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Chronic minocycline treatment improves hippocampal neuronal structure, NMDA receptor function, and memory processing in *Fmr1* knockout mice

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ABSTRACT

Fragile X Syndrome (FXS) is the most common inherited cause of intellectual disability, and is the leading known single-gene cause of autism spectrum disorder. FXS patients display varied behavioural deficits that include mild to severe cognitive impairments in addition to mood disorders. Currently there is no cure for this condition, however minocycline is becoming commonly prescribed as a treatment for FXS patients. Minocycline has been reported to alleviate social behavioural deficits, and improve verbal functioning in patients with FXS; however, its mode of action is not well understood. Previously we have shown that FXS results in learning impairments that involve deficits in *N*-methyl-D-aspartate (NMDA) receptor-dependent synaptic plasticity in the hippocampal dentate gyrus (DG). Here we tested whether chronic treatment with minocycline can improve these deficits by enhancing NMDA receptor-dependent functional and structural plasticity in the DG. Minocycline treatment resulted in a significant enhancement in NMDA receptor function in the dentate granule cells. This was accompanied by an increase in PSD-95 and GluN2A and GluN2B subunits in hippocampal synaptoneurosome fractions. Minocycline treatment also enhanced dentate granule cell dendritic length and branching. In addition, our results show that chronic minocycline treatment can rescue performance in novel object recognition in FXS mice. These findings indicate that minocycline treatment has both structural and functional benefits for hippocampal cells, which may partly contribute to the pro-cognitive effects minocycline appears to have for treating FXS.

1. Introduction

Fragile X Syndrome (FXS) is a genetic disorder and the most common form of inherited intellectual impairment and autism spectrum disorder that can be attributed to a single gene. It is produced by a mutation in *Fmr1* gene, which encodes Fragile X mental retardation protein (FMRP). FMRP, in turn, is important for the synthesis and regulation of synaptic proteins that contribute to cognitive and affective processes (Darnell and Klann, 2013; Zalfa et al., 2003). Not surprisingly, FXS patients display a varied number of behavioural deficits that include mild to severe cognitive impairment, mood disorders, language problems and social anxiety (Leigh et al., 2013; Paribello et al., 2010; Winarni et al., 2012). Although different medications have therapeutic effects for some specific FXS symptoms (Schaefer et al., 2015), there remains a need for effective treatments that target the varied behavioural sequelae associated with FXS, particularly those involving cognitive deficits.

Emerging clinical evidence has indicated that minocycline, a broad spectrum tetracycline antibiotic, is able to rescue synaptic and behavioural abnormalities in FXS patients (Dziembowska et al., 2013; Leigh et al., 2013; Paribello et al., 2010; Winarni et al., 2012). Minocycline appears to improve verbal functioning and alleviate some of the social and emotional deficits in children and adolescents with FXS (Leigh et al., 2013; Paribello et al., 2010; Winarni et al., 2012). The beneficial effects of this drug for FXS have also been documented in several preclinical studies, where minocycline has been shown to improve the cognitive and social performance of *Fmr1* knockout (KO) mice in specific behavioural tasks (Dansie et al., 2013; Rotschafer et al., 2012; Yau et al., 2016a, 2016b). There is also some evidence to suggest that minocycline may help to promote dendritic spine maturation in the hippocampus (Bilousova et al., 2009), however the exact mechanisms underlying minocycline's actions have yet to be fully elucidated.

The loss of FMRP has a significant impact on the hippocampus, a brain structure that plays an important role in the modulation of

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cognitive and affective behaviours (Bostrom et al., 2016a). *Fmr1* KO mice exhibit a number of hippocampal deficits that include: abnormal adult neurogenesis; increases in immature spine numbers, impaired synaptic plasticity, and delayed dendritic development within hippocampal sub-regions (Bostrom et al., 2016a; Eadie et al., 2012, 2009; Huber et al., 2002). Enhanced long-term depression (LTD), mediated by group 1 metabotropic glutamate receptors (mGluR), has been proposed as a possible mechanism for FXS (Bear et al., 2004), however clinical trials have failed to establish the effectiveness of any mGluR-based therapeutic regime (Berry-Kravis et al., 2011). Conversely, we, and others, have previously shown that targeting NMDA receptor function can enhance synaptic plasticity and alleviate some cognitive deficits in *Fmr1* KO mice (Bostrom et al., 2015; Eadie et al., 2012; Franklin et al., 2014; Yau et al., 2016a, 2016b). In the current experiments we examined whether chronic minocycline treatment can improve both hippocampal synaptic structure and NMDA receptor function in *Fmr1* KO mice.

2. Materials and methods

2.1. Animals

Male *Fmr1* hemizygous knockout mice (*Fmr1* KO) and WT littermates (C57Bl/6J genetic background) were generated by breeding female *Fmr1* heterozygous mice with male WT mice. All experiments were carried out in accordance with international standards on animal welfare, guidelines set by the Canadian Council on Animal Care, and approved by the Animal Care Committee at the University of Victoria.

2.2. Genotyping

Ear snips were collected and genotyping was conducted using a DNA isolation kit (Invitrogen, Ontario, Canada) and polymerase chain reaction (PCR) analysis. The PCR was performed as described previously (Bostrom et al., 2016b; Eadie et al., 2012; Yau et al., 2016a, 2016b) using the following cycling parameters: denaturation at 94 °C for 5 min, followed by 35 cycles of 60 s at 94 °C, 90 s at 65 °C, and 150 s at 72 °C. Primers used for probing the *Fmr1* KO allele (800 base pairs) were: M2 = 5′-ATCTAGTCAYGCTATGGATATCAGC-3′ and N2 = 5′-GTGGGCTCTATGGCTTCTGAGG-3′. Primers for probing the WT allele (450 base pairs) were: S1 = 5′-GTGGTTAGCTAAAGTGAGGATGAT-3′ and S2 = 5′-CAGGTTTGTTGGGATTAACAGATC-3′.

2.3. Minocycline treatment

Minocycline was administered directly to female mice and their litters through the drinking water. Treatment commenced 3 days after a litter was born (30 mg/kg/day in dark, amber-colored bottles), as we previously performed (Yau et al., 2016a, 2016b). This method of minocycline administration has been previously shown to yield detectable concentrations of minocycline in the blood of adult mice and in the breast milk of lactating dams (Lee et al., 2006; Lin et al., 2005). The volume of consumed water was monitored daily and was comparable between study groups (data not shown). After the offspring were weaned, minocycline administration continued in the drinking water of the offspring until they reached 2 months of age. This protocol for continued minocycline treatment has been shown to be effective for reducing anxiety, ultrasonic vocalization deficits and severity of autogenic seizures in adult *Fmr1* KO mice (Bilousova et al., 2009; Rotschafer et al., 2012).

2.4. Electrophysiological recordings

2.4.1. Slice preparation

To obtain hippocampal slices, mice were first anesthetized with isoflurane and then rapidly decapitated. The brain was then quickly removed in a high Mg^{2+}, low Ca^{2+} artificial cerebrospinal fluid (ACSF, consisting of (in mM) 123 NaCl, 25 $NaHCO_3$, 3 KCl, 1.25 $NaH_2PO_4{\cdot}H_2O$, 1 $CaCl_2$, 6 $MgCl_2$, 10 Glucose) that was chilled to 4 °C and well oxygenated (95% O_2/5% CO_2). Transverse hippocampal slices (350 μm) were sectioned in this ACSF using a Vibratome 1500 (Ted Pella Inc., Redding, California, U.S.A.). Following sectioning, individual slices were transferred to a holding chamber and incubated in continuously oxygenated normal ACSF (nACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, and 10 dextrose, pH 7.3 at 30 °C, All slices were allowed to recover for a minimum of 1 h before any recordings commenced.

2.4.2. Whole cell recordings

Whole cell recordings were obtained using an Axopatch 200B amplifier and signals were digitized using a DigiData 1440A connected to a personal computer that was running Clampex 10.3 software (Molecular Devices, Sunnyvale, CA). All drugs were dissolved in nACSF before bath application with perfusion rate of 2 mL/min. Whole-cell recordings were made with a borosilicate glass electrode (5–7 MΩ) using an intracellular solution that contained (in mM): 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 7 phosphocreatine, 0.3 Na_3GTP, 2 Mg_2ATP, 10 QX-314, 0.2–0.4% biocytin, pH 7.3. The liquid conjunction potential was adjusted to 9.5 mV.

Synaptic responses were evoked using a concentric bipolar electrode placed visually in the middle molecular layer (medial perforant path) of the superior blade of the dentate gyrus. Dentate granule cells with series resistance larger than 30 MΩ or a change of series resistance larger than 30% over the course of the experiment were not included in the data set. To examine AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor-mediated excitatory postsynaptic currents (EPSCs) using voltage-clamp, DG granule cells were held at −70 mV during the delivery of a 0.05 ms current pulse as previously described (Yau et al., 2016a, 2016b). NMDA receptor-mediated EPSCs were obtained by holding cells at +40 mV, while delivering the same 0.05 ms pulses described previously (Yau et al., 2016a, 2016b). The peak current for EPSC's evoked at −70 mV was used to obtain the maxima for AMPA receptor mediated-EPSCs. NMDA receptor-mediated EPSCs were measured at +40 mV, and the maxima was measured 50 ms following the delivery of the stimulating pulse to ensure no contamination by any residual AMPA receptor-mediated response. AMPA/NMDA ratios were calculated by dividing the maximum AMPA receptor-mediated EPSC by the maximum NMDA receptor-mediated EPSC.

2.5. Biocytin staining and dendritic analysis

Biocytin (0.2–0.4%) was added to the intracellular solution prior to whole-cell recordings and allowed to diffuse into neurons over the time course of the recording. After each recording, the patch pipette was quickly, but carefully, removed from the slice to preserve neuron integrity and minimize biocytin diffusion to surrounding cells (Christie et al., 2000). Brain slices were immediately fixed in 4% paraformaldehyde at 4 °C overnight and then washed thoroughly with 0.01 M phosphate buffer (PBS). After incubation with streptavidin-conjugated Cy3 (1:500 in PBS) at 4 °C for 2 days, slices were then washed three times with PBS, mounted onto 2% gelatin-coated slides and cover-slipped using an anti-fading mounting medium (SouthernBiotech). Cells were visualized using a fluorescence microscope (Olympus, Melville, New York) and a 40× objective. As described previously, we only included cells in the study if they possessed intact dendrites, with no signs of cuts or damage (Kannangara et al., 2015, 2014). All cells that met our inclusion criteria were traced using Neurolucida (MBF Bioscience, Williston, VT) with the experimenter blinded to the group identity of the cell. Dendritic complexity was quantified using dendritic length, number of intersections within Sholl radii (10 μm concentric circles), and mean dendritic length/number of