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ORIGINAL PAPER

NNZ-2566, a Novel Analog of (1–3) IGF-1, as a Potential Therapeutic Agent for Fragile X Syndrome

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Abstract Fragile X syndrome (FXS) is the most common form of inherited intellectual disability. Previous studies have implicated mGlu5 in the pathogenesis of the disease, and many agents that target the underlying pathophysiology of FXS have focused on mGluR5 modulation. In the present work, a novel pharmacological approach for FXS is investigated. NNZ-2566, a synthetic analog of a naturally occurring neurotrophic peptide derived from insulin-like growth factor-1 (IGF-1), was administered to *fmr1* knockout mice correcting learning and memory deficits, abnormal hyperactivity and social interaction, normalizing aberrant dendritic spine density, overactive ERK and Akt signaling, and macroorchidism. Altogether, our results indicate a unique disease-modifying potential for NNZ-2566 in FXS. Most importantly, the present data implicate the IGF-1 molecular pathway in the pathogenesis of FXS. A clinical trial is under way to ascertain whether these findings translate into clinical effects in FXS patients.

Keywords Fragile X syndrome · Insulin growth factor 1 · NNZ-2566 · Behavior · Biomarkers

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Introduction

Fragile X syndrome (FXS) is a monogenic neurodevelopmental disorder caused by the 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 macroorchidism (Hagerman 1997). FXS is caused by mutations in the *fmr1* gene, triggering partial or complete gene silencing and partial or complete lack of the fragile X mental retardation protein (FMRP) (Oostra and Willemsen 2003). Mutant *fmr1* knockout (KO) mice recapitulate this phenotype and represent a preclinical model for the assessment of putative drug treatments (Yan et al. 2005). Novel therapeutics active in this preclinical model have shown potential benefit during clinical studies in FXS (Levenga et al. 2011; Michalon et al. 2012). Despite recent advances in understanding the pathophysiology of FXS, there is still no cure for this condition; current treatment is symptomatic. Preclinical research is essential in the development of potential therapeutic agents.

Both *fmr1* KO mice and patients with FXS show altered neuronal dendritic spine morphology (Irwin et al. 2000; Nimchinsky et al. 2001). Dendritic spine morphology is under the control of the PI3K–Akt–mTOR and Ras–MAPK signaling pathways (Kumar et al. 2005). These pathways

are aberrantly activated in the *fmr1* KO mouse and in patients with FXS (Sharma et al. 2010; Hoeffer et al. 2012). At the same time, loss of *fmr1* function in astrocytes induces the FXS neuronal phenotype, and normal astrocytes can rescue this abnormality (Jacobs and Doering 2010).

We hypothesized that a pharmacological intervention able to modulate neuroinflammation, astrocyte, and glial function might rescue the PI3K–Akt–mTOR and Ras–MAPK signaling pathway abnormalities in the *fmr1* KO mouse. We further predicted that rescue of signaling pathway deficits would reduce neuronal dendritic spine deficits and improve functional outcomes in this model of FXS.

Insulin-like growth hormone (IGF-1) is an endogenous hormone that has important effects on CNS development (D'Ercole and Ye 2008). IGF-1 is cleaved by plasma and brain peptidases, one product of this cleavage being a terminal tripeptide, (1–3) IGF-1 (Sara et al. 1989). The tripeptide, GPE, is capable of crossing the blood–brain barrier (Baker et al. 2005), where it retains strong neurotrophic efficacy and has behavior-modifying activity (Guan and Gluckman 2009). GPE increases the activity of the Ras–PI3K–Akt–mTOR in glia and decreases the activity of the Ras–MAPK pathway in neurons (Corvin et al. 2012). GPE decreases astrogliosis and neuroinflammation (Svedin et al. 2007), and rescues function in the MeCP2 mouse model of Rett syndrome (Tropea et al. 2009) in which microglial function is pathogenic (Maezawa and Jin 2010; Derecki et al. 2012).

NNZ-2566 is a peptidase-resistant analog of (1–3) IGF-1, the terminal tripeptide of IGF-1, that is both orally available and brain-penetrant (Bickerdike et al. 2009). NNZ-2566 shares the same profile of action as GPE, regulating neuroinflammation, preventing apoptosis, and being neuroprotective (Wei et al. 2009). This profile of effect is suitable for testing the hypothesis we formulated. Further, NNZ-2566 is a clinical-stage compound available for investigation in patients.

Furthermore, NNZ-2566 does not interact with metabotropic or ionotropic glutamate receptors at therapeutic concentrations and therefore represents a different therapeutic approach to novel therapeutics currently the subject of clinical investigation in FXS, such as mGluR5 receptor negative allosteric modulators (Levenga et al. 2011) or indirect inhibitors of glutamate release such as the $GABA_B$ receptor antagonist arbaclofen (Henderson et al. 2012).

In the present study, we have investigated the effects of NNZ-2566 in the *fmr1* KO mouse model in vivo to test whether intraperitoneal administration can reverse FXS phenotypes in a fully developed brain. Our results suggest beneficial effects in a wide range of symptoms and a disease-modifying potential for NNZ-2566 in FXS. Studies were performed using doses known to produce therapeutically relevant plasma levels compared to human studies (Neuren Pharmaceuticals Ltd, unpublished).

Experimental Procedures

Animals

Fmr1 KO mice (The Dutch-Belgium Fragile X Consortium 1994) were initially obtained from the Jackson Laboratory, and wild-type (WT) littermates were generated on a C57BL/6J background and repeatedly backcrossed onto a C57BL/6J background for more than eight generations. The *fmr1* KO mice were housed in groups of the same genotype in a temperature- and humidity-controlled room with a 12-h light–dark cycle (lights on 7 a.m.–7 p.m.). Testing was conducted during the light phase. Food and water were available ad libitum. Testing was conducted on *fmr1* KO mice and their WT littermates. Ten mice per treatment group, 14 weeks of age, were used for behavioral experiments. Mice were housed in commercial plastic cages, and experiments were conducted in line with the requirements of the UK Animals (Scientific Procedures) Act, 1986. All experiments were conducted with experimenters blind to genotype and drug treatment.

Drug Treatment

NNZ-2566 was dissolved in saline at a dose of 100 mg/kg for in vivo experiments.

Determination of Akt and ERK Activity

A once-daily intraperitoneal dose of 100 mg/kg NNZ-2566 was administered during 28 days to groups of $N = 5$ *fmr1* KO and WT mice. Animals were killed at time points of 15, 30, 60, and 240 min following the last administration after 28 days, and 12 days after 28 days of administration. At killing, brain and plasma samples were taken for analysis.

Phosphorylated ERK and Akt protein expression was measured by Western blot as previously described by Lopez Verrilli et al. (2009). The following antibodies were used such as anti-phosphospecific antibodies against Akt (1/1000) and kinase (ERK) 1/2 (1/2000) (Cell Signaling Technology, Danvers, MA, USA). Total Akt and ERK 1/2 protein content (phosphorylated) were evaluated by blotting membranes with anti-phospho-Akt (1/1000) and anti-phospho-ERK antibodies (1/2000) (Cell Signalling Technology, Danvers, MA, USA). Akt or ERK phosphorylation was normalized to protein content in the same sample and expressed as % of change with respect to basal conditions, considering basal levels as 100 %. Protein loading was evaluated by stripping and re-blotting membranes with β-actin antibody (1/1000) (Sigma-Aldrich, St. Louis, MO, USA). Lymphocyte phospho-Akt and phospho-ERK were determined by flow cytometry.

Neuronal Dendritic Morphology

Dendritic spine formation was investigated using in vitro cell culture. Hippocampal cell cultures were prepared from WT and *fmr1* KO fetal mice (14–16 days of gestation). Briefly, mice were killed by cervical dislocation under chloroform anesthesia and dissociated hippocampal cells plated in 15-mm multiwell vessels (BD Biosciences, San Jose, CA, USA). A plating medium of MEM-Eagle's salts (supplied glutamine-free) supplemented with 10 % fetal bovine serum was used. Cultures were kept at 37 °C in a humidified 5 % CO_2 atmosphere. After 3 days in vitro (DIV), green fluorescent protein (GFP) was used to monitor dendritic spine morphogenesis during the time course of culture (Ethell and Yamaguchi 1999; Ethell et al. 2001; Henkemeyer et al. 2003). Dendritic spines are usually formed between 7 and 14 DIV. By 14 DIV, most dendritic protrusions are spines; however, their maturation continues until 21 DIV. We utilized a compartmentalized culture system, based on a microfluidic chamber. NNZ-2566 is a positive control (the mGluR5 antagonist 2-methyl-6-phenylethynyl-pyridine or *MPEP*) and vehicle controls on dendritic spine density in *fmr1* KO and WT hippocampal primary cell cultures. We treated *fmr1* KO and WT cultures at 17 DIV. Morphological analysis was conducted using standard image analysis techniques.

Macroorchidism

The effect of 28 days of once-daily treatment of 100 mg/kg i.p. NNZ-2566 was assessed on testis weight in groups of 10 *fmr1* KO and WT mice starting at age 14 weeks.

Behavioral Analysis

Testing was conducted over 12 days commencing on the first day following treatment cessation in groups of 10 mice. The following tests were used following 28 days of once-daily intraperitoneal administration of 100 mg/kg NNZ-2566:

Open Field

The open-field apparatus was used to test multiple processes including anxiety/hyperactivity and habituation to a novel environment, one of the most elementary forms of learning, in which decreased exploration as a function of repeated exposure to the same environment is taken as an index of memory. This is normally studied in two sessions of exposure to the open field, occurring at 10 min and 24 h.

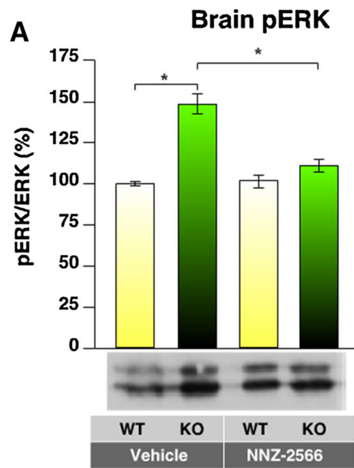
The apparatus was a gray PVC-enclosed arena 50 × 30 cm divided into 10 cm^2. Mice were brought to the experimental room 5–20 min before testing. A mouse was placed into a corner square facing the corner and observed for 3 min. The number of squares entered (whole body) (locomotor activity) and rears (both front paws off the ground, but not as part of grooming) were counted. The latency to the first rear was also noted. The movement of the mouse around the field was recorded with a video-tracking device for 3 min (version NT4.0, Viewpoint). The latency for the mouse to enter the brightest, central part of the field, total time spent in this central region, and total activity (as path length in cm) were recorded.

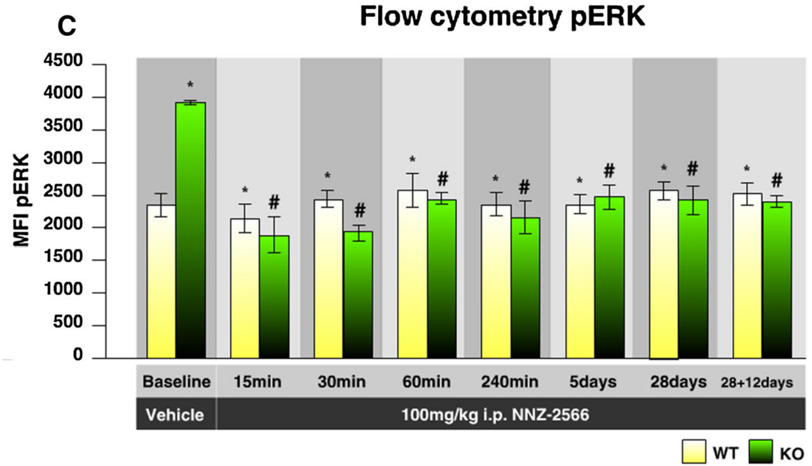
Elevated Plus Maze

The elevated plus maze (EPM) apparatus consists of horizontal open arms and closed arms, crossed in the middle perpendicularly to each other, and a center area, raised above floor height. The EPM apparatus was built according to the description of Lister (1987). Briefly, the apparatus used comprises two open arms (25 × 5 × 0.5 cm) across from each other and perpendicular to two closed arms (25 × 5 × 16 cm) with a center platform (5 × 5 × 0.5 cm). The open arms have a very small (0.5 cm) wall to decrease the number of falls, whereas the closed arms have a high (16 cm) wall to enclose the arm. Mice have access to all arms and are allowed to move freely between them. Mice were tested for 5 min, and their behavior was recorded. Measures taken included time spent in the arms and the center of the maze, and number of arm entries.

Successive Alleys

The successive alleys apparatus consisted of four successive, linearly arranged, increasingly anxiogenic alleys made of painted wood: each succeeding alley was painted a lighter color, has lower walls, and/or was narrower than the previous alley. Each alley was 25 cm long. Alley 1 had 25-cm-high walls, was 8.5 cm wide, and was painted black. A 0.5-cm step down led to alley 2 that was again 8.5 cm wide, but had 1.3-cm-high walls, and was gray. A 1.0-cm step down led to alley 3 that was 3.5 cm wide, had 0.8-cm-high walls, and was white. A 0.4-cm step down led to alley 4 that was also white, 1.2 cm wide with 0.2-cm-high walls. The apparatus was elevated by anchoring the back of alley 1 to a stand, 50 cm high. Padding was provided under arms 3 and 4 in case a mouse fell off. The latency to first enter each alley (all four feet), the time spent there, and the total number of alleys entered (going away from or toward alley 1) were recorded during a total test time of 300 s. The successive alleys test has the advantage over the EPM of bracketing a wider range of anxiety.


A
Brain pERK
pERK/ERK (%)
175
150
125
100
75
50
25
0
*
*
WT KO WT KO
Vehicle NNZ-2566


C
Flow cytometry pERK
MFI pERK
4500
4000
3500
3000
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2000
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1000
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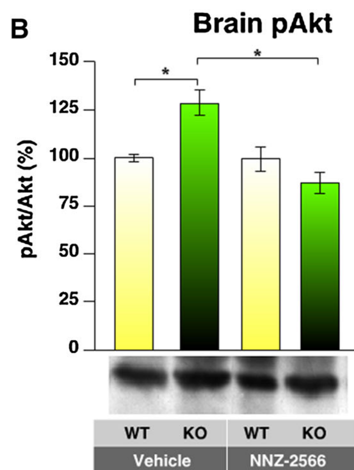
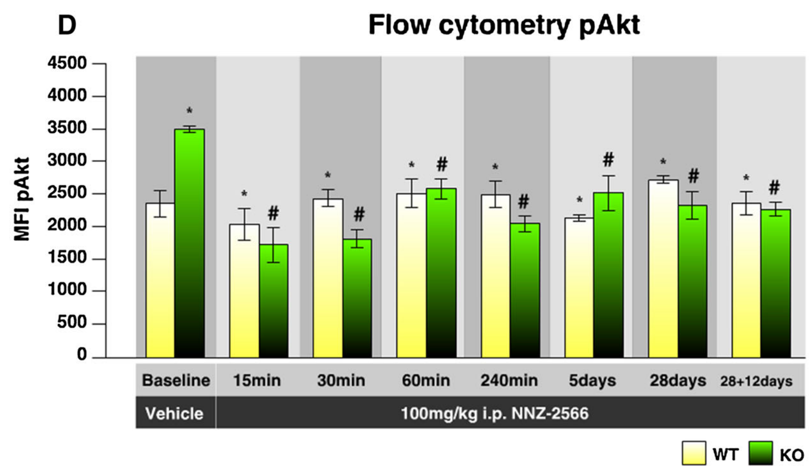
Vehicle 100mg/kg i.p. NNZ-2566
WT KO


B
Brain pAkt
pAkt/Akt (%)
150
125
100
75
50
25
0
*
*
WT KO WT KO
Vehicle NNZ-2566


D
Flow cytometry pAkt
MFI pAkt
4500
4000
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3000
2500
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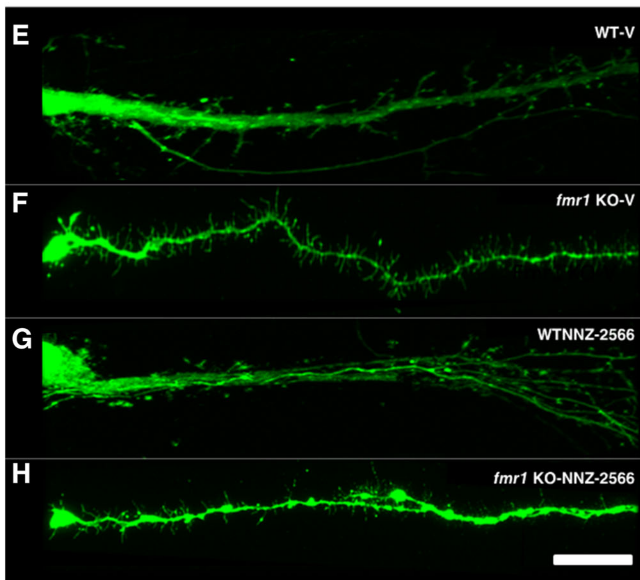
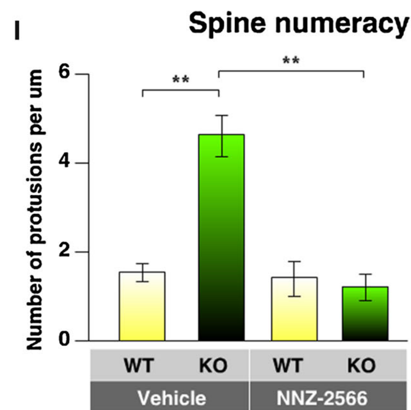
1500
1000
500
0
Baseline 15min 30min 60min 240min 5days 28days 28+12days
Vehicle 100mg/kg i.p. NNZ-2566
WT KO


E
WT-V
F
fmr1 KO-V
G
WTNNZ-2566
H
fmr1 KO-NNZ-2566
20um


I
Spine numeracy
Number of protusions per um
6
4
2
0
**
**
WT KO WT KO
Vehicle NNZ-2566

◀**Fig. 1** NNZ-2566 corrects abnormal intracellular signaling and corrects dendritic spine phenotype. **a, b** Representative Western blots of phospho-ERK (**a**) and phospho-Akt (**b**) from brain lysates from wild type and *fmr1* knockout mice treated for 28 days once daily with 100 mg/kg i.p. NNZ-2566 or with vehicle, and killed after 12 days of behavioral testing. Data are mean + SEM, $N = 5$ mice per time point. *$p < 0.05$. **c, d** The effect of intraperitoneal treatment with 100 mg/kg NNZ-2566 in wild type or *fmr1* knockout mice starting at age 14 weeks, on lymphocyte levels by flow cytometry of phospho-ERK (**c**) and phospho-Akt (**d**). Animals were killed 15 min after vehicle treatment ("baseline"), 15, 30, 60, and 240 min after a single dose of 100 mg/kg i.p. NNZ-2566, 15 min after 5 or 28 days treatment with 100 mg/kg i.p. once-daily NNZ-2566 or 12 days after 28-day treatment with 100 mg/kg i.p. once-daily NNZ-2566. MFI is mean fluorescence intensity. *$p < 0.05$ comparing baseline for wild-type and *fmr1* knockout mice. # $p < 0.05$ compared to *fmr1* knockout baseline value. **e, f, g, h** Photomicrographs of dendritic spine morphology in wild-type and *fmr1* knockout mouse hippocampal cells (obtained at E14–E16 and cultured to 14–21 DIV). **i** *Panel* **i** shows quantitation of spine numbers (data are mean ± SEM) measured as number of spines per micrometer. Spine numbers are increased in vehicle-treated *fmr1* knockout mice compared to vehicle-treated wild-type mice (**$p < 0.01$)

Contextual Fear Conditioning

Testing involved placing the animal in a novel environment (dark chamber), providing an aversive stimulus (a 1-s electric shock, 0.2 mA, to the paws), and then removing it. The conditioning chamber we used was from Kinder Scientific, USA.

Social Interaction

The apparatus was a test arena/cage within the same order of magnitude of size as the adult's home cage. Typically, this is a 40 × 23 × 12 cm cage, with a Perspex lid to facilitate viewing the mice. It should have fresh wood chipping on the floor. Preferably also give it a background mouse odor by putting in some non-experimental mice before starting to test. Mice were transferred to experimental room 10–15 min prior to test to wake them up. Simultaneously, both a test subject and a juvenile were placed in the test cage. The total duration and number of bouts of social investigation, defined as sniffing and close following (<2 cm from the tail) of the stimulus juvenile, were measured for 3 min.

Marble Burying

Transparent plastic cages were filled with a 10-cm deep layer of sawdust on top of which 10 glass marbles were placed in two rows. Each animal was left undisturbed in such a cage for 30 min, after which the number of marbles that were buried to at least two-thirds of their depth was recorded.

Nesting

This test was performed in the same individual cages as above. Normal bedding covered the floor to a depth of 0.5 cm. Each cage was supplied with a "Nestlet," a 5 cm^2 of pressed cotton batting (Ancare). Mice were placed individually into the nesting cages 1 h before the dark phase, and the results were assessed the next morning. The nests were assessed on a 5-point scale, and the amount of un-torn Nestlet was weighed.

Statistics

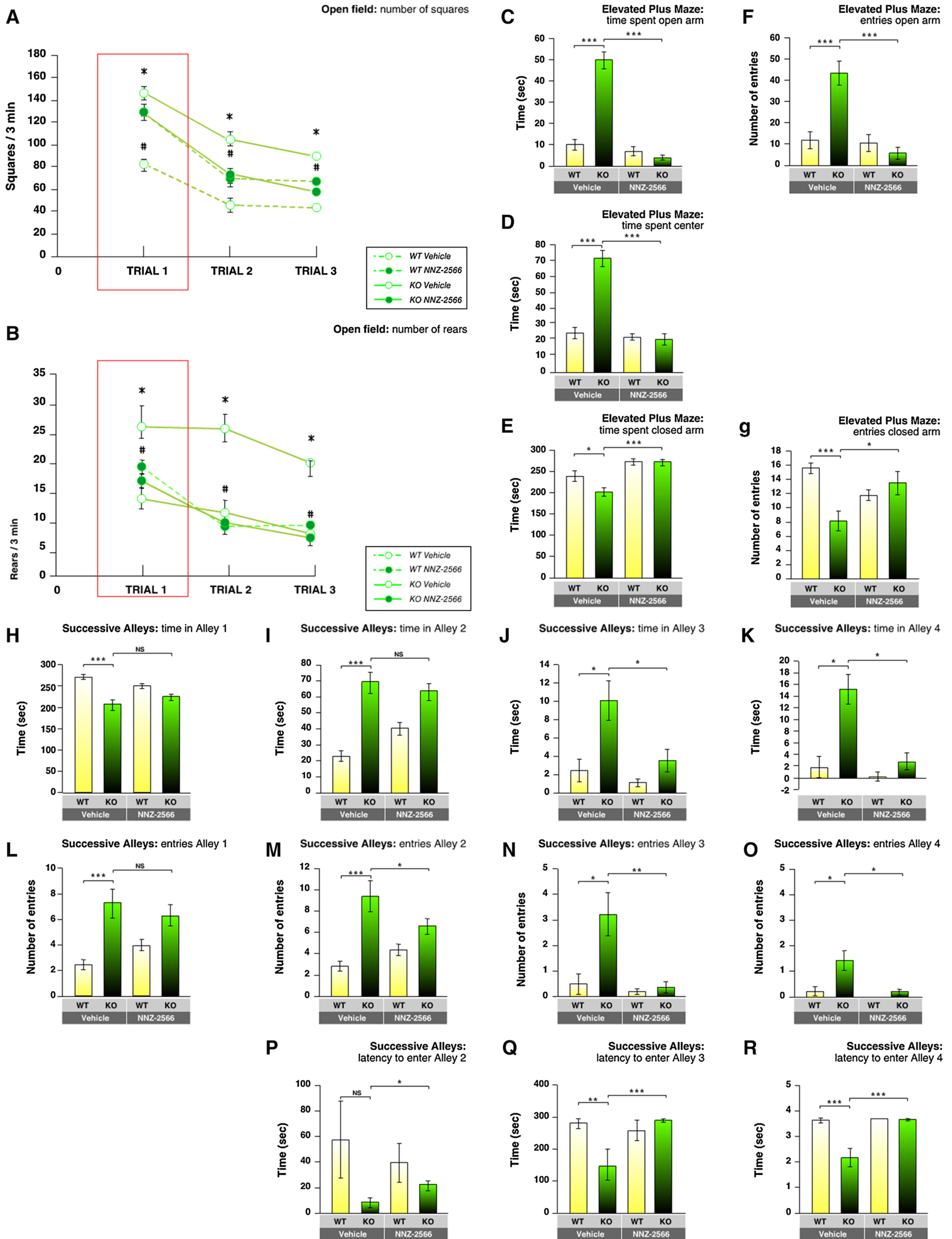
Parametric data were analyzed using two-way ANOVAs (genotype and sex as between-subject factors). Where data violated assumptions of normality or equality of variance, transformations (log 10 or square root) were utilized. For repeated-measures ANOVAs, homogeneity of variance was tested using Mauchly's test of sphericity, and where this was violated, Huyn–Feldt corrections were used. Nonparametric data were analyzed using Mann–Whitney *U* tests. A *p* value <0.05 was considered statistically significant throughout. Testis weight was analyzed with a three-way ANOVA with genotype, treatment, and age as independent factors, and the corresponding effect sizes are reported.

Results

Correction of Abnormal Intracellular Signaling

Levels of phospho-ERK and phospho-Akt in brains of WT and *fmr1* KO mice were assayed by quantitative Western blot (Fig. 1a, b, respectively) following 28 days of treatment and 12 days of the behavioral testing described below. *Fmr1* KO mice showed increases in both brain phospho-ERK and phospho-Akt compared to WT mice ($p < 0.001$ for both proteins). Administration of 100 mg/kg i.p. NNZ-2566 had no effect on the levels of brain phospho-ERK or phospho-Akt in WT mice. In *fmr1* KO mice, treatment with NNZ-2566 significantly reduced brain phospho-ERK and phospho-Akt.

Levels of phospho-ERK and phospho-Akt in lymphocytes of WT and *fmr1* KO mice were assayed using flow cytometry (Fig. 1c, d, respectively). *Fmr1* KO mice showed increases in both peripheral phospho-ERK and phospho-Akt compared to WT mice ($p < 0.001$ for both proteins). Administration of 100 mg/kg i.p. NNZ-2566 had no effect on the levels of peripheral phospho-ERK or phospho-Akt in WT mice. In *fmr1* KO mice, treatment with NNZ-2566 significantly reduced peripheral phospho-ERK and phospho-Akt at all time points tested ($p < 0.001$


A
Open field: number of squares
Squares / 3 min
TRIAL 1
TRIAL 2
TRIAL 3
WT Vehicle
WT NNZ-2566
KO Vehicle
KO NNZ-2566
B
Open field: number of rears
Rears / 3 min
C
Elevated Plus Maze: time spent open arm
D
Elevated Plus Maze: time spent center
E
Elevated Plus Maze: time spent closed arm
F
Elevated Plus Maze: entries open arm
g
Elevated Plus Maze: entries closed arm
Time (sec)
Number of entries
WT KO WT KO
Vehicle NNZ-2566
H
Successive Alleys: time in Alley 1
I
Successive Alleys: time in Alley 2
J
Successive Alleys: time in Alley 3
K
Successive Alleys: time in Alley 4
L
Successive Alleys: entries Alley 1
M
Successive Alleys: entries Alley 2
N
Successive Alleys: entries Alley 3
O
Successive Alleys: entries Alley 4
P
Successive Alleys: latency to enter Alley 2
Q
Successive Alleys: latency to enter Alley 3
R
Successive Alleys: latency to enter Alley 4
NS

◀**Fig. 2** Correction of anxiety/hyperactivity. **a, b** Initial exposure to *open field* (*Trial 1*), assessing either distance travelled (**a**) or rearing (**b**). The effect of 28 days of once-daily treatment with 100 mg/kg i.p. NNZ-2566 or vehicle on responses in novel environments in groups of $N = 10$ *fmr1* knockout or wild-type mice starting at age 14 weeks. Data are mean ± SEM. *$p < 0.01$ comparing vehicle-treated wild-type mice to vehicle-treated *fmr1* knockout mice. # $p < 0.05$ comparing NNZ-2566-treated *fmr1* knockout mice to vehicle-treated *fmr1* knockout mice. NNZ-2566-treated *fmr1* knockout mice did not differ statistically from NNZ-2566-treated wild-type mice. NNZ-2566 treatment increased the distance travelled in wild-type mice ($p < 0.05$). **c, d, e, f, g** *Elevated plus maze* assessing time spent in open arm (**c**), time spent in center (**d**), time spent in closed arm (**e**), open-arm entries (**f**), and closed-arm entries (**g**). Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. NNZ-2566 treated *fmr1* knockout mice did not differ significantly from NNZ-2566-treated wild-type mice. **h, i, j, k, l, m, n, o, p, q, r** *Successive alleys* assessing time spent in alleys 1, 2, 3, and 4 (**h, i, j,** and **k**, respectively), number of entries to alleys 1, 2, 3, and 4 (**l**, **m**, **n,** and **o**, respectively), and latency to enter alleys 2, 3, and 4 (**p**, **q**, and **r**, respectively). Data are mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

comparing NNZ-2566-treated to vehicle-treated values for *fmr1* KO mice at all time points) including the 15-min time point and when measurement was taken 12 days after cessation of treatment. These data show that NNZ-2566 produced a rapid onset of normalization of peripheral signaling abnormalities that persisted after treatment, mirroring effects seen in the brain.

Correction of Dendritic Spine Phenotype

Neurons from *fmr1* KO mice (Fig. 1f) show significantly increased spine density compared to WT mice (Fig. 1e)—an effect that is reversed by in vitro treatment with NNZ-2566 (50 nM) as seen comparing vehicle-treated *fmr1* KO mouse neurons (Fig. 1g) to NNZ-2566-treated *fmr1* KO mouse neurons (Fig. 1h). Treatment with the 50-nM dose produced a significant reduction in spine numbers ($p < 0.001$) as shown in Fig. 1i.

Correction of Anxiety/Hyperactivity

The open field is a novel arena that is sensitive to changes in anxiety (frightened animals are less active on initial exposure). During the initial exposure to the open-field test (Trial 1), *fmr1* KO mice traversed more squares ($p < 0.01$) (Fig. 2a) and reared more ($p < 0.01$) (Fig. 2b) than WT mice, thus showing hyperactivity in comparison with the WT vehicle control group. *Fmr1* KO mice treated with NNZ-2566 showed significant reductions in the total distance travelled when compared to the WT NNZ-2566-treated control group, indicating a statistically significant reduction in hyperactivity in the *fmr1* KO animals with NNZ-2566.

The elevated plus-maze test examines anxiety and locomotor activity. It is a + shape, with two-walled (closed) opposing arms (offering protection and therefore preferred by most mice) while the other two opposing arms have no walls (open). The test is based on the natural aversion of mice for open and elevated areas (Deacon et al. 2006). WT mice spent more time in the closed arms than in the open arms (compare Figs. 2c vs. 2e). The number of entries into each arm type showed a similar pattern (compare Figs. 2f vs. 2g). Vehicle-injected *fmr1* KO mice entered the open arms more frequently ($p < 0.001$) (Fig. 2f) and the closed arms less frequently ($p < 0.001$) (Fig. 2g) than their WT vehicle-injected littermates. They also spent more time in the center, deciding whether to enter an open arm.

In contrast, the *fmr1* KO mice injected with 100 mg/kg of NNZ-2566 showed a decreased number of open-arm entries compared to vehicle-treated *fmr1* KO mice ($p < 0.001$). NNZ-2566-treated *fmr1* KO mice made a significantly increased number of closed-arm entries ($p < 0.001$) compared to vehicle-treated *fmr1* KO mice, suggesting that differences in open-arm behavior do not simply reflect general changes in locomotor activity (Fig. 2d). *Fmr1* KO mice are hyperactive in the open arms but little different in the closed arms, the latter being a better index of general activity (Rodgers and Johnson 1995). This clearly suggests a low anxiety phenotype in the *fmr1* KO corrected by the administration of NNZ-2566.

The successive alleys test consists of four linear, successive, increasingly anxiogenic alleys (Deacon 2013). The *fmr1* KO mice injected with vehicle displayed a significantly shorter latency to enter the first open alley ($p = 0.05$) (Fig. 2p) and spent significantly more time in the open alleys 2–4 ($p < 0.05$) than WTs (Fig. 2h–k), indicating less anxiety than their WT vehicle-treated littermate controls. They also made more alley entries (Fig. 2l–o) ($p < 0.001$). *Fmr1* KO mice treated with NNZ-2566 showed a significant reduction in entering alleys 3 and 4 ($p < 0.05$) (Fig. 2m, n). These results essentially mirror the plus-maze results: hyperactivity and lower anxiety, corrected by the administration of NNZ-2566.

Correction of Learning and Memory Deficits

The open-field test was also used to assess potential effects on short- (Fig. 3a) and long-term memories (Fig. 3b). Following an initial exposure to the novel open field on Trial 1, subsequent exposures to the now familiar conditions after 10-min (Trial 2) and 24-h (Trial 3) detected habituation effects would depend on short- (Fig. 3a) and long (Fig. 3b)-term memories, respectively.

In the open-field Trial 2, we observed that vehicle-treated *fmr1* KO mice exhibited elevated locomotor activity when compared to WT vehicle-treated mice, suggesting a mnemonic failure ($p < 0.001$). The increased

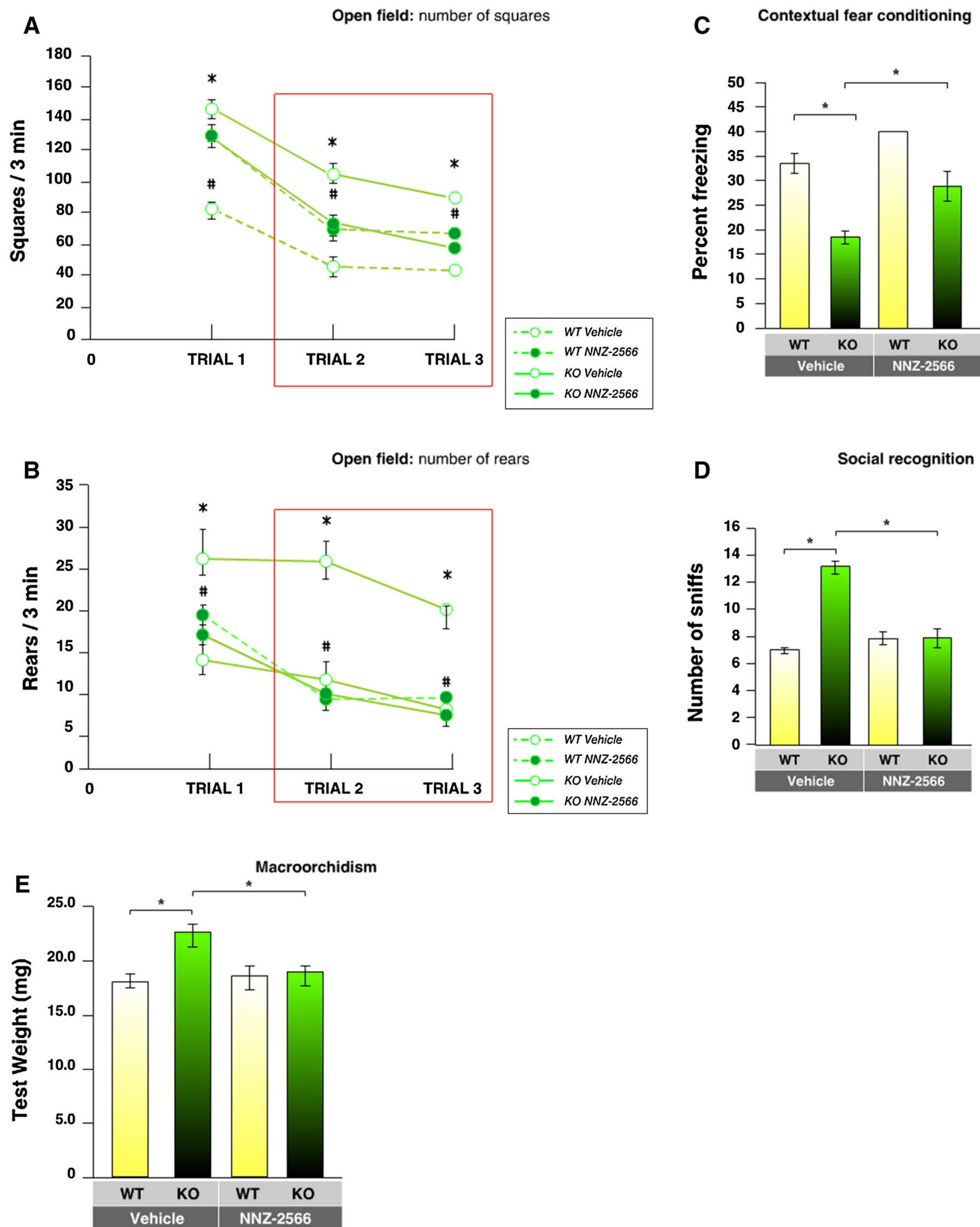


Fig. 3 **a, b, c** Effect of NNZ-2566 on learning and memory. Short- (**a**) and long (**b**)-term memories as assed by habituation to open field during 10 min (Trial 2) or 24 h (Trial 3) after initial exposure. Data are mean ± SEM. *$p < 0.01$ comparing vehicle-treated wild-type mice to vehicle-treated *fmr1* knockout mice. $^{\#}$ $p < 0.05$ comparing NNZ-2566-treated *fmr1* knockout mice to vehicle-treated *fmr1* knockout mice. Contextual fear conditioning (**c**) assessed by percent freezing in an environment previously associated with a 1-s electric shock at 0.2 mA to the paw. Data are mean ± SEM. *$p < 0.05$. NNZ-2566-treated *fmr1* knockout mice did not differ significantly from NNZ-2566-treated wild-type mice. **d** The effect of NNZ-2566 on social recognition as assessed by the number of sniffs of an introduced conspecific. Data are mean ± SEM. *$p < 0.05$. NNZ-2566-treated *fmr1* knockout mice did not differ significantly from NNZ-2566-treated wild-type mice. **e** The effect of NNZ-2566 on macroorchidism. The effect of 28 days of once-daily treatment of 100 mg/kg i.p. NNZ-2566 on macroorchidism in groups of $N = 10$ *fmr1* knockout and wild-type mice starting at age 14 weeks, assessing testis weight. Data are mean ± SEM. *$p < 0.001$. NNZ-2566-treated *fmr1* knockout mice did not differ significantly from NNZ-2566-treated wild-type mice. Vehicle- and NNZ-2566-treated wild-type mice did not differ statistically

locomotor activity was corrected in the *fmr1* KO mice when they were injected for 4 weeks with NNZ-2566, suggesting an improvement of short-term memory (Fig. 3a) ($p < 0.01$).

In the open-field Trial 3, locomotor activity was lower in the WT vehicle-treated mice (Fig. 3b) demonstrating habituation to the open field, reflecting long-term memory function. However, the *fmr1* KO vehicle-injected mice showed approximately similar locomotor activity as in Trial 1, indicating an absence of habituation to the open field, i.e., a deficit in long-term learning and memory. When the test was performed in *fmr1* KO after treatment with NNZ-2566, mice showed significantly reduced locomotor activity, indicating a significant improvement in learning and long-term memory ($p < 0.001$). *Fmr1* KO NNZ-2566-treated mice did not display a significant difference in the number of rears when compared with the WT mice treated with NNZ-2566 control group; both showed habituation. The open-field 24-h habituation test showed a significant improvement in learning and long-term memory in the *fmr1* KO mice after 4 weeks of treatment with NNZ-2566.

The dependent measure used in contextual fear conditioning was a freezing response following a pairing of an unconditioned stimulus (foot shock), with a conditioned stimulus, a particular context. 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. Vehicle-treated *fmr1* KO mice showed a significantly lower percentage of freezing behavior on exposure to conditioned context compared to WT mice ($p < 0.001$) (Fig. 3c). Under acute stress conditions, *fmr1* KO mice treated with NNZ-2566 overcame their learning deficit, exhibiting a similar percentage of freezing to the NNZ-2566 and vehicle-treated WT mice. Therefore, NNZ-2566 treatment normalizes the deficit seen in contextual fear conditioning in *fmr1* KO mice, reflecting a reversal of a cognitive deficit characteristic of *fmr1* KO mice.

Normalization in Social Interaction

Mice are a social species, which engage in easily scored social behaviors. Social interaction in mice was evaluated by recording the amount of time spent sniffing a novel mouse. Vehicle-treated *fmr1* KO mice displayed heightened sniffing of a presented mouse compared to vehicle-treated WT controls ($p < 0.05$) (Fig. 3d), suggesting a dysfunction in social behavior. This effect was reversed by 28-day treatment with NNZ-2566. Sniffing behavior of NNZ-2566-treated *fmr1* KO animals differed significantly from vehicle-treated *fmr1* KO mice ($p < 0.05$) and was not significantly different to WT mice. Our data show that NNZ-2566 normalized the abnormalities in social recognition seen in *fmr1* KO mice.

Correction of Species-Typical Behaviors

Nesting

For small rodents, nests are important in heat conservation as well as reproduction and shelter. For the *fmr1* KO vehicle-injected animals, the nesting median score was around 2–3, significantly lower performance than the WT controls vehicle-treated mice, which was around 4–5, as shown in Table 1. However, for the *fmr1* KO mice injected with NNZ-2566, nest scores were in the same range (they scored 4–5) as the WT controls NNZ-2566-treated mice (score 4–5). This test shows a hippocampal-dependent improvement in the *fmr1* KO NNZ-2566-treated group.

Marble Burying

Mice spontaneously dig in many substrates in the laboratory. Digging behavior is easily quantified by placing marbles on the surface of the cage bedding; as the mice dig in the bedding, the marbles, being heavier, become buried. Table 1 shows that *fmr1* KO mice engaged in less marble burying than WT mice. After 28 days of treatment with NNZ-2566, *fmr1* KO mice did not differ from WT mice in digging represented by the total number of "hidden" marbles. We conclude that NNZ-2566 treatment normalized marble burying behavior in *fmr1* KO animals.

Correction of Macroorchidism

Testis weight was measured in vehicle- and NNZ-2566-treated WT and *fmr1* KO mice (Fig. 3e). Macroorchidism was evident in vehicle-treated *fmr1* KO mice compared to WT mice, as reflected by a statistically significant 12–28 %

Table 1 Effect of NNZ-2566 on species-typical behaviors

Group	Wild-type vehicle	*Fmr1* knockout vehicle	Wild-type NNZ-2566	*Fmr1* knockout NNZ-2566
Marbles buried out of 10 (median)	9	4	10	8
Nest building score (median)	4.5	2.5	4.5	4.5

increased testis weight ($p < 0.001$). NNZ-2566 had no effect on testis weight in WT mice, but significantly reduced the macroorchidism seen in *fmr1* KO mice, which differed significantly from vehicle-treated *fmr1* KO mice ($p < 0.001$).

Discussion

This study investigates the impact of NNZ-2566, a synthetic analog of a naturally occurring neurotrophic peptide derived from insulin-like growth factor-1 (IGF-1), in the *fmr1* KO mouse at a preclinical level. The profile of effect of NNZ-2566 is consistent with a rescue of the core phenotype of FXS—administration to the *fmr1* KO transgenic mouse model normalizes brain IGF-1 (potentially a key molecular pathology), abrogates abnormalities in Akt/ERK signalling implicated in neuronal plasticity, the dendritic spine morphology that accompanies abnormal plasticity, and rescues cognition, social behavior, hyperactivity, and species-typical activities. This broad profile of effect has some persistence of effect in that the data show a maintained effect persisting for 14 days beyond 28-day treatment, although duration of effect in patients with FXS remains to be investigated.

The dose selected for the treatment of *fmr1* KO mice in this study (100 mg/kg) was expected to result in plasma levels in mice that would approximate the Cmax and daily exposure (AUC_{0-24}) equivalent to the top human dose (70 mg/kg b.i.d.) in planned clinical trials in human subjects and that have been efficacious in other animal models. NNZ-2566 readily accesses the brain from plasma, as shown by Bickerdike et al. (2009). Treatment with NNZ-2566 at this dose ameliorated the functional deficits observed in this mouse model. Indeed, in some instances, functional abnormalities were completely normalized. At the same time, NNZ-2566 was essentially devoid of effect in WT mice.

The broad profile of effect of NNZ-2566 in rescuing all aspects of the behavioral phenotype of the *fmr1* KO mouse and the ready access of NNZ-2566 to the brain from plasma are consistent with an effect across many brain regions. The data of Corvin and collaborators suggest that glial cells are more directly responsive to (1–3) IGF and presumably NNZ-2566 (Corvin et al. 2012). It is known that glia and astrocytes are key regulators of neuronal plasticity, and that loss of the FMRP protein in these accessory cells is necessary and sufficient to induce the FXS neuronal phenotype (Jacobs and Doering 2010). Therefore, it is proposed that the specific effect of (1–3) IGF-1, and therefore NNZ-2566, on accessory cells is sufficient to bring about a homeostatic normalization of glial phenotype that subsequently relates to a normalization of neuronal plasticity. The normalization of brain Akt/ERK function seen in the *fmr1* KO mouse following administration of NNZ-2566 is a reflection of the normalization of neuronal plasticity seen following microglial stabilization of synapses.

The present data confirm that *fmr1* KO mice show hyperactivity, cognitive deficits, altered social behavior, and differences in species-typical behaviors. These functional differences occurred in parallel to increased activity of the Ras–MAPK and PI3K–Akt–mToR pathways in the central nervous system, as is seen in patients with FXS (Hoeffer et al. 2012). The effect of NNZ-2566 in *fmr1* KO mice was accompanied by a normalization of activation of the Ras–MAPK and PI3K–Akt–mToR activation seen in the central nervous system. This raises the possibility that the functional deficits seen in the *fmr1* KO mice are at least partially caused by underlying signaling deficits. In support of this suggestion, the effect of NNZ-2566 on behavioral function is preceded by the normalization of abnormalities of brain signaling pathways. Indeed, the effect of NNZ-2566 on hyper-phosphorylation of ERK and Akt is very rapid, occurring within 15 min of administration. The reversal of brain signaling pathway abnormalities was reflected in a correlated reversal of peripheral activation of Ras–MAPK and PI3K–Akt–mToR signaling seen in *fmr1* KO mice. NNZ-2566 was without effect in the periphery of WT mice.

Our experiments have shown that NNZ-2566 acts on dendritic spine numeracy and in this way plays an important role in brain development and plasticity, strongly promoting synaptic maturation in the hippocampus. Marble burying and nesting are sensitive and robust tests of species-typical behavior have been shown to be the characteristic of hippocampal dysfunction (Deacon and Rawlins 2005; Deacon 2006a, b). The strong impairment on marble digging and nesting observed in the *fmr1* KO mice complements the impairments in spatial learning and memory, which are well-established effects of hippocampal lesions. Interestingly, the *fmr1* KO mice treated with NNZ-2566 did not differ from WTs on these tests, indicating a significant improvement in hippocampal-related behaviors.

Our cognitive tests demonstrate a powerful impact of NNZ-2566 administration on memory and learning behavior. The *fmr1* KO mice, after 4 weeks of treatment with NNZ-2566, showed a significant improvement in short- and long-term memories as suggested by the open-field 10-min and 24-h habituation tests. The *fmr1* KO mice treated with NNZ-2566, under acute stress conditions, overcame their learning deficit, exhibiting a similar percentage of freezing to the vehicle-treated WT mice, in the contextual fear-conditioning test. Altogether, our data reflect a reversal of the cognitive deficit characteristic of the *fmr1* KO mice.

Understanding how the brain processes social information and regulates social behavior helps us understand psychiatric disorders specifically affecting social behavior. Social recognition reflects the ability of mice to identify and remember conspecifics. Social recognition is assessed as a decrease in spontaneous investigation behaviors observed in a mouse re-exposed to a familiar conspecific. In our studies, the *fmr1* KO mouse fails to habituate to, or recognize, a stimulus mouse. This deficit in social memory is not due to problems with general olfactory processing. Social recognition in the *fmr1* KO mouse was fully restored by NNZ-2566.

NNZ-2566 treatment was shown to normalize the hyperactivity observed in the *fmr1* KO mice as observed in the EPM and successive alleys tasks. In the plus maze, however, the lower time/entries into the closed arms, regarded as the best measure of general activity, were not markedly different to WT mice, apparently contradicting the open-field data. This may have been due to the competing large increase in open-arm activity.

Overall, our behavioral findings provide direct evidence that NNZ-2566 has a robust effect on hippocampal species-typical behavior, learning and memory, sociability, and hyperactivity. In addition, our data show that NNZ-2566 treatment of WT mice results in no apparent side effects. Altogether, these findings offer impetus to potential therapies of both hyperactivity and cognitive symptoms in FXS patients.

Taken together, our data provide evidence for the potential of NNZ-2566 to correct a comprehensive range of behavioral, cellular, and neuroanatomical phenotypes in *fmr1* KO mice closely related to FXS patients' symptoms. These data have provided the rationale for the implementation of a Phase II clinical study of NNZ-2566 in FXS patients. Furthermore, this new therapeutic agent implicates the IGF-1 regulatory pathway in the pathogenesis of FXS, thus introducing a novel pharmacological approach, different to those currently based on the mGluR theory of FXS.

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