Review Article

Matrix Metalloproteinases and Minocycline: Therapeutic Avenues for Fragile X Syndrome

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Fragile X syndrome (FXS) is the most common known genetic form of intellectual disability and autism spectrum disorders. FXS patients suffer a broad range of other neurological symptoms, including hyperactivity, disrupted circadian activity cycles, obsessive-compulsive behavior, and childhood seizures. The high incidence and devastating effects of this disease state make finding effective pharmacological treatments imperative. Recently, reports in both mouse and \textit{Drosophila} FXS disease models have indicated that the tetracycline derivative minocycline may hold great therapeutic promise for FXS patients. Both models strongly suggest that minocycline acts on the FXS disease state via inhibition of matrix metalloproteinases (MMPs), a class of zinc-dependent extracellular proteases important in tissue remodeling and cell-cell signaling. Recent FXS clinical trials indicate that minocycline may be effective in treating human patients. In this paper, we summarize the recent studies in \textit{Drosophila} and mouse FXS disease models and human FXS patients, which indicate that minocycline may be an effective FXS therapeutic treatment, and discuss the data forming the basis for the proposed minocycline mechanism of action as an MMP inhibitor.

1. Introduction

Fragile X syndrome (FXS), the most common heritable cause of intellectual disability and autism spectrum disorders [1], has a prevalence of roughly 1 in 4000 males and 1 in 6000 females [2, 3]. While a hallmark of FXS is low IQ (\(\sim 40\)), patients present with a wide spectrum of behavioral, physical, and neurological symptoms [4–6]. Behavioral problems include hyperactivity and hypersensitivity to sensory stimuli, anxiety and mood disorders, disrupted sleep patterns, defects in cognitive learning and memory consolidation, and impaired social skills [1, 4, 5, 7–14]. Often, a speech deficit is the first symptom leading to a FXS clinical diagnosis [4, 5]. Although there are usually not significant non-neurological medical impairments associated with the syndrome, FXS patients typically display male macroorchidism, macrocephaly with prominent ears and a long, thin face, joint hypermobility, and flat feet [4, 5, 15–17]. Elevated electroencephalogram (EEG) activity is characteristic, and epileptic seizures are present in \(\sim 20\%\) of FXS patients, typically with remittance by adulthood [18].

FXS is caused by loss of the \textit{fragile X mental retardation 1} (\textit{FMR1}) gene product, FMRP [19], typically due to expansion (>200) of the CGG trinucleotide repeat in the 5\textsuperscript{\prime}-untranslated gene region, leading to subsequent hypermethylation and gene silencing [20]. FMRP is an mRNA-binding protein known to regulate mRNA stability, trafficking, and translation [21–26], with roles in the activity-dependent regulation of synaptic development and plasticity [26–33]. Numerous studies support the “mGluR theory of FXS” that suggests enhanced group 1 metabotropic glutamate receptor 5 (mGluR5) signaling is responsible for deficits in synaptogenesis, dendritic spine morphology, long-term potentiation (LTP) and depression (LTD) in the disease state [5, 27, 28, 30–32, 34–43]. Consequently, many studies have focused on mGluR inhibitors, such as 2-methyl-6-phenylethynyl-pyridine (MPEP), as a therapeutic intervention for FXS [31, 38–40, 43–47], with considerable
success. For example, in the Drosophila disease model, MPEP effectively prevents cellular synaptic deficits and behavioral learning and memory impairments [31, 42, 45, 46]. While some mGluR inhibitors cannot be used in FXS patient treatment due to toxicity and bioavailability limitations (e.g., MPEP), other drugs, such as the selective mGluR5 inhibitor fenobam, are currently in human clinical trials [48, 49]. Lithium, an inhibitor of GSK3β, a downstream effector of mGluR5 signaling, is also being taken to clinical trials with promising results as a potential FXS therapeutic treatment [50].

In addition to the promise of mGluR5 pathway interventions, several recent reports suggest minocycline as another potential avenue of FXS therapeutic treatment [51–54]. Used for decades as an antibiotic and acne treatment, the second-generation, semisynthetic tetracycline derivative minocycline has a long half-life, highly lipophilic characteristics, and easily crosses the blood-brain barrier [55]. In addition to its antibiotic actions, minocycline also functions as an anti-inflammatory agent via inhibition of several molecules, including COX-2, iNOS, and P38 MAPK, and as an anti-apoptotic agent via inhibition of caspases, among many other putative modes of action [55, 56]. Central to its role in FXS, minocycline is known to inhibit matrix metalloproteinases (MMPs), a family of secreted and membrane-tethered zinc-dependent extracellular proteases with roles in tissue remodeling and intercellular signaling [57–60]. Functioning through one or more of these modes of action, minocycline has been shown to have neuroprotective effects [55] and has been previously suggested to be useful in the treatment of several neurodegenerative diseases, including multiple sclerosis [61], amyotrophic lateral sclerosis (ALS) [62, 63], Huntington’s disease [64], Parkinson’s disease [65], and Alzheimer’s disease [66].

In the FXS disease state, several recent studies have proposed that minocycline exerts its therapeutic effects via MMP inhibition [51, 53]. In humans, a diverse array of at least 24 MMPs functions to cleave components of the extracellular matrix (ECM), including both secreted and cell membrane proteins [57–59]. In general, MMPs contain a pro domain that is cleaved to activate the extracellular protease, an enzymatic zinc-containing catalytic domain, a linker domain, and a hemopexin domain [57–59]. MMPs are part of the metzincin family of proteases, named for a conserved methionine residue and zinc in the protease catalytic sites, which includes ADAMS (a disintegrin and metalloproteinase) and ADAM proteases with thrombospondin motifs (ADAMTSs) [57–59, 67]. In the central nervous system, MMPs have been implicated in axonal guidance, synaptogenesis, neurotransmission, synaptic plasticity, and behavioral learning [57, 58, 67]. MMPs are endogenously inhibited by tissue inhibitors of MMPs (TIMPs), with 4 family members in humans [68, 69]. Along with MMPs, TIMPs are also expressed in the central nervous system, where they also regulate synaptic mechanisms and behavioral outputs [67–69].

In this paper, we review recent evidence that minocycline acts as an effective therapeutic treatment in FXS genetic animal models and human FXS patient clinical trials. We then summarize current data supporting an “MMP inhibition mechanism” by which minocycline may remediate the FXS disease state. Finally, we consider possible pathways through which the MMP/TIMP and FMRP pathways could intersect in the formation of FXS pathogenesis.

2. Minocycline in Fragile X Animal Models

Minocycline was first revealed as a possible FXS therapeutic treatment in the mouse disease model [51]. The FMR1 knockout (KO) mouse is a well-validated model of the human disease state [30, 40, 51, 71, 72], which displays macroorchidism, hyperactivity, and some learning/memory deficits [71–73]. As with cortical postsynaptic dendritic spine morphogenesis defects in FXS patient autopsies [74, 75], FMR1 KO mice exhibit an immature dendritic spine profile with more long, thin filopodia-like spines and a proportional lack of mature short, stubby, mushroom-like spines [76–79]. This synaptic maturation/overgrowth defect has been reported to vary in severity/penetrance in different brain regions and may be developmentally transient, with the defect being most prominent during neural circuit refinement stages of early postnatal development [80, 81]. The dendritic spine phenotype of FMR1 KO mice has been linked with defects in postsynaptic group 1 mGluR signaling activity, as with defects in synaptic plasticity [82]. This postsynaptic dendritic spine defect has long been considered the FXS neuroanatomical hallmark and was the logical choice to first assay the effects of minocycline treatment.

In 2009, Bilusova and colleagues provided the first evidence for the therapeutic effectiveness of minocycline in the FMR1 KO mouse with a thorough examination of dendritic spine profiles of hippocampal neurons both in vitro and in vivo [51]. This study showed that 20 μM minocycline promoted maturation of dendritic spines in control hippocampal neuronal cultures. In FMR1 KO neurons, minocycline treatment in culture (20 μM for 17 hours) or fed to mice (30 mg/kg/day in drinking water) shifted the immature dendritic spine profiles towards normal dendritic spine profiles (Table 1). It is important to note that no change was reported in dendritic spine length or the total number of spines between FMR1 KO untreated and treated conditions; rather, there was solely a proportional shift of the number of immature to mature spines upon minocycline treatment [51]. Following this neuroanatomical analysis, the same study examined the effect of minocycline treatment on FMR1 KO mouse behavior [51]. Minocycline was again fed to newborn mice via their drinking water at levels of 30 mg/kg/day. At 3 weeks of age, treated FMR1 KO mice were found to be less anxious in an elevated plus maze assay (Table 1). Moreover, minocycline treatment resulted in better memory in a passive Y maze, compared to untreated FMR1 KO mice [51]. Additionally, a follow-up study was very recently conducted by this same group [70]. In FMR1 KO adult male mice, it was found that the rate of ultrasonic vocalizations (USVs) produced during mating is reduced. No other acoustic property deficits were identified. Interestingly, minocycline treatment restored a normal rate of USVs in the FMR1 KO...
Table 1: Summary of recent minocycline treatment trials in Drosophila and mouse disease models, and human clinical studies. The columns display the systems, minocycline dosages, phenotypes tested, treatment outcomes and side effects.

| Model  | Dosage                        | Phenotypes tested                                                                 | Treatment effects                                                                 | Side effects           | Study                                      |
|--------|-------------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------|-------------------------------------------|
| Drosophila | 20 μM in larvae; 1 mM in adult (oral feeding) | Syaptic structure of NMI, sLNv, and MB neurons                                    | Prevention of all neuroanatomical defects                                        | None                   | Siller and Broadie, 2011 [53]             |
| Mouse  | 20 μM for 17 hrs in vitro; 30 mg/kg/day in vivo | Immature dendritic spine profiles, anxiety, memory defects, decreased rate of USVs | More mature dendritic spine profiles, less anxious, memory improvements, increased rate of USVs | More mature dendritic spines | Bilosuova et al., 2009; [51] Rotschafer et al., 2012 [70] |
| Human  | 50 mg BID (low dose) 100 mg BID (high dose) | Behavioral symptoms                                                              | Better language and social communication skills, less anxiety, more attentive; Better irritability, stereotypy, hyperactivity, inappropriate speech subscales on ABC-C | GI issues, diarrhea, loss of appetite, dizziness, headaches | Utari et al., 2010; [54] Paribello et al., 2010 [52] |

Mouse (Table 1). Thus, minocycline was shown to be effective at ameliorating both neuroanatomical and behavioral FXS defects in the mouse disease model.

Recently, a study employing the Drosophila FXS disease model corroborated and expanded these data, reinforcing the therapeutic potential of minocycline treatment [53]. Over more than a decade of research, the Drosophila system has been firmly established as a highly validated model of the human disease state [25, 26, 31, 42–47, 83–99]. Drosophila contains a single FMR1 homolog, dFMR1, compared to the three Fragile X family genes present in mammals (FMR1, FXR1, and FXR2). Only human FMR1 rescues the broad range of neurological phenotypes caused by dFMR1 KO, with human FXR1/2 having no activity, showing that FMR1 function has been evolutionarily conserved and that human FMR1 requirements can be effectively dissected in the Drosophila FXS disease model [84, 99]. Like human patients and the mouse model, dFMR1 KO flies exhibit synaptic overgrowth and synaptic immaturity in a range of neural circuits, including motor neurons [84, 86, 99], clock neurons [84, 87, 88, 99], and learning/memory neurons [26, 42]. Likewise mimicking the human disease condition, dFMR1 null animals display macroorchidism and deficits in spermatogenesis, hyperactivity and circadian arrhythmicity, and strong deficits in learning formation and memory consolidation [46, 83, 85, 87, 88, 92]. The breadth and FXS disease relevance of these phenotypes makes Drosophila an excellent system to examine minocycline’s effectiveness in treating the disease state.

In 2011, Siller and Broadie provided a follow-up study examining the therapeutic effectiveness of minocycline in the dFMR1 KO in a thorough examination of synaptic architecture in a range of disparate neural circuit types [53]. Synapse structure was analyzed in three locations: (1) the well-characterized glutamatergic neuromuscular junction (NMJ) in the peripheral musculature, (2) the pigment dispersing factor (PDF) neuropeptidergic small ventrolateral (sLNv) clock neurons in the central brain, and (3) Kenyon cell neurons of the brain mushroom body (MB) learning and memory center (Table 1). In all three circuit types, the dFMR1 KO displays the same characteristic synaptic overgrowth and over elaboration, including an expanded synaptic arbor domain, increased synaptic branching, and increased supernumerary synaptic boutons with prominent structurally immature synaptic sites [25, 26, 42, 47, 86, 87]. Minocycline was fed to dFMR1 KO mutants in their food during larval development in the range of 2–20 μM and to adults at the higher dosage of 1 mM (Table 1), resulting in a dosage-dependent improvement of synaptic architecture towards the wildtype state [53]. Interestingly, the CNS synaptic deficits responded better to minocycline treatment than the NMJ defects, although the reason for this difference is currently unknown. At the NMJ, minocycline treatment completely prevented the accumulation of immature synaptic boutons and partially prevented the overabundance of mature boutons, but had no effect on the defect in synaptic branching in dFMR1 KOs [53]. In the brain, both in clock neurons and in MB Kenyon cells, minocycline treatment both prevented the excess synaptic branching and completely rescued the overelaboration of synaptic boutons in dFMR1 KOs (Table 1). Unlike the mouse study, the Drosophila study found no effect of minocycline treatment on wild-type synaptic architecture [53]. Moreover, the Drosophila study contained no behavioral analyses. However, at a neuroanatomical level, the Drosophila study corroborates the findings of the mouse study and adds further evidence for the effectiveness of minocycline as a broad-spectrum FXS therapeutic treatment in multiple classes of neural circuits [51, 53].

3. Minocycline in Fragile X Patients

Taken together, the two animal model studies strongly suggest minocycline may be an effective FXS treatment. However, the obvious question still remains: does the drug effect translate to human FXS patients? In 2010, two studies began to provide insight into this question with early clinical trials [52, 54]. Utari and colleagues studied 50 FXS patients, given minocycline for 2 weeks or longer, mainly to assess
the safety of the drug as a FXS treatment [54]. Of the 50 patients examined, 21 reported side effects, with the most common being gastrointestinal problems, including diarrhea and loss of appetite (Table 1). Most patients reported side effects as mild. One patient did experience coloring of the nails, but none reported tooth discoloration. Of the FXS patients followed throughout the course of the study, most displayed improvements in several areas, including language, attention, social communication, and anxiety [54]. A small subset of patients exhibited worsening in two areas: hyperactivity and moodiness (Table 1). Thus, this study suggests minocycline may be effective in FXS treatment with only mild side effects, and the results warrant a followup, controlled study to more closely examine minocycline effectiveness.

Paribello and colleagues performed an open-label, add-on minocycline treatment trial with 19 FXS patients aged 13–32 years followed for an 8-week treatment period [52]. One patient dropped out due to side effects, and two other patients developed asymptomatic seroconversion of antinuclear antibodies, a diagnostic in autoimmune disorders. None of the other patients reported serious side effects that were attributed to the minocycline treatment, although dizziness, headaches, sleepiness, and diarrhea were reported as mild side effects (Table 1). In this trial, significant improvements occurred in behavioral outcomes using the Aberrant Behavior Checklist-Community Edition (ABC-C) [52]. Four out of five subscale scores showed significant improvement, including the irritability subscale, which was used as the primary outcome measure, and stereotypy, hyperactivity, and inappropriate speech subscales, which were employed as secondary outcome measures (Table 1). Positive results using the clinical global improvement scale (CGI) and the visual analog scale for behavior (VAS) as measures were also reported for the majority of minocycline-treated FXS patients [52]. Interestingly, given the choice to extend minocycline treatment for 1 year, 18 of the 19 families independently decided to continue treatment based on their perceptions of behavioral improvements. Taken together with the Utari study, both sets of data strongly suggest that minocycline is a relatively safe and potentially effective treatment for FXS patients [52, 54]. However, a double-blind, placebo-controlled clinical treatment trial is still necessary to provide concrete evidence that minocycline is a positive and effective FXS therapy.

4. Mechanism of Action: MMP Inhibition

The 2009 mouse FXS model study provides good evidence that minocycline acts through inhibition of secreted matrix metalloproteinase-9 (MMP-9) [51]. In other neurological disorders, such as multiple sclerosis, minocycline has similarly been found to be effective as an MMP-9 inhibitor [55]. Bilousova and colleagues performed both Western Blot and gel zymography analyses to assess differences in MMP-9 levels and enzymatic (gelatinase) activity in the mouse hippocampus [51]. In FMR1 KO (P7) mice, levels of active MMP-9 as well as MMP-9 gelatinase activity were both increased compared to controls (Figure 1). Importantly, minocycline treatment of the FMR1 KO decreased both hippocampal active MMP-9 protein levels and hippocampal gelatinase activity towards the wildtype condition [51]. Interestingly, MMP-9 treatment of wildtype hippocampal cell cultures induced immature dendritic spine profiles with a greater proportion of long, thin filopodia-like dendritic spines, mimicking the FMR1 KO state [51], a finding that has since been validated via genetic methods [100]. Together, this evidence suggests that upregulation of secreted active MMP-9 is a novel aspect of the molecular pathology of FXS and that MMP-9 inhibition is the mechanism of action of minocycline in alleviating FXS phenotypes (Figure 1).

In the follow-up Drosophila study, Siller and Brodie greatly extended testing of this MMP hypothesis, taking advantage of the fly’s relative genetic simplicity (2 MMPs in Drosophila compared to 24 in mammals; 1 TIMP in Drosophila compared to 4 in mammals) [53, 57, 58, 60, 101]. The two Drosophila MMPs include secreted MMP-1 and membrane anchored MMP-2, with a good antibody probe available for MMP-1 only. MMP-1 expression levels and gelatinase activity showed no significant differences in the dFMR1 KO compared to control, at least at the NMJ synapse with immunocytochemistry and in situ zymography and in whole-brain Western Blots (Figure 1) [53]. Nevertheless, to begin to test the MMP hypothesis, the endogenous TIMP inhibitor was genetically overexpressed in the dFMR1 KO background to mimic the proposed minocycline effect. At the NMJ, TIMP overexpression was highly efficacious in suppressing the synaptic structural overelaboration characterizing the dFMR1 KO, restoring the synaptic branching and excess mature and immature bouton formation to the wildtype condition [53]. Conversely, TIMP overexpression causes early developmental lethality and tracheal deformation prior to death [60, 102, 103], and dFMR1 removal bidirectionally suppressed these TIMP overexpression phenotypes. Importantly, a dFMR1; MMP-1 double KO mutant displayed the same reciprocal suppression of phenotypes, with prevention of dFMR1 KO synaptic architecture defects and rescue of MMP-1 KO tracheal defects and early lethality [53]. Together, these data provide excellent evidence for a specific genetic interaction between the TIMP/MMP-1 and FMRP pathways (Figure 1). Taken with the previous mouse FXS model study showing specific upregulation of secreted MMP-9, reduced upon minocycline treatment, the combined data set strongly suggests that minocycline is inhibiting MMPs to exert its alleviatory actions on FXS phenotypes [51, 53].

A critical question is to determine how the FMRP and TIMP/MMP pathways intersect (Figure 1). FMRP is best known as a negative translational regulator, and, therefore, it is possible that FMRP directly inhibits MMP expression, resulting in MMP upregulation in the FXS disease state. Based on known FMRP functions, this interaction could happen at the level of regulating MMP mRNA stability or translation, or FMRP could secondarily influence MMP protein function, secretion, or localization via acting on MMP-interacting proteins (Figure 1). However, numerous more indirect interactions between the two pathways are also
possible. FXS is a disease of enhanced glutamate receptor signaling, associated with defects in synaptic morphogenesis and plasticity (LTD/LTP) [35, 37]. Several studies have shown that NMDA glutamate receptor signaling causes local MMP-9 release, leading to MMP-mediated synaptic plasticity events [104–106], presumably via extracellular proteins, such as integrins, laminins, cadherins, β-dystroglycan, brevican, and tenascin-R, which have all been implicated in hippocampal LTP [57, 58, 67] (Figure 1). Indeed, recent work implicates integrin β1 signaling as the mechanism by which MMP-9-mediated changes in dendritic spine morphology occur [100]. Furthermore, MMP-dependent synapse remodeling can be blocked by NMDA receptor inhibitors, and NMDA receptor activity has been shown to increase MMP-9 activity, suggesting another possible link [58]. Moreover, MMPs play roles in axonal-dendritic structural remodeling [58, 67]; for example, with MMP-9 present in mammalian dendritic spines and MMP-1 present at the Drosophila glutamatergic NMJ [53, 107, 108]. MMP-9 KO mice display deficiencies in hippocampal LTP, and other changes, for example, after spinal cord injury, can induce elevated gelatinase activity, showing that careful control of MMP expression levels is critical to synaptic regulation [105, 109]. In addition, MMP inhibition may lead to TIMP signaling changes due to decreased levels of MMP-bound TIMP versus increased levels of free unbound TIMP [110]. For example, the balance between MMP-7 and TIMP-1 was recently shown to be important for pro-nerve growth factor (NGF) cleavage and neuroprotection following kainite-induced seizures [111], which could possibly provide a link with seizure manifestations in FXS patients. Thus, while it is unclear how the MMP and FMRP pathways intersect, it is clear that a number of intriguing possibilities need to be explored (Figure 1).

5. Conclusions and Future Directions

FXS is a devastating neurological disease characterized by a broad spectrum of cellular and behavioral symptoms [4, 5]. While much attention has been focused on mGluR5 inhibitors as a potential avenue of disease treatment [31, 45–47], a significant amount of new evidence from Drosophila [53], mouse [51], and human studies [52, 54] suggests that the common tetracycline derivative minocycline may be a new and highly effective treatment alternative (Table 1). These recent reports indicate that minocycline may be a broad-spectrum treatment with only mild side effects. The clear next step is to pursue a double-blind, placebo-controlled FXS clinical trial of minocycline effectiveness. Importantly, it may be critical to test minocycline in young children as it is probable that treatment effectiveness may be linked, in part, to developmentally transient events of neural circuit formation and/or refinement. Of course, it is to be hoped that the inherent plasticity of the nervous system will also make adult minocycline treatments effective. Thus, minocycline holds the real possibility of being an accessible and cost-effective broad treatment for the disease. Importantly, minocycline has long been FDA approved, greatly facilitating its rapid dissemination to the FXS community. This is indeed an exciting development for families afflicted by this devastating neurological disease.

Both Drosophila and mouse studies point toward MMP inhibition being the mechanism of minocycline action in
FXS [51, 53]. However, another possibility that must be considered arises from the fact that minocycline also functions as an antibiotic by inhibiting bacterial translation, and a similar function could be predicted to antagonize the effect of losing FMRP translational repression, causing elevated translation [21]. Although this should not be a consideration at the levels of minocycline used in the recent FXS studies (Table 1), which are the same dosages as used for treatment of acne and bacterial infections, such as neurosyphilis [112, 113], it remains a strong possibility to be investigated as potentially FXS patients may be more sensitive toward minocycline treatment than healthy individuals. Significant research into minocycline’s effects on eukaryotic ribosomes, especially in FXS, has not been done and must be performed extensively to determine if the minocycline mechanism of action is through its ability to regulate translation. In addition, the available evidence does not rule out the possibility of other mechanisms of minocycline action, such as p53 MAPK regulation [55, 114, 115]. Nevertheless, the recent Drosophila study strongly indicates specific genetic interplay between the MMP and FMRP pathways that should be the focus of investigation in the immediate future [53]. It will be important to fully study the roles of TIMP and MMPs in the context of synaptic development/refinement and to define overlapping and distinct synaptic functions of the different MMP family members (i.e., membrane-anchored versus secreted). Moreover, a great limitation in the recent Drosophila study was that it only examined synaptic structure defects, and it is imperative to extend the work to the examination of minocycline/MMP involvement at the level of synaptic function/plasticity and behavioral outputs in the Drosophila FXS model. Besides the neuronal mechanisms, it is also important to note that the genetic interaction between MMP and FMRP occurs also in nonneuronal tissues, because MMP-1 nonneuronal phenotypes and overall lethality are rescued by dFMR1 removal [53]. A similar nonneuronal FMR1 function is also revealed by the joint symptoms of FXS patients, strongly implicating an ECM component of the human disease state. Moving forward, it will be important to understand how TIMP/MMP dysfunction fits into the larger picture of FXS pathogenesis, to provide both a greater understanding of TIMP/MMP roles at the synapse, as well as to bring to light possible new therapeutic targets for FXS.

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