Contribution of mGluR5 to pathophysiology in a mouse model of human chromosome 16p11.2 microdeletion

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Human chromosome 16p11.2 microdeletion is the most common gene copy number variation in autism, but the synaptic pathophysiology caused by this mutation is largely unknown. Using a mouse with the same genetic deficiency, we found that metabotropic glutamate receptor 5 (mGluR5)-dependent synaptic plasticity and protein synthesis was altered in the hippocampus and that hippocampus-dependent memory was impaired. Notably, chronic treatment with a negative allosteric modulator of mGluR5 reversed the cognitive deficit.

Autism spectrum disorder (ASD) is characterized by behavioral, cognitive and language impairment. Over the past decade, studies on monogenetic syndromes with high prevalence of ASD, such as fragile X (FX) and tuberous sclerosis (TS), have provided insights into the pathophysiology of diseases that can cause autism1. For example, it has been shown that altered signaling downstream of metabotropic glutamate receptor 5 (mGluR5) has a pivotal role in the pathogenesis of FX, and that genetic and pharmacological modulation of mGluR5 can ameliorate numerous impairments in FX animal models2.

Chromosomal copy number variations (CNVs) have been associated with 5–10% of patients with ASD3–4. Variation at human chromosome 16p11.2 is the most common of these and accounts for approximately 0.5–1% of all ASD cases4. The affected region harbors ~27 annotated protein-coding genes, many of which are expressed in the brain5,6. The common clinical presentations in individuals carrying chr16p11.2 microdeletion are language impairment, intellectual disability (ID), ASD, anxiety, attention deficit hyperactive disorder (ADHD) and epilepsy7. Recent studies on animal models of human chr16p11.2 microdeletion have revealed morphological, behavioral and electrophysiological deficits8–10, but the synaptic pathophysiology remains largely unexplored.

Using electrophysiology, biochemistry and behavioral tests, we characterized hippocampal function of a mouse model for human chr16p11.2 microdeletion8. We uncovered alterations in mGluR5-mediated synaptic plasticity, Arc expression, basal protein synthesis and hippocampus-dependent learning that are reminiscent of previous observations in mouse models of syndromic autism and ID1. Notably, we were able to ameliorate the behavioral abnormalities using an mGluR5-negative allosteric modulator (NAM), 2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethyl)pyridine (CTEP), suggesting a pathophysiology that is shared with FX.

Mutant mice (termed 16p11.2 df/+), engineered to be heterozygous null at the region of chromosome 7qF3 that is syntenic to human chromosome 16p11.2 (ref. 8), were backcrossed for 5–10 generations to C57BL/6N mice (Charles River) to allow a comparison of synaptic physiology with previous studies1,2. As noted previously3, loss of the genes in this region can compromise survival, and we found that this effect was amplified in successive generations on the C57BL/6N background (Supplementary Fig. 1).

We first characterized basal synaptic transmission at the Schaffer collateral–CA1 synapse using hippocampal slices from 4–5-week-old mice and found no difference from wild type (WT) in input-output or paired-pulse facilitation (PPF) (Supplementary Fig. 2). To investigate NMDA receptor–dependent synaptic plasticity, we induced long-term potentiation (LTP) with theta-burst stimulation (TBS), and long-term depression (LTD) with low-frequency (1 Hz) stimulation (LFS). Again, there was no difference from WT (Fig. 1a,b), suggesting that basic excitatory synaptic transmission and plasticity mechanisms are intact in the mutant mice.

We next assayed mGluR5-mediated LTD (mGluR-LTD). mGluR-LTD was induced by either brief application of the mGluR1/5 agonist 3,5-dihydroxyphenylglycine (DHPG) or applying a series of paired pulses at 50-ms interval (PP-LFS)1. We again found no difference between WT and the 16p11.2 df/+ mutant with either induction protocol (Fig. 1c–f).

A distinctive property of mGluR-LTD in WT animals is a requirement for mRNA translation at the time of induction11. In slices from Fmr1–/– mice, however, mGluR-LTD is unaffected by translation inhibitors12, as basal synaptic synthesis of LTD-regulatory proteins such as Arc13–15 is elevated downstream of constitutive mGluR5 activity as a result of a loss of the translational repressor FMRP16. We were therefore compelled to investigate the protein synthesis dependence of mGluR LTD in the 16p11.2 df/+ mice and discovered a marked difference from WT. Similar to FX model mice, mGluR-LTD in the 16p11.2 df/+ mice was unaffected by cycloheximide (CHX) (Fig. 1d,f).

As reported previously in WT animals, DHPG induces LTD via two mechanisms: a postsynaptic reduction in AMPA receptors and a presynaptic reduction in glutamate release probability. Only the postsynaptic mechanism is CHX sensitive1. To test whether the different sensitivity of LTD to CHX in the mutant is a result of a qualitatively

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different expression mechanism, we analyzed PPF at the beginning and end of each DHPG-LTD experiment. No difference was observed between the WT and mutant slices (Supplementary Fig. 3). These findings point to a deficiency in postsynaptic regulation of protein synthesis in the 16p11.2 df/+ mice.

We next tested the mutant mice in two hippocampus-dependent behavioral assays, contextual fear conditioning (CFC) and inhibitory avoidance (IA), which have been shown in previous studies to reveal cognitive impairments in Fmr1<sup>+/y</sup> mice<sup>1,2</sup>. CFC requires intact mGluR5 signaling and new protein synthesis at the time of conditioning<sup>12</sup>. In this assay, mice were exposed to a distinctive environmental context in which a foot shock is delivered; 24 h later, the mice were returned to either the same (familiar) or a different (novel) context (Fig. 2a). WT mice expressed fear memory by freezing in the familiar context and demonstrated an ability to discriminate different contexts by freezing less in the novel context. In contrast, the mutant mice showed significantly less freezing in the familiar context (P = 0.0018) and were unable to distinguish between the familiar and novel context (Fig. 2b). Mutant and WT mice exhibited comparable sensitivity to foot shock (Fig. 2c), suggesting that the difference in freezing at 24 h was a result of an impairment in memory formation in the mutant mice.

![Figure 1](https://example.com/figure1.png)

**Figure 1** mGluR-LTD is protein synthesis independent in 16p11.2 df/+ mice. (a) TBS-LTP was unchanged in mutant (n = 9 animals, 16 slices) compared with WT (n = 7 animals, 15 slices) mice. (b) LFS-LTD was unchanged in mutant (n = 6 animals, 10 slices) compared with WT (n = 6 animals, 10 slices) mice. (c,d) The magnitude of DHPG-LTD was comparable in hippocampal slices from the WT (n = 17 animals, 18 slices) and mutant (Mut, n = 16 animals, 20 slices) mice in the absence of CHX. However, CHX blocked DHPG-LTD in WT slices (n = 17 animals, 21 slices), but not mutant slices (n = 16 animals, 20 slices) (two-way ANOVA, genotype × CHX, P = 0.0074). (e,f) The magnitude of PP-LFS-LTD was comparable in hippocampal slices from WT (n = 12 animals, 14 slices) and mutant (n = 8 animals, 10 slices) mice in the absence of CHX. CHX significantly attenuated PP-LFS-LTD in the WT slices (n = 12 animals, 14 slices), but not mutant slices (n = 8 animals, 9 slices) (two-way ANOVA, genotype × CHX, P = 0.013). Representative field excitatory postsynaptic potential (fEPSP) traces (average of ten sweeps) were taken at the times indicated by numerals. All data are plotted as mean ± s.e.m.

![Figure 2](https://example.com/figure2.png)

**Figure 2** 16p11.2 df/+ mice exhibit deficits in hippocampal-associated CFC and IA. (a) CFC experimental design. Mutant mice showed significantly less freezing in the familiar context compared with WT (unpaired t test, P = 0.0018). Although WT mice were able to distinguish a novel from familiar context (unpaired t test, P < 0.0001), the mutant mice were impaired (unpaired t test, P = 0.2840). Two-way ANOVA, genotype × context, P = 0.0166. (b) Mutant and WT mice had the same running response to foot shock during the training session (unpaired t test, P = 0.6234). (c) Mutant mice were impaired in IA acquisition (WT versus Mut, 0 h versus 6 h, repeated measures two-way ANOVA, P = 0.0108; WT versus Mut at 6 h, post hoc unpaired t test, P = 0.0033). Unlike WT (6 h versus 48 h, post hoc paired t test, P = 0.0101), mutant mice showed no extinction of fear memory (WT versus Mut, 6 h versus 48 h, repeated measures two-way ANOVA, P = 0.0197; Mut, 6 h versus 48 h, post hoc paired t test, P = 0.6278). (e) CTEP treatment ameliorated behavioral deficits in mutant mice in IA. In mutant mice, CTEP treatment enhanced acquisition (Mut + Veh versus Mut + CTEP, 0 h versus 6 h, repeated measures two-way ANOVA, P = 0.0016; Mut + Veh versus Mut + CTEP at 6 h, post hoc unpaired t test, P = 0.0013) and extinction of fear memory (Mut + Veh versus Mut + CTEP, 6 h versus 48 h, repeated measures two-way ANOVA, P = 0.0039; Mut + Veh, 6 h versus 48 h, post hoc paired t test, P = 0.4281; Mut + CTEP, 6 h versus 48 h, post hoc paired t test, P = 0.0140). There was no statistically significant difference between WT + Veh and Mut + CTEP at 6 h (unpaired t test, P = 0.3471). In WT mice, CTEP had no effect on either acquisition (WT + Veh versus WT + CTEP, 0 h versus 6 h, repeated measures two-way ANOVA, P = 0.6564) or extinction of fear memory (WT + Veh versus WT + CTEP, 6 h versus 48 h, repeated measures two-way ANOVA, P = 0.9882). All data are plotted as mean ± s.e.m. with individual values superimposed.
In the IA assay, mice received a foot shock following entry into the dark side of a two-chamber box (Fig. 2d). Memory strength and extinction were measured as the latency to enter the dark side when given the opportunity at 6-, 24- and 48-h intervals\textsuperscript{2,18}. The 16p11.2 df/+ mice showed impaired acquisition and extinction of IA memory. Similar IA deficits in Fmr1\textsuperscript{-/-} mice have been ameliorated by chronic post-adolescent treatment with the mGluR5 NAM CTEP\textsuperscript{2}. We therefore repeated the IA assay on WT and mutant mice that were treated every second day with CTEP (2 mg per kg of body weight, orally) or vehicle for 4 weeks\textsuperscript{2}. Although treatment had no effect in the WT mice, it corrected the deficits in the mutants both in terms of acquisition and extinction (Fig. 2e).

We were motivated to investigate the possibility that FX and 16p11.2 microdeletion have shared pathophysiology for several reasons, including the fact that four deleted genes are targets of 16p11.2 microdeletion disorder and this gives rise to cognitive impairment. In the Fmr1\textsuperscript{-/-} mouse, bulk protein synthesis in hippocampal slices is elevated and corrected by manipulations of mGluR5 signaling (MVP, CDIP7 and MAPK3) or protein turnover (KCTD13\textsuperscript{19}). The most straightforward prediction from our results is that synaptic protein downstream of mGluR5 is exaggerated by the 16p11.2 microdeletion and this gives rise to cognitive impairment. In the Fmr1\textsuperscript{-/-} mouse, bulk protein synthesis in hippocampal slices is elevated and corrected by manipulations of mGluR5 (refs. 16,18). However, we found that basal protein synthesis in hippocampal slices in the 16p11.2 df/+ mice was reduced, which might be explained by the decrease in ERK pathway activity (Fig. 3). Nevertheless, immunoblots for Arc protein showed a significant increase ($P = 0.0191$). The mGluR5-dependent synthesis of Arc protein normally gates LTD and synapse elimination\textsuperscript{3-15}. Thus, constitutive elevation of Arc could render mGluR-LTD insensitive to acute inhibition of protein synthesis and contribute to cognitive impairment.

These findings support two important conclusions. First, the data suggest that some cognitive and neuropsychiatric symptoms of 16p11.2 microdeletion disorder arise from altered synaptic signaling that is amenable to targeted drug therapy. Second, the data strengthen the hypothesis that multiple causes of ASD and ID converge on common pathophysiological processes, and one of these is the synaptic regulation of protein synthesis\textsuperscript{20}.

Figure 3 16p11.2 df/+ mice exhibit a decrease in basal protein synthesis which is accompanied by an increase in Arc protein levels. (a) Metabolic labeling of hippocampal slices revealed a significant reduction of basal protein synthesis in 16p11.2 df/+ compared to WT mice (unpaired t test, $P = 0.0210$). (b) MVP, ERK1 and pERK1 were decreased in 16p11.2 df/+ mice relative to WT mice (unpaired t test, $P = 0.0036$, $P = 0.0001$ and $P = 0.0013$ respectively), whereas ERK2 and pERK2 levels were comparable between 16p11.2 df/+ and WT mice (unpaired t test, $P = 0.9091$ and $P = 0.8568$ respectively). Arc protein levels were significantly increased in 16p11.2 df/+ mice as compared with WT mice (unpaired t test, $P = 0.0191$). All data are plotted as mean ± s.e.m. with individual values superimposed; $n$ indicates number of animals.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.F.B. and D.T. conceived and designed the study. M.F.B. and A.J.H. supervised the study. D.T. performed hippocampal electrophysiology and contextual fear conditioning. D.T. and A.J.H. performed inhibitory avoidance test. L.I.S. performed hippocampal protein synthesis and immunoblot experiments. D.T., A.J.H., L.I.S. and M.F.B. wrote the manuscript. L.L. and G.J. provided CTEP. A.M.A.M. provided the 16p11.2 df/+ mice before publication and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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**ONLINE METHODS**

**Animals.** A mouse line carrying a microdeletion of mouse chromosome 7qF3, the syntenic region of human chr16p11.2, was generously provided by A. Mills (Cold Spring Harbor Laboratory)\(^1\). In the present study, F3 heterozygous mutant male mice on a mixed 129/C57BL/6 background were backcrossed 5–10 generations to C57BL/6 mice from Charles River Laboratory. Mice were group-housed on a 12-h on/12-h off light/dark cycle. All experiments were conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee at the Massachusetts Institute of Technology. During backcrossing, we observed a steady decline in the percentage of heterozygous mutant allele. All experiments were performed by an experimenter blind to genotype.

**Reagents.** S-3,5-dihydroxyphenylglycine (S-DHPG) was purchased from Sigma-Aldrich. Aliquots of DHPG were prepared in H\(_2\)O as 1,000× stock (50 mM) and used within 7 d of preparation. Cycloheximide (CHX) was purchased from Sigma-Aldrich, prepared fresh in H\(_2\)O as 1,000× stock (60 mM) and used on the same day of preparation. CTEP, [2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine], was synthesized at Hoffmann-La Roche, formulated as a micro-suspension prepared in 0.9% saline/0.3% Tween-80, and administered by oral gavage at a dose of 2 mg per kg of body weight.

**Hippocampal electrophysiology.** Electrophysiological experiments were performed at the Schaffer collateral–CA1 synapse of dorsal hippocampal slices (400 µm thick) prepared from P28–35 male mice using experimental protocols previously described\(^1\). Input-output functions were determined by incrementally (10–100 µA) stimulating the Schaffer collaterals and recording the resulting fEPSP response. Paired-pulse facilitation was conducted by applying two stimulus pulses (stimulus 1 and 2) at varying inter-stimulus-intervals (ISIs). Facilitation was measured by taking the ratio of the fEPSP slope in response to stimulus 2 to that of stimulus 1. LTP was induced using TBS delivered in two trains with a 30 s interval. Each train was composed of 50-ms bursts of stimuli (100 Hz) delivered at 5 Hz for 1 s. NMDA receptor–dependent LTD was elicited with LFS composed of 900 pulses at 1 Hz. For DHPG-LTD, slices were incubated in artificial cerebrospinal fluid (ACSF) in the presence or absence of the protein synthesis inhibitor cycloheximide (±CHX, 60 µM, 40 min), and mGlur5 was activated by bath application of DHPG (50 µM, 5 min). Synaptic responses were followed for an additional 60 min following DHPG application. Paired pulse facilitation was assessed 30 min before, and 60 min following, DHPG application in all slices used in DHPG-LTD experiments (Fig. 1c.d). For PP-LFS, slices were incubated in ACSF containing APS (50 µM) ± CHX for 30 min. mGlur5-LTD was then induced by application of 1,200 paired-pulse stimulation (50-ms ISI) at 1 Hz, and synaptic responses were recorded for an additional 60 min in the presence of APS. Statistical significance was determined using repeated measures two-way ANOVA and post hoc Bonferroni tests.

**Metabolic labeling.** Metabolic labeling of new protein synthesis was performed as previously described\(^1\). Male P28–32 littermate (WT) and 16p11.2 (d/+ mice were anesthetized with isoflurane and the hippocampus was rapidly dissected into ice-cold ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 10 dextrose, 1 MgCl\(_2\), 2 CaCl\(_2\), saturated with 95% O\(_2\) and 5% CO\(_2\)). Hippocampal slices (500 µm) were prepared using a Stooling Tissue Slicer and transferred into 32.5°C ACSF (saturated with 95% O\(_2\) and 5% CO\(_2\)) within 5 min. Slices were incubated in ACSF undisturbed for 3.5–4 h to allow for recovery of basal protein synthesis. Actinomycin D (25 µM) was then added to the recovery chamber for 30 min to inhibit transcription, after which slices were transferred to fresh ACSF containing –10 mCi ml\(^{-1}\) [\(^{35}\)S] Met/Cys (PerkinElmer) for an additional 30 min. Slices were then homogenized, and labeled proteins isolated by TCA precipitation. Samples were read with a scintillation counter and subjected to a protein concentration assay (Bio-Rad). Data was analyzed as counts per minute per microgram of protein, normalized to the [\(^{35}\)S] Met/Cys ACSF used for incubation and the average incorporation of all samples analyzed and then normalized to percent WT for each experiment. Statistical significance was determined using unpaired t tests.

**Immunoblotting.** Immunoblotting was performed according to established methods using primary antibodies to Arc (1:700, Synaptic Systems #156002), p-ERK1/2 (1:2,000, Thr202/Tyr204 #9101, Cell Signaling Technology), ERK1/2 (1:1,000, Cell Signaling Technology #9102) or MVP (1:500, Abcam #ab97311). Arc, MVP, ERK1 and ERK2, as well as ERK1 and ERK2 phosphorylation were measured by densitometry (Quantity One), and quantified as the densitometric signal of each protein divided by the total protein signal (determined by Memcode staining) in the same lane. Statistical significance was determined using unpaired Student’s t tests.

**CFC.** CFC was performed in a Freezeframe Chamber (Coulbourn Instrument) as previously described\(^1\). Briefly, 6–12-week-old WT and mutant male mice were fear conditioned on day 1 and the subsequent percentage of time spent freezing in either the familiar or a novel context was determined 24 h later. On the day of conditioning, animals were allowed to explore the behavioral chamber for 3 min, followed by delivery of a 2-s 0.8-mA foot shock. Mice remained in the context for 15 s after the shock before returning to their home cages. Fear response was assessed 24 h later. To determine context specificity of the conditioned response, mice trained on day 1 were separated into two groups on day 2: one group was tested in the same training context (familiar context), and the other tested in a novel context. The novel context was created by varying spatial cues, floor material and lighting of the testing chamber. The percentage of time a mouse spent freezing during a testing session of 4 min on day 2 was the behavioral readout. To determine if WT and mutant mice had the same sensitivity to foot shock, the distance traveled by each subject during the 2-s foot shock and 1 s immediately following was recorded. Statistical significance was determined using repeated measures two-way ANOVA and post hoc Student’s t tests.

**IA test.** IA tests were performed with a passive avoidance apparatus (Ugo Basile Passive Avoidance Apparatus, Step-through model for mouse) as previously described with modification\(^1\). In all IA tests, a mouse was subjected to one training session (0 h) and three subsequent testing sessions (6, 24 and 48 h). In each session, a mouse was first placed in a LED-illuminated “START” compartment of a two-compartment test apparatus for 30 s before the partition door opening. Upon entering the dark compartment the door was closed immediately. The subject remained in the dark compartment for 60 s before being taken out. During the training session, a 2-s foot shock of 0.4 mA was delivered. No shock was delivered in the three testing sessions. The latency to enter the dark compartment was recorded to assess baseline level (0 h), acquisition of fear memory (6 h) and memory extinction (24 h and 48 h). Male mice of 6–10 weeks of age were used in IA tests without drug treatment. In IA tests with CTEP treatment, 4–6-week-old male mice were divided into four groups according to genotype (WT or mutant) and drug treatment (vehicle or CTEP). Administration of vehicle or CTEP (2 mg per kg) was initiated at the age of 4–6 weeks, given as one dose every 48 h by oral gavage, and continued for 4 weeks before IA testing by an experimenter blind to both genotype and drug condition. Statistical significance was determined using repeated measures two-way ANOVA and post hoc Student’s t tests.

The **Supplementary Methods Checklist** is available.