors (SP, SAW in preparation). These early effects may have ramifications for later developmental events such as axon elongation.

Loss of Usp9x also reduced the size of the hippocampus by 75% (Fig. 5). Hippocampal reduction was observed at late embryonic stages though not as severe as in the adult. An increase in apoptosis was observed in E18.5 and P0 Emx1-Usp9x<sup>2/Y</sup> in the hippocampal region and may, in part, contribute to the reduction. A potential candidate mediating this effect is the anti-apoptotic protein Mcl1, which can be stabilised by Usp9x, at least in lymphoma and cultured cell lines [68]. However, deletion of Mcl1 results in a more severe loss of neural cells throughout the entire CNS. Therefore Usp9x may only be a rate-limiting factor of Mcl1 levels in the hippocampus. The hippocampal phenotype, in particular the reduction in size, disorganisation of the CA3 region, together with the absence or reduction of the corpus callosum, is similar to that observed in Doublecortin (Dcx) and Doublecortin-like (Dckl) double knock-out mice [69]. Dcx is an Usp9x interacting protein, though not a substrate, as it is not ubiquitylated [16]. Instead it is proposed that Dcx traffics Usp9x along axonal microtubules. A mutation in Dcx, which is unable to bind specifically to Usp9x, was detected in a patient with lissencephaly, suggesting this interaction is important in human cortical development [16].

Given the similarity in phenotype in the mouse knockout models, it may be that Usp9x is also required for the migration of hippocampal neurons, especially those destined for the CA3 region. The disorganisation of nascent neurons in the cortical plate in embryonic cerebral cortex (Fig. 1) also supports a role for Usp9x in neuronal migration. However, this may be independent of Dcx as its expression was not altered in the cerebral cortex in the absence of Usp9x (Figure S3).

The Bmp and Wnt signalling pathways at the cortical hem in the medial neocortex are essential for the development of the hippocampus [70,71]. As a member of the Tgf-β family of signalling proteins, Smad4 is required in the signal-receiving cell to facilitate Bmp signalling. As noted above Usp9x neurons did not respond to Tgf-β raising the possibility that defective Bmp signalling may contribute to the reduction in hippocampal growth as has been suggested [72]. Usp9x can also stabilise β-catenin, which is a second messenger for the canonical Wnt signalling pathway. Therefore Wnt signalling may also be defective in the hippocampal anlage. A more detailed analysis of Usp9x regulation of hippocampal development is required.

**USP9X** has already been linked to a number of human neurodevelopmental and neurodegenerative disorders. USP9X is linked to lissencephaly, via its interaction with DCX [16], and USP9X is a candidate gene in X-linked intellectual disability and...
epilepsy [22]. Usp9x also deubiquitylates mono-ubiquitylated alpha-synuclein raising the possibility it may play a role in the progression of neurodegenerative diseases such as Parkinson’s disease [73]. This study indicates that Usp9x regulation of brain development likely involves multiple pathways and elucidation of these mechanisms may shed light on a number of human conditions involving aberrant neural development and/or function.

Supporting Information

Figure S1 Deletion of Usp9x exon 3 and protein in Nes-Usp9x−/Y embryos. (A) PCR detected removal of exon 3 in genomic DNA isolated from Nes-Usp9x−/Y. E18.5 embryos. (B) Immunoblot analysis of whole brain lysate revealing decreased levels of Usp9x protein in E18.5 Nes-Usp9x−/Y embryos identified by PCR. Residual levels of full length Usp9x probably reflect the presence of non-neural cells in brain lysates. (C-F) Usp9x antibody staining of neurocortex of E12.5 (C,D) and E14.5 (E,F) wild-type (Nes-Usp9x+/+; C,E) and knockout (Nes-Usp9x−/−; D,F) embryos. Residual amounts of Usp9x was detected in E12.5 Nes-Usp9x−/− neural tissue (D), but Usp9x was unable to be detected by E14.5 (F). Representative images from n = 4 for each genotype at each embryonic stage.

Figure S2 Usp9y expression is not induced in the absence of Usp9x. Immunoblot analysis of whole brain lysate revealing decreased levels of Usp9y protein in E18.5 Nes-Usp9x−/Y embryos identified by PCR. Residual levels of full length Usp9y probably reflect the presence of non-neural cells in brain lysates. (C-F) Usp9y antibody staining of neurocortex of E12.5 (C,D) and E14.5 (E,F) wild-type (Nes-Usp9y+/+; C,E) and knockout (Nes-Usp9y−/−; D,F) embryos. Residual amounts of Usp9y was detected in E12.5 Nes-Usp9y−/− neural tissue (D), but Usp9y was unable to be detected by E14.5 (F). Representative images from n = 4 for each genotype at each embryonic stage.

Figure S3 Loss of Usp9x does not affect Doublecortin in the developing cerebral cortex. Immunofluorescence staining of Doublecortin (Dcx) in the presence (A,C,E,G,I) or absence (B,D,F,H,J) of Usp9x. At each stage three Nes-Dcx-Usp9x−/Y (A,C) and Nes-Dcx-Usp9x+/Y (B,D) brains stained with Usp9x antibody (red) and DAPI (blue) to detect nuclei. Usp9x is absent form the cerebral cortex (B) but present at the same level in the striatum as control littermates (C,D). (TIF)

Figure S4 Loss of Usp9x increases neural apoptosis. 10 μm coronal cryosections of medial neocortex from E18.5 Nes-Usp9x embryos stained with antibodies to cleaved caspase 3 (red) to identify cells undergoing apoptosis. Nuclei are stained with DAPI (blue). (TIF)

Figure S5 Emx1-cre deletion of Usp9x. 10 μm coronal cryosections of 7 week Emx1-Usp9x+/Y (A,C) and Emx1-Usp9x−/Y (B,D) brains stained with Usp9x antibody (red) and DAPI (blue) to detect nuclei. Usp9x is absent form the cerebral cortex (B) but present at the same level in the striatum as control littermates (C,D). (TIF)

Figure S6 Loss of Usp9x does not affect the apoptosis of cultured hippocampal neurons. Wildtype (Usp9x+/+; n = 3) or knockout (Usp9x−/−; n = −3) hippocampal neuronal cultures were grown in-vitro for 8 days. A. Representative immunofluorescent images showing cells stained for the apoptotic marker activated caspase 3 (green), the neuronal marker βIII-tubulin (red) and cell nuclei counterstained with DAPI (Blue). B. The percentage of caspase3 positive (+ve) cells in wildtype and Usp9x null cultures. At least 1000 cells were scored per experiment. p = 0.39 by Students 2-tailed unpaired t-test. (TIF)

Acknowledgments

The authors thank Lynn Tolley and Claire Homan for technical assistance.

Author Contributions

Conceived and designed the experiments: SS LAJ SP JG LJR AMS SAW. Performed the experiments: SS LAJ. Analyzed the data: SS LAJ SP JG LJR AMS SAW. Wrote the paper: SS LAJ SAW.

References

1. Hicke L, Schubert HL, Hill CP (2005) Ubiquitin-binding domains. Nat Rev Mol Cell Biol 6: 610-621.
2. Andersen KM, Hofmann K, Hartmann-Petersen R (2005) Ubiquitin-binding proteins: similar, but different. Essays Biochem 41: 49-67.
3. Tuoc TC, Stoykova A (2010) Roles of the ubiquitin-proteosome system in neurogenesis. Cell Cycle 9: 3174–3180.
4. Rogers N, Paine S, Bedford L, Layfield R (2010) Review: the ubiquitin-proteasome system: contributions to cell death or survival in neurodegeneration. Neuropathol Appl Neurobiol 36: 113–124.
5. Imai Y, Soda M, Takahashi R (2008) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem 283: 363-373.
6. Lim KL, Tan JM (2007) Role of the ubiquitin proteosome system in Parkinson’s disease. BMC Biochem 8 Suppl 1: S13.
7. Oddo S (2008) The ubiquitin-proteosome system in Alzheimer’s disease. J Cell Mol Med 12: 363-373.
8. Pasienski GM (2001) Use of cDNA microarray in the search for molecular markers involved in the onset of Alzheimer’s disease dementia. J Neurosci Res 63: 471–476.
9. Reyes-Turcu FE, Ventrí KH, Wilkinson KD (2009) Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem 78: 363–397.
10. Katz EJ, Isaacs M, Crosas B (2010) A new map to understand deubiquitination. Biochem Soc Trans 38: 21-28.
11. Millard SM, Wood SA (2006) Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes. J Cell Biol 173: 463–468.
12. DiAntonio A, Hasghiphi AP, Portman SL, Lee JD, Amaranto AM, et al. (2001) Ubiquitination-dependent mechanisms regulate synaptic growth and function. Nature 412: 449–452.
13. Staropoli JF, Abelowich A (2005) The ubiquitin-proteasome pathway is necessary for maintenance of the postmitotic status of neurons. J Mol Neurosci 27: 173-183.
14. Todi SV, Paulson HL (2011) Balancing act: deubiquitinating enzymes in the nervous system. Trends in Neurosciences 34: 370-382.
15. Fricouart G, Kappeller C, Saillour Y, Fauchereau F, Rodríguez MS, et al. (2005) Doublecortin interacts with the ubiquitin protease DUBX, which associates with microtubules in neuronal processes. Mol Cell Neurosci 28: 153–164.
16. Jolly LA, Taylor V, Wood SA (2009) Usp9X Enhances the Polarity and Self-renewal of Embryonic Stem Cell-derived Neural Progenitors Mol Biol Cell 10: 1091.
17. Jones MH, Furlong RA, Burkin H, Chalmers JJ, Brown GM, et al. (1996) The Drosophila developmental gene fat facets promotes a human homologue in Xp11.4 which escapes X-inactivation and has related sequences on Yq11.2. Hum Mol Genet 5: 1695–1701.
18. Wood SA, Pascoe WS, Ru K, Yamada T, Hinchenhain J, et al. (1997) Cloning and expression analysis of a novel mouse gene with sequence similarity to the Drosophila fat facets gene. Mech Dev 63: 29–38.
19. Xu J, Taya S, Kaibuchi K, Arnold AP (2005) Spatially and temporally specific degradation in neuronal function and dysfunction. Nat Rev Neurosci 9: 826-838.
20. Overstreet E, Fitch E, Fischer JA (2004) Fat facets and Liquid facets promote Drosophila eye and embryo development. Development 27: 175–183.
21. Chastagner P, Israel A, Brou C (2008) AIP4/Itch Regulates Notch Receptor Degradation in the Absence of Ligand. PLOS one 3: e2735.
22. Tuoi SV, Vennstrom B, Lauerman A, Westermark B, Arvidsson A, et al. (2009) A novel mouse gene with sequence similarity to the Drosophila developmental gene fat facets has a human homologue in Xp11.4 which escapes X-inactivation and has related sequences on Yq11.2. Hum Mol Genet 5: 1695–1701.
23. Tarpey PS, Smith R, Pleasance E, Whibley A, Edkins S, et al. (2009) A systematic, large-scale resequencing screen of X-chromosome coding exons in mental retardation. Nat Genet 41: 535–543.
24. Chastagner P, Israel A, Brou C (2008) AIP4/Itch Regulates Notch Receptor Degradation in the Absence of Ligand. PLOS one 3: e2735.
49. Pramparo TY, Yingling Y, Hirotsune J, Wynshaw-Boris S (2010) Novel
48. Piper M, Barry G, Hawkins J, Mason S, Lindwall C, et al. (2010) NFIA controls
46. Tronche F, Kellendonk C, Kretz O, Gass P, Asigbey K, et al. (1999) Disruption of
47. Chen X, Zhang B, Fischer JA (2002) A specific protein substrate for a
debiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev
16: 209–294.
45. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
43. Xu Z, Xia B, Gong Q, Bailey J, Groves B, et al. (2010) Identification of a
39. Ikeda W, Nakanishi H, Miyoshi J, Mandai K, Ishizaki H, et al. (1999) Afadin: A
38. Zhadanov AB, Provance DW Jr, Speer CA, Coffin JD, Goss D, et al. (1999)
37. Stinchfield MJ, Takaesu NT, Quijano JC, Castillo AM, Tiusanen N, et al. (2012)
36. Zechner D, Fujita Y, Hulsken J, Muller T, Walther I, et al. (2003) beta-Catenin
32. Mouchantaf R, Azakir BA, McPherson PS, Millard SM, Wood SA, et al. (2006)
30. Yoon K, Gaiano N (2005) Notch signaling in the mammalian central nervous
29. Choe EA, Liao L, Zhou JY, Cheng D, Duong DM, et al. (2007) Neuronal
28. Chen X, Zhang B, Fischer JA (2002) A specific protein substrate for a
debiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev
16: 209–294.
26. Taya S, Yamamoto T, Nakanishi H, Miyoshi J, Mandai K, Ishizaki H, et al. (1999) Afadin: A
25. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
24. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
23. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
22. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
21. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
20. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.
19. Perez-Mancera PA, Rust AG, van der Weyden L, Kristiansen G, Li A, et al.