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BRIEF REPORT

# USP9X deletion elevates the density of oligodendrocytes within the postnatal dentate gyrus

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**ABSTRACT**

Neural stem cells (NSCs) within the adult hippocampal dentate gyrus reside in the subgranular zone (SGZ). A dynamic network of signaling mechanisms controls the balance between the maintenance of NSC identity, and their subsequent differentiation into dentate granule neurons. Recently, the ubiquitin-specific protease 9 X-linked (USP9X) was shown to be important for hippocampal morphogenesis, as mice lacking this gene exhibited a higher proportion of proliferating NSCs, yet a decrease in neuronal numbers, within the postnatal dentate gyrus. Here we reveal that *Usp9x*-deficiency results in the upregulation of numerous oligodendrocytic and myelin-associated genes within the postnatal hippocampus. Moreover, cell counts reveal a significant increase in oligodendrocyte precursor cells and mature oligodendrocytes per unit volume of the mutant dentate gyrus. Collectively, these findings indicate that USP9X may regulate NSC lineage determination within the postnatal SGZ.

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NSCs within the adult hippocampus maintain the capacity to undergo neurogenesis throughout life.[1] The continual supply of new neurons arising from the neurogenic niche of the SGZ is crucial for learning,[2] memory,[3] and spatial navigation.[4] To ensure the stem cell pool is maintained throughout adulthood, NSCs predominantly remain in a state of quiescence and self-renew when they undergo proliferation.[5,6] Under normal conditions in the adult brain, NSCs within the SGZ predominantly undergo neurogenic differentiation to generate dentate granule neurons.[1] However, these NSCs are multipotent,[7] as they retain the ability to produce astrocytes,[8] and can be induced to generate oligodendrocytes via virally-mediated reprogramming.[9,10] Multiple intrinsic and extrinsic signaling mechanisms act in cohesion to determine the fate of NSCs within the SGZ and ensure proper hippocampal neurogenesis. Critically, disruption at any stage of these processes can have profound consequences on the development and function of the hippocampus, leading to cognitive deficits[11] and psychiatric disorders.[12] Therefore, it is imperative to understand the underlying mechanisms governing the maintenance of the NSC pool and their path to neurogenic differentiation.

The deubiquitylating enzyme USP9X has previously been shown to play a pivotal role with relation to NSC biology. USP9X promotes the self-renewal of embryonic stem cell-derived NSCs *in vitro*.[13] *In vivo*, USP9X is highly expressed throughout the developing central nervous system,[14] the postnatal hippocampus,[15] and within the adult neurogenic niches.[13] The conditional ablation of *Usp9x* from NSCs within the embryonic forebrain (via *Emx1-Cre*-mediated deletion) results in a number of cortical abnormalities,[14] most notably the severe reduction in the size of the postnatal hippocampus and dentate gyrus, evident as early as one week after birth.[15] We recently analyzed cellular populations within the postnatal dentate gyrus of these *Usp9x*-deficient mice and showed a significant decrease in the total number of NSCs, neuroblasts and mature neurons.[15] Interestingly, when we examined the proportion of NSCs that were quiescent versus those that were proliferating, we found a significantly higher proportion of proliferating NSCs in the SGZ of *Usp9x*-deficient mice, and a concomitant decrease in

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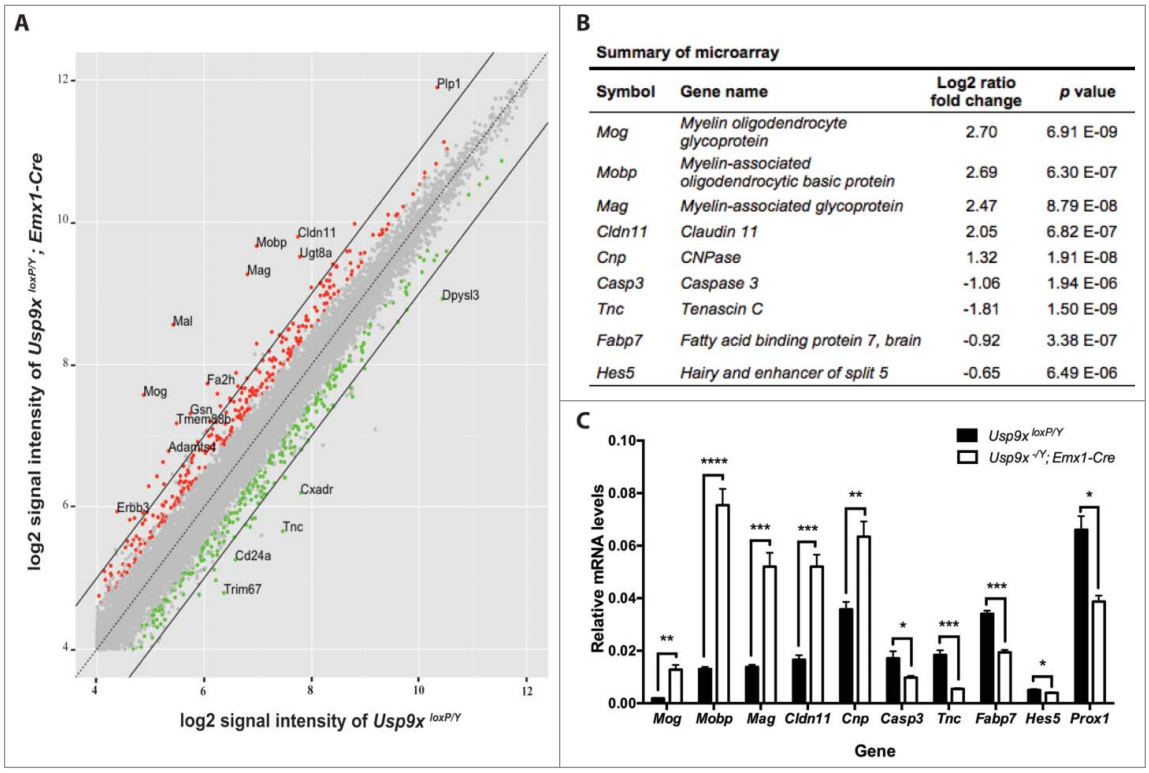
the proportion of quiescent NSCs, compared to controls.[15] Together, these findings indicate that *Usp9x*-deficiency culminates in abnormal neurogenesis within the postnatal SGZ, which contributes to, at least in part, the reduced size of the dentate gyrus. However, it remains unclear as to why the lack of *Usp9x* leads to an increase in the relative proportion of proliferating NSCs within the SGZ but a reduced number of neuronal cells in the dentate granule cell layer. In this paper, we posited that the lack of *Usp9x* might result in the premature differentiation of NSCs toward the astrocytic or oligodendrocytic lineage.

To test the hypothesis that *Usp9x*-deficiency leads to precocious differentiation of NSCs into glia, we performed microarray-based transcriptomic profiling on hippocampi from mice in which *Usp9x* had been conditionally ablated from neural progenitors within the dorsal telencephalon using an *Emx1-Cre* allele. These mice will be referred to as *Usp9x*$^{-/Y}$; *Emx1-Cre* mice from here onwards, while littermate controls mice will be referred to as *Usp9x*$^{loxP/Y}$ mice. Microarray analysis of postnatal day (P) 14 mutant and control hippocampi revealed that over 600 genes were differentially regulated in the mutant hippocampus. Significance of differentially expressed transcripts were identified at $p < 0.05$ and a log2 ratio fold change of $<-0.5$ or $>0.5$. Notably, many of the genes that were upregulated in the mutant hippocampi were related to the oligodendrocytic lineage, including *myelin oligodendrocyte glycoprotein* (*Mog*),[16] *myelin-associated oligodendrocytic basic protein* (*Mobp*),[17] *myelin associated glycoprotein* (*Mag*),[18] *CNPase* (*Cnp*),[18] and *claudin-11*[19] (*Cldn11*; Fig. 1A, B). In contrast, a number of factors related to NSC self-renewal and neurogenesis were significantly downregulated, including *Tenascin C* (*Tnc*),[20] *fatty acid binding protein 7, brain* (*Fabp7*),[21] and *hairy and enhancer of split 5*[22] (*Hes5*; Fig. 1B). This is consistent with the previously reported role for USP9X in neural progenitor cell




| Symbol | Gene name | Log2 ratio fold change | *p* value |
|---|---|---|---|
| *Mog* | *Myelin oligodendrocyte glycoprotein* | 2.70 | 6.91 E-09 |
| *Mobp* | *Myelin-associated oligodendrocytic basic protein* | 2.69 | 6.30 E-07 |
| *Mag* | *Myelin-associated glycoprotein* | 2.47 | 8.79 E-08 |
| *Cldn11* | *Claudin 11* | 2.05 | 6.82 E-07 |
| *Cnp* | *CNPase* | 1.32 | 1.91 E-08 |
| *Casp3* | *Caspase 3* | -1.06 | 1.94 E-06 |
| *Tnc* | *Tenascin C* | -1.81 | 1.50 E-09 |
| *Fabp7* | *Fatty acid binding protein 7, brain* | -0.92 | 3.38 E-07 |
| *Hes5* | *Hairy and enhancer of split 5* | -0.65 | 6.49 E-06 |

**Figure 1.** Lack of *Usp9x* results in the upregulated mRNA expression levels of oligodendrocyte and myelin associated genes within the hippocampus at P14. (A) Signal intensity plot showing the differentially expressed genes for comparison between *Usp9x*$^{loxP/Y}$ control mice vs. *Usp9x*$^{-/Y}$; *Emx1-Cre* mutant mice. Only the genes that were statistically ($p < 0.05$) and magnitudually (log2 ratio fold change $<-0.5$ or $> 0.5$) differentially expressed were colored. Upregulated genes are illustrated in red and downregulated genes are illustrated in green. All other genes are shown in gray. The dotted diagonal line shows equal intensity across the experimental conditions while the solid lines correspond to a log2 ratio fold change of $<-1$ or $> 1$. (B) Summary of the microarray results, showing the gene symbol, gene name, log2 ratio fold change, and *p* value of key significantly misregulated genes in the hippocampus of mutant mice at P14. (C) qPCR validation of the microarray results, demonstrating increased relative levels of oligodendrocyte and myelin associated mRNAs, as well as reduced levels of neurogenic mRNAs, in the hippocampus of mutant mice at P14. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, *t*-test.

self-renewal,[13] and the reduction in dentate granule neurons within the postnatal *Usp9x*$^{-/Y}$; *Emx1-Cre* dentate gyrus.[15] Validation of these microarray data was performed using quantitative polymerase chain reaction (qPCR) on cDNA generated from mRNA isolated from independent hippocampal samples from P14 mice. This analysis confirmed that the relative expression of these oligodendrocyte-associated genes was significantly elevated in mutant samples compared to controls (Fig. 1C). Interestingly, expression of *caspase 3* (*Casp3*) was significantly reduced (Fig. 1B, C). As this factor is expressed in apoptotic cells,[23] this suggests that the smaller dentate gyrus phenotype of *Usp9x*-deficient mice is unlikely to be related to abnormal cell death. This finding was supported by immunocytochemical analysis of cleaved caspase 3 expression, which revealed fewer apoptotic cells in the mutant dentate gyrus in comparison to controls at P14 (data not shown). It should be noted, however, that a decrease in cell death may simply be a reflection of the fact that there are reduced numbers of new-born neurons present in the mutant hippocampus. Finally, the qPCR analysis also confirmed that the expression of *Tnc, Fabp7, Hes5* and the dentate granule neuron marker *prospero homeobox protein-1* (*Prox1*)[24] was significantly reduced in the mutant in comparison to the controls (Fig. 1C). Collectively, these data are consistent with previous reports into the phenotype of *Usp9x*-deficient mice,[15] suggesting that in the absence of *Usp9x*, NSC self-renewal and neurogenesis are impaired. Moreover, these data further suggest that *Usp9x*-deficient NSCs in the SGZ, which normally produce neurons *in vivo*,[1] may potentially be biased to produce myelinating oligodendrocytes in the absence of this factor.

To investigate whether the elevated expression of oligodendrocyte-associated genes was reflected at a cellular level, we next performed immunocytochemical analyses of P14 *Usp9x*$^{-/Y}$; *Emx1-Cre* and *Usp9x*$^{loxP/Y}$ mice using antibodies against oligodendrocyte transcription factor 2 (Olig2), which identifies all cells within the oligodendrocytic lineage[25] and platelet-derived growth factor receptor $\alpha$ (PDGFR$\alpha$), which, in conjunction with Olig2, labels oligodendrocyte precursor cells.[26,27] We performed co-immunofluorescence labeling with these 2 antibodies on hippocampal tissue from P14 mutant and control brains, followed by confocal microscopy, and counted cells of the oligodendrocyte lineage (Olig2$^+$), oligodendrocyte precursor cells (Olig2$^+$/PDGFR$\alpha^+$) and mature oligodendrocytes (Olig2$^+$/PDGFR$\alpha^-$) (Fig. 2A-H). As oligodendrocytes differentiating from NSCs within the SGZ migrate into the hilus of the dentate gyrus,[9] we counted both the total number of immuno-positive cells within the hilus, as well as normalizing cell counts relative to the volume of the respective hilar regions. This analysis revealed that the total number of cells within the oligodendrocytic lineage, including oligodendrocyte precursor cells and mature oligodendrocytes, was reduced in *Usp9x*-deficient mice in comparison to controls (Fig. 2I). This is consistent with the markedly reduced size of the dentate gyrus within *Usp9x*$^{-/Y}$; *Emx1-Cre* mice at P14 (Fig. 2A, E).[15] Interestingly, however, normalized counts relative to the volume of the hilar region revealed that there were significantly elevated numbers of Olig2$^+$ cells per mm$^3$ in P14 *Usp9x*$^{-/Y}$; *Emx1-Cre* mice in comparison to *Usp9x*$^{loxP/Y}$ controls, a finding reflected in the relative increase of oligodendrocyte precursor cells and mature oligodendrocytes per unit volume (Fig. 2J). Furthermore, although the absence of *Usp9x* leads to a higher density of oligodendrocytic cells in the dentate gyrus, there seems to be no effect on the ability of oligodendrocyte precursor cells to differentiate and mature. A similar analysis of astrocytes was performed using co-labeling of astrocytic markers glial fibrillary acidic protein (GFAP) and s100 calcium-binding protein $\beta$ (s100$\beta$; Fig. 3A-H).[28] We found that the total number of astrocytes in the mutant was reduced in *Usp9x*-deficient mice in comparison to controls (Fig. 3I). However, there was no significant change in the number of astrocytes per unit volume of the mutant hilar region in comparison to wild-type controls at P14 (Fig. 3J). When considered in light of the elevated proportion of proliferating NSCs and the reduced number of neuronal cells in the dentate gyrus of P14 *Usp9X*-deficient mice,[15] these findings indicate that the absence of *Usp9x* may result in the abnormal production of oligodendrocytic cells instead of neurons within the postnatal hippocampus.

NSCs residing within the SGZ of the postnatal and adult dentate gyrus do not normally produce oligodendrocytes, instead these cells predominantly generate Prox1-expressing dentate granule neurons,[1,16] as well as a small proportion of astrocytes.[8] However, the SGZ NSCs can be directed to differentiate into oligodendrocytes, both *in vitro* and *in vivo*.[9,10] Indeed, a recent report revealed that retrovirally-driven expression of key oligodendrocytic genes, including *Olig2, Sox10* and *Ascl1*, within the dentate gyrus of adult

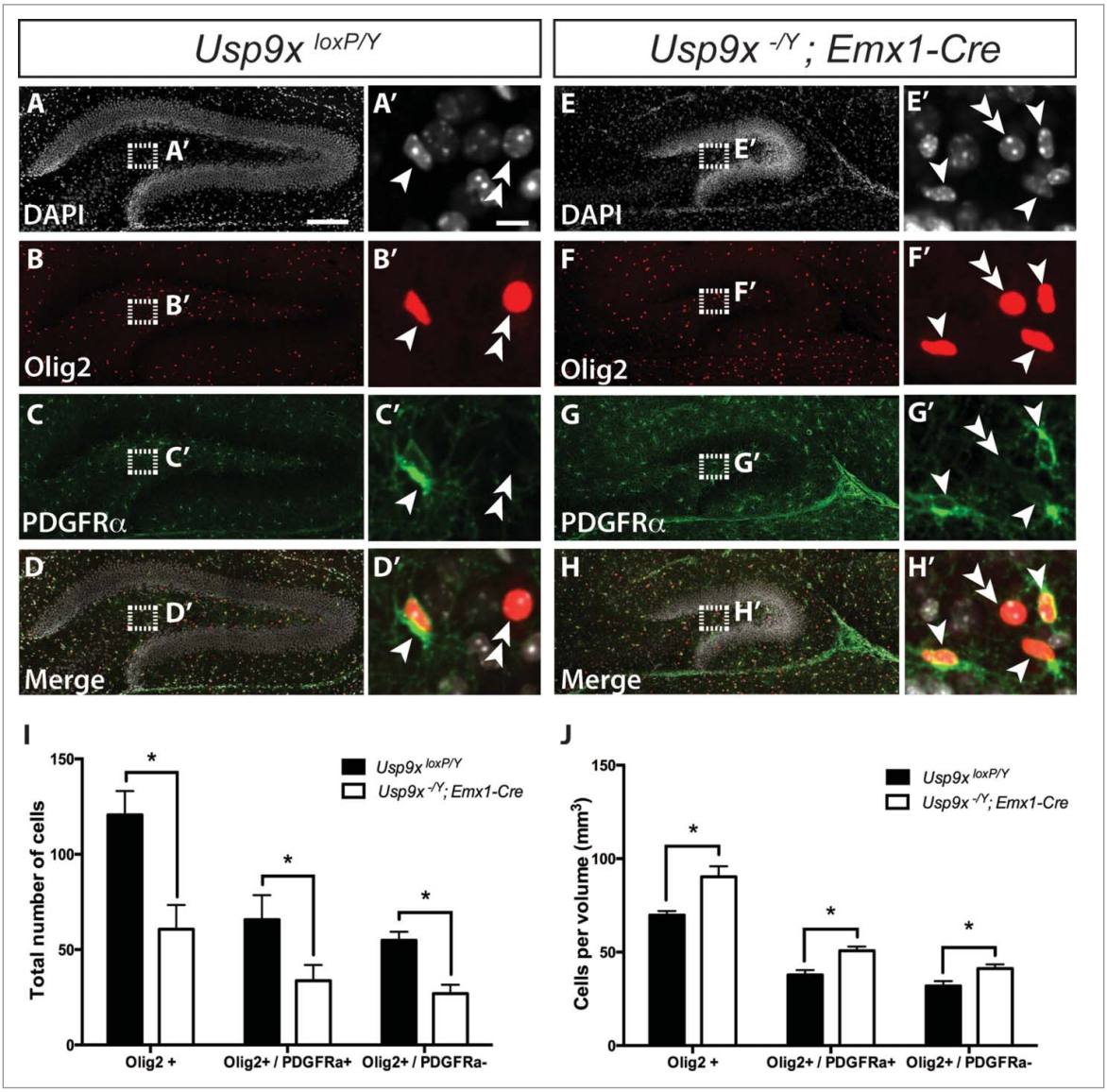



**Figure 2.** Increased density of oligodendrocytes in the dentate gyrus of *Usp9x*$^{-/Y}$*; Emx1-Cre* mice. Co-immunofluorescence labeling and confocal microscopy was performed on hippocampal sections of *Usp9x*$^{loxP/Y}$ (A–D) and *Usp9x*$^{-/Y}$*; Emx1-Cre* (E–H) at P14. Cell nuclei were labeled with DAPI (A, E). Oligodendrocyte precursors were defined as cells expressing both Olig2 (red in (B)and F) and PDGFR$\alpha$ (green in C and G). Mature oligodendrocytes were defined as cells that only expressed Olig2. The merged panels are shown in (D, H). The insets reveal a higher magnification view of the boxed region showing oligodendrocyte precursor cells (arrowheads) and mature oligodendrocytes (double arrowheads). Quantification of labeled cells was performed within the hilar region of the dentate gyrus. Total numbers of Olig2$^+$ oligodendrocytes, including Olig2$^+$/PDGRF$\alpha^+$ precursors and Olig2$^+$/PDGRF$\alpha^-$ mature oligodendrocytes in the mutant mice were significantly reduced compared to controls (I). Normalized cell counts relative to the volume of the hilar region revealed a significant increase in oligodendrocytic cells per mm$^3$ within the mutant compared to controls, including elevated numbers of oligodendrocyte precursors and mature oligodendrocytes (J). $^*p < 0.05$, *t*-test. Scale bar in (A): (A-H) – 150 $\mu$m; (A′): (A′-H′) —10 $\mu$m.

mice was sufficient to induce the differentiation of hippocampal NSCs into oligodendrocyte precursor cells and myelinating mature oligodendrocytes.[10] How could the loss of *Usp9x* culminate in oligodendrogenesis? At this stage this is unclear, however previous reports of interactions between USP9X and members of the Notch signaling pathway,[29] coupled with the reduced expression of Notch pathway members revealed here (*Hes5*, *Fabp7* and *Tnc*) suggests that a primary role for USP9X, in the context of NSCs, is the maintenance of their identity and self-renewal capacity. Identification of why the loss of *Usp9x* potentially biases NSCs toward oligodendrocytic differentiation will require a more comprehensive analysis of the USP9X interactome, coupled with proteomic analysis of NSCs lacking this enzyme. The importance of such

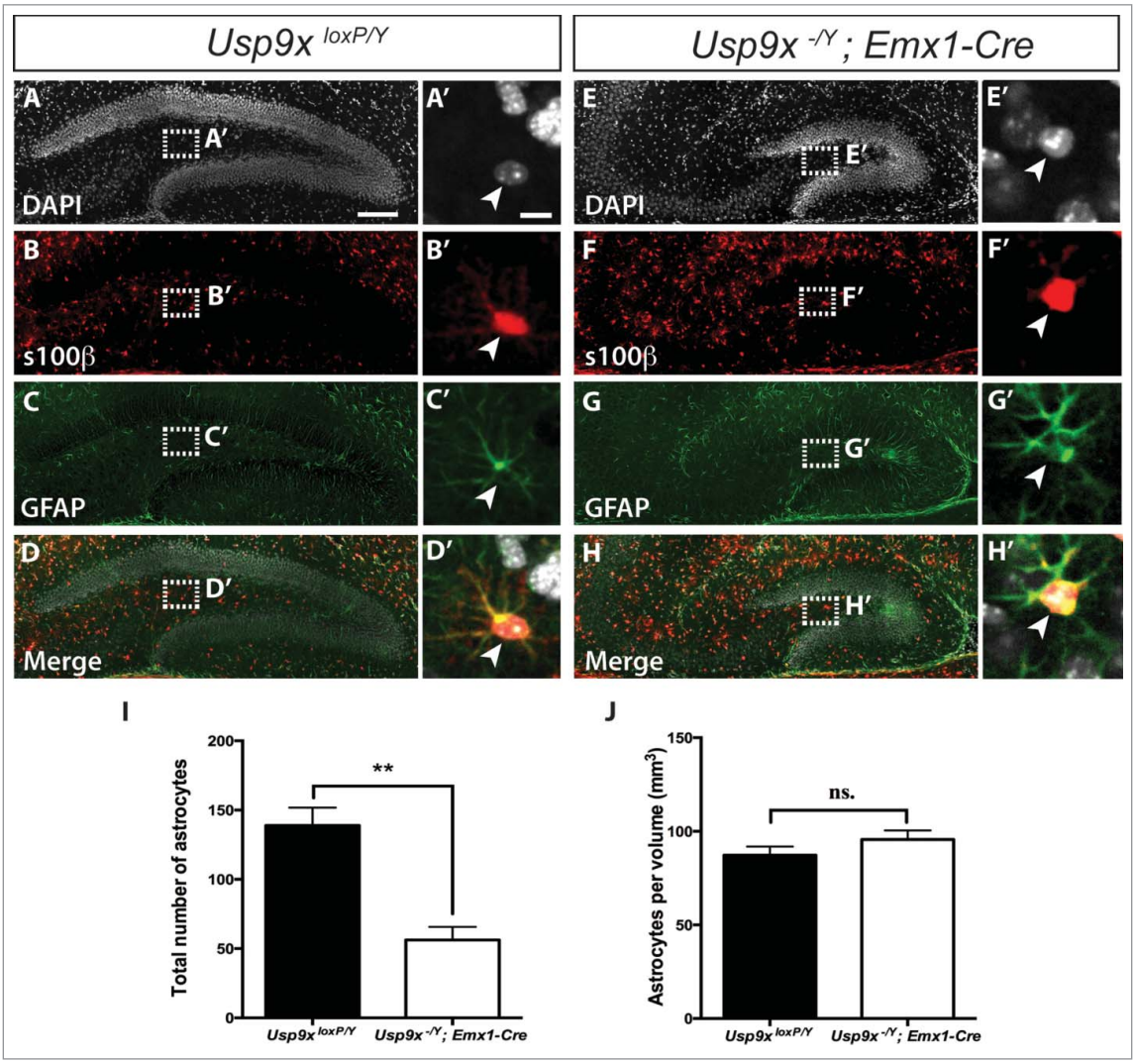



**Figure 3.** Astrocytic cell density did not change in the dentate gyrus of *Usp9x*$^{-/Y}$*; Emx1-Cre* mice. Co-immunofluorescence labeling and confocal microscopy was performed on hippocampal sections of *Usp9x*$^{loxP/Y}$ (A–D) and *Usp9x*$^{-/Y}$*; Emx1-Cre* (E–H) at P14. Cell nuclei were labeled with DAPI (A, E). Astrocytes were defined as cells expressing both s100$\beta$ (red in B and F) and GFAP (green in C and G). The merged panels are shown in (D, H). The insets reveal a higher magnification view of the boxed region showing astrocytes (arrowheads). Quantification of labeled cells was performed within the hilar region of the dentate gyrus. There were significantly fewer total numbers of GFAP$^{+}$/s100$\beta^{+}$ astrocytes in the mutant mice compared to controls (I). Normalized cell counts relative to the volume of the hilar region revealed no significant changes in astrocytes per mm$^3$ within the mutant compared to controls (J). (ns) Not significant, $^{**}p < 0.001$, *t*-test. Scale bar in (A): (A-H) – 150 $\mu$m; (A′): (A′-H′) −10 $\mu$m.

future studies is emphasized by the fact that aberrant USP9X expression has been associated with a number of human neurological disorders that exhibit abnormalities in hippocampal function, including X-linked intellectual disability,[30] epilepsy,[31] and Parkinson's disease.[32]

Although our findings implicate USP9X in the inhibition of oligodendrocytic differentiation of NSCs within the SGZ, there are caveats to this interpretation of the data. Firstly, although we noted a correlation between *Usp9x*-deficiency and the production of oligodendrocytes, the data presented here does not confirm that the absence of USP9X in NSCs directly leads to their differentiation into oligodendrocytes. To address this, lineage tracing experiments using the *Usp9x* conditional allele crossed with an inducible NSC-specific Cre driver (e.g. *Nestin Cre ER*$^{T2}$),[33] coupled with ethynyl deoxyuridine (EdU) labeling to identify proliferating cells, could be used to directly demonstrate if *Usp9x*-deficient cells generate oligodendrocytes within the postnatal dentate gyrus hilus. Moreover, proliferating oligodendrocyte precursor

cells are also found distributed throughout non-neurogenic areas of the hippocampus.[34] Thus, although we counted cells only within the hilar region of the dentate gyrus, it is similarly unclear as to whether the generation of oligodendrocytes in the *Usp9x*-deficient hippocampus was a result of NSCs in the SGZ abnormally differentiating toward the oligodendrocytic lineage instead of a neurogenic path, or whether the lack of *Usp9x* influenced the proliferation of these randomly distributed oligodendrocyte precursors throughout the hippocampus. Again, the use of an inducible NSC-specific Cre driver in future studies will clarify this question. Overall, given the expression of USP9X by NSCs within the adult neurogenic niches, as well as the reported role for this enzyme in maintaining NSC self-renewal *in vitro*,[13] our findings support the hypothesis that USP9X plays an important role in regulating NSC biology within the postnatal dentate gyrus.

## Methods

### Animal breeding

Mice were bred as we described previously.[15] Male mice lacking the *Usp9x* allele inherited the *Emx1-Cre* allele (referred to as $Usp9x^{-/Y}$; *Emx1-Cre*), while Cre-negative males were used as controls (referred to as $Usp9x^{loxP/Y}$). All mouse breeding was performed under the ethical clearance approved by Griffith University Animal Ethics Committee. All experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and were carried out in accordance with The University of Queensland Institutional Biosafety committee.

### Microarray

Four $Usp9x^{-/Y}$; *Emx1-Cre* and 4 $Usp9x^{loxP/Y}$ animals were used for microarray analyses. RNA was isolated from dissected hippocampus using an RNase kit (Qiagen). Prior to microarray, the integrity of the RNA was verified on an Agilent Bioanalyzer RNA Nano 6000. The Affymetrix Mouse Gene 2.0 ST microarray was performed at the Ramaciotti Center for Genomics, The University of New South Wales, Australia. Data was processed, quantile normalized and differentially expressed transcripts were identified at $p < 0.05$ and log2 ratio fold change of $<-0.5$ or $>0.5$ using R/BioConductor limma package,[35] at the QFAB Bioinformatics, The University of Queensland, Australia. False discovery rate (FDR) correction was performed on the *p*-values.[36]

### Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed on hippocampal tissue from P14 animals (6 *Usp9x−/Y; Emx1-Cre* and 4 $Usp9x^{loxP/Y}$) using standard protocol as we described previously.[15] Gene expression was calculated using $-\Delta\Delta$ Ct-method relative to the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*). All the samples were tested in triplicate, and each experiment was repeated a technical triplicate. Primer sequences used were:

*Mog*_Forward:5′GACCTGCAGGAGGATC GTAG3′
*Mog*_Reverse: 5′ACCAAGAAGAGGCAGCAATG3′
*Mobp*_Forward: 5′AATGAGAGCAAGACAAGCGG3′
*Mobp*_Reverse: 5′TCCTTGGCCATTTTCTGACT3′
*Mag*_Forward: 5′CGGGTTGGATTTTACCACAC3′
*Mag*_Reverse: 5′CTGCCTTCAACCTGTCTGTG3′
*Cldn11*_Forward: 5′GCTGGGGTGCTCCTTATTCT3′
*Cldn11*_Reverse: 5′CAACCTGCGTACAGCGAGTA3′
*Cnp*_Forward: 5′GTTCTGAGACCCTCCGAAAA3′
*Cnp*_Reverse: 5′CCTTGGGTTCATCTCCAGAA3′
*Casp3*_Forward: 5′TGCTGGTGGGATCAAAGC3′
*Casp3*_Reverse: 5′TGAATCCACTGAGGTTTTGTTG3′
*Tnc*_Forward: 5′AGTCCAGGACAGACGGAAAC3′
*Tnc*_Reverse: 5′AAAACCATCAGTACCACGGC3′
*Fabp7*_Forward: 5′ CGGACAATGCACATTCAAG3′
*Fabp7*_Reverse: 5′TCTTTGCCATCCCACTTCTG3′
*Hes5*_Forward: 5′CCAGGAAAACCGACTG3′
*Hes5*_Reverse: 5′AACTCCTGCTCCAGCAGCA3′
*Prox1*_Forward: 5′GGCATTGAAAAACTCCCGTA3′
*Prox1*_Reverse: 5′GCTATACCGAGCCCTCAACA3′

*Gapdh*_Forward: 5′
GCACAGTCAAGGCCGAGAAT3′
*Gapdh*_Reverse: 5′
GCCTTCTCCATGGTGGTGAA3′

### *Immunocytochemistry labeling and image analysis*

Preparation of tissue sections and immunocytochemical labeling was performed as described.[15] Briefly, P14 brains were embedded in noble agar and sectioned on a coronal plane at 50 $\mu$m using a vibratome. Primary antibodies used include: Olig2 (polyclonal rabbit, 1:400, EMD Millipore), PDGFR$\alpha$ (polyclonal goat, 1:100, R&D Systems), cleaved caspase 3 (polyclonal rabbit, 1:200, Cell Signaling) and GFAP (monoclonal mouse, 1:400, EMD Millipore). Corresponding secondaries antibodies included donkey 488, Cy3, 555 (Jackson), and s100$\beta$ (rabbit conjugated AlexaFluor 647) before being counter-stained with 4′, 6-diamidino-2-phenylindole (DAPI). All image acquisition and analysis was performed as previously described.[15] For all analyses we had 6 rostro-caudal sequential hippocampal sections, with each section containing the left and right hippocampi, per animal to image and count. Cell counts for Olig2$^+$/PDGFR$\alpha^-$ cells, Olig2$^+$/PDGFR$\alpha^+$, and GFAP$^+$/S100b$^+$ cells were performed within the hilar region of the hippocampal dentate gyrus in a 10 $\mu$m z-stack, which consisted of 10 consecutive 1 $\mu$m-thick optical sections. The hilus was defined by the area below the SGZ and between the superior and inferior blades of the dentate gyrus. The normalized cell counts were calculated by dividing the number of cells by the volume of the respective hilar region. Student's *t*-tests were used to compare all quantification datasets, where we used n = 3 animals per genotype at the age of P14. Statistical significance was established at a *p*-value of $< 0.05$. Error bars represent standard error of the mean (SEM). Data analysis was performed blind to the genotype of the sample.

## Abbreviations

| | |
|---|---|
| GFAP | glial fibrillary acidic protein |
| NSC | neural stem cell |
| Olig2 | oligodendrocyte transcription factor 2 |
| PDGFR$\alpha$ | platelet-derived growth factor $\alpha$ |
| qPCR | quantitative polymerase chain reactions |
| s100$\beta$ | s100 calcium-binding protein $\beta$ |
| SGZ | subgranular zone |
| USP9X | ubiquitin-specific protease 9 X-linked |

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