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Title: TCF7L2 activation is required for myelin regeneration in 5-FU-induced demyelinating mice

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Abstract:

Previous studies have showed that 5-FU (5-fluorouracil) could cause delayed myelin degeneration by inducing oligodendrocytes death. However, it is not clear whether 5-FU-induced demyelination is recoverable. Here, we demonstrated that 5-FU-induced demyelination could occur in the corpus callosum of different ages of mice. Inconsistent with a delayed demyelination in the white matter of adult mice, 5-FU could result in acute damage to oligodendrocytes in the adolescent mice white matter. The spontaneous myelin repair could emerge after 5-FU withdrawal both in the adolescent and adult mice. However, the recovery period of myelin-injured adolescent mice was faster than adults. Our data identified that the transcription factor TCF7L2 was markedly re-activated and re-appeared in cerebral white matter of injured mice. In addition, TCF7L2 was sufficient for oligodendrocytes proliferation and differentiation *in vitro*. Taken together, our data uncovered that 5-FU-induced demyelination was recoverable and TCF7L2 might be a potentially therapeutic target in 5-FU-induced myelin damage.

Key words: TCF7L2, oligodendrocytes, 5-FU, remyelination

1. Introduction

5-FU (5-Fluorouracil) and its derivatives, including 5'-Deoxy-5-fluorouridine, floxuridine, tegafur and capecitabine, are widely used for the treatment of multiple solid cancers[1-3]. Clinical treatment with 5-FU and its derivatives often leads to several adverse effects. Cerebral white matter lesions are one of the adverse effects of 5-FU therapy for cancer, which is associated with oligodendrocytes damage[4-6]. Several studies indicate that 5-FU treatment could induce oligodendrocytes death by way of apoptotic process and degeneration of central nervous system (CNS) white-matter tracts, further cause a syndrome of delayed myelin damage in the CNS of adult mice[7-9]. However, it is unclear whether the toxic effects of 5-FU on oligodendrocytes or myelin sheaths are reversible after 5-FU withdrawal.

The CNS oligodendrocyte myelination/remyelination processes are tightly regulated by a network of transcriptional and post-transcriptional factors[10-14]. TCF7L2, which is regulated by Wnt signaling pathway, is an essential transcription factor for oligodendrocyte development. TCF7L2 is transiently expressed during development and downregulated in adulthood in oligodendroglial lineage cells[15]. Ye et al has demonstrated that TCF7L2 is critical for oligodendrocytes differentiation by a gain-of-function study *in* ovo[16]. Lürbke A et al has shown that TCF7L2 is re-expressed in oligodendrocytes in a subset of MS (demyelinating diseases) patients[17]. TCF7L2 re-expression is specific to remyelinating lesions generated by many different chemicals induced demyelination in different adult CNS regions. Up-regulation of TCF7L2 in the white matter of neonatal mice is also observed after exposure to chronic hypoxia[18]. However, the underlying role of TCF7L2 in the remyelination of 5-FU-lesioned mice brain is still unknown.

Here, we employed the adolescent and adult mice to identify the demyelinating injury induced by 5-FU. Furthermore, spontaneous remyelination was observed in 5-FU-treated mice. We found the time and severity of 5-FU-induced demyelination in the adolescent mice were different from the adults. TCF7L2 may play a positive role in the remyelination after 5-FU withdrawal.

2. Materials and Methods

2.1 Animals and Reagents

3-4w-old and adult C57BL/6 mice (over P60) obtained from SHANGHAI SLAC LABORATORY ANIMAL CO. LTD.) were used in this study. Healthy adolescent or adult mice were randomly divided into saline and 5-FU-treated group, respectively. The adult mice were treated with 5-FU (TIANJIN KINGYORK GROUP CO., LTD,). or saline for 5 days and collected tissues at 1 dpl (day post lesion), 8 dpl and 22 dpl, respectively. The adolescent mice were injected with 5-FU for 4 times and collected tissues at 1 dpl, 5 dpl and 22 dpl. Each group of mice number in different stages was 6. The animal studies have been approved by the Institutional Animal Care & Use Committee (IACUC) at Zhejiang University.

2.2 Histology

Mice brains were removed and fixed with 4% paraformaldehyde (PFA) for 1hr at 4℃ and embedded in O.C.T. compound (Tissue-Tek) for cryo-sections. The following primary antibodies were used for immunohistology: CC1 (Oncogene Research, OP80), MBP (Covance, SMI-94R), TCF7L2 (Cell signaling, #2565S), Then sections were incubated with secondary antibodies conjugated to Cy2, Cy3 and Cy5 (Jackson ImmunoResearch) for 1hr. At last microscopy was performed using a Zeiss LSM510 Meta fluorescence microscope. For observation of myelin sheath, the optic nerves of mice were dissected and fixed in fresh fixative overnight at 4℃. Tissues were rinsed in PBS, postfixed in 1% OsO_4 in PBS for 1 hr, dehydrated in a graded ethanol series, infiltrated with propylene oxide, and embedded in Epon. Measurements were made on electron micrograph from the control and the experimental groups[19].

2.3 Primary Oligodendroglial Cell Culture

OPCs were purified by differential shaking of mixed glial cell cultures as previously described[20]. Briefly, cerebral cortices at P2 Sprague Dawley rats were dissected, minced, and digested. Dissociated cells were counted and plated in poly-D-lysine-coated 75 cm^2 flasks (5×10^6 cells per flask), and maintained in

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin. After the cells were confluent (7~10 days), the flasks were shaken for 2 h on an orbital shaker (200 rpm) at 37 °C to remove microglia and debris. Medium was replaced and cultures were shaken overnight, OPCs were harvested by centrifugation and re-suspended in growth medium (N2 supplemented with PDGF-AA and bFGF). Four to five days after plating, the OPCs were used for the experiments. The primary OPCs were transduced with lenti-virus and assayed for immunocytochemistry analysis. The MBP+ cell numbers were measured by counting.

2.4 transient transfection and immunostaining

The mouse oligodendrocyte precursor cell line Oli-neu was graciously provided by Dr. Richard Lu (UT Southwestern). The Oli-neu cells were maintained in growth medium consisting of DMEM, which supplemented with 1% horse serum and 1% N2. Oli-neu cells were transfected with control and TCF7L2 and/or TCF7L2-EN expressing plasmid using Lipofectamine 2000 (Invitrogen) and assayed 48 h post-transfection for qRT-PCR.

For immunostaining of 5'-ethynyl-2'-deoxyuridine (EdU, Life Technologies) and PDGFRα (BD Bioscience, 558774), Oli-neu cells were transfected with TCF7L2 or control plasmid, cells were incubated with EdU 1 hr before harvest, then fixed and permeabilized cells, and EdU and PDGFRα staining was performed according to the manufacturer's instructions.

2.5 Quantitative Real-time PCR

Total RNAs were purified from cells transfected with TCF7L2 or TCF7L2-EN using TRizol reagent according to the manufacturer's instructions (Invitrogen). RNA was transcribed to cDNA with First-Strand cDNA Synthesis Kit (GE Healthcare). Quantitative RT-PCR was carried out using the ABI Fast Dx Real-time PCR instrument (Perkin-Elmer Applied Biosystems), and the relative gene expression was normalized to internal control such as *Gapdh*. The PCR primer sequences are available upon request[13].

2.6 Statistical Analysis

Quantifications were carried out from at least three independent experiments; data were calculated as mean ± SD in the graphs. The significance of differences between two sets of data was determined by one way ANOVA, and $p < 0.05$ was considered statistically different.

3. Result

3.1 Remyelination occurred spontaneously after 5-FU withdrawal in adult mice

To demonstrate whether the remyelinating process happened after 5-FU withdrawal, the demyelination was induced by short-term exposure (five consecutive days) of adult mice with 5-FU, then 5-FU was discontinued to occur the spontaneous remyelination (Fig. 1A). The expression of MBP (a mature marker of oligodendrocytes) was slightly decreased in 5-FU-treated mice at the first day post lesion (1dpl), indicating that mild damage had begun in these animals. Subsequently, a dramatic reduction of MBP was observed in 5-FU-injected cerebral white matter at 8 dpl, suggesting that 5-FU treatment could result in the delayed demyelination, which is consistent with previous study[9]. However, MBP expression was significantly increased in 5-FU-injected mice after 22-day recovery (Fig. 1B), demonstrating that spontaneous remyelination could occur after long-term withdrawal of 5-FU.

To further identify the myelin-forming function of oligodendrocytes, electron microscopy analysis was conducted to examine myelin sheath assembly in the CNS after 5-FU treatment. In contrast to abundance of myelinated axons that were observed in adult mice without 5-FU treatment (Fig. 1C upper), the axons morphology was seemingly elongated in the optic nerve of 5-FU-injected mice at 1 dpl. Subsequently, 5-FU injection resulted in a remarkable delayed damage to myelin sheath at 8 dpl, which is in accordance with the decreased expression of MBP (Fig. 1C middle). Then we asked whether 5-FU-induced demyelination was reversible, we collected the optic nerves at 22 dpl. The axons from 22 dpl optic nerves appeared to remyelinate significantly compared with demyelinated axons at 8 dpl (Fig. 1C bottom). Collectively, these data revealed that delayed myelin degeneration caused by 5-FU in adult mice could remyelinate spontaneously within one month.

3.2 Acute demyelination in 5-FU-treated adolescent mice recovered more quickly

To compare the difference of 5-FU-caused demyelination/remyelination between adult and adolescent mice, the adolescent mice were treated with 5-FU for 4 times and the brain were collected at the indicated time (Fig. 2A). Inconsistent with the delayed

demyelination in adult mice, the expression of MBP was immediately and significantly decreased in 5-FU-treated cerebral white matter of adolescent mice at 1dpl (Fig. 2B left), indicating that oligodendrocyte damage induced by 5-FU was more susceptible in adolescent mice. Subsequently, MBP-positive cells was remarkably increased at 5 dpl compared with acute injury of white matter at 1 dpl (Fig. 2B middle). After long-term recovery (22 dpl), high MBP protein levels were detected in adolescent mice with 5-FU injection in contrast to low levels in 5 dpl mice, which was almost the same as the control mice (Fig. 2B right). These data suggested that 5-FU-induced demyelination in adolescence was faster and more severe than adult mice and the remyelination also quickly appeared after 5-FU withdrawal in adolescent mice.

3.3 TCF7L2 was activated in remyelinating lesions

Previous studies have shown that the oligodendrocyte-specific factor TCF7L2 is a critical transcription factor involved in oligodendrocyte remyelination[21, 22]. To identify whether TCF7L2 was involved in the remyelinating process after 5-FU withdrawal, immunostaining was applied to detect the expression of TCF7L2 in the corpus callosum of adolescent and adult mice. In contrast to robust TCF7L2 and CC1 (oligodendrocyte marker) expression in saline-treated adolescent white matter, 5-FU treatment remarkably reduced CC1 level and immediately diminished the expression of TCF7L2 in CC1-expressing oligodendrocytes (Fig. 3A, D). However, TCF7L2 seemed to be re-activated at 5 dpl and 8 dpl in white matter tracts of adolescent and adult mice, respectively. (Fig. 3B, C). In contrast to low CC1 expression at 1 dpl, CC1+ oligodendrocytes were obviously increased 5 days post-withdrawal of 5-FU (Fig. 3D and Fig. S1). The quantification analysis indicated that the percentage of TCF7L2+ cells among CC1+ cells was decreased from 54.6% ±8.1% to 2.8% ±3.1% after 4-day-5-FU treatment (Fig. 3E left), but the proportion was increased to 49.4% ±7.5% in 5-FU-treated adolescent mice at 5 dpl compared to control mice (38.2% ±4.5%, Fig. 3E right). These data suggest that TCF7L2 could be activated in the white matter lesions of adolescent and adult mice after 5-FU withdrawal.

3.4 TCF7L2 promoted oligodendrocytes proliferation and differentiation

Although we observed the TCF7L2 is activated in 5-FU-injured oligodendrocytes, we asked if TCF7L2 was sufficient for OPCs survival and differentiation. To address this issue, we cultured an oligodendroglial precursor cells (Oli-neu) and then transfected these cells with control (pCDNA3.1) and TCF7L2-expressing plasmid, respectively. The proliferation of OPCs was detected by immunostaining, we observed increased number of EdU+ or PDGFRa+ cells after 96 h-transfection of TCF7L2 (Fig. 4A, B). We also detected PDGFRa and Olig2 (lineage marker) by qRT-PCR after transfected TCF7L2 or TCF7L2-EN (a dominant negative vector of TCF7L2). Transfection with TCF7L2 and TCF7L2-EN plasmids was about 12-fold increase and 70% decrease of TCF7L2 expression compared to control cDNA transfection, respectively (Fig. 4C and D). In contrast to control cDNA, TCF7L2 overexpression increased 9-fold level of *Pdgfrα*, but TCF7L2-EN transfection significantly downregulated *Pdgfrα* expression (Fig. 4E), which indicating TCF7L2 was related with OPCs proliferation.

To further identify whether TCF7L2 could promote OPCs differentiation, we isolated OPCs from neonatal rat cortex and maintained these cells in oligodendrocyte growth medium in the presence of PDGF-AA, and transduced with lentivirus expressing green fluorescent protein (GFP) and GFP-TCF7L2, then stained for MBP after 3-day infection. In control group, spontaneous OPC differentiation detected as MBP+ cells was less than 5/area. In contrast, TCF7L2 overexpression resulted in a drastic increase of MBP+ mature oligodendrocytes (Fig. 4F, G). Besides, we found that a 5-fold and 6-fold upregulation of myelin gene *Mbp* and of the genes encoding important differentiation activator *Olig2* in TCF7L2-transfected cells compared to control in Oli-neu cells by qPCR (Fig. 4H). Taken together, these data revealed that TCF7L2 could promote OPCs proliferation and differentiation.

4. Discussion

The current study investigated if 5-FU-induced demyelination/remyelination could be happened in the corpus callosum and the potential candidate involved in the myelin

sheath repair. Inconsistent with a delayed demyelination in the adult mice, 5-FU could result in immediate damage to cerebral white matter of adolescent mice. Similarly, spontaneous remyelination occurred in adolescent mice was faster than in adults. Meanwhile, we identified the transcription factor TCF7L2 was markedly re-activated in 5-FU-injured corpus callosum. In addition, TCF7L2 was sufficient for oligodendrocytes proliferation and differentiation *in vitro*. Our data uncovered that 5-FU-induced white matter degeneration was recoverable and TCF7L2 might be a potentially therapeutic target in 5-FU-induced white matter damage.

Previous evidences have revealed that 5-FU could induce apoptosis and suppress proliferation of cells in the neurogenic regions of brain including the hippocampus and white matter tracts[23]. Furthermore, treatment with 5-FU could cause oligodendrocytes death *in vitro* and a syndrome of delayed myelin damage in the CNS[7]. However, two issues about 5-FU-induced oligodendrocytes damage still needed to be elucidated. Firstly, is 5-FU-induced oligodendrocytes damage reversible both in adolescent and adult mice? If so, the gene required for remyelination in 5-FU-lesioned white matter is still unknown. Our data confirm that the adolescent mice catch an acute injury on myelin, it can be inferred that an easy attack on the CNS myelination in adolescent mice is due to immature oligodendrocyte of mice during the developmental stage. The demyelination caused by 5-FU could be self-repaired after 5-FU withdrawal both in adolescent and adult mice, and remyelination in adolescent mice is faster than in adults, indicating 5-FU-caused demyelination/remyelination is closely related with the age of mice.

Numerous studies have revealed the importance of TCF7L2 during developmental myelination as well as remyelination[18]. TCF7L2, which is emerging before the formation of the myelin sheaths and disappeared at the end of oligodendrocytes maturation, is specifically expressed in oligodendrocyte lineage cells by a time dependent way[15]. Weng et al demonstrated that TCF7L2 was associated with 5-FU-caused demyelination in adolescent mice. After 5-FU injection, the expression of TCF7L2 is decreased significantly in *vivo* and in *vitro*[24]. Besides, TCF7L2 re-expression is specific to remyelinating lesions generated by many different

chemicals induced in different adult CNS regions, however, the underlying role of TCF7L2 in 5-FU-damged oligodendrcoytes remylination is not fully understood. In our present study, accompanied with severely decreased MBP expression, oligodendrocyte-specific TCF7L2 expression is significantly decreased in the cerebral white matter of 5-FU-injected adolescent mice, consistent with our previous study (Weng et al, 2014). After 5-FU withdrawal, TCF7L2 rapidly appears and increases in damaged white matter of 5-FU-treated adolescent and adult mice during the recoverable stage. Interestingly, mRNA level of OPC marker *Pdgfrα* is regulated by TCF7L2 or TCF7L2-EN overexpression, which indicating TCF7L2 is related with OPC proliferation. We further observe that TCF7L2 could increase the expression of oligodendrocytes differentiation marker, indicating TCF7L2 is also required for oligodendrocyte differentiation. Therefore, our results emphasize that stage-specific oligodendroglial regulators TCF7L2 plays a crucial role in oligodendrocyte remyelination by promoting oligodendrocyte proliferation and differentiation. Activation of TCF7L2 may provide a future effective ways to promote brain repair in 5-FU-treated patients with severe demyelinating diseases of the CNS.

5. Conclusion

This study demonstrated that 5-FU-induced demyelination is spontaneously recoverable both in adolescent and adult mice. In addition, we found that the transcription factor TCF7L2 was markedly re-activated and re-appeared in cerebral white matter injured mice, which indicating TCF7L2 may play a positive role in the remyelination after 5-FU withdrawal.

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Conflict of interest statement

The authors declare no conflicts of interest.

Author contributions

X.C Yang and Q.J Weng designed experiment and wrote paper; X.C Yang, B Yang, B.Q Tan, J Wang and Mengting Zhao collected data; Q.J Weng, Q.J He, B.Q Tan, Yan Hu and J.J Wang analyzed data.

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Figure legends

Figure 1 Spontaneous remyelination in adult mice

(A) Schematic diagram shows the administration scheme of 5-FU in adult mice. dpl: day post lesion.

(B) Immunohistochemistry of white matter tracts from adult mice using antibodies to MBP (n=6).

(C) Electron micrographs of the optic nerve of vehicle and 5-FU-injected adult mice (n=6). Low-power and High-power images are showing in upper and bottom panels, respectively.

Scale bars in B, 25 μm, in C left, 2 μm, right, 1 μm.

Figure 2 Acute demyelination and remyelination in adolescent mice

(A) Schematic diagram shows the administration scheme of 5-FU in adolescent mice.

(B-C) Immunohistochemistry of white matter tracts from adolescent mice using antibodies to MBP after 1 dpl, 5 dpl (B) and 22 dpl (C), respectively. Mice number of each stage is 6.

Scale bar in B, 25 um.

Figure 3 Activation of TCF7L2 in remyelination lesions

(A-B) The white matter tracts immuolabeled by antibodies to TCF7L2 and CC1 after 1 dpl (A) and 5 dpl (B) in adolescent mice (n=6). Arrows indicate CC1+/TCF7L2+ co-labeled cells.

(C) Immunostaining for TCF7L2 and CC1 in the corpus callosum from control and 5-FU-treated adult mice at 8 dpl. Arrows indicate CC1+/TCF7L2+ co-labeled cells.

(D) Quantification of the number of CC1+ cell per area in A. Data represent mean± SD, n>3. * $p < 0.05$, one-way ANOVA

(E) Quantification of the percentage of TCF7L2+ among CC1+ oligodendrocytes in corpus callosum of adolescent mice in A, B. Data represent mean ± SD, n>3. * $p < 0.05$, one-way ANOVA

Scale bars in A, B, C, 50 um.

Figure 4 The effect of TCF7L2 on oligodendrocytes

(A) Immunocytochemistry using antibodies to EdU and PDGFRα on Oli-neu cells. Arrows indicate EdU+/PDGFRa+ co-labeled cells.

(B) Quantification of the number of EdU+ and PDGFRα+ cells in A. Data represent mean ± SD, n>3. * $p < 0.05$, one-way ANOVA.

(C-D) Histogram shows the qRT-PCR analysis of tcf7l2 expression from RNAs isolated from Oli-neu cells transfected with or without TCF7L2/TCF7L2-EN plasmid. Data represent mean ± SD from at least three independent experiments.

(E) Histogram shows the qRT-PCR analysis of *pdgfa* expression from RNAs isolated from Oli-neu cells transfected with or without TCF7L2/TCF7L2-EN cDNA. Data represent mean ± SD, n>3 (* $p < 0.05$, one-way ANOVA).

(F) Immunostaining is performed with antibodies to MBP in primary OPCs infected with GFP or GFP-TCF7L2 expressing lenti-virus. Arrows indicate MBP+/TCF7L2+ co-labeled cells.

(G) Quantification of the number of MBP-positive oligodendrocytes in F. Data represent mean ± SD, n>3. * $p < 0.05$, one-way ANOVA.

(H) Histogram shows the qRT-PCR analysis of mbp and olig2 expression from RNAs isolated from Oli-neu cells transfected with or without TCF7L2-EN plasmid. Data represent mean ± SD, n>3 (* $p < 0.05$, one-way ANOVA).

Supplemental figure legends

Supplemental figure 1

The white matter tracts immuolabeled by antibodies to CC1 after 1 dpl and 5 dpl in adolescent mice. Arrows indicate CC1+ cells.

Scale bars 50 um.

Figure 1

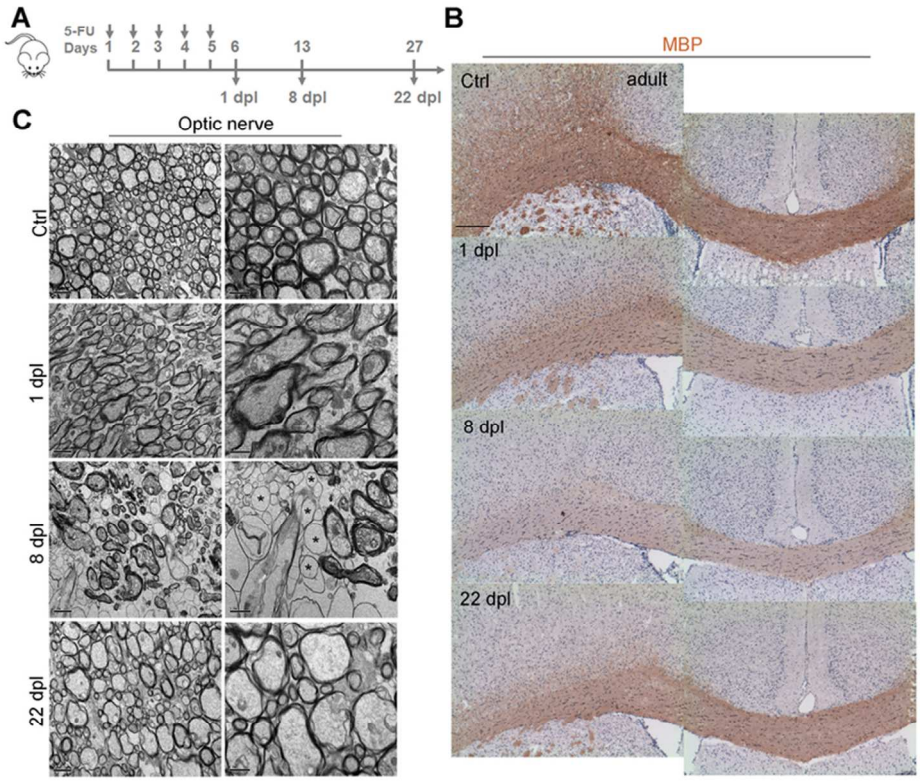

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Figure 2

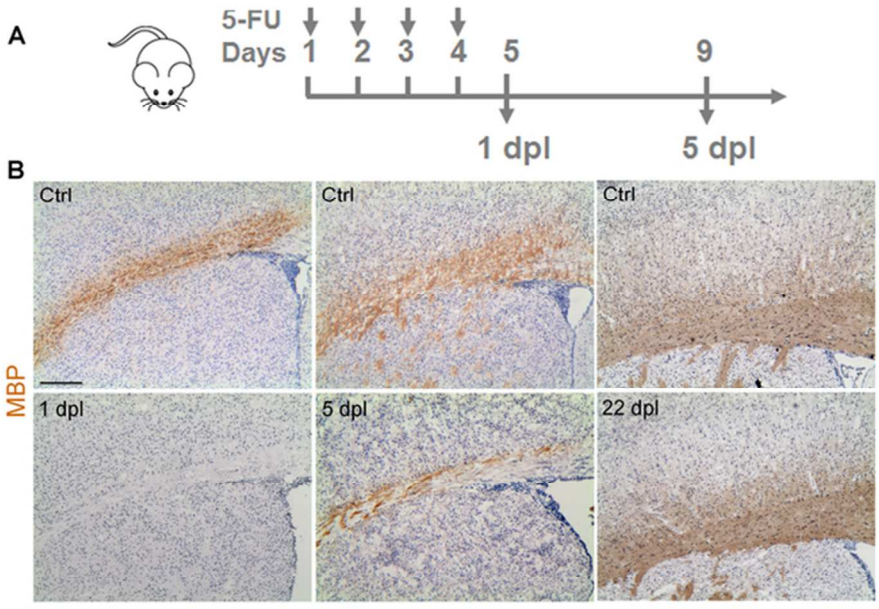

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Figure 3

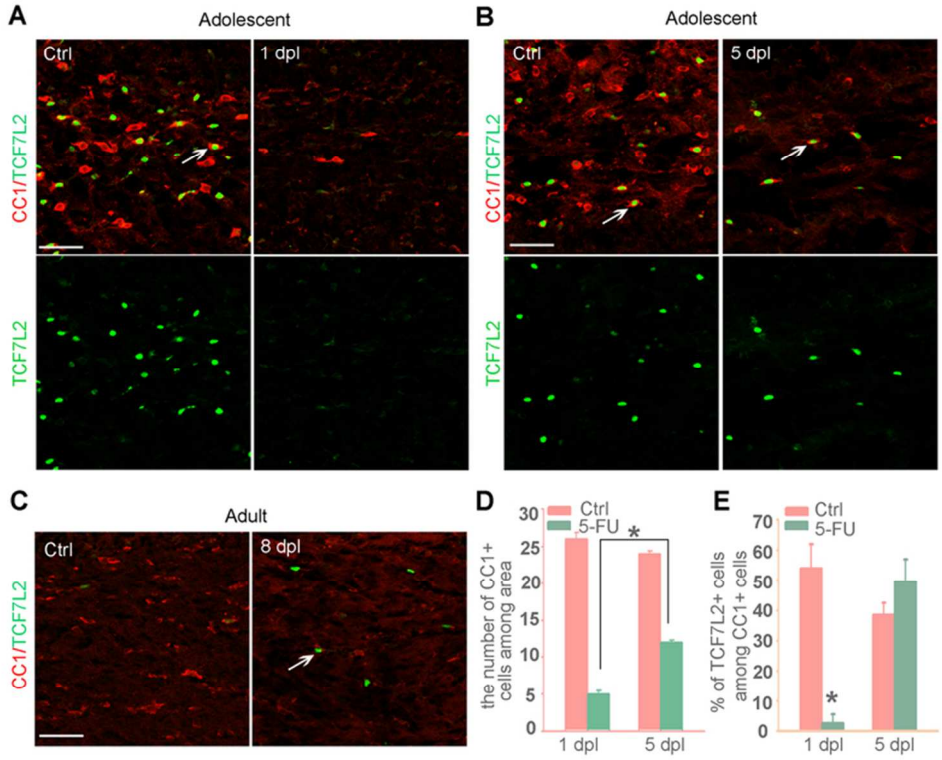

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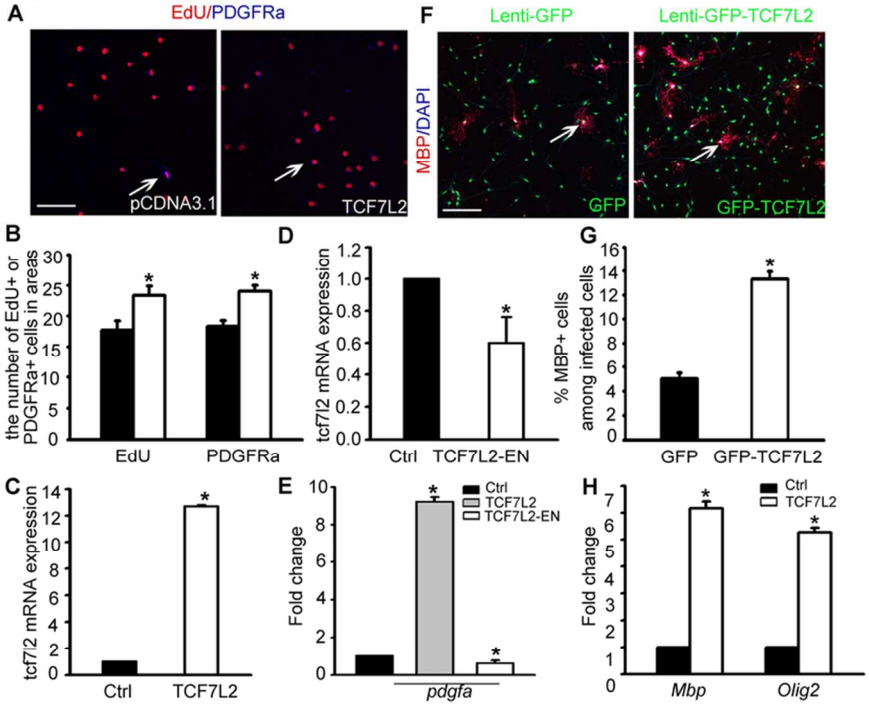
Figure 4



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