The Impact of NNZ-2591 on the *fmr1* Knockout Mouse Model of Fragile X Syndrome

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Background

Fragile X syndrome is a neurodevelopmental disorder caused by mutation of the fragile X mental retardation 1 (*fmr1*) gene, and characterized by intellectual disability, social anxiety, attention-deficit hyperactivity disorder and abnormal physical characteristics such as macro-orchidism (enlarged testes). Mutant *fmr1* knockout (KO) mice recapitulate this phenotype and represent a preclinical model for assessment of putative drug treatments.

The current study evaluated the potential of NNZ-2591 to reverse the Fragile X phenotype exhibited by *fmr1* KO mice.

Drug Treatment

Fmr1 KO and wild-type mice (C57BL/6J background) were dosed with either vehicle or NNZ-2591 (30 mg/kg i.p.) 1/day, starting at 14 weeks of age, for 28 days. Various behavioral and anatomic outcomes were assessed following treatment.

Results

At baseline, *fmr1* KO mice manifested numerous phenotypic changes compared with wild-type mice, including: *decreased hyperactivity* in the open-field ($p < 0.001$) and successive alley tests ($p < 0.001$); *increased contextual-fear conditioned memory* ($p < 0.001$); *increased social sniffing* ($p < 0.001$); *decreased dendritic spine density* and *decreased phosphorylation of ERK and Akt* ($p < 0.001$). Treatment with NNZ-2591 significantly ameliorated all of these aberrant features of the *fmr1* KO mouse phenotype.

Open Field Activity

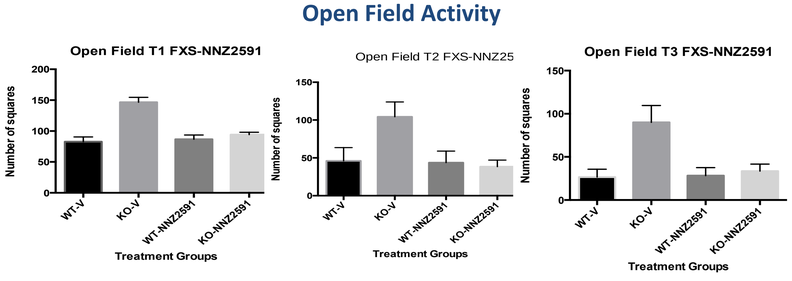

Figure 1. *Open field (OF).* Fmr1 *KO mice show hyperactivity at basal time (T1), as measured by squares crossed which is reversed by treatment with NNZ-2591.* T1 corresponds to the OF at basal time as a measure of hyperactivity, T2 corresponds to the OF performed 10 minutes after T1, as a measure of short term memory and T3 corresponds to the OF performed 24 hours later after T2, as a measure of Long term memory. NNZ2591 significantly reduced hyperactivity and improves short and long term memory in the FXS mice.

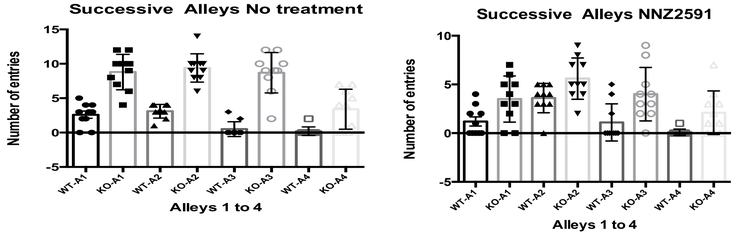

Figure 2. *Successive alley test. Wild-type mice show diminishing propensity to enter successive alleys that are increasingly neophobic (lighter, lower walled as the mouse progresses from alley 1 through alley 4). Fmr1 KO mice show significantly greater impartiality, most likely due to hyperactivity. NNZ-2591 reverses this phenotype.*

Contextual Fear Conditioning: Test of memory and learning

Contextual fear conditioning is the most basic of the conditioning procedures. It involves taking an animal and placing it in a novel environment, providing an aversive stimulus, and then removing it. When the animal is returned to the same environment, it generally will demonstrate a freezing response if it remembers and associates that environment with the aversive stimulus. Freezing is a species-specific response to fear, which has been defined as "absence of movement except for respiration". This may last for seconds to minutes depending on the strength of the aversive stimulus, the number of presentations, and the degree of learning achieved by the subject. Contextual fear conditioning test is used to examine both hippocampus-dependent memory and learning.

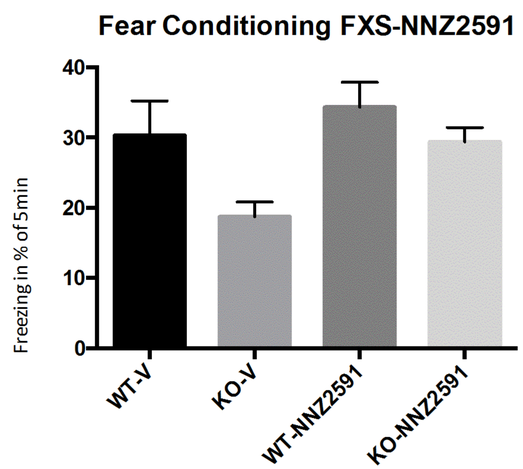

Figure 3. *Assessments of cognition and memory.* Fmr1 *KO mice show decreased behavioural freezing when reintroduced to an aversive environment (contextual fear conditioning). NNZ-2591 significantly improves learning and memory in the FXS mice.*

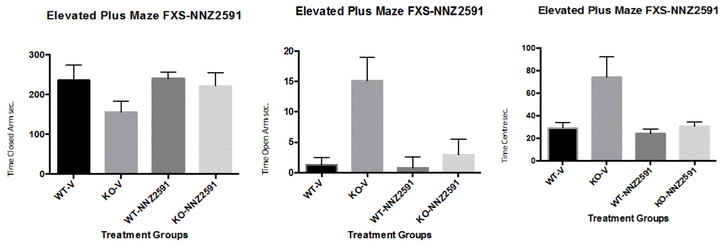

Figure 4. *Assessment of behavior in the elevated plus maze test.. Fmr1 KO mice show increased entries of the 'open' arm, which can indicate reduced anxiety. However, the considerable increase seen in time spent in the center of the maze suggests the KO the mice spend an exaggerated time choosing which arm to enter, and then make an impartial decision. This behavioural profile may therefore represent impaired cognition or memory. NNZ-2591 treatment completely normalised this profile.*

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Hippocampal Dendritic Spine Morphology

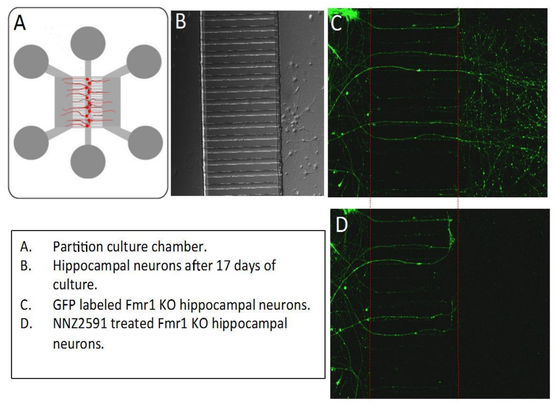

Figure 5. *Photomicrographs of dendritic spine morphology in wild-type and fmr1 KO mouse hippocampal cells (obtained at E17.5 and cultured to 21 DIV). Dissociated hippocampal cells were plated in 15 mm multi-well vessels and a plating medium of Neurobasal medium (suppli ed B27) was supplemented with 10% fetal bovine serum. After 7 days (culture conditions: 37 °C in humidified 5% CO_2), green-fluorescent protein (GFP) was applied to monitor dendritic spine morphogenesis during culture. Dendritic spines are usually formed between 16 and 17 days in vitro (DIV). Fmr1 KO significantly decreased spine density by in vitro treatment with NNZ-2591 5 nM (0.25 ± 0.03) and 50 nM (0.27 ± 0.10).*

ERK and Akt Phosphorylation

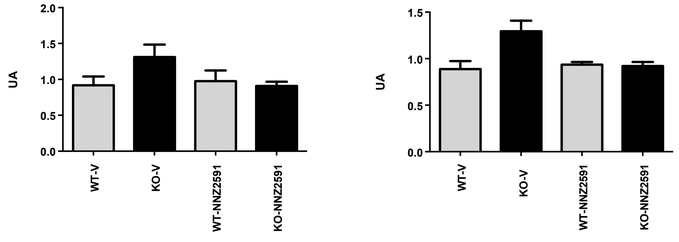

Figure 6*. Western blot analysis was conducted on extracellular-signal-regulated kinase (ERK), and Akt from wild-type and fmr1 KO mouse lymphocytes (obtained ex vivo, following 28 day treatment with either vehicle or NNZ-2591). ERK is a classical MAPK signal transduction protein, responsible for growth factor transduction, proliferation, cytokine response to stress and apoptosis. Akt is a key component in the PI3K/Akt/mTOR signalling pathway. Excess activation (phosphorylation) of both has been implicated in Fragile X Syndrome and autism-spectrum disorder. Fmr1 KO increased ERK and Akt activation was reversed by treatment with NNZ-2591.*

Conclusions

NNZ-2591 treatment for 28 days appears to normalize the phenotype of *fmr1* KO mice. The efficacy of the drug was observed not only in behavioral studies but also in studies of dendrite morphology and ERK/Akt activation. Taken together, these data suggest that the novel small molecule, NNZ-2591, may represent a potentially important treatment for Fragile X syndrome. Further studies are ongoing to expand our understanding of the mechanism of action of NNZ-2591 in *fmr1* KO mice.