The Impact of NNZ-2566 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.

NNZ-2566 is a peptidase-resistant analogue of the terminal tripeptide of IGF-1, and is currently in clinical development for the treatment of traumatic brain injury and autism spectrum disorders. The current study evaluated the potential of NNZ-2566 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-2566 (100 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: hyperactivity in the open-field ($p < 0.01$) and successive alley tests ($p < 0.01$); decreased contextual-fear conditioned learning ($p < 0.01$); increased social sniffing ($p < 0.01$); macro-orchidism ($p < 0.01$); increased dendritic spine density and increased phosphorylation of brain ERK and Akt ($p < 0.01$). Treatment with NNZ-2566 significantly ameliorated all of these aberrant features of the *fmr1* KO mouse phenotype.

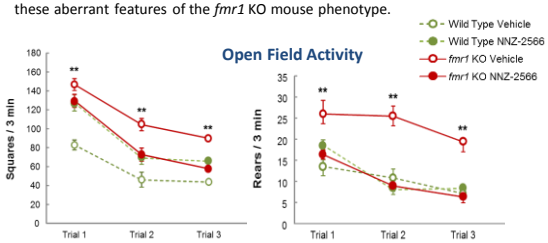

Figure 1. *Open field activity.* Fmr1 *KO mice show hyperactivity, as measured by squares crossed (left panel) and rears (right panel), which are reversed by treatment with NNZ-2566.*

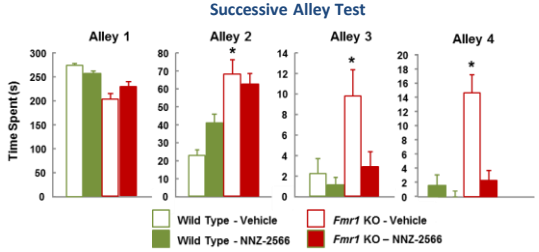

Figure 2. *Successive alley test. Wild-type mice show diminishing propensity to enter successive alleys that are increasingly neophobic (lighter, lower walled as the mouse progresses from alley 1 through alley 4). Fmr1 KO mice show significantly greater impartiality, most likely due to hyperactivity. NNZ-2566 reverses this phenotype.*

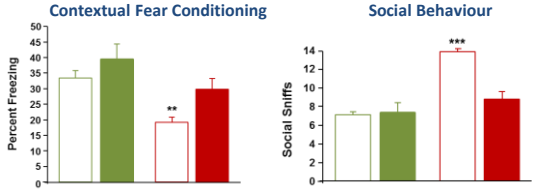

Figure 3. *Assessments of cognition and memory. Fmr1 KO mice show decreased behavioural freezing when reintroduced to an aversive environment (contextual fear conditioning), as well as enhanced sniffing of a reintroduced conspecific (social memory) – indicative of learning and memory impairment. NNZ-2566 reverses both of these effects.*

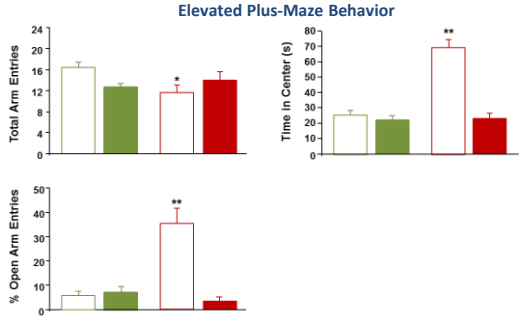

Figure 4. *Assessment of behavior in the elevated plus-maze test. Fmr1 KO mice show increased entries of the 'open' arm, which can indicate reduced anxiety. However, the considerable increase seen in time spent in the center of the maze suggests the KO mice spend an exaggerated time choosing which arm to enter, and then make an impartial decision. This behavioural profile may therefore represent impaired cognition or memory. NNZ-2566 treatment completely normalised this profile.*

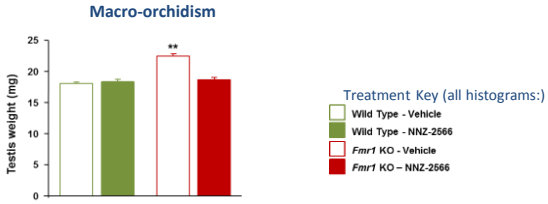

Figure 5. *An anatomical feature of Fragile X syndrome, also observed in fmr1 KO mice, is enlarged testes (macro-orchidism). This aberrant phenotype was clearly observed in the current mouse study, and was reversed by 28 day treatment with NNZ-2566 (100 mg/kg, i.p.)*

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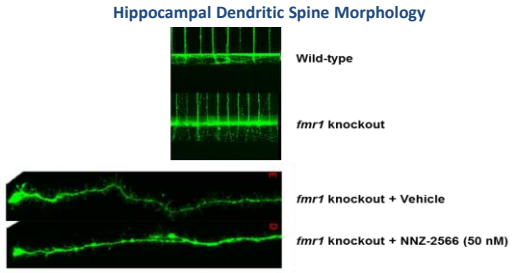

Figure 5. *Photomicrographs of dendritic spine morphology in wild-type and fmr1 KO mouse hippocampal cells (obtained at E14-E16 and cultured to 14-21 DIV). Dissociated hippocampal cells were plated in 15 mm multi-well vessels and a plating medium of MEM-Eagle's salts (supplied glutamine free) was supplemented with 10% fetal bovine serum. After 3 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 7 and 14 days in vitro (DIV). By 14 DIV most dendritic protrusions are spines; however, their maturation continues until 21 DIV. Fmr1 KO significantly increased spine density - an effect that is reversed by in vitro treatment with NNZ-2566 (50 nM).*

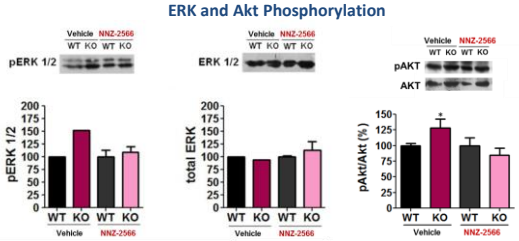

Figure 6. *Western blot analysis was conducted on extracellular-signal-regulated kinase (ERK), and Akt from wild-type and fmr1 KO mouse brain (obtained ex vivo, following 28 day treatment with either vehicle or NNZ-2566). ERK is a classical MAPK signal transduction protein, responsible for growth factor transduction, proliferation, cytokine response to stress and apoptosis. Akt is a key component in the PI3K/Akt/mTOR signalling pathway and regulates cellular survival and metabolism by binding and regulating many downstream effectors, such as Nuclear Factor-κB (NfκB) and Bcl-2 family proteins. Excess activation (phosphorylation) of both has been implicated in autism spectrum disorders. Fmr1 KO increased ERK and Akt activation in the current study. This effect was reversed by treatment with NNZ-2566.*

Conclusions

NNZ-2566 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. Significantly, a complete reversal of macro-orchidism was also seen following NNZ-2566, indicative of a potential for disease-modifying effects involving not just the CNS but also other tissues that are affected adversely in Fragile X Syndrome. Taken together, these data suggest that the novel small molecule, NNZ-2566, 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-2566 in *fmr1* KO mice, and to extend studies to other models of autism spectrum disorders.