Effects of Chronic Minocycline Treatment on Neurogenesis in *Fmr1* KO Mice

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1. Introduction

Fragile X syndrome (FXS) is an X-linked genetic disorder resulting from over-expansion of CGG trinucleotide repeats within the Fragile X Mental Retardation 1 (*Fmr1*) gene, inhibiting the activity of its transcript, Fragile X Mental Retardation protein (FMRP) (Garber *et al*. 2008). FMRP has been shown to regulate translation by binding a subset of mRNAs important in synaptic plasticity, and therefore plays an important role in cognitive development (Barber *et al*. 2008). Thus, those with FXS suffer cognitive disabilities, including learning and memory loss (Terracciano *et al*. 2005).

Minocycline is a drug traditionally used in bacterial infections (Brogden *et al*. 1975). In recent literature minocycline has been shown to possess neuroprotective ability in common neurological diseases (Plane *et al*. 2010) such as Parkinson's and Huntington's diseases, as well as FXS. Furthermore, our own behavioral tests using minocycline-treated FXS mice show improved learning and memory compared to controls. However, the methodology behind these improved cognitive functions in minocycline treated FXS mouse models (*Fmr1* KO mice) is lacking in literature and requires further studies.

In this experiment we investigate neurogenesis, the growth and development of nervous tissue, via cell proliferation and neuronal differentiation in the dentate gyrus (DG) of minocycline treated *Fmr1* KO mice as a possible mechanism for the improved cognitive functions. We hypothesize that minocycline could up-regulate cell proliferation and or neuronal differentiation in the DG, and we investigate this by staining Ki67$^+$, PCNA$^+$, and DCX$^+$ cells using immunohistochemistry then counting these in a manual sample-blinded manner. Cell counts for minocycline treated *Fmr1* KO mice are compared to water-treated/ wild-type littermate controls.

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2. Materials and Methods

30mg/kg minocycline in drinking water → Sacrifice and Perfusions → Slice brains at 30µm/slice → Staining → Mounting → Counting

Subjects & Experimental Conditions:
C57Bl/7K background *Fmr1* KO mice with wild type littermates, water/minocycline treated.

Minocycline Treatment:
Minocycline was implemented in drinking water at a dosage of 30mg/kg from postnatal day 3 until sacrifice at 2 months. Drinking water was changed daily, and mice were re-weighed weekly.

Perfusions:
Transcardial perfusions of 0.9% saline followed by 4% paraformadehyde were performed. The brains were extracted then stored overnight in 4% PFA followed by storage in 30% sucrose prior to slicing & staining.

Slicing:
Brains were sliced on a Leica vt1000s Vibratome at 30µm/slice for easy visualization of staining.

Staining:
Free-floating immunohistochemistry was performed. For Ki67, PCNA and DCX, 1:500 rabbit anti-Ki67, 1:100 mouse anti-PCNA, and 1:200 goat anti-DCX were used respectively as primary antibodies. For Ki67 and PCNA, 1:200 biotin-conjugated goat anti-rabbit and anti-mouse IgG were used as secondary antibodies respectively, and 1:200 Cy5-conjugated anti-goat was used for DCX. Slices were developed with 3,3'-diaminobenzidine (DAB) for 3 minutes and stored at 4^{o}C for at least 2 days before mounting.

-Ki67 is a cellular marker for proliferation not shown in G_o cells.
-PCNA is a DNA clamp for DNA polymerase δ.
-DCX is a microtubule associated protein expressed by neuronal precursor cells.

Mounting:
Stained slices were mounted on 2% gelatin-coated slides and dehydrated by immersing them in solutions of 50%, 70% and 100% ethanol sequentially, then finally CitriSolv, each for 5 minutes. Slides were cover slipped with Permount^{TM} before counting.

Counting:
Slices were counted for Ki67$^+$, PCNA$^+$, and DCX$^+$ cells separately for both hemispheres of the brain in the dentate gyrus (DG) in a sample-blinded manner using an Olympus CX21LED light microscope at 40X magnification, with only cell bodies completely inside the confines of the DG counted (See Figure 1).

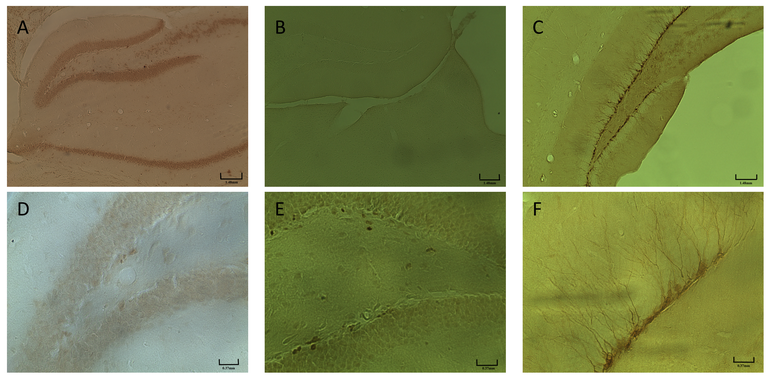

Figure 1. 10x fields of view for counting Ki67, PCNA and DCX (A,B,C respectively) and 40x fields of view for counting Ki67, PCNA, and DCX (D,E,F respectively). Cells were only counted as positive cells if the entire cell body was stained dark brown and was completely in the confines of the dentate gyrus. The fields of view for D, E, and F yielded cell counts of 3, 11, and 42 respectively.

3. Results

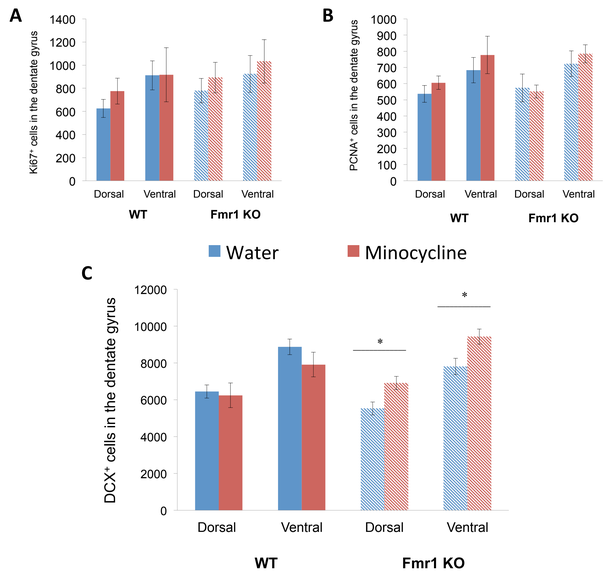

Figure 2. Effects of minocycline on neurogenesis staining for Ki67, PCNA, and DCX. Total number of Ki67$^+$ (A), PCNA$^+$ (B), or DCX$^+$(C) cell counts in the dentate gyrus (DG) of water or minocycline (30mg/kg) treated *Fmr1* KO/WT littermate control for the dorsal and ventral portions of the DG. The total number of cells was calculated as the average Ki67$^+$/PCNA$^+$/DCX$^+$ cells in one 30µm slice multiplied by the average number of 30µm slices in the dorsal or ventral portions of the DG (42, 83 respectively). Counting done at 40X magnification. Bars represent group means, error bars are SEM. * indicates a p-value < 0.01. WT/*Fmr*1 KO water & WT/*Fmr*1KO mino had n = 10,11,5,10 respectively.

4.Discussion & Conclusion

Discussion:

In this experiment, we investigated the effects of chronic minocycline treatment on neurogenesis via cell proliferation (Ki67/PCNA) and neuronal differentiation (DCX) in the dentate gyrus of *Fmr1* KO mice, as behavioral data in the literature has shown minocycline rescues many of the cognitive deficits shown in FXS, in both mouse models (Bilousova *et al*. 2009) as well as human subjects (Leigh *et al*. 2013). We found that there were no statistically significant differences in the number of Ki67$^+$ (Figure 2A) or PCNA$^+$ (Figure 2B) cells across all experimental groups (*Fmr1* KO/WT, water/minocycline treated) in the dentate gyrus for the dorsal and ventral portions of the hippocampus. However, we found that the number of DCX$^+$ cells showed an increasing trend (Figure 2C) in the minocycline treated group for *Fmr1* KO mice compared to the water treated group, but no difference between the WT groups suggesting neuronal differentiation may play a role in improved cognitive functions in *Fmr1* KO mice, but not cellular proliferation. In comparison to other studies, Mattei *et al*. 2014 found that minocycline normalizes neurogenesis in a schizophrenia model, and Ekdahl *et al*. 2003 found that minocycline restores impaired neurogenesis in inflammation. Thus, it is possible that minocycline may also play a role in increasing neurogenesis in *Fmr1* KO mice given behavioral data, as well as comparative studies.

Conclusion:

The improved cognitive functions of *Fmr1* KO mice given minocycline treatment (30mg/kg) could be in part due to increased neurogenesis, specifically increased neuronal differentiation in the dentate gyrus.