

R-Baclofen Reverses Cognitive Deficits and Improves Social Interactions in Two Lines of 16p11.2 Deletion Mice

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Human chromosome 16p11.2 microdeletion is among the most common gene copy number variations (CNVs) known to confer risk for intellectual disability (ID) and autism spectrum disorder (ASD) and affects an estimated 3 in 10 000 people. Caused by a single copy deletion of ~27 genes, 16p11.2 microdeletion syndrome is characterized by ID, impaired language, communication and socialization skills, and ASD. Studies in animal models where a single copy of the syntenic 16p11.2 region has been deleted have revealed morphological, behavioral, and electrophysiological abnormalities. Previous studies suggested the possibility of some overlap in the mechanisms of pathophysiology in 16p11.2 microdeletion syndrome and fragile X syndrome. Improvements in fragile X phenotypes have been observed following chronic treatment with *R*-baclofen, a selective agonist of GABA_B receptors. We were therefore motivated to investigate the effects of chronic oral *R*-baclofen administration in two independently generated mouse models of 16p11.2 microdeletion syndrome. In studies performed across two independent laboratories, we found that chronic activation of GABA_B receptors improved performance on a series of cognitive and social tasks known to be impaired in two different 16p11.2 deletion mouse models. Our findings suggest that *R*-baclofen may have clinical utility for some of the core symptoms of human 16p11.2 microdeletion syndrome.

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INTRODUCTION

Several conceptual frameworks have been introduced to explain how a growing list of diverse gene mutations and environmental factors manifest as similar neurodevelopmental disorders with intellectual disability (ID) and autism. Among these is the prominent theory that autistic features arise from alterations in the balance of excitation to inhibition in mature cortical microcircuitry (Gogolla *et al*, 2009; Rubenstein and Merzenich, 2003). Another is the concept, based on research in animal models of several genetically defined syndromic forms of ID and autism spectrum disorder (ASD), that synaptic function and plasticity are impaired by dysregulation of neuronal proteostasis (Bear *et al*, 2004; Kelleher and Bear, 2008; Louros and Osterweil, 2016). These frameworks are not mutually exclusive. Consequences of altered protein synthesis regulation could include derailed development of

excitation–inhibition (E–I) balance, and because neuronal protein synthesis is under tight regulation by glutamatergic synaptic transmission, an increased E–I ratio could manifest as aberrant protein synthesis. In either case, there is a strong rationale for examining treatment strategies based on boosting inhibition relative to excitation, at least for some causes of ID and ASD.

To date, this approach has been most fully explored in animals and humans with fragile X syndrome (FXS). FXS is caused by silencing of the *FMRI* gene that encodes the mRNA-binding protein FMRP. It has been shown that one important function of FMRP is to repress activity-dependent neuronal protein synthesis (Santoro *et al*, 2012). In animal models in which the gene has been knocked out, altered protein synthesis and a large number of disease phenotypes can be corrected either by reducing glutamate signaling via metabotropic glutamate receptor 5 (mGluR5) (Dolen *et al*, 2007; Michalon *et al*, 2012; Stoppel *et al*, 2017) or by boosting inhibition via GABA_B receptors (Henderson *et al*, 2012; Qin *et al*, 2015). The GABA_B agonist used for these preclinical studies was *R*-baclofen, the active enantiomer of baclofen. Racemic baclofen has been extensively used clinically for the treatment of spasticity in cerebral palsy and multiple sclerosis (Ertzgaard *et al*, 2017; Rizzo *et al*, 2004). The well-known safety profile of the racemic mixture made *R*-baclofen an attractive clinical candidate for early interventions in neurodevelopmental disorders (the clinical

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formulation of *R*-baclofen is also called arbaclofen or STX209). Several clinical trials have now been completed for both FXS and idiopathic autism (Berry-Kravis *et al*, 2017a,c; Berry-Kravis *et al*, 2012; Veenstra-VanderWeele *et al*, 2017). Although no significant improvements over placebo were observed using the predetermined primary end points, efficacy signals were observed on physician-rated global assessments in all four trials. Interestingly, the nominally significant change observed in the autism trial was driven by a strong response in a subset of participants (Veenstra-VanderWeele *et al*, 2017). These individuals may have responded to treatment because they represent a subset that shares some pathophysiological features with FXS.

In an effort to understand the degree to which different identified causes of autism and ID converge onto a common set of pathophysiological processes, over the past several years our laboratories have compared several different genetically defined mouse models (Auerbach *et al*, 2011; Barnes *et al*, 2015; Dhame *et al*, 2017; Portmann *et al*, 2014; Silverman *et al*, 2015; Silverman *et al*, 2012; Tao *et al*, 2016; Tian *et al*, 2015; Yang *et al*, 2015a,b). Among these were mouse models of human chromosome 16p11.2 microdeletion. Humans with 16p11.2 microdeletion syndrome are hemizygous in a region comprising ~27 protein-coding genes and show impairments in adaptive function that prominently include ID. Full-scale IQ is decreased by ~2 SD, such that 70% of deletion carriers have an IQ < 85 (Zufferey *et al*, 2012). Additional features of 16p11.2 microdeletion syndrome can include speech and language disruptions and ASD, along with an increased incidence of seizures, larger head size and body weight, developmental coordination and anxiety disorders (Bernier *et al*, 2017; Hanson *et al*, 2015; Hippolyte *et al*, 2016; Moreno-De-Luca *et al*, 2015). Several mouse models have been created that carry a heterozygous deletion of the same genes on mouse chromosome 7qF3, the syntenic region of human chr16p11.2. Studies in one of these revealed, surprisingly, a synaptic plasticity phenotype that closely resembles the *Fmr1*^{-/-} mouse (Tian *et al*, 2015). These findings prompted us to ask if behavioral phenotypes in these mice might similarly be improved by *R*-baclofen, in which case this treatment might be considered for further investigation in the well-characterized 16p11.2 subgroup of patients.

Three models of the human 16p11.2 deletion have been established and characterized (Arbogast *et al*, 2016; Horev *et al*, 2011; Portmann *et al*, 2014). These mice all carry highly similar (with subtle differences in regional overlap) heterozygous deletion of the syntenic region of human chromosome 16p11.2 (Arbogast *et al*, 2016; Horev *et al*, 2011; Portmann *et al*, 2014). Confidence in a drug response in an animal model is strongest when phenotypes and therapeutic efficacy replicate across laboratories. Corroborative findings from two separately generated lines of mice with the same mutation offer an even higher standard of reproducibility. Here we employ two independent lines of 16p11.2 deletion mice (termed 16p11.2 *df/+*), tested in two independent laboratories, to evaluate the therapeutic potential of *R*-baclofen on autism-relevant behavioral phenotypes.

MATERIALS AND METHODS

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Mice and treatment. A mouse line carrying a microdeletion of mouse chromosome 7qF3, the syntenic region of human chr16p11.2, was generously provided by Professor Alea Mills from the Cold Spring Harbor Laboratory (Horev *et al*, 2011). To supplement the colony, F3 heterozygous 16p11.2 heterozygous male mice on a mixed 129/C57BL/6 background were backcrossed >3 generations to C57BL/6J mice from Charles River Laboratory. All mice were group-housed on a 12 h on/12 h off light/dark cycle. Both male and female 16p11.2 *df/+* mice were crossed to male or female wild-type (WT) mice to generate *+/+* and *df/+* littermates. There was no discernable difference in survival between *+/+* and *df/+* littermates; however, progeny did not follow Mendelian ratios as previously described (Tian *et al*, 2015). All littermates were weaned at P21 and only male mice were used for behavioral testing. Most animals were tested on open field habituation, novel object recognition, and context discrimination, in that order. Because the context discrimination task required more animals, an additional cohort was exposed to that task. All experiments were conducted in accordance with the rules and regulations of the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at MIT. All experiments were performed by an experimenter blind to genotype and treatment.

To provide continuous exposure and obviate the need for daily injections, *R*-baclofen (generously supplied by the Simons Foundation Autism Research Initiative) was administered in the drinking water as described by Henderson *et al* (2012). As mice were group housed in mixed genotype cages, individual consumption volumes could not be determined. However, administration in the drinking water at 0.5 mg/ml has been shown to provide on average a brain exposure equivalent to 6 mg/kg administered by intraperitoneal injection twice a day, which is sufficient to engage GABA_B receptors and reduce excessive protein synthesis in the *Fmr1*^{-/-} mice. Vehicle (drinking water) and *R*-baclofen-treated water were given *ad libitum* and changed daily for the duration of the experiment. Animals were given vehicle or *R*-baclofen for at least 12 days prior to behavioral testing. Vehicle or *R*-baclofen administration continued in the drinking water throughout behavioral testing, which was conducted in the sequence described below.

Open field habituation task. Open field habituation task was performed in a 40 × 40 × 40 cm³ box during 2 × 15-min habituation sessions, spaced 1–2 h apart. Animals at 8–12 weeks of age were placed in the behavior box and allowed to explore freely during each habituation session. Sessions were recorded and locomotor activity was tracked using Plexon's CinePlex Studio and analyzed using Plexon's CinePlex Editor and code written in MATLAB. For analysis, the box was divided into two zones: an 'inner' zone (containing the inner 30 × 30 cm² center square) and an 'outer' zone (the outermost area 5 cm from the walls). Total distance traveled was computed as the sum of distance covered over the course of each habituation session.

Novel object recognition. The novel object recognition task was adapted from experiments previously described (Leger *et al*, 2013). Animals at 8–12 weeks of age were habituated to a 40 × 40 × 40 cm³ box during 2 × 15-min sessions, spaced 1–2 h apart. Animals were returned to their home cage in between session. Twenty-four hours after habituation, animals were exposed to two identical objects for 2 × 10 min exploration sessions in the same box, spaced 1–2 h apart. Animals were required to explore each object for at least 10 s (for a total of at least 20 s) in the first session to be included in the subsequent sessions. Twenty-four hours after object exploration, one object was replaced with a novel object and the animals were allowed to explore the objects for 10 min. Time spent exploring was recorded during this exploration period and was characterized by sniffing within 2 cm of each object or directly touching the objects. Time spent climbing or on top of the objects was not included. Familiar and novel object and side placement was randomly assigned, by animal. Discrimination index was calculated as ((time spent exploring novel object)/(time spent exploring novel object+time spent exploring familiar object)).

Contextual discrimination task. Context discrimination was performed in a Freezeframe Chamber (Coulbourn Instrument) as previously described (Auerbach *et al*, 2011; Ehninger *et al*, 2008). Briefly, 6–12-week-old wild-type and 16p11.2 heterozygous 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 being returned 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 (taping pink cage cards on the walls of the environment), floor material (grid rather than barred), and lighting (red light rather than white light) of the testing chamber. The percentage of time a mouse spent freezing during the 3 min test session on day 2 was the behavioral readout. Statistical significance was determined using repeated measures two-way ANOVA and *post hoc* Student's *t*-tests.

Statistical analysis. Object recognition data were analyzed using paired *t*-test, comparing the number of seconds spent sniffing each object within genotype and within treatment, during the familiarization and novel object recognition phases. Discrimination index was analyzed using unpaired *t*-tests and two-way ANOVA, genotype × treatment. Contextual discrimination task data were analyzed using unpaired *t*-tests (for pairwise comparisons), comparing the percentage of time spent freezing between mice placed in the familiar context *vs* the novel context by genotype. Additionally, data were analyzed using a three-way ANOVA for genotype, treatment, and context. Open field habituation data were analyzed using a paired *t*-test to compare the

percentage of time spent in the inner and outer zones within each group and an unpaired *t*-test to compare distance travelled or zone preference between groups. Additionally, within each treatment condition, data were analyzed using a three-way ANOVA for genotype, zone, and session. Within the vehicle-treated cohort, additional pairwise multiple comparison procedures were also performed (Holm–Sidak method).

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Mice and treatment. 16p11.2 deletion mice, generated and generously provided by Thomas Portmann and Ricardo Dolmetsch at Stanford University, were rederived at the University of California Davis in Sacramento. Wild-type (+/+) female mice were mated with heterozygous (+/–) males to produce +/+ and +/- littermates of both sexes for behavioral testing. The reciprocal cross was not employed because pup survival was low in litters born to female +/- dams. Genotypes were identified by visualizing mCherry expression with a DFP-1 dual fluorescent protein flashlight (<http://www.nightsea.com/products/dfp-1/> Nightsea, Bedford, MA), as previously described (Yang *et al*, 2015a, b). Wild-type pups were weaned at 21 days of age and heterozygous mice were weaned at up to 35 days to reduce nutrition-related fatalities. Mice were housed by sex in cages of 2–4 littermates, maintained in a temperature-controlled vivarium, lights on at 0700 hours and off at 0700 hours, with *ad libitum* access to food. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the University of California Davis Institutional Animal Care and Use Committee.

R-baclofen (generously supplied by the Simons Foundation Autism Research Initiative) was delivered in the home cage drinking water via Falcon 50 ml conical centrifuge tubes, beginning 1 week before the start of behavioral testing. Vehicle or R-baclofen administration continued in the drinking water throughout behavioral testing, which was conducted in the sequence described below. Drinking water contained either a 1% saccharin solution (control) or solution of 0.5 mg/ml R-baclofen in 1% saccharin (experimental). Saccharin was added to improve palatability of R-baclofen. Animal weight was measured and recorded daily to monitor possible dehydration using an Ohaus CL201 portable balance. Amount of drinking water consumed per cage was measured daily, using the gradation lines on the Falcon tubes. As mice were group housed in mixed genotype cages, individual consumption volumes could not be determined. Fresh vehicle and R-baclofen solutions were added daily. R-baclofen treatment reduced consumption and body weights in both genotypes during the initial days of treatment, after which volumes consumed were similar to untreated water, and body weights returned to a normal trajectory in all groups. Behavioral tests were conducted during the light phase of the circadian cycle, between ages 2 and 5 months.

Object location memory. On day 1, each mouse was placed in an empty white plastic chamber (40 × 40 cm²) and allowed to explore for 30 min to habituate to the chamber. A vertical

stripe of black duct tape was placed on one wall of the chamber to facilitate subsequent spatial memory. On day 2, the subject mouse was placed in the same empty arena for a second habituation session of 10 min duration. The mouse was removed, and two identical objects were placed in the chamber ~12 cm from the wall and ~18 cm apart. The mouse was then placed back into the chamber and allowed to explore the test arena containing the two identical objects for 10 min. After this familiarization session, the subject mouse was removed from the arena and placed into an empty holding cage in a different room for 1 h. Objects were cleaned in an Alconox bath and chambers were cleaned with ethanol between the familiarization and novel object recognition test sessions. One of the objects was replaced in its original location, and the other object was moved to a new location, ~18 cm forward from its initial location. After the 1 h interval, the subject mouse was returned to the testing chamber and allowed to explore both objects for 5 min. Time spent sniffing each object was recorded and scored by the Noldus Ethovision software. Object location memory was defined as spending significantly more time sniffing the relocated object than sniffing the object that remained in its original location. The novel object location was counter-balanced (left or right) to prevent side bias.

Male–female reciprocal social interactions and vocalizations. Adult 16p11.2 *+/+* and B6 female mice in estrus were used as stimulus mice for adult male 16p11.2 deletion mice. Each female mouse was used as a partner for a maximum of three male subject mice. Each trial was run in a clean cage ($36.9 \times 15.6 \times 13.2 \text{ cm}^3$) with an 0.5 cm layer of bedding under dim red lighting in a sound-attenuating chamber. One female stimulus mouse and one male subject mouse were placed in the cage and behaviors were recorded with a video camera for 5 min. Interactions were scored from videos by human observers blind to genotype and treatment, using the Noldus Observer XT 8.0 event recording software. Social parameters scored in the male subjects included nose-to-nose sniff (sniffing snout region of partner), nose-to-anogenital sniff (sniffing anogenital region of partner), and follow (walking closely behind partner). Ultrasonic vocalizations were recorded during the 5 min session using an ultrasonic microphone (Avisoft UltraSoundGate) inside the sound attenuating chamber and subsequently scored using Avisoft SASLab Pro.

General exploratory locomotion. To assess potential drug effects on general activity, locomotion was assessed during a 10 min exploration session in the same chambers used for the object location memory task. Total distance traveled was scored with the Noldus Ethovision software.

Statistical analysis. Object location memory data were analyzed using paired *t*-tests, comparing the number of seconds spent sniffing each object within genotype and within treatment, during the familiarization and novel object recognition phases, as previously described (Vogel-Ciernia and Wood, 2014). Male–female social interaction data were analyzed using an unpaired *t*-test, comparing treatments within genotype for each parameter. Locomotor activity data were analyzed using one-way analysis of variance followed

by Tukey's multiple comparisons *post hoc* test. Body weight and daily consumption were analyzed using two-way repeated-measures analysis of variance.

RESULTS

Two independently generated 16p11.2 microdeletion model mice (termed 16p11.2 *df/+*) were employed in our studies. The Mills mice (Horev *et al*, 2011) were used for all studies conducted in the Bear laboratory at MIT and the Dolmetsch mice (Portmann *et al*, 2014) were used for all studies conducted in the Crawley laboratory at UCSD. Before describing the effects of *R*-baclofen, here we briefly review the deficits that have been previously reported in these two lines of mice.

The Mills mice display poor survival as pups, smaller body weights, higher home cage activity, and an unusual descent from the cage ceiling in some individuals (Brunner *et al*, 2015; Horev *et al*, 2011). Normal sociability and preference for social novelty were observed in 16p11.2 *df/+* mice in the three-chambered social approach task, with no genotype differences detected on reciprocal social interactions or male sniffing and vocalizing in response to female urine (Brunner *et al*, 2015). Cognitive studies of the Mills mice revealed deficits in contextual fear conditioning, passive avoidance, and novel object recognition (Brunner *et al*, 2015; Pucilowska *et al*, 2015; Tian *et al*, 2015), while T-maze acquisition and reversal did not differ between genotypes (Brunner *et al*, 2015). Lower open field activity and anxiety-related tendencies in the elevated plus-maze were reported in the Mills line (Pucilowska *et al*, 2015).

The Dolmetsch line of 16p11.2 deletion mice similarly displayed poor pup survival, with body weights significantly lower than wild-type littermates throughout life, while other measures of general health remained normal throughout the lifespan in the surviving heterozygotes (Portmann *et al*, 2014). Circling and jumping motor stereotypies were seen in a small subset of individuals (Portmann *et al*, 2014; Yang *et al*, 2015b). Social behaviors were normal on both three-chambered social approach and juvenile reciprocal social interactions but reduced on parameters of male–female reciprocal social interactions and accompanying ultrasonic vocalizations, as compared with wild-type male littermate controls (Portmann *et al*, 2014; Yang *et al*, 2015b). Cognitive deficits were prominent in the Dolmetsch 16p11.2 *df/+* mice on recognition tasks, including novel object recognition, object location memory, preference for social novelty, and touchscreen pairwise visual discrimination acquisition and reversal (Portmann *et al*, 2014; Yang *et al*, 2015a). No differences between genotypes were detected on contextual fear conditioning; water maze could not be conducted owing to poor swimming abilities (Portmann *et al*, 2014; Yang *et al*, 2015a).

MIT Studies on Mills Mice

Object recognition. Using the Mills 16p11.2 deletion mice (Horev *et al*, 2011; Tian *et al*, 2015), we first examined the effects of chronic *R*-baclofen treatment using an object recognition memory task. Mice were allowed to explore an arena with two identical objects for two 10-min exploration

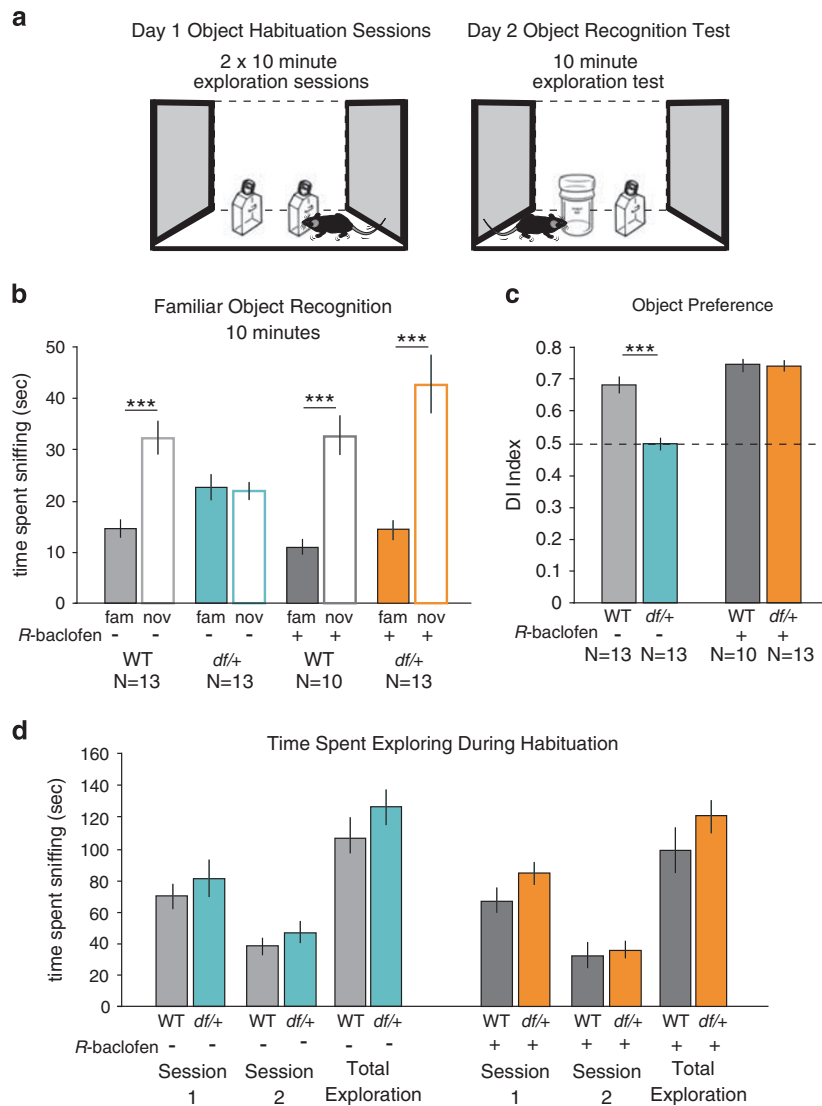


Figure 1 Chronic *R*-baclofen treatment restores novelty detection deficits in an object recognition task in 16p11.2 *df/+* mice. (a) Experimental design of novel object recognition task. (b) Vehicle-treated 16p11.2 *df/+* mice show impaired novelty detection on experimental test day 2 when presented with a familiar and novel object compared with vehicle-treated WT (WT veh, fam vs nov: $t(12) = 6.51$; $p < 0.001$; *df/+* veh, fam vs nov: $t(12) = 0.467$; $p = 0.649$; WT *R*-baclofen, fam vs nov: $t(9) = 7.68$; $p < 0.001$; *df/+* *R*-baclofen, fam vs nov: $t(12) = 6.54$; $p < 0.001$). (c) As depicted in panel (b), vehicle-treated 16p11.2 *df/+* mice show no preference for the novel or familiar object, exploring each approximately equally, whereas WT mice treated animals favor the novel object, as determined by calculating discrimination index (WT, veh vs *df/+*, veh: $t(24) = 6.49$; $p < 0.001$). In comparison, similar to vehicle-treated WT animals, *R*-baclofen-treated WT and 16p11.2 *df/+* mice show significantly more exploration of a novel object compared with a familiar object, as depicted by a discrimination significantly > 0.5 . Chronic *R*-baclofen treatment restores novelty detection in 16p11.2 *df/+* mice, making them indistinguishable from WT mice (WT, *R*-baclofen vs *df/+*, *R*-baclofen: $t(21) = 0.063$; $p = 0.950$). Two-way ANOVA, genotype: $F = 23.0$, $p < 0.001$; treatment: $F = 62.4$, $p < 0.001$; genotype \times treatment: $F(3,45) = 22.2$, $p < 0.001$. (d) There is no significant difference in time spent exploring each of two identical objects during the first object habituation session in either genotype regardless of treatment nor is there any significant difference in total time spent exploring during either habituation session (WT veh vs *df/+* veh, session 1: $t(24) = 0.802$, $p = 0.430$; WT veh vs *df/+* veh, session 2: $t(24) = 1.100$, $p = 0.282$; WT veh vs *df/+* veh, total: $t(24) = 1.20$, $p = 0.241$; WT *R*-baclofen vs *df/+* *R*-baclofen, session 1: $t(21) = 1.84$, $p = 0.079$; WT *R*-baclofen vs *df/+* *R*-baclofen, session 2: $t(21) = 0.614$, $p = 0.546$; WT *R*-baclofen vs *df/+* *R*-baclofen, total: $t(21) = 1.64$, $p = 0.116$). All data for all figures are plotted as mean \pm SEM; n indicates the number of animals. *** $p < 0.001$, *R*-baclofen vs vehicle within genotype.

sessions, spaced 1–2 h apart. Twenty-four hours later, one of the familiar objects was replaced with a novel object and a discrimination index was calculated as (time spent sniffing the novel object)/(cumulative time spent sniffing the novel + familiar object) across a 10-min testing session (Figure 1a). Vehicle-treated WT mice showed a strong preference for the novel object. In comparison and similar to previous reports, vehicle-treated 16p11.2 *df/+* mice did not show a preference

for the novel object, exploring the familiar and novel object equally (Figures 1b and c). Chronic treatment with *R*-baclofen had no effect on preference for a novel object in WT mice but completely restored novelty detection in 16p11.2 *df/+* mice (Figures 1b and c). To ensure that our findings were not confounded by differences in total overall exploration during the habituation sessions to the familiar object, we scored total sniffing during the two exploration

sessions on day 1 and observed no differences across genotype or treatment (Figure 1d).

Context recognition. We next examined the effects of chronic R-baclofen administration on cognitive performance in an aversive hippocampus-associated context recognition task in the 16p11.2 *df/+* mice. In this assay, mice were exposed to a distinctive environmental context in which they receive a foot shock (The acute behavioral response to foot shock in the Mills 16p11.2 *df/+* mice was previously shown to be no different from WT (Tian et al, 2015), suggesting comparable sensory sensitivity.). Twenty-four hours later, the mice were returned to either the same (familiar) or a different (novel) context on the testing day (Figure 2a). Confirming previous observations, vehicle-treated 16p11.2 *df/+* mice exhibited profound memory deficits on the testing day, as evidenced by lack of freezing in the familiar context where they had previously received a foot shock (Figure 2b). 16p11.2 *df/+* mice treated with R-baclofen froze significantly more in the familiar context on testing day, comparable to WT mice treated with either vehicle or R-baclofen, demonstrating that the drug treatment restored the ability to both acquire and recall the association between context and an aversive event (Figure 2b).

Open field habituation. In order to investigate habituation to a context, we tracked the activity of the 16p11.2 *df/+* mice across two 15-min recording sessions, spaced 1–2 h apart. During each habituation session, we used the Cineplex monitoring software to monitor activity throughout a 40 × 40 cm² environment. We used MATLAB to analyze the time spent in two predefined zones (outer, inner) of the behavior arena across sessions (Figure 3a). We first measured the total distance (pooled across sessions) traveled by WT and 16p11.2 *df/+* mice as an indicator of hyperactivity. Vehicle-treated 16p11.2 heterozygous mice

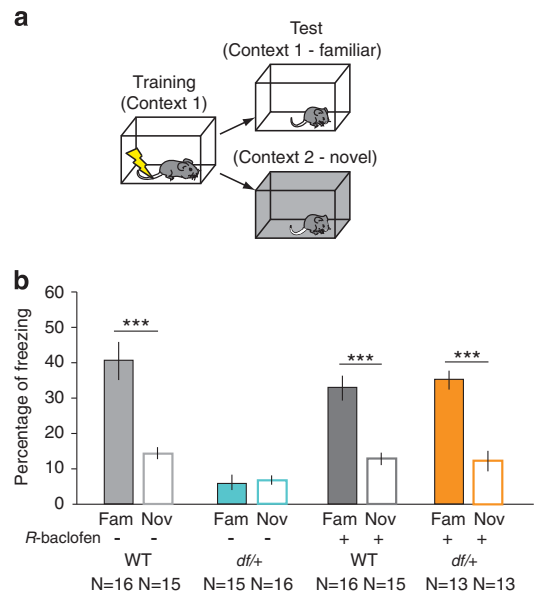


Figure 2 Chronic R-baclofen treatment restores memory deficits in a context-dependent aversive learning task in 16p11.2 *df/+* mice. (a) Experimental design of context-dependent discrimination task. (b) Vehicle-treated 16p11.2 *df/+* mice show no difference in freezing in the familiar vs novel context and significantly less freezing in the familiar context compared with WT mice (*df/+* veh, nov vs fam: $t(29) = 0.227$, $p = 0.822$; WT, veh vs *df/+*, veh: $t(29) = 7.05$, $p < 0.001$). Vehicle- and R-baclofen-treated WT mice are able to distinguish a novel from familiar context (WT veh, nov vs fam: $t(29) = 5.70$, $p < 0.001$; WT R-baclofen, nov vs fam: $t(28) = 5.29$, $p < 0.001$). Upon chronic treatment with R-baclofen, 16p11.2 *df/+* mice are able to distinguish a novel from familiar context as indicated by significantly increased freezing in the familiar context compared with novel context (*df/+* R-baclofen, nov vs fam: $t(24) = 6.03$, $p < 0.001$). Three-way ANOVA, genotype: $F = 30.7$, $p < 0.001$; treatment: $F = 7.86$, $p < 0.01$; context: $F = 82.0$, $p < 0.001$; genotype × treatment: $F = 22.2$, $p < 0.001$; genotype × context: $F = 11.5$, $p < 0.001$; treatment × context: $F = 3.01$, $p = 0.086$; genotype × treatment × context: $F(7,111) = 17.7$, $p < 0.001$. *** $p < 0.001$, R-baclofen vs vehicle within genotype.

Figure 3 16p11.2 *df/+* mice show habituation deficits in an open field task, which is reversed by chronic R-baclofen treatment. (a) Experimental design of open field habituation task. (b) 16p11.2 *df/+* mice treated with vehicle show statistically significant hyperactivity compared with WT animals treated with vehicle in the open field as indicated by distance traveled over the course of both habituation sessions (veh vs *df/+* veh: $t(22) = 4.33$, $p < 0.001$). WT animals treated with R-baclofen show significantly increased locomotion compared with vehicle-treated animals (WT veh vs R-baclofen: $t(22) = 2.89$, $p < 0.01$). There is no statistical significance between: 16p11.2 *df/+* animals treated with vehicle or R-baclofen (*df/+* veh vs R-baclofen: $t(22) = 0.248$, $p = 0.807$; WT); WT animals treated with R-baclofen and 16p11.2 heterozygous animals treated with vehicle (*df/+* veh vs WT R-baclofen: $t(22) = 0.010$, $p = 0.992$) or WT animals and 16p11.2 heterozygous treated with R-baclofen (WT R-baclofen vs *df/+* R-baclofen: $t(22) = 0.206$, $p = 0.839$). (c) Across genotypes and treatments, animals travel a greater distance in the first habituation session compared with the second habituation session (WT veh, session 1 vs session 2: $t(11) = 6.85$, $p < 0.001$; *df/+* veh, session 1 vs session 2: $t(11) = 9.58$, $p < 0.001$; WT R-baclofen, session 1 vs session 2: $t(11) = 4.81$, $p < 0.001$; *df/+* R-baclofen, session 1 vs session 2: $t(11) = 4.56$, $p < 0.001$). (d) Vehicle-treated WT and 16p11.2 *df/+* mice exhibit similar behavior during the first habituation session. During the second session, WT mice spend significantly more time in the outer zone of the arena, whereas 16p11.2 *df/+* mice do not spend significantly more time in either the inner or outer zones of the arena (WT vs *df/+*, Session 1—outer zone, $t(22) = 1.32$, $p = 0.201$; WT vs *df/+*, Session 1—inner zone, $t(22) = 1.32$, $p = 0.201$; WT vs *df/+*, Session 2—outer zone, $t(22) = 5.83$, $p < 0.001$; WT vs *df/+*, Session 2—inner zone, $t(22) = 5.83$, $p < 0.001$; WT, Session 1, inner vs outer, $t(11) = 0.782$, $p = 0.450$; *df/+*, Session 1, inner vs outer, $t(11) = 1.13$, $p = 0.281$; WT, Session 2, inner vs outer, $t(11) = 9.90$, $p < 0.001$; *df/+*, Session 2, inner vs outer, $t(11) = 0.820$, $p = 0.430$). Three-way genotype × zone × session: $F(7,88) = 16.867$, $p < 0.001$. Pairwise multiple comparison procedure (Holm–Sidak method): genotype within session 1, WT vs *df/+*: $t = 0.00005$, $p = 1.00$; zone within session 1, inner vs outer: $t = 0.131$, $p = 0.896$; genotype within inner—session 2, WT vs *df/+*: $t = 5.82$, $p < 0.001$; genotype within outer—session 2, WT vs *df/+*: $t = 5.82$, $p < 0.001$; zone within WT, session 2: $t = 12.9$, $p < 0.001$; zone within *df/+*, session 2: $t = 1.24$, $p = 0.218$. (e) R-baclofen-treated WT and 16p11.2 *df/+* mice exhibit similar behavior during the first habituation session. During the second session, both WT mice and 16p11.2 *df/+* mice spend significantly more time in the outer zone of the arena (WT vs *df/+*, session 1—outer zone, $t(22) = 0.142$, $p = 0.889$; WT vs *df/+*, session 1—inner zone, $t(22) = 0.142$, $p = 0.889$; WT vs *df/+*, session 2—outer zone, $t(22) = 0.106$, $p = 0.917$; WT vs *df/+*, session 2—inner zone, $t(22) = 0.106$, $p = 0.917$; WT, Session 1, inner vs outer, $t(11) = 1.21$, $p = 0.252$; *df/+*, Session 1, inner vs outer, $t(11) = 1.60$, $p = 0.138$; WT, Session 2, inner vs outer, $t(11) = 2.56$, $p = 0.026$; *df/+*, Session 2, inner vs outer, $t(11) = 3.19$, $p < 0.01$). Three-way genotype × zone × session: $F(7,88) = 0.055$, $p = 0.814$). (f) Dolmetsch 16p11.2 *df/+* mice in UCD lab: Total distance traveled was recorded using the Noldus Ethovision software. No significant effect of drug treatment was detected on exploratory activity during a 10-min open field session in the same chambers used for the object location memory task. One-way ANOVA ($F(3,81) = 3.22$; $p = 0.027$, Tukey's multiple comparison test $p = 0.027$ for the comparison of WT vehicle vs *df/+* vehicle only. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, R-baclofen vs vehicle within genotype.

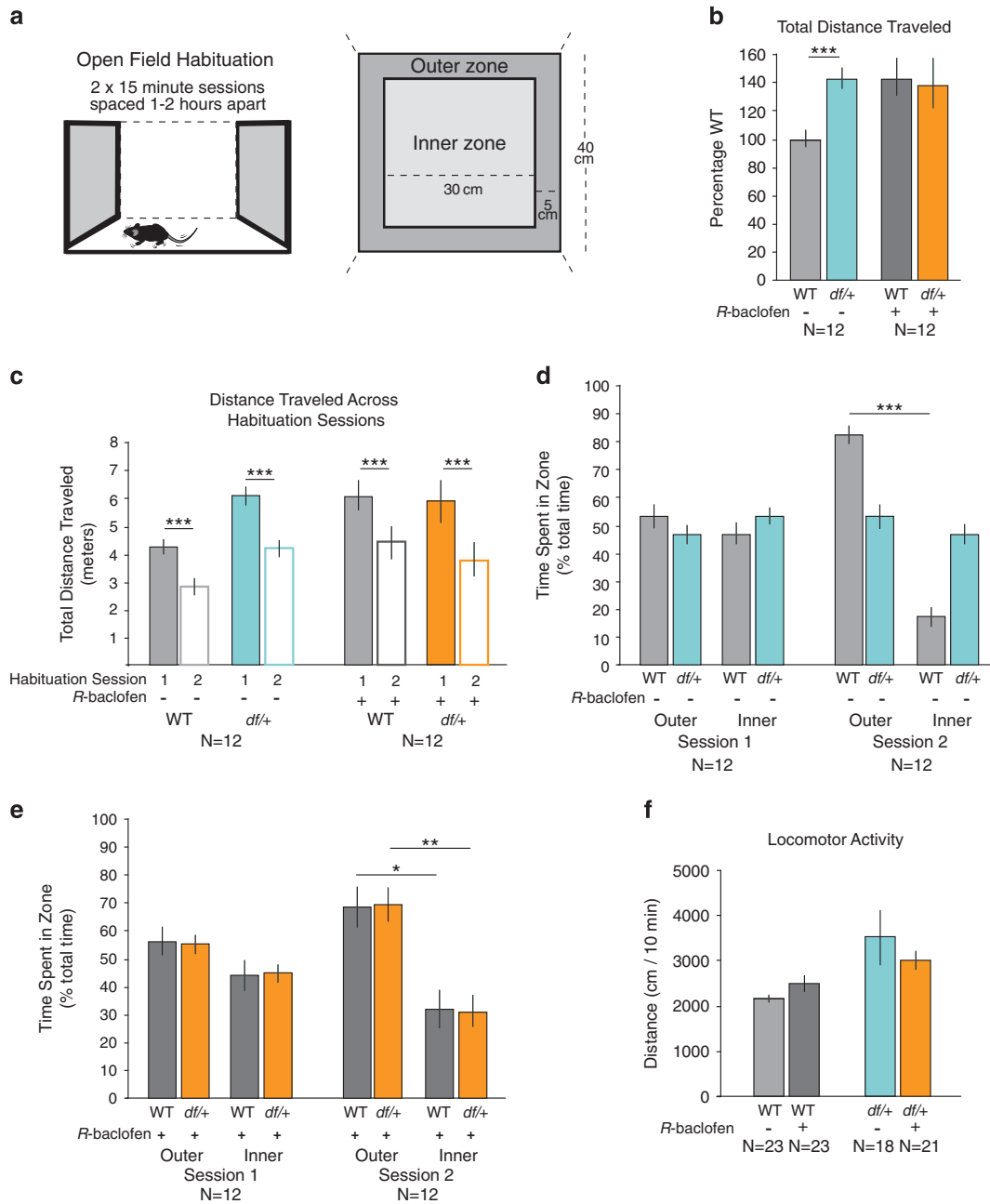
traveled a significantly greater distance in the arena compared with vehicle-treated WT mice across both habituation sessions, indicative of a generalized hyperactivity in these mice. Chronic administration of R-baclofen had no effect on locomotion in 16p11.2 *df/+* but rather surprisingly induced hyperactivity in WT mice to a degree comparable to the treated and untreated 16p11.2 *df/+* mice (Figure 3b).

Across all genotypes and treatments, mice spent more time exploring the context during the first recording session compared with the second session (Figure 3c). Zone-specific analysis revealed that vehicle-treated WT and 16p11.2 *df/+* mice explored the inner and outer zone of the environment roughly equally during the first 15-min session. However, vehicle-treated WT mice spend significantly more time near

the walls in the outer zone of the arena during the second 15-min session, whereas 16p11.2 *df/+* mice spend roughly equal time exploring the outer and inner zones of the arena during the second session, potentially reflecting a context recognition deficit across sessions in these mice (Figure 3d). Interestingly, chronic R-baclofen administration does not affect exploration patterns of WT mice but restores normal exploration in 16p11.2 *df/+* mice, leading to increased time spent in the outer zone and decreased time spent exploring the inner zones in the second exploration session (Figure 3e).

UCD Studies on Dolmetsch Mice

Locomotor activity. Exploratory activity was tested in a standard open field to evaluate potential sedative effects of



R-baclofen. Consistent with studies on Mills mice, treatment did not significantly affect open field locomotor activity in WT or 16p11.2 *df/+* Dolmetsh mice at the dose employed in these studies (Figure 3f).

Object location memory. During the familiarization phase of the object location memory assay, no innate side bias for object position was detected in mice of either genotype treated with vehicle or R-baclofen (Figure 4a). During the object location test phase, WT mice administered vehicle spent significantly more time sniffing the object in the novel location compared with time spent sniffing the object in the familiar location (Figure 4b). 16p11.2 *df/+* mice administered vehicle did not exhibit significant object location memory, but 16p11.2 *df/+* mice administered R-baclofen spent significantly more time sniffing the object in the novel location compared with the object in the familiar location. WT mice administered R-baclofen displayed increased exploration of both objects but interestingly did not display object location memory, a finding to be further investigated in future studies.

Male–female reciprocal social interactions and vocalizations. Lower scores in 16p11.2 deletion males interacting with estrous females were significantly ameliorated by R-baclofen treatment on several parameters (Figure 5). 16p11.2 *df/+* males administered R-baclofen showed significantly greater nose-to-anogenital sniffing as compared with vehicle treatment (Figures 5c and d). Time spent following was also higher in 16p11.2 *df/+* mice that received R-baclofen as compared with vehicle (Figures 5e and f). Interestingly, R-baclofen significantly decreased these social behaviors in the wild types. R-baclofen had no significant effect on nose-to-nose sniffing in 16p11.2 deletion males but significantly decreased nose–nose sniffing in WT males (Figure 5a). R-baclofen also had no effect on the number of ultrasonic vocalizations emitted by 16p11.2 *df/+* males but significantly reduced the number of ultrasonic vocalizations in wild type (Figure 5g).

DISCUSSION

16p11.2 deletion syndrome includes symptoms of intellectual impairment and autism (Zufferey *et al*, 2012). Cognitive and social deficits were previously detected in three independently generated lines of 16p11.2 deletion mice (Arbogast *et al*, 2016; Brunner *et al*, 2015; Portmann *et al*, 2014; Pucilowska *et al*, 2015; Tian *et al*, 2015; Yang *et al*, 2015a,b). We employed two of these lines to test the hypothesis that the GABA_B agonist R-baclofen could improve performance in behavioral domains relevant to symptoms of the human 16p11.2 deletion syndrome. Our two separate laboratories used the same R-baclofen dose and treatment time course to test 16p11.2 deletion mice and their wild-type littermates on distinct but complementary behavioral assays. This parallel, corroborative approach was undertaken to maximize rigor and reproducibility in evaluating the efficacy of a drug treatment in preclinical studies.

Chronic R-Baclofen Treatment Corrects Several Cognitive Defects in 16p11.2 *df/+* Mice

Impaired novelty detection in the two assays used here, object recognition and object location memory, is one of the most robust and reproducible phenotypes associated with all three current 16p11.2 heterozygous deletion mouse models. Similar to previous studies, we found profound deficits in both novel object recognition and object location memory in the Mills and Dolmetsh mouse lines, respectively (Brunner *et al*, 2015; Pucilowska *et al*, 2015). Chronic administration of R-baclofen significantly improved novelty detection in both 16p11.2 *df/+* mouse models in these recognition memory tasks. Furthermore, in the Mills 16p11.2 *df/+* mouse model, chronic R-baclofen treatment also corrected severely impaired context discrimination.

It was reported previously that open field locomotor behavior fails to habituate in the Mills 16p11.2 *df/+* mice over the course of 15 min (Pucilowska *et al*, 2015). By comparing the locomotion of 16p11.2 *df/+* mice and wild-type littermates across two 15-min open field habituation sessions spaced 1–2 h apart, we were also able to identify an additional behavioral deficit in the Mills mouse line. Wild-type mice spent less time in the center of the environment during the second recording session, exhibiting characteristic thigmotaxic behavior. In contrast, the activity of 16p11.2 *df/+* mice was indistinguishable between recording sessions. This difference may represent a deficit in recognition of a familiar environment, although we acknowledge that there could be multiple interpretations of the findings, such as increased impulsivity or decreased anxiety. Nevertheless, regardless of the interpretation, 16p11.2 heterozygous mice treated with chronic R-baclofen exhibited behavior that was virtually indistinguishable from wild-type mice treated with either vehicle or R-baclofen.

Chronic R-Baclofen Improves Social Behaviors in 16p11.2 *df/+* mice

We previously reported reduced reciprocal social interactions by male 16p11.2 *df/+* mice on parameters of anogenital sniffing and following an estrous female, along with fewer ultrasonic vocalizations during the social session (Yang *et al*, 2015b). Here we replicated the social and vocalization phenotypes during male–female interactions and detected elevations of anogenital sniffing and following in 16p11.2 deletion males treated with R-baclofen, while vocalizations remained low.

Effects of R-Baclofen on Locomotor Activity

Neither laboratory observed any improvement in the hyperlocomotion exhibited by the 16p11.2 *df/+* mice with chronic R-baclofen administration. In fact, the Bear (but not Crawley) laboratory observed a paradoxical increase in locomotion in treated wild-type mice. Although at high doses R-baclofen is known to induce a sedative effect in humans and mice (Henderson *et al*, 2012), this clearly was not observed in our studies at the dose regimen employed. The fact that such a dramatic improvement in cognition, along with improvement in two parameters of social behavior, is observed at doses that fail to cause sedation suggests that R-baclofen has a broad therapeutic window. On the other hand, the finding that the hyperlocomotion and vocalization phenotypes were not

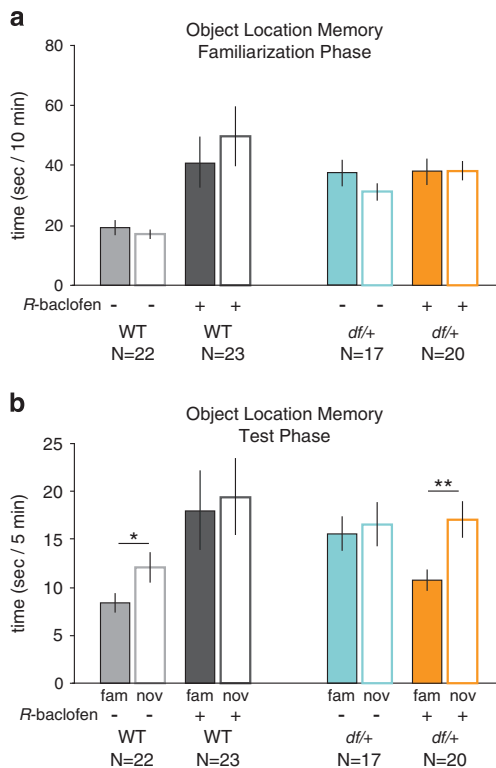


Figure 4 16p11.2 *df/+* mice displayed a learning and memory deficit in object location memory, which was reversed by *R*-baclofen treatment. *R*-baclofen (0.5 mg/ml), indicated by '+', rescued the deficit in object location memory in 16p11.2 *df/+* mice. (a) During the familiarization phase, no side bias was detected (WT, veh: $t(21) = 0.864$; $p = 0.398$, NS; *df/+*, veh: $t(16) = 1.31$; $p = 0.208$, NS; WT, *R*-baclofen: $t(22) = 1.97$; $p = 0.062$, NS; *df/+*, *R*-baclofen: $t(19) = 0.001$, $p = 0.995$, NS). (b) During the test phase, WT mice administered vehicle, indicated by '-', spent significantly more time sniffing the object in the novel location (Nov) compared with time spent sniffing the object in the familiar location (Fam), while 16p11.2 *df/+* mice administered vehicle did not exhibit significant object location memory (WT, veh: $t(21) = 2.34$; $p = 0.029$; *df/+*, veh: $t(16) = 0.305$; $p = 0.764$, NS). 16p11.2 *df/+* mice administered *R*-baclofen spent significantly more time sniffing the object in the novel location compared with the object in the familiar location (*df/+*, *R*-baclofen $t(19) = 3.00$, $p < 0.01$). WT administered *R*-baclofen increased exploration of both objects and did not display the expected object location memory (WT, *R*-baclofen $t(22) = 0.641$; $p = 0.528$). * $p < 0.05$, ** $p < 0.01$, *R*-baclofen vs vehicle within genotype.

improved by our treatment indicates that not all aberrant behavioral phenotypes associated with 16p11.2 deletion mice respond to this treatment.

Mechanism of Action

At this point, we can only speculate how *R*-baclofen treatment improves cognitive and social phenotypes in adult 16p11.2 *df/+* mice. Our experiments were motivated in part by an earlier study in which we found a synaptic phenotype in the hippocampus that resembles what is observed in the *Fmr1*^{-/-} mice. Specifically, the data showed that 16p11.2 *df/+* mice also have aberrant activity-dependent protein synthesis regulation downstream of mGluR5 and increased hippocampal expression of Arc protein, a molecular marker of elevated neuronal activity. Moreover, as in the *Fmr1*^{-/-} mice, impairments in hippocampus-dependent fear memory could be ameliorated by treatment with an mGluR5-selective

negative allosteric modulator (NAM) (Tian *et al*, 2015). In fragile X, *R*-baclofen was advanced as a treatment because it can also normalize protein synthesis (Henderson *et al*, 2012; Qin *et al*, 2015), presumably by inhibiting glutamate release. An additional motivation for our experiments in 16p11.2 *df/+* mice was the previous finding that *R*-baclofen treatment can also improve sociability and repetitive behaviors in two additional mouse models of autism, the BTBR and C58 inbred strains (Silverman *et al*, 2015). BTBR mice have reduced GABAergic neurotransmission (Gogolla *et al*, 2014; Han *et al*, 2014), and deficits in both strains also are improved by treatment with an mGluR5 NAM (Silverman *et al*, 2012).

As a working hypothesis, we suggest that some cognitive and social deficits in 16p11.2 deletion mice arise from an ongoing disturbance in neuronal protein synthesis regulation that can be ameliorated with chronic drug treatment in adults. Two genes in the 16p11.2 region, MAPK3 and Major Vault Protein (MVP), are directly involved in signaling pathways downstream of mGlu5 signaling, and several other genes in this region are targets of FMRP (Crepel *et al*, 2011; Kolli *et al*, 2004; Paspalas *et al*, 2008). MAPK3 codes for ERK1, a key regulator of mGluR5-dependent protein synthesis, and levels of ERK1, ERK1 phosphorylation, and basal protein synthesis are reduced in the Mills 16p11.2 *df/+* mice (Tian *et al*, 2015). We note that baclofen, in addition to inhibiting presynaptic glutamate release, can stimulate ERK1/2 phosphorylation in hippocampal neurons by activation of dendritic GABA_B receptors (Vanhoose *et al*, 2002). Conversely, MVP is thought to be a negative regulator of ERK1/2 (Kolli *et al*, 2004), and a rare inherited deletion in the 16p11.2 region confined to MVP, CDIPT1, SEZ6L2, ASPHD1, and KCTD13 has been shown to be associated with ASD (Crepel *et al*, 2011). More work is necessary to delineate how 16p11.2 microdeletion causes cognitive and social deficits and understand how these impairments can be reversed by GABA_B receptor activation.

Clinical Implications

Our findings that *R*-baclofen improved performance on three cognitive tasks and reversed deficits on two measures of male-female reciprocal social interactions suggest the drug may have therapeutic utility in humans with 16p11.2 microdeletion syndrome. Racemic baclofen has been used clinically for many years for the treatment of spasticity in children and adults with cerebral palsy, and placebo-controlled trials in fragile X and autism using *R*-baclofen have shown that the drug is safe and well tolerated in patients with other developmental brain disorders.

Prior to the current study, the strongest preclinical rationale for use of *R*-baclofen for autism and ID derived from work in fragile X. However, despite promising findings in mice (Henderson *et al*, 2012; Qin *et al*, 2015), three placebo-controlled clinical trials failed to show any significant improvements using the predetermined metrics chosen as the primary end points, various subscales of the Aberrant Behavior Checklist (ABC) (Berry-Kravis *et al*, 2017b; Berry-Kravis *et al*, 2012). Nevertheless, efficacy was suggested by improvements in physician-rated global assessments and several secondary end points that included other ABC subscales. Notably, and regrettably, cognition was not

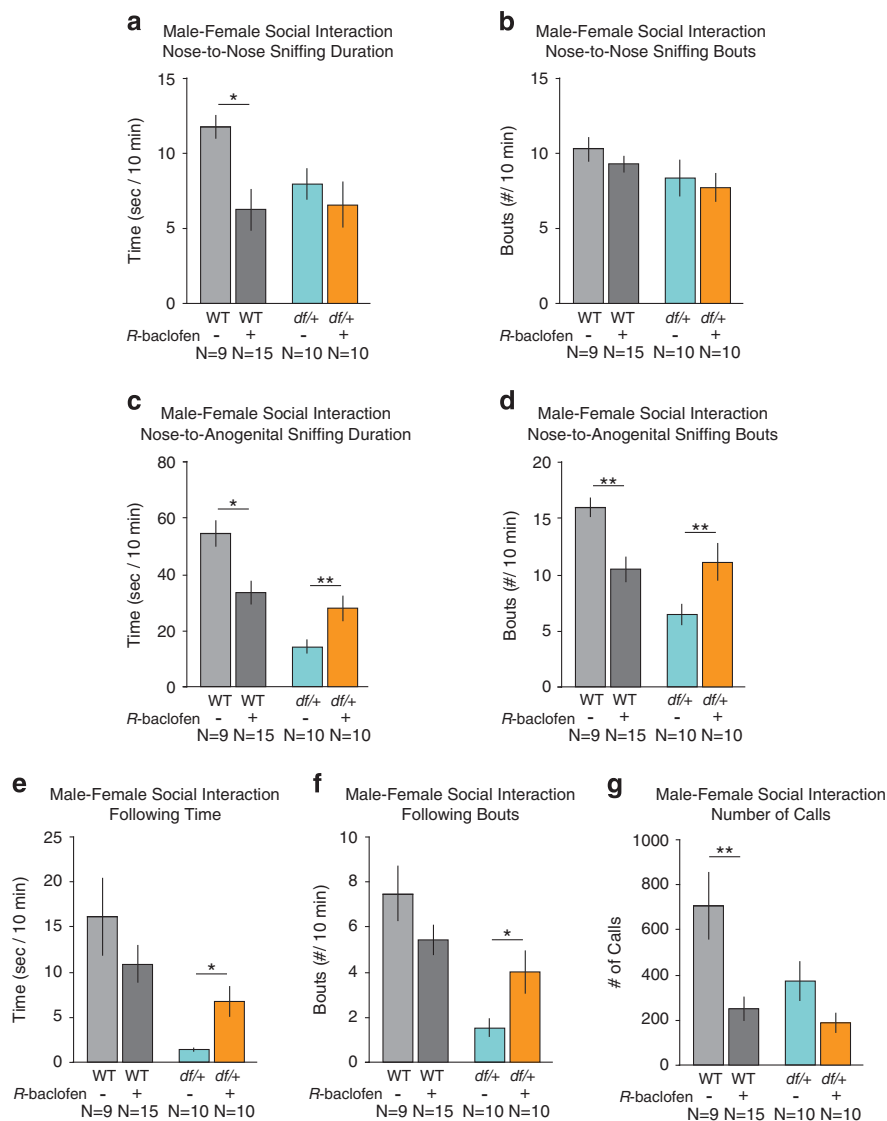


Figure 5 16p11.2 *df/+* male mice displayed lower scores on some parameters of male–female social interactions, which were reversed by *R*-baclofen treatment. Male 16p11.2 *df/+* mice engaged in less reciprocal social interactions with an estrous female on some parameters, as compared with wild-type littermate controls. *R*-baclofen increased sociability in 16p11.2 *df/+* male mice. Although *R*-baclofen did not significantly affect nose-to-nose sniffing in 16p11.2 *df/+* mice (a, b: *df/+*: $t(18) = 0.129$; $p = 0.899$, NS; WT: $t(22) = 2.12$; $p < 0.05$), *R*-baclofen treatment significantly increased nose-to-anogenital sniffing in 16p11.2 *df/+* mice on measures of time spent (c: *df/+*: $t(18) = 2.14$; $p < 0.05$, WT: $t(22) = 0.913$, $p = 0.371$, NS) and number of bouts (d: *df/+*: $t(22) = 2.94$, $p < 0.01$; WT: $t(18) = 1.12$, $p < 0.05$) and on time spent following the female (e: *df/+*: $t(18) = 2.66$, $p < 0.05$; WT: $t(22) = 1.16$; $p = 0.260$, NS) and the number of following bouts (f: *df/+*: $t(18) = 2.15$, $p < 0.05$; WT: $t(22) = 1.52$, $p = 0.143$, NS). (g) *R*-baclofen had no effect on the number of ultrasonic vocalizations emitted by 16p11.2 *df/+* males ($t(18) = 1.56$, $p = 0.134$; NS) but significantly reduced the number of ultrasonic vocalizations in WT ($t(22) = 2.87$; $p < 0.01$). Decreased scores appeared in WT treated with *R*-baclofen on measures of time spent in nose-to-nose sniffing, nose-to-anogenital sniffing, and the number of vocalizations, for unknown reasons, which do not appear to include sedation. * $p < 0.05$, ** $p < 0.01$, *R*-baclofen vs vehicle within genotype.

assessed in these studies. These findings illustrate one of the great challenges in translational neuroscience—because the effects of a particular gene mutation do not manifest behaviorally the same in all individuals, improvements can be difficult to capture across the patient population in any single symptom domain. The same genetic disruption does not always cause the same symptoms (eg, autism in fragile X), and the same symptoms may be caused by many different gene disruptions (eg, autism in the general population).

16p11.2 copy number variation was originally discovered in studies of autistic patients (Sebat *et al*, 2007; Weiss *et al*,

2008). Subsequent studies revealed variable penetrance of autistic phenotypes in deletion carriers but consistent deficits overall in traits related to adaptive skills (Zufferey *et al*, 2012). Full-scale intelligence quotient was reported to be ~2 SD lower, on average, in carriers compared with unaffected relatives, with 20% satisfying criteria for a DSM-IV diagnosis of ID (IQ < 70). To the extent that preclinical phenotypic improvements in mice can be used to guide end point and patient selection in humans, our findings—obtained in two independent laboratories in different 16p11.2 deletion mouse strains—suggest that *R*-baclofen should be explored for the treatment of cognitive phenotypes in affected humans.

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