Published in final edited form as:

Genes Brain Behav. 2019 September ; 18(7): e12593. doi:10.1111/gbb.12593.

Loss of CELF6 RNA binding protein impairs cocaine conditioned place preference and contextual fear conditioning

Susan E. Maloney^{1,2,3}, Michael A. Rieger^{1,2}, Ream Al-Hasani^{4,5}, Michael R. Bruchas^{2,4,6}, David F. Wozniak^{2,3}, Joseph D. Dougherty^{1,2,3}

¹Department of Genetics, Washington University School of Medicine, St. Louis, MO 63110, USA

²Department of Psychiatry, Washington University School of Medicine, St. Louis, MO 63110, USA

³Intellectual and Developmental Disabilities Research Center, Washington University School of Medicine, St. Louis, MO 63110, USA

⁴Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO 63110, USA

⁵Department of Pharmaceutical and Administrative Sciences, St. Louis College of Pharmacy, St. Louis, MO 63110, USA

⁶Department of Neuroscience, Washington University School of Medicine, St. Louis, MO 63110, USA

Abstract

In addition to gene expression differences in distinct cell types, there is substantial post-transcriptional regulation driven in part by RNA binding proteins (RBPs). Loss-of-function RBP mutations have been associated with neurodevelopmental disorders, such as Fragile-X syndrome and syndromic autism. Work done in animal models to elucidate the influence of neurodevelopmental disorder-associated RBPs on distinct behaviors has revealed a connection between normal post-transcriptional regulation and conditioned learning. We previously reported cognitive inflexibility in a mouse model null for the RBP CUG-BP, Elav-like factor 6 (CELF6), which we also found to be associated with human autism. Specifically, these mice failed to potentiate exploratory hole-poking behavior in response to familiarization to a rewarding stimuli. Characterization of *Celf6* gene expression revealed high levels in monoaminergic populations such as the dopaminergic midbrain populations. To better understand the underlying behavioral disruption mediating the resistance to change exploratory behavior in the holeboard task, we tested three hypotheses: Does *Celf6* loss lead to global restricted patterns of behavior, failure of immediate response to reward, or failure to alter behavior in response to reward (conditioning). We found the acute response to reward was intact, yet *Celf6* mutant mice exhibited impaired conditioned learning to both reward and aversive stimuli. Thus, we found that the resistance to change by the *Celf6* mutant in the holeboard was most parsimoniously explained as a failure of conditioning, as the mice had blunted responses even to potent rewarding stimuli such as cocaine. These findings further support the role of RBPs in conditioned learning.

Keywords

Celf6; RNA binding protein; cocaine; conditioned place preference; fear conditioning; mouse; behavior; holeboard; reward; resistance to change

Introduction

We previously investigated the role of the RNA binding protein (RBP) CUG-BP, Elav-like factor 6 (CELF6) in behavioral circuits posited to be relevant to autism. Initially, we became interested in *Celf6* because of its robust expression in serotonin (5-HT)-expressing cells (Dougherty *et al.* 2013), and a focused analysis of human polymorphisms in 5-HT-expressed transcripts suggested an association between common variants of human *CELF6* and autism. We additionally detected one rare inherited variant of *CELF6* resulting in a premature stop codon in a male proband with autism. Thus, we were motivated to generate *Celf6* homozygous null (*Celf6*^{-/-}) male mice and characterize their behavior using a battery of tasks designed to assess general abilities and screen for deficits that may be related to social and communicative interactions and repetitive/restrictive behavior patterns (Dougherty *et al.* 2013). Behavioral impairments were observed in two tasks. First, *Celf6*^{-/-} pups exhibited a robust decrease in ultrasonic vocalizations in response to isolation from the dam and littermates, a phenotype suggested to reflect early social communicative deficits and disturbances in neurodevelopment. Second, as adults, *Celf6*^{-/-} mice displayed a different response pattern compared to littermate controls in the holeboard task, a measure used to assess exploration and olfactory preference, and how they may be related to perseverative behavior (Moy *et al.* 2008; Ghoshal *et al.* 2012). Specifically, *Celf6*^{-/-} mice failed to exhibit control-like levels of potentiation of their hole-poking responses following familiarization with a rewarding stimulus during this task, suggesting a resistance to change phenotype. Accompanying these findings, we demonstrated decreased levels of monoamine neurotransmitters in the adult *Celf6*^{-/-} brain using mass spectrometry (Dougherty *et al.* 2013).

Subsequent deep characterization of the temporal and spatial expression patterns of *Celf6* RNA and protein in the brain demonstrated these gene products are present early in a subset of neurons during neurodevelopment and into adulthood, peaking around birth (Maloney *et al.* 2016). Specifically, robust expression of the protein was observed in the neuromodulatory transmitter-expressing nuclei, including noradrenergic and dopaminergic populations, and, as expected, 5-HT cells. There was also robust expression in the hypothalamus, cholinergic cells of the striatum, and other aspects of the limbic system. Together with the mass spectrometry data, *Celf6* gene expression patterns suggest behavioral circuits that require heavy involvement of the monoamines may be particularly vulnerable in the absence of *Celf6*.

Mice modeling the loss of another RBP, Fragile-X Mental Retardation Protein (FMRP), show clear deficits in behavioral circuits controlling learning and social behaviors (Santos *et al.* 2014). Loss of FMRP in humans results in Fragile-X syndrome (FXS), the most common form of intellectual disability (ID) and syndromic autism (Abekhouk & Bardoni 2014).

Both FMRP and its binding partners, cytoplasmic FMR interacting proteins 1 and 2 (CYFIP1 and CYFIP2), have been shown to underlie pathological reward responses and compulsive-like behaviors. These include desensitized responses to cocaine administration, lack of cocaine-induced conditioning, and binge-eating (Kumar *et al.* 2013; Smith *et al.* 2014; Kirkpatrick *et al.* 2017), as well as disrupted effects on learning in response to aversive stimuli during fear conditioning (Santos *et al.* 2014). These studies suggest there may be some consistent link between disruptions in processing of rewarding and aversive stimuli, and the development of autism-like behaviors, an idea not inconsistent with some human theories of autism (Neuhaus *et al.* 2010; Kohls *et al.* 2012, 2013).

While recent human genomic research has not identified any new variants of *CELF6* in autism, mutations in this gene clearly alter behavior. Thus, we sought to further understand the role *Celf6* may play in additional behavioral circuits and to determine if it, like FMRP, was essential for normal conditioning. We tested three hypotheses in an effort to elucidate underlying behavioral disruptions mediating the lack of potentiation in the holeboard task. We specifically addressed if loss of this RBP leads to global restricted patterns of behavior, failure of immediate response to reward, or failure to alter behavior in response to reward/aversive stimulus (conditioning). Our behavioral analyses help provide insight into the core deficit underlying the resistance to change phenotype, as well as into the behavioral circuits most vulnerable following loss of this RBP.

Material and Methods

Animals

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Washington University in St. Louis in accordance with guidelines from the National Institutes of Health (NIH). Mice were housed in translucent plastic cages measuring 28.5 cm x 17.5 cm x 12 cm with corncob bedding and standard lab diet and water freely available. The colony room lighting was a 12:12 h light/dark cycle with relative humidity (50%) and room temperature (~20–22°C) controlled. All mice were all group-housed. For all experiments, adequate measures were taken to minimize any pain or discomfort. *Celf6* mutant mice (<https://www.jax.org/strain/028389>; RRID:IMSR_JAX:028389) were generated on the C57BL/6 background by deletion of exon 4 of the *Celf6* gene as previously described (Dougherty *et al.* 2013). Heterozygous breeding pairs were used to generate *Celf6*^{+/+}, *Celf6*^{+/-}, and *Celf6*^{-/-} littermates. Offspring were genotyped using standard reagents and primers for amplification of the region spanning exons 3 and 4: forward, ATCGTCCGATCCAAGTGAAGC and reverse, CTCCTCGATATGGCCGAAGG.

Five independent cohorts were used in this study. See Table 1 for sample sizes, including sex distribution, for each cohort.

Drugs

For conditioned place preference, cocaine was obtained from the Division of Comparative Medicine pharmacy at Washington University School of Medicine and dissolved in 0.9%

sterile saline at a concentration of 0.15mg/100uL, and injected at a volume per mass of 10 ml/kg.

Behavioral Tasks

Experimenters were blinded to experimental group designations during behavioral testing. Order of and age at testing were chosen to minimize effects of stress and previous behavioral tests. All behavioral testing occurred during the light phase. See Table 1 for details. A female experimenter conducted all behavioral testing. Cohort 1 served to replicate and extend the original resistance to change findings in the Holeboard Exploration/Olfactory Preference task to include both *Celf6*^{-/-} and WT males and females. Mice in Cohort 1 were also assessed in several tasks aimed at probing the behavioral domain underlying the resistance to change deficit. Because our original findings were in male mice (Dougherty *et al.* 2013), Cohort 2 extended our examination to include *Celf6*^{+/-} males as well as *Celf6*^{-/-} and WT littermates. Cohorts 3, 4 and 5 extended testing in independent cohorts of *Celf6* mutants and WT littermates to tasks that require drug exposure and/or are considered stressful, which can potentially influence behavior on subsequent tests, specifically cocaine conditioned place preference and the conditioned fear task. Due to the interesting findings in the conditioned fear task, we subsequently examined Cohort 2 in this task to identify possible phenotypes in the *Celf6*^{+/-} mice and extended our study to include Cohort 5 to examine female phenotypes in this task.

Holeboard Exploration/Olfactory Preference task.—The Holeboard Exploration/Olfactory Preference task was used, as previously described (Moy *et al.* 2008; Dougherty *et al.* 2013), to assess the influence of *Celf6* loss on cognitive flexibility and resistance to change behavior patterns as previously shown (Dougherty *et al.* 2013). The apparatus is a computerized holeboard (41 × 41 × 38.5 cm) with eight equidistant holes in the floor (Learning Holeboard; MotorMonitor, Kinder Scientific, LLC, Poway, CA, USA). Beam breaks quantified frequency and duration of holepokes (2 cm deep) and dips (1 cm deep). Each mouse received a 30-min habituation session during which the holes contained no odorants. The following day, a 20-min test day 1 session was conducted during which three of the corner holes were baited, as shown in Figure 1a, with a familiar odorant (fresh corncob bedding), a novel odorant (mint or coconut extract diluted onto a filter) or a novel, putatively rewarding odorant (chocolate chips or 33% sweetened condensed milk in water). The odorants were contained in a cup at the bottom of the hole (7 cm deep) and access blocked by a plastic mesh cap. The configuration of the odorant-containing and empty corner holes was counterbalanced within and across groups. Immediately following completion of test day 1, the mice were familiarized with the novel, rewarding odorant in their home cages (either 4–7 chocolate chips or 33% sweetened condensed milk in the drinking water), which was repeated 24 h later. Test day 2 was conducted 48 h after test day 1 using the same procedures as test day 1 with a different configuration of odorant-containing and empty corner holes (Figure 1a). Exploration was quantified as total holepoke frequencies and olfactory preference was assessed by quantifying differences in holepoke frequencies between odorant-containing or empty corner holes. All odorant-containing cups were cleaned with mild soap and water and the entire apparatus was cleaned with a 2% chlorohexidine diacetate solution (Nolvasan, Zoetis, Parsippany-Troy Hills, NJ).

Morris Water Maze.—Spatial memory acquisition and retention was assessed in the Morris water maze (MWM) as we previously described (Dougherty *et al.* 2013; Maloney *et al.* 2019). Briefly, the mice received two days each of four cued trials during which the platform was visible and tagged with a red tennis ball on a wooden dowel rod. Each animal was placed in the galvanized steel pool (120cm in diameter) filled with opaque water in the quadrant opposite the visible platform (11.5cm in diameter) and allowed 60 sec to find the platform and another 30 sec on the platform. Trials were separated by one h. These trials served to identify any non-associative differences between genotypes that may confound interpretation of the acquisition and retention trials, as well as to train the mice to perform in the task. Three days later, the mice received five days each of two blocks of two place trials during which the platform was submerged 2 cm and extra-maze cues used to aid the animals in spatial learning. For each trial block, the mice received two trials separated by 30–90 sec. The blocks of two trials were separated by 2 hours. The place trials were used to evaluate spatial acquisition performance. One h following the completion of the last place trial, a 60 sec probe trial was used to examine the memory retention of the mice by removing the platform and quantifying time spent in each quadrant and crossings of the previous platform location while the animal searched for the escape platform. The swim pathway of each animal was tracked with ANY-maze tracking software (Stoelting Co., Wood Dale, IL), connected to a digital video camera mounted to a PC computer.

Learning Set and Massed-trial Reversal MWM.—To examine cognitive flexibility in reversal-style MWM tasks different to that previously used with this model (Dougherty *et al.* 2013), we used both the learning set MWM (spaced trials) and the massed-trial reversal MWM. The pool and tracking set up was the same as that for the typical MWM. The procedure for the learning set MWM was as previously described (Ghoshal *et al.* 2012). Briefly, the mice received four trials each of five consecutive days, lasting 60 sec maximum and segregated by 30 sec inter-trial interval (ITI), and blocked into pairs separated by one h (Figure 2a). At completion of each trial, the animal received 15 sec on the platform followed by 30 sec in a holding cage. On each test day, the platform was moved to a new location in the pool. Short-term working memory was assessed by the performance on average during the second trials each day and general acquisition levels were determined by examining differences between Trial 1 and Trial 4 on each test day. For the Massed-trial reversal MWM, each animal received 20 massed acquisition trials, to match the number of trials conducted during a typical MWM acquisition training. Trials were conducted in blocks of four (60 sec maximum, 30 sec and 15 min ITIs) with a 15 min inter-block interval (IBI; Figure 2a). One h following the acquisition trials, four reversal trials were conducted in blocks of two with a 30 sec ITI and 15 min IBI.

Marble Burying Task.—Marble burying behavior in mice serves as a proxy for repetitive and perseverative digging behavior (Thomas *et al.* 2009; Angoa-Pérez *et al.* 2013), and possibly anxiety-related behavior. Our procedure was previously described (Maloney *et al.* 2018). Briefly, mice were placed in a transparent enclosure (47.6 × 25.4 × 20.6 cm) with fresh clean Aspen bedding and 20 clear marbles. After 30 minutes of free exploration, the animal was removed and two independent observers scored buried marbles using the criterion for buried if two-thirds of the marble was covered by bedding, and the average

score of buried marbles was analyzed. Testing was conducted under dim overhead lighting. The correlation between observers' scores for all marble burying experiments in this study was $r > .94$, $p < .000005$. In between animals, the enclosure and all marbles were cleaned thoroughly with 70% ethanol.

Spontaneous Alternation T-maze.—The spontaneous alternation T-Maze was used to assess perseverative exploratory behavior and was previously described (Maloney *et al.* 2018). Testing was conducted under dim overhead lighting. The opaque acrylic apparatus comprised a 20×8.7 cm start chamber with two radiating arms, each measuring 25×8.7 cm. Testing consisted of 10 consecutive trials, with a maximum length of two min. After a two min habituation to the start box, the door was then raised and the animal allowed to choose either the right or left arm of the maze. An arm choice was determined when the animal entered the arm with all four paws. The door to that arm was then closed, and the animal allowed to explore for five seconds and then allowed to return to the start box. If the animal did not quickly move back to the start box, it was gently guided by placement of a hand or object behind the animal. After five seconds, the start box door was again lifted to start the next trial. After 10 consecutive trials, the animal was returned to its home cage and the apparatus cleaned thoroughly with a chlorohexidine diacetate solution (Nolvasan, Zoetis, Parsippany-Troy Hills, NJ). Each of the trials was scored as an alternation, a non-alternation or no choice trial. The percent of trials exhibiting an alternation was compared between groups.

Sucrose Preference Test.—The sucrose preference test is designed to assess an animal's responsiveness to natural reward, and a lack of sensitivity to this reward is suggested to model anhedonia (Barrot *et al.*, 2002; Monteggia *et al.*, 2007). The testing apparatus consisted of a transparent enclosure ($47.6 \times 25.4 \times 20.6$ cm) equipped with two water bottles (Small Animal Water Bottle, Great Choice; $16.5 \times 7.6 \times 3.8$ cm) hanging on either end (Figure 3a). A thin layer of fresh corncob bedding and five food pellets were placed on the floor. For habituation, the homecage water bottle was removed one h prior to testing to motivate drinking during the session. Two 1-h sessions were conducted on two consecutive days (Figure 3b). During the first, both water bottles were filled with 100 mL of water only, and during the second, both were filled with 100 mL of 1% sucrose in water. The bottles were weighed before and after testing to approximate liquid consumed. The next day, a 48-h testing session began, during which one bottle contained 100 mL of 1% sucrose water and the other 100 mL of water only. The location of the water vs. sucrose bottles was counterbalanced across groups. At 24 h, the bottles were weighed, filled with new water or 1% sucrose, and moved to the opposite side of the chamber to prevent side bias effects.

1-h Locomotor Activity/Exploration.—A 1-h locomotor activity/exploration test was conducted to assess the general activity, exploratory behavior, and anxiety-related levels of the mice as we previously described (Dougherty *et al.* 2013). Briefly, the mice were evaluated over a 1-h period in transparent enclosures ($47.6 \times 25.4 \times 20.6$ cm) surrounded by metal frames containing 4×8 matrices of photobeam. Computer software (MotorMonitor, KinderScientific, LLC, Poway, CA) quantified horizontal and vertical beam breaks as ambulations and rearings, respectively. the movement of the animal within a 33×11 cm

central zone and a bordering 5.5 cm peripheral zone. General activity (total ambulations), exploration (rearing) and measures of anxiety-related behavior (including time spent, distance traveled and entries made into the central area) were analyzed. Each enclosure was cleaned with 70% ethanol solution between each mouse.

Sensorimotor battery.—Balance, strength, and coordination were evaluated by a battery of sensorimotor measures using previously published procedures (Dougherty *et al.* 2013) to identify any sensorimotor issues that might confound interpretation of subsequent tests. The battery included walking initiation, ledge, platform, pole, and inclined and inverted screen tests. An observer manually recorded time in hundredths of a sec for each test. Two trials were conducted for each test and the average of the two yielded a single time used in the analyses. To avoid exhaustion effects, the order of the tests during the first set of trials was reversed for the second set of trials. All tests lasted a maximum of 60 sec, except for the pole test, which lasted a maximum of 120 sec. Walking initiation was assessed by placing the mouse on a flat surface inside a square measuring 21×21 cm, and recording the time to leave the square (i.e. all four limbs concurrently outside of the square). The ledge test required the mouse to balance on a clear acrylic ledge, measuring 0.50 cm wide and standing 37.5 cm high. Time the mouse remained on the ledge was recorded. During the platform test, the mouse used basic balance ability to remain on a wooden platform measuring 1.0 cm thick and 3.3 cm in diameter and elevated 27 cm above the floor. The time the mouse was able to balance on the platform was recorded. The pole test was used to evaluate fine motor coordination. The mouse was placed head upward on a vertical pole with a finely textured surface and the time taken by the mouse to turn downward 180° and climb to the bottom of the pole was recorded. The 60° , 90° , and inverted screen tests assessed a combination of coordination and strength. The mouse was placed head oriented downward in the middle of a mesh wire grid measuring 16 squares per 10 cm, elevated 47 cm and inclined to 60° or 90° . The time required by the mouse to turn upward 180° and climb to the top of the screen was recorded. For the inverted screen test, the mouse was placed head oriented downward in the middle of a mesh wire grid measuring 16 squares per 10 cm, elevated 47 cm, and, when it was determined the mouse had a proper grip on the screen, it was inverted to 180° . The time the mouse was able to hold on to the screen without falling off was recorded.

Cocaine Conditioned Place Preference.—We assessed reward conditioning in our model using the cocaine conditioned place preference (CPP) based on previously published methods (Bruchas *et al.* 2011; Al-Hasani *et al.* 2013). The acrylic CPP apparatus comprises three chambers: two outer chambers measuring $26 \times 26 \times 26$ cm each connected via door openings to a center chamber measuring $7.5 \times 26 \times 26$ cm (Figure 4a). Each outer chamber was marked with either horizontal or vertical black and white stripes (2.5 cm wide). Each apparatus was placed in a custom-made expanded PVC board sound- and scent-attenuating box ($70.5 \times 50.5 \times 60$ cm) equipped with a video camera (700 TVL 960H day/night wide angle board camera, Supercircuits Security, Austin TX) mounted above and connected to a computer for tracking of the animals during testing via BnC cables and a Quad processor (Real time color quad processor, Supercircuits Security, Austin TX). The CPP procedure occurred on five consecutive days. (Figure 4b). For the pre-conditioning trial on day 1, each animal was allowed free access to the entire apparatus for 30 mins. For each the 3

conditioning days (which occurred on procedure days 2 – 4), each animal received a 30-min saline-paired control trial and, 4 h later, a 30-min drug-paired conditioning trial. For the saline-paired control trial, each animal received a 10 ml/kg subcutaneous (s.c.) saline injection and was isolated in either the vertical or horizontal outer chamber for 30 min. For the drug-paired conditioning trial, each animal received either a 10 ml/kg s.c. saline injection (control mice) or a 15 mg/kg s.c. cocaine injection and was isolated in the opposite chamber as the saline-paired control trial. Drug (cocaine versus saline) was counterbalanced between genotypes and the mice were randomly assigned to either the vertical or horizontal striped chamber. For the post-conditioning trial on day 5, each animal was again allowed free access to the entire apparatus for 30 min. The apparatus was cleaned in between each mouse with 2% chlorohexidine diacetate disinfectant solution (Nolvasan, Zoetis Animal Health, Florham Park, NJ). Time spent in each chamber during pre- and post-conditioning trials as well as distance traveled during the conditioning trials was monitored using ANY-maze tracking software (Stoelting Co., Wood Dale, IL). Cocaine conditioning was measured by the conditioning score (time in the drug-paired chamber post-conditioning trial minus pre conditioning trial) (Tzschentke 2007).

Conditioned Fear Test.—Fear conditioning was evaluated in our mice following our previously described procedure (Dearborn *et al.* 2015; Maloney *et al.* 2019). Briefly, each mouse was habituated to and tested in an acrylic chamber (26 × 18 high x 18 cm) containing a metal grid floor, an LED light bulb and an inaccessible peppermint odorant. The chamber light turned on at the start of each trial and remained illuminated. The testing session on day 1 was 5 min during which time an 80 dB tone (white noise) sounded for 20 sec at 100 sec, 160 sec and 220 sec. A 1.0 mA shock (unconditioned stimulus; UCS) was paired with the last two sec of the tone (now conditioned stimulus; CS). The baseline freezing behavior during the first two min and the freezing behavior (conditioned response; CR) during the last three min was quantified through the computerized image analysis software program FreezeFrame (Actimetrics, Evanston, IL). The testing session on day 2 was for 8 min. The light was illuminated during the entire trial and no tones or shocks were presented. This procedure allowed for the evaluation of freezing behavior (CR) in response to the contextual cues associated with the shock stimulus (UCS) from day 1. The testing session on day 3 was 10 min in duration and the context of the chamber was changed to an opaque acrylic-walled chamber containing a different (coconut) odorant. The 80 dB tone (CS) began at 120 sec and lasted the remainder of the trial. Freezing behavior to habituation to the new context (pre-CS) was quantified during the first two min. Freezing behavior (CR) to the auditory cue (CS) associated with the shock stimulus (UCS) from day 1 was quantified during the remaining 8 min. Shock sensitivity was evaluated following testing as previously described (Maloney *et al.* 2019).

Statistical Analysis

All statistical analyses were performed using the IBM SPSS Statistic software (v.24). One-way and factorial ANOVAs, included repeated measures (rm) ANOVAs were used where appropriate. The Huynh-Feldt correction was applied to violations of the sphericity assumption for rmANOVAs. With a statistically significant interaction between main factors, simple main effects were calculated to provide clarification of statistical significance

between-genotype and within-genotype differences. Multiple comparisons were Bonferroni adjusted. Tukey's HSD or the Games-Howell method was used as post hoc tests. Probability value for all analyses was $p < .05$. Data from each of the five cohorts were first analyzed independently across cohorts. Where cohorts showed differences in significance meta-analysis were applied to summarize across studies (Holeboard total pokes outcome variable and fear conditioning freezing in males).

Results

Independent replication of the resistance to change phenotype observed in *Celf6*^{-/-} mice.

We previously demonstrated *Celf6* homozygous mutant mice exhibit a phenotype of resistance to change their behavioral response patterns during the holeboard exploration/olfactory preference task (holeboard) as well as decreased levels of monoamines (Dougherty *et al.* 2013). In the present work, we conducted additional behavioral analyses to provide a better understanding of this phenotype. This involved testing three hypotheses relevant to the interpretation of the original behavioral phenotyping results observed in the *Celf6* mutants based on our previous holeboard results. Testing the first hypothesis included a further assessment of our assertion that resistance to change is a reliable phenotype of *Celf6* mutant mice. This entailed testing them and littermate controls on several tasks including an evaluation of their performance on the original holeboard procedure, as well as on learning set and massed-trials MWM protocols, marble burying and spontaneous alternation in a T-maze. The second hypothesis involved evaluating the possibility that *Celf6* mutant mice do not find sweetened foods immediately rewarding, since this may have contributed to the lack of shift in their odor preference to the sweetened food following familiarization with it during the original holeboard test. The sucrose preference test was used for this purpose. The third hypothesis focused on the possibility that mice lacking the *Celf6* protein may have intact immediate responses to rewarding stimuli but their associative learning or conditioning capabilities are compromised and that this condition precludes altering future behavior based on positive reinforcement. To test the third hypothesis, we assessed the performance of the mice on the conditioned fear and cocaine-CPP tasks.

As a precursor to these analyses, we attempted to replicate the published holeboard findings in our mutant model with a second, independent cohort consisting of *Celf6*^{-/-} and WT littermates (Cohort 1), this time including both males and females. The addition of females allowed us to examine if the resistance to change phenotype observed in *Celf6*^{-/-} male mice in this assay extended to *Celf6*^{-/-} females. These mice were first tested following a similar holeboard procedure as previously published (Dougherty *et al.* 2013) using a different novel odorant (mint extract) and novel rewarding odorant (33% sweetened condensed milk in water; Figure 1a). Previous research demonstrated mice will acquire self-administration of 33% sweetened condensed milk in water which is indicative of reward (Thomsen & Caine 2011). Following familiarization with the sweetened solution, the *Celf6*^{-/-} mice once again failed to exhibit the WT-like robust potentiation of hole-poking and change in olfactory preference behaviors. A test day x genotype interaction was observed for total hole poke frequency, $F(1,18)=11.931$, $p=.003$ (Figure 1b), due to WT mice displaying more total hole pokes on test day 2 compared to *Celf6*^{-/-} mice, $F(1,36)=9.881$, $p=.006$. In addition, WT

mice exhibited more hole pokes on test day 2 relative to test day 1, $F(1,18)=7.890$, $p=.024$, whereas *Celf6*^{-/-} mice did not. The change in olfactory preference behavior was quantified by a test day x genotype interaction, $F(1,18)=6.920$, $p=.017$ (Figure 1c). WT mice exhibited more hole pokes into the odorant-containing holes overall during test day 2 compared to *Celf6*^{-/-} mice, $F(1,144)=13.174$, $p=.0008$, which is most pronounced in the hole containing familiar bedding, $F(1,144)=11.398$, $p=.007$. WT mice did not show as great of an increase over *Celf6*^{-/-} mice in hole-poking into the novel odorant and rewarding odorant hole following milk familiarization as was reported previously following chocolate chip exposure. This difference between cohorts may be due to different olfactory properties between the milk and chocolate chips, and also between the novel woodchip bedding (used in Dougherty *et al.* 2013) and mint extract used here. We did not observe a statistically significant interaction involving genotype and sex, indicating that both male and female mice of each genotype performed comparably in this task. On test days 1 and 2, both WT and *Celf6*^{-/-} mice poked more in the hole containing familiar bedding versus the empty corner hole ($p<.012$), indicating the mice were not anosmic and confirming the previous holeboard findings.

To further understand if the reduction in exploration in the *Celf6*^{-/-} mice is due to time spent engaging in repetitive behaviors, we examined consecutive dips in any single hole, which has been previously described as a sign of stereotypy (Makanjuola *et al.* 1977a, 1977b). A dip is recorded when the animal's head passes at least 1 cm down the hole whereas a poke is recorded when the head passes at least 2 cm. We examined dips as our measure of continuous perseveration to ensure we included all entries into the hole made by the animal, thus accounting for any entries into the hole that did not register as a full poke. No differences between genotypes were observed for mean number of consecutive dips into any single hole (Figure 1d) or maximum number of consecutive dips (test day 1, *Celf6*^{-/-}: $M=5.25$, $SD=2.38$, WT: $M=3.6$, $SD=1.65$; test day 2, *Celf6*^{-/-}: $M=4.25$, $SD=2.70$, WT: $M=4.1$, $SD=1.37$), indicating the *Celf6*^{-/-} mice did not repetitively dip their heads into any one hole at a greater frequency than their WT littermates. Thus, the decreased exploration is likely explained by a different behavioral response, or lack thereof, in the *Celf6*^{-/-} mice.

The holeboard was repeated using an all-male cohort that also included *Celf6*^{+/-} mice to determine gene dosage effect on this phenotype (Cohort 2). In addition, we did not familiarize half of this cohort with chocolate chips to determine the necessity of this familiarization for the potentiation of hole-poking behavior. Coconut extract replaced the mint extract as the novel odorant. Surprisingly, we did not observe the robust increase in general exploratory hole poking behavior in the WT male mice following familiarization with chocolate chips, such that *Celf6*^{-/-}, *Celf6*^{+/-} and WT littermates exhibited the same exploratory behavior on test day 2, regardless of familiarization (Figure S1a). Olfactory preferences were changed with familiarization; however, this was independent of genotype. (Figure S1b). During test day 1, no difference in olfactory preference was observed between mice that went on to receive familiarization compared to those that did not. All mice exhibited a similar olfactory preference, $F(2,287,121.220)=124.305$, $p<.000005$ (main effect of hole) such that the hierarchy was familiar bedding > chocolate chips > coconut extract=empty ($p<.001$). A significant hole x familiarization interaction, $F(2,705,143.387)=5.750$, $p=.001$, was observed for test day 2. Mice that did not receive familiarization continued to

show a preference, $F(3,51)=20.120$, $p<.000005$, for the familiar bedding over all other holes ($p<.000005$). Mice that received familiarization showed a strong preference, $F(3,51)=34.345$, $p<.000005$, for both the familiar bedding and the now familiar chocolate chips over both the empty and coconut extract holes ($p<.005$), while actually showing an aversion to the coconut odorant by poking significantly more in the empty hole ($p=.002$). When directly compared, mice that received familiarization to the chocolate chips poked more frequently into the hole containing the chocolate chips on test day 2 than unfamiliarized mice, $F(2,226)=8.806$, $p=.012$. Animals in Cohort 2 were younger than both Cohort 1 or the previously published cohort (Dougherty *et al.* 2013). It may be that the influence of *Celf6* loss on exploratory behavior in response to familiarization with a rewarding substance is greater in older animals, thus accounting for the lack of effect in Cohort 2 here.

To understand how this cohort may differ from the previous two and to get an estimate of the overall effect of *Celf6* mutation on Holeboard behavior after reward familiarization, we conducted a meta-analysis of total holepokes from all three cohorts (Dougherty *et al.* 2013 cohort, Cohort 1 and Cohort 2; since only Cohort 2 included *Celf6*^{+/-}, this genotype was excluded from the meta-analysis). We analyzed the combined datasets using a rmANOVA with genotype and cohort as between-subjects. The test day x genotype interaction was again observed, $F(1,56)=10.264$, $p=.002$, $f=.43$ with observed power of .88. All combined, WT mice exhibited a greater number of holepokes following reward familiarization compared to *Celf6*^{-/-} littermates, $F(1,112)=14.901$, $p=.0004$, $f=.36$ (Figure S1c). Thus, overall the effect of *Celf6* loss on this behavior is on the large end of moderate. The test day x genotype x cohort interaction, $F(2,56)=4.205$, $p=.020$, $f=.39$, confirmed the difference between WT and *Celf6*^{-/-} mice holepokes on test day 2 in the Dougherty *et al.* 2013 cohort, $F(1,112)=14.672$, $p=.001$, $f=.36$, and Cohort 1, $F(1,112)=12.043$, $p=.004$, $f=.33$, but not in Cohort 2, $F(1,112)=0.323$, $p=.571$, $f=.05$ (Figure S1d). To understand if age might be playing a role in the lack of effect of mutation in Cohort 2, we examined age in months at testing between the cohorts, and found Cohort 2 was significantly younger than the other two cohorts, $F(2,56)=23.271$, $p<.000005$, $f=.91$ (Dougherty *et al.* 2013, $M=7.40$, $SD=1.19$; Cohort 1, $M=5.95$, $SD=0.95$; Cohort 2, $M=5.05$, $SD=1.10$). These data indicate *Celf6* loss has a largely moderate effect on activity potentiation in response to a reward, and that age may also play a large role in exploration potentiation in this task.

***Celf6*^{-/-} mice do not show overall repetitive patterns of behaviors.**

The original premise for the holeboard task was based on clinical work showing repetitive interests in children with autism often result in a reduction of novel exploration, and that tasks of exploration in mice may be a way to model this (Pierce & Courchesne 2001; Moy *et al.* 2008). Thus, the holeboard offered a way to measure exploratory differences as well as olfactory preferences and hole-poking perseverations. To understand if the lack of potentiated exploration in our *Celf6* mutant mice is due to repetitive interests/patterns of behavior in general, and to distinguish any reward learning from the perseverative behavior, we conducted three different tasks designed to measure inability to shift behavioral patterns that lack a strong reward stimulus, as well as a task designed to test an animal's responsiveness to a natural reward (Barrot *et al.* 2002). This allowed us to assess repetitive

behavior patterns in the *Cellf6*^{-/-} mice using three distinct motivators: escape from water, digging compulsion, and novel environment exploration.

We originally observed a tendency towards a failed initial reversal in the MWM by *Cellf6*^{-/-} mice (Dougherty *et al.* 2013), therefore we evaluated Cohort 1 mice in two MWM paradigms that contain more extensive reversal trials to measure resistance to change utilizing the motivation to escape the water and end swimming. We used both the learning set MWM and massed-trial reversal MWM to test reversal after a 24-h or 1-h inter-trial interval (ITI) between acquisition and reversal, respectively. Including both test paradigms allows for cognitive flexibility assessment following both spaced and massed trial presentation. It has been suggested that spaced learning may induce better memory acquisition than massed trials (Sisti *et al.* 2007). Thus, reversal learning may be differentially impacted by the type of trial learning used prior to reversal. Testing was separated by approximately two months. No significant sex interactions with genotype were observed during any MWM performance, therefore data are collapsed for sex. As a control for intact spatial learning and retention, we tested *Cellf6*^{-/-} and WT littermates in the standard MWM paradigm one week prior to learning set MWM and replicated the initial findings of WT-like spatial learning and retention performance in the *Cellf6*^{-/-} animals (Figure S2a–c). During the learning set MWM, the platform was placed in a new location each day for five consecutive days of testing with four trials each day (Figure 2a). This paradigm required proper reversal performance each new test day, therefore, we were able to assess the reversal performance of the *Cellf6* mutant mice across five consecutive days. Performance during trial 2 each day represents a measure of short-term working memory because the mouse must recall the platform location from the immediately preceding trial rather than the learned platform location from the previous test day. WT and *Cellf6*^{-/-} mice exhibited similar path lengths to the escape platform during average trial 2 performance, $F(1,18)=.143$, $p=.710$ (Figure 2b). All mice also demonstrated intact general acquisition performance quantified by the daily decrease in escape path length from trial 1 to trial 4 on average within WT, $F(1,18)=39.433$, $p=.000006$, and *Cellf6*^{-/-}, $F(1,18)=39.155$, $p=.000007$, mice (Figure 2c) with no difference between genotypes, $F(1,18)=.365$, $p=.553$. *Cellf6*^{-/-} mice acquired each new platform location in a manner comparable to WT littermates without demonstrating a failure to reverse to the new locations.

Similar results were obtained during massed-trial reversal MWM. In this task, all mice received four blocks of two sets of two acquisition trials each, followed by one block of two sets of two reversal trials, with a one-h IBI (Figure 2a). This paradigm required proper reversal performance one h following mass acquisition trials, therefore, we were able to assess the reversal performance of the *Cellf6* mutant mice immediately following massed acquisition. *Cellf6*^{-/-} mice traveled comparable escape path length distances during both acquisition trials, $F(1,18)=0.320$, $p=.579$, and reversal trials, $F(1,18)=0.013$, $p=.912$ (Figure 2d). Once again, *Cellf6*^{-/-} mice did not demonstrate a failure to reverse to the new platform location. No differences between genotypes were observed for swimming speeds for any MWM procedure, indicating the *Cellf6* mutation did not influence ability to swim. Overall, these reversal MWM data indicate the *Cellf6*^{-/-} mice did not demonstrate a cognitive inflexibility following a 24-h or 1-h acquisition to reversal interval in a MWM paradigm.

Next, we investigated the resistance to change phenotype of the *Celf6* mutant mice by assessing repetitive patterns of behavior in low-stress tasks that use ethologically natural behaviors, digging and exploratory alternation, as motivation to perform. During the marble burying task, *Celf6*^{-/-} mice did not demonstrate repetitive digging behavior, and actually displayed a tendency to bury fewer marbles than WT littermates, $F(1,18)=3.127$, $p=.094$. Interestingly, a significant genotype x sex interaction, $F(1,18)=4.926$, $p=.040$, revealed the genotype difference was driven by the low numbers of marbles buried by the *Celf6*^{-/-} females in comparison to WT females, $F(1,18)=8.945$, $p=.016$ (Figure 2e). No difference was observed between males. Similarly, the male-only Cohort 2 mice did not differ in repetitive burying behavior between genotypes, $F(2,55)=.543$, $p=.584$ (Figure 2f). During spontaneous alternation T-maze, *Celf6*^{-/-} mice alternated at the same percent as the WT littermates, $F(1,18)=0.014$, $p=.907$ (Figure 2g). No significant effects involving sex were observed. The performance of *Celf6* mutant mice relative to their WT littermates indicates these mice do not exhibit repetitive behavior patterns as assessed in these tasks. Taken together with the learning set and massed-trial reversal MWM data, we can infer that in the absence of a strongly rewarding stimulus, the *Celf6* mutant mice do not exhibit restricted patterns of behavior that could account for the reduced exploration in the holeboard task. Therefore, we next turned to assessing both acute and learned responses of *Celf6*^{-/-} mice to rewarding stimuli.

***Celf6*^{-/-} mice are responsive to natural reward.**

The above findings of negative overall repetitive patterns of behavior suggest the exposure to the rewarding stimulus may play a key role in the resistance to change phenotype observed in *Celf6*^{-/-} mice in the holeboard. It is possible loss of *Celf6* alters response to reward. To address this, we assessed the performance of our Cohort 1 mice in the sucrose preference test (Figure 3a,b), a task designed to test an animal's responsiveness to natural reward (Barrot *et al.* 2002) in an acute setting that does not require learning. Sucrose was used because mice find it rewarding and will self-administer it (Alsiö *et al.* 2011), and it can be dissolved in drinking water. No difference in volume of liquid consumed was observed between *Celf6*^{-/-} and WT mice during habituation sessions with free access to either pure drinking water or to 1% sucrose water (Figure 3c). During the 48-h preference test in which the mice were given a choice between a bottle of pure water or a bottle of 1% sucrose water, both *Celf6*^{-/-}, $F(1, 18)=55.044$, $p<.000005$, and WT, $F(1,18)=31.367$, $p=.000026$, mice consumed more 1% sucrose water than pure water, with no difference between genotypes in amount consumed of either type of water (Figure 3d). Within this sucrose preference paradigm, *Celf6*^{-/-} mice did not demonstrate a disturbance in response to a natural reward in an acute setting. This suggests that the resistance to change behavior by the *Celf6* mutants in the holeboard was not a result of decreased sensitivity to the rewarding properties of sweet foods.

Interestingly, a significant bottle x genotype interaction, $F(1,18)=4.884$, $p=.040$, for time spent near the drinking bottles during each habituation session was observed. *Celf6*^{-/-} mice did not exhibit a WT-like increase in time spent near the drinking bottles during the sucrose habituation compared to water habituation, $F(1,18)=6.208$, $p=.023$ (Figure 3e), despite entering this area at a similar rate (Figure 3f). During the 48-h testing, this difference in time

spent and entries into the zones directly below the drinking bottles containing 1% sucrose or pure water (Figure 3g,h) was no longer observed; possibly due to the longer duration of the test. The differences observed during habituation are likely due to a decrease in overall locomotor activity observed in the *Celf6*^{-/-} mice. Indeed, *Celf6*^{-/-} mice exhibited lower locomotor activity and exploratory rearing compared to WT mice during the habituation sessions, $F(1,18)=10.587$, $p=.004$ and $F(1,18)=16.770$, $p=.0007$, respectively (Figure 3i). Compared to WT performance over the 48 h of preference testing, the *Celf6*^{-/-} mice exhibited a trend towards less locomotor activity, $F(1,18)=3.463$, $p=.079$, and significantly less exploratory rearing, $F(1,18)=7.416$, $p=.014$ (Figure 3j). Results from prior assessment of Cohort 1 *Celf6*^{-/-} mice in the 1-h locomotor activity/exploration test mirror the results in the sucrose preference task. When tested two months earlier in identical chambers (although lacking the drinking bottles), *Celf6*^{-/-} mice again exhibited a trend towards hypoactivity. Analysis of total horizontal ambulations across the 60-min test session yielded a genotype x ten-min-block interaction, $F(5,90)=2.659$, $p=.027$, yet the differences between the genotypes during the individual 10-min blocks did not reach significance after correction for multiple comparisons (specifically, the third 10-min block; Figure S3a). These results again suggest the *Celf6*^{-/-} mice are marginally hypoactive in response to a novel environment. A significant effect of genotype was observed for rearing $F(1,18)=5.347$, $p=.033$, indicating a persistent decrease in exploratory behavior by the *Celf6*^{-/-} mice (Figure S3b). No differences in the duration spent or entries made into the center zone of this task, indicating no differences in anxiety-like behaviors (Figure S3c). Overall, these findings are contradictory to the published cohort (Dougherty *et al.* 2013), in which we observed no differences compared to WT littermates in activity or exploratory levels in the 1-h locomotor activity/exploration test. This suggests the inclusion of females may be driving the effects observed in Cohort 1. However, no significant main effects of sex or sex x genotype interactions were observed. We tested Cohort 2 in the 1-h locomotor activity/exploration test, again composed entirely of males, but of greater sample size to increase the power of our analyses compared to Cohort 1. Similar to our original cohort, we did not observe any differences between genotypes in activity-, exploratory-, or anxiety-related behaviors in this cohort (Figure S3d-f). We do not think the decreased rearing observed in our Cohort 1 is a result of decreased hindlimb strength, as the *Celf6* mutant mice of both Cohorts 1 and 2 performed similarly to WT mice during a battery of strength, coordination and balance. Specifically, *Celf6*^{-/-} and *Celf6*^{+/-} mice were able to initiate movement, balance atop a ledge and small platform, climb down a pole, and navigate vertical and inverted wire screens comparably to WT littermates (Figure S3g-p). However, the initial lack of increase in time near the rewarding drinking bottle suggests a possible disruption in conditioning to the reward in our mutant mice, but that interpretation may be confounded by altered locomotor activity levels. To address this and possible associative learning deficits outside of the spatial memory domain, we next examined our mice in conditioning paradigms.

***Celf6*^{-/-} mice demonstrate a robust conditioned learning deficit.**

We next sought to understand if an associative learning deficit, particularly in conditioning, underlie the failure of the *Celf6*^{-/-} mice to potentiate hole-poking in response to familiarization with a reward and to increase the time spent near the sucrose water bottle. We

tested the behaviors of our mutant mice and their WT littermates in conditioning paradigms, including conditioned place preference for cocaine and the conditioned fear task.

To assess conditioning in response to reward in our *Celf6* mutant mice, we used an independent cohort of females and males tested only in cocaine CPP at two to four months of age (Cohort 3; Figure 4a,b). Because of the drug exposure, these mice were not used for any other behavioral testing. Sex did not result in a significant main or interaction effect in our two-way ANOVA (sex and genotype as factors), therefore the data are presented collapsed across sex. Cocaine exposure resulted in significant preference for the drug-paired chamber in the majority of mice, $F(1,49)=8.415$, $p=.006$ (main effect of drug; Figure 4c), however conditioned place preference differences between WT and mutant mice were observed. When compared to saline-only mice, the cocaine-treated *Celf6*^{-/-} mice failed to show conditioning to the drug-paired chamber $F(1,49)=0.33$, $p=.857$, unlike cocaine-exposed WT and *Celf6*^{+/-} littermates, $F(1,49)=6.798$, $p=.036$ and $F(1,49)=7.087$, $p=.030$, respectively. Repeated cocaine exposures in mice results in locomotor sensitization (Eisener-Dorman *et al.* 2011), therefore we also analyzed distance traveled in the drug-paired chamber during the three conditioning days to investigate the acute responses of our mice to cocaine exposure. Only cocaine-exposed WT mice show a significant change in locomotor activity compared to saline-exposed mice, $F(1,49)=18.805$, $p=.0002$, which occurred on conditioning days 2 and 3, $F(1,147)=11.978$, $p=.002$ and $F(1,147)=31.763$, $p<.00005$. *Celf6* mutant mice locomotor activity in response to repeated exposures of cocaine was not different than that of saline-treated mutants, $F(2,49)=3.465$, $p=.039$ (main interaction effect of genotype x drug; Figure 4d) suggesting that the absence of *Celf6* may alter plasticity mechanisms in these circuits. Activity levels for cocaine-exposed *Celf6* mutants, along with WT littermates, were different on the last conditioning day as compared to the first, $p<.012$, despite a lack of difference from saline controls. Importantly, no saline-exposed mice altered locomotor activity across the conditioning days and no differences between genotypes were observed within the saline-exposed mice, thus indicating the activity differences should be interpreted with respect to the response to cocaine and do not reflect underlying hypoactivity in the *Celf6* mutants in this task. Overall, the CPP results here indicate a clear lack of cocaine-induced conditioning by *Celf6* mutant mice.

Where the cocaine CPP task tests the animal's response to a rewarding stimulus, the conditioned fear paradigm uses a rodent's innate response of freezing in fear to a learned aversion to a stimulus (cue-dependent fear) or an environment (context-dependent fear) (Curzon *et al.* 2009). Thus, this test was employed to see if this conditioning deficit is isolated to reward or if it extends to aversive stimuli. It also serves to assess emotion-associated learning in our *Celf6* mutant animals. We tested an independent cohort of male *Celf6*^{-/-} and WT littermates to initially investigate fear conditioning in our mutants (Cohort 4). All mice exhibited comparable baseline freezing behaviors (Figure 4e). During the pairing of shock and tone/context, a significant genotype x min interaction, $F(1,210, 21.799)=5.825$, $p=.017$, was observed due to a reduction in freezing behavior by the *Celf6*^{-/-} mice during min 5, $p=.003$. This suggests a diminished reaction to shock in the mutant animals. During contextual fear conditioning testing on day 2, animals of each genotype demonstrated contextual conditioning by displaying significantly more freezing during the average of mins 1 and 2 during day 2 relative to day 1 (WT, $F(1,9)=18.424$, $p=.002$;

Cellf6^{-/-}, $F(1,9)=15.703$, $p=.003$). However, *Cellf6*^{-/-} mice showed attenuated contextual conditioning over the full eight mins of testing. A significant min x genotype interaction was observed, $F(5.655,101.790)=3.115$, $p=.009$. WT mice froze significantly more than *Cellf6*^{-/-} mice during mins 4 and 5, $p<.024$ (Figure 4f). The difference during mins 2 and 3 did not stand up to multiple comparison correction ($p<.040$, uncorrected). No significant effects involving genotype were observed for baseline freezing during day 3 indicating comparable baseline freezing behavior in the novel context prior to tone (Figure 4g). No significant effects of genotype were observed for auditory cue conditioning as demonstrated by comparable freezing during mins 3–10 on day 3, during which the tone from day 1 was again presented. These initial fear conditioning data indicate the *Cellf6*^{-/-} mice present an attenuated contextual conditioning phenotype, and extends the reward conditioning deficit to include aversive stimuli.

To understand if the heterozygous mutants present with the same conditioning deficits, we tested our independent male replication cohort (Cohort 2) comprised of heterozygous as well as homozygous mutants and their WT littermates in the conditioned fear test. The sample sizes of this independent cohort also served to increase our statistical power as compared to Cohort 4. As before, during day 1, our mutants showed comparable baseline freezing to that of WT littermates. Unlike in Cohort 4, *Cellf6*^{-/-} mice did not demonstrate a blunted reaction during the later mins of the tone/shock pairing (Figure 4h). At this increased statistical power we were able to detect a significant main effect of genotype, $F(2,50)=10.501$, $p=.0002$, during contextual fear conditioning testing on day 2, revealing the *Cellf6*^{-/-} mice froze significantly less overall than both the WT and *Cellf6*^{+/-} mice (Figure 4i). No significant effects involving genotype were observed for baseline freezing in a novel context during day 3 prior to tone exposure. Overall, a significant genotype effect, $F(2,50)=11.169$, $p=.0001$, indicated WT and *Cellf6*^{+/-} mice froze significantly more than *Cellf6*^{-/-} mice during presentation of the auditory cue (Figure 4j). A significant min x genotype interaction, $F(10.208,255.205)=2.229$, $p=.016$, was also observed for freezing behavior during the presentation of the auditory cue. WT and *Cellf6*^{+/-} mice froze significantly more than *Cellf6*^{-/-} mice during mins 5, 6, 7, and 8 ($p<.025$), although min 7 did not surpass Bonferroni correction.

To determine if the conditioning deficit in response to an aversive stimulus extended to females, we examined the performance in this task of a fifth independent cohort that included females, also including heterozygous mice. During day 1, our female mutants showed comparable freezing to that of female WT littermates during baseline and during the later mins of the tone/shock pairing (Figure 4k). During contextual fear conditioning testing on day 2, we detected a significant main effect of genotype, $F(2,27)=3.506$, $p=.044$, indicating WT females froze more than either the *Cellf6*^{+/-} or *Cellf6*^{-/-} females (Figure 4l). In addition, all female mice showed contextual conditioning by freezing more on average during min 1 and 2 on day 2 as compared to that on day 1, $F(1,27)=52.941$, $p<.000005$. During baseline freezing in a novel context on day 3 prior to tone exposure, WT females froze more during min 2 compared to both *Cellf6*^{+/-} or *Cellf6*^{-/-} females (minute x genotype interaction, $F(2,27)=3.875$, $p=.033$; minute 2 simple main effect, $F(2,54)=5.093$, $p=.018$). *Cellf6* mutant females exhibited comparable freezing behavior to WT littermates during presentation of the auditory cue on day 3 (Figure 4m). We conducted a meta-analysis of all

males from Cohorts 2, 4, and 5 to understand the overall effect of *Celf6* loss on contextual and cued fear conditioning. No significant effect of *Celf6* mutation was observed during baseline or training on day 1, although there is a marginal effect of genotype during training, $F(2,99)=2.841$, $p=.063$, likely driven by minute 5 (Figure S4a). *Celf6*^{-/-} males exhibited an deficit to contextual conditioning on day 2 compared to both *Celf6*^{+/-} and WT males (main effect of genotype, $F(2,99)=4.596$, $p=.012$; Figure S4b). Again, no differences were observed for baseline freezing in the new context on day 3. Both *Celf6*^{+/-} and *Celf6*^{-/-} exhibited overall impaired freezing in response to the cue on day 3 when collapsed across all 8 minutes (main effect of genotype, $F(2,99)=9.626$, $p=.0002$; Figure S4c). A significant genotype x minute interaction, $F(10.59,524.12)=1.964$, $p=.032$, revealed WT males froze significantly more during minutes 4 and 6 compared to both *Celf6*^{+/-} and *Celf6*^{-/-} mice, $F(2,792)=6.093$, $p=.016$ and $F(2,792)=10.178$, $p=.0003$, and just *Celf6*^{-/-} males during minutes 5 and 8, $F(2,792)=8.159$, $p=.024$ and $F(2,792)=7.959$, $p=.003$. The significant differences during minute 7 did not hold after Bonferroni correction for 8 comparisons. These data indicate the impact of *Celf6* loss in males on fear conditioning may be stronger than in females. However, the greater sample size of males may contribute to our ability to detect both contextual and cued deficits in males and only contextual differences in females. The differences in freezing behavior observed by the *Celf6* mutants in this task across all cohorts were not due to altered sensitivity to shock in the *Celf6* mutant animals as comparable shock current (mA) to exhibit response behaviors (flinch and escape behavior) were observed between *Celf6* mutants and WT littermates (Supplemental Table 1).

Our findings from three independent cohorts in the conditioned fear test suggest that complete loss of the *Celf6* protein results in a contextual, and to a lesser extent an auditory-cued, fear conditioning deficit. On the whole, the results of the conditioned fear task complement that of cocaine CPP and further support the conclusion that *Celf6*^{-/-} mice demonstrate deficits in conditioning, whether to rewarding or aversive stimuli.

Discussion

Mice lacking the RBP *Celf6* demonstrated a resistance to change phenotype in the holeboard exploration/olfactory preference task, while exhibiting relatively normal performance across other tasks measuring sensorimotor, spatial learning and memory, anxiety-like, and social approach behaviors (Dougherty *et al.* 2013). The resistance to change phenotype in the holeboard task is suggested to model reduced novel exploration that accompanies repetitive interests in children with autism (Moy *et al.* 2008). We sought to understand the core behavioral deficit that drives this potentiation failure in our mice by testing three hypotheses: global restricted interests, failure of immediate response to reward, or failure to alter behavior in response to reward (conditioning). A global restriction of interests or tendency to exhibit repetitive behavior patterns is not present in mice lacking *Celf6*. This suggests the reduction in exploration in response to familiarization is not secondary to time spent perseverating. A normal response to acute reward was observed in *Celf6* mutants, indicating reward insensitivity was not the root of the lack of potentiation. While the acute response to reward was not perturbed, when that reward was included in a learning paradigm, the *Celf6* mutant mice showed impaired conditioning. This disruption to conditioning extended to aversive, emotion-related stimuli, as presented in the conditioned fear paradigm. Thus, we

found that the resistance to change by the *Celf6* mutant in the holeboard was most parsimoniously explained as a failure of conditioning, as the mice had blunted responses even to potent rewarding stimuli such as cocaine.

It was previously reported that both Balb/c and BTBR mice failed to show a potentiation after familiarization in the holeboard exploration/olfactory preference task (Moy *et al.* 2008). They also showed comparatively low levels of overall exploratory nose poking, which was attributed to the high levels of anxiogenic behaviors (Moy *et al.* 2008). Other work has shown, when compared to C57BL/6 mice, the Balb/c mice failed to acquire cocaine CPP (Belzung & Barreau 2000), while the BTBR mice exhibited normal methamphetamine CPP (Kosaki & Watanabe 2016). However, both Balb/c and BTBR mice exhibited blunted conditioned fear responses compared to C57BL/6J mice (Chen *et al.* 1996; MacPherson *et al.* 2008; Kosaki & Watanabe 2016). In contrast, NR1-NMDA receptor subunit (NR1) hypomorphic mice, which underexpress NR1, exhibited enhanced nose poke responses into the hole containing the chocolate chips in this holeboard task, with overall holepoking patterns different from WT littermates (Moy *et al.* 2008). In conditioning paradigms, conditional deletion of NR1 from cells expressing either dopamine (DA) or corticotropin-releasing hormone resulted in normal cocaine CPP yet impaired food CPP and actually enhanced conditioned fear responses, respectively (Engblom *et al.* 2008; Zweifel *et al.* 2009; Gafford *et al.* 2014). These findings, along with those we presented here, suggest conditioning capabilities may be intertwined with the resistance to change phenotype in the holeboard. In particular, the familiarization to the rewarding food item likely requires a conditioning-like response to alter the behavior in the post-familiarization holeboard session.

RBPs provide post-transcriptional regulation and have the ability to variously impact splicing, transcript stability, translational efficiency, and RNA localization. RNA localization allows for local protein synthesis, which is required for proper learning and memory function (Kang & Schuman 1996; Antion *et al.* 2008; Wang *et al.* 2009; Redondo & Morris 2011); thus it is no surprise that animal models null for RBPs that regulate translation exhibit learning and memory deficits. As mentioned above, FXS is the most common form of ID and results from loss of the RBP FMRP. The murine model of FXS, *Fmr1*-deficient mice, demonstrates learning and memory functions similar to what we have reported in the *Celf6*-deficient mice: specifically, normal spatial acquisition and retention in the Morris water maze, yet impaired fear conditioning (Santos *et al.* 2014) and impaired cocaine CPP with blunted response to the locomotor sensitization effects of cocaine (Smith *et al.* 2014).

Other models of RBP dysregulation also show interesting conditioning phenotypes. For example, expression of the RBP HuD, a neuronal-specific RBP linked to stabilization and translation of transient mRNAs during brain development and plasticity (Bolognani *et al.* 2007), is induced by neuronal activity, and has been linked to seizures and other activity-dependent regulatory networks (Oliver *et al.* 2017). Murine models of HuD overexpression (Hud-OE) have elucidated its role in conditioning. Cocaine CPP is enhanced in HuD-OE models, and HuD mRNA and protein is elevated in the nucleus accumbens (NAc) following cocaine CPP in wild-type, inbred animals (Oliver *et al.* 2017). Yet, impaired fear conditioning has been observed in a dose-response manner in HuD-OE mice (Bolognani *et al.* 2007). Translin is a RBP located in neuronal dendrites and regulator of mRNA transport

and translation (Stein *et al.* 2006). Translin null females show enhanced fear conditioning while males show impaired spatial acquisition in the Morris water maze, yet both sexes demonstrate robustly reduced anxiogenic behaviors and region-specific reductions in monoamines (Stein *et al.* 2006). The authors suggest translin may affect monoamine signaling pathways or synthesis. Reductions in monoamine levels appear to be a theme in models of RBP dysregulation, suggesting a strong role for RBPs in proper function of circuits heavily involving monoamine activity.

Cocaine CPP requires DA-driven activation of D1 medium spiny GABA-ergic neurons of the NAc (Wang *et al.* 2014). Activation of the hippocampal dentate gyrus is sufficient to induce contextual fear conditioning in genetic models of memory loss (Perusini *et al.* 2017). These areas central to conditioning responses express at least low-levels of Celf6 (Maloney *et al.* 2016), yet these areas also require monoamine activity for proper circuit function (Fadok *et al.* 2009; Heath *et al.* 2015). Celf6 is highly expressed in the monoamine-expressing cell populations, specifically the locus coeruleus (noradrenergic), raphe nuclei (serotonergic) and the ventral tegmental area and substantia nigra (dopaminergic; Maloney *et al.* 2016). In addition, levels of these neurotransmitters are reduced in brains that lack Celf6 (Dougherty *et al.* 2013). Thus, loss of Celf6 specifically in these populations may be sufficient to recreate the conditioning deficits seen in constitutive *Celf6* mutants. Future studies are planned to address this question.

Drugs of abuse act heavily on the DA system, and DA is released into the NAc during presentations of both rewarding and aversive stimuli, suggesting that this system underlies both forms of conditioning (Young *et al.* 2005). In reward-based conditioning, such as that observed in drug abuse, DA activity in the NAc is altered in addicted subjects in response to drug-conditioned cues. The blunted locomotor sensitization observed in *Celf6* mutants indicates the cocaine-induced effects on DA usually associated with this behavior might be altered in terms of how those effects impact long-term potentiation in the ventral tegmental area and NAc circuits (Thomas *et al.* 2001). Indeed, the locomotor data suggest the *Celf6* mutants may be less sensitive to cocaine as compared to WT controls. Due to the noted fear conditioning deficit (Figure 4a-m) and the fact that cocaine-induced locomotion was not different in the CPP assay (Figure 4d), the deficit in cocaine CPP is unlikely to be caused by reduced drug sensitivity. However, a future dose-response analysis will be needed to determine if a subtle cocaine sensitivity difference might contribute to the magnitude of the noted CPP deficit. The conditioning deficits and monoamine impact observed in models of RBP polymorphisms have led to the investigation of the role these proteins play in addiction (Bryant & Yazdani 2016). In addition, molecular targets of RBPs have been shown to play key roles in the behavioral sequela of addiction. Cyfip2, a binding partner of FMRP, is a regulator of acute and sensitized responses to cocaine (Kumar *et al.* 2013), and is a plausible candidate for mediating the blunted responses to cocaine observed in both *Fmr1*-deficient and potentially *Celf6*-deficient mice.

Identifying the molecular targets and the molecular function of an RBP can also help elucidate the mechanism by which it acts in the central nervous system to disrupt behavior, and identify potential therapeutics. Much has been done in this regard with FMRP, both through CLIP (Darnell 2012) and loss of function studies (De Rubeis *et al.* 2014). For

example, mGluR has been a main target for therapeutic strategies due to the increased long-term depression observed in *Fmr1*-deficient mice, which is mGluR5-dependent (Santos *et al.* 2014). Agents that decrease mGluR5 activity and its downstream signaling have rescued FXS-related phenotypes in *Fmr1*-deficient mouse models. Many of the receptor signaling pathways that influence FMRP activity at the synapse, which in turn are dysregulated in the absence of FMRP, have been identified and pharmacologically targeted, such as NMDA, AMPA, BDNF, and mTOR (Bagni *et al.* 2012). In contrast, the molecular targets of Celf6 are just now being studied (Rieger *et al.* 2018), and its molecular function is beginning to be elucidated. In *in vitro* assays it has been shown to have some activity as a splicing factor (Ladd *et al.* 2004), but localization in the central nervous system includes predominant cytoplasmic labeling (Maloney *et al.* 2016), consistent with non-nuclear roles in regulating RNA stability. Integrating these behavioral and molecular findings, and coupling them with the already known patterns of Celf6 expression could provide opportunities to define novel pathways that modulate rewarding and aversive conditioning using this model. We establish here that the RBP Celf6 is required for normal conditioning both to rewarding and aversive stimuli, and that this requirement is clearly not mediated by anhedonia. Moving forward, these mice will be valuable to further examining the molecular targets and cellular circuits that underlie reward and fear conditioning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was funded by W.M. Keck Fellowship in Molecular Medicine from Washington University in St. Louis to SEM and the NIH (R01DA033396 to MRB; K99/R00DA038725 to RA; 1R21DA041883-01, R21DA038458, 5U01MH109133-03, 5R01MH107515-03, R00NS067239 to JDD). JDD is a NARSAD Independent Investigator. The authors declare no competing financial interests.

We would like to thank Katherine B. McCullough for behavioral data collection, Karen L. O'Malley, PhD and Steven K. Harmon for access to equipment and training, and Katherine B. McCullough, Kayla Nygaard and Emily Wasserman for copyediting.

References

- Abekhoukh S & Bardoni B (2014) CYFIP family proteins between autism and intellectual disability: links with Fragile X syndrome. *Front Cell Neurosci* 8; 81:1–8. [PubMed: 24478626]
- Al-Hasani R, McCall JG & Bruchas MR (2013) Exposure to chronic mild stress prevents kappa opioid-mediated reinstatement of cocaine and nicotine place preference. *Front Pharmacol* 4.
- Alsö J, Nordenankar K, Arvidsson E, Birgner C, Mahmoudi S, Halbout B, Smith C, Fortin GM, Olson L, Descarries L, Trudeau L-É, Kullander K, Lévesque D & Wallén-Mackenzie Å (2011) Enhanced Sucrose and Cocaine Self-Administration and Cue-Induced Drug Seeking after Loss of VGLUT2 in Midbrain Dopamine Neurons in Mice. *J Neurosci* 31, 12593–12603. [PubMed: 21880920]
- Angoa-Pérez M, Kane MJ, Briggs DI, Francescutti DM & Kuhn DM (2013) Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *J Vis Exp JoVE*.
- Antion MD, Merhav M, Hoeffler CA, Reis G, Kozma SC, Thomas G, Schuman EM, Rosenblum K & Klann E (2008) Removal of S6K1 and S6K2 leads to divergent alterations in learning, memory, and synaptic plasticity. *Learn Mem Cold Spring Harb N* 15, 29–38.
- Bagni C, Tassone F, Neri G & Hagerman R (2012) Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest* 122, 4314–4322. [PubMed: 23202739]

- Barrot M, Olivier JDA, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC, Zachariou V & Nestler EJ (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc Natl Acad Sci U S A* 99, 11435–11440. [PubMed: 12165570]
- Belzung C & Barreau S (2000) Differences in Drug-Induced Place Conditioning Between BALB/c and C57Bl/6 Mice. *Pharmacol Biochem Behav* 65, 419–423. [PubMed: 10683481]
- Bolognani F, Qiu S, Tanner DC, Paik J, Perrone-Bizzozero NI & Weeber EJ (2007) Associative and spatial learning and memory deficits in transgenic mice overexpressing the RNA-binding protein HuD. *Neurobiol Learn Mem* 87, 635–643. [PubMed: 17185008]
- Bruchas MR, Schindler AG, Shankar H, Messinger DI, Miyatake M, Land BB, Lemos JC, Hagan CE, Neumaier JF, Quintana A, Palmiter RD & Chavkin C (2011) Selective p38 α MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. *Neuron* 71, 498–511. [PubMed: 21835346]
- Bryant CD & Yazdani N (2016) RNA-binding proteins, neural development and the addictions. *Genes Brain Behav* 15, 169–186. [PubMed: 26643147]
- Chen C, Kim JJ, Thompson RF & Tonegawa S (1996) Hippocampal lesions impair contextual fear conditioning in two strains of mice. *Behav Neurosci* 110, 1177–1180. [PubMed: 8919020]
- Curzon P, Rustay NR & Browman KE (2009) Cued and Contextual Fear Conditioning for Rodents In Buccafusco JJ (ed), *Methods of Behavior Analysis in Neuroscience*, Frontiers in Neuroscience, CRC Press/Taylor & Francis, Boca Raton (FL).
- Darnell R (2012) CLIP (Cross-Linking and Immunoprecipitation) Identification of RNAs Bound by a Specific Protein. *Cold Spring Harb Protoc*.
- De Rubeis S, He X, Goldberg AP, Poultney CS, Samocha K, Cicek AE, Kou Y, Liu L, Fromer M, Walker S, Singh T, Klei L, Kosmicki J, Fu S-C, Aleksic B, Biscaldi M, Bolton PF, Brownfeld JM, Cai J, Campbell NJ, Carracedo A, Chahrour MH, Chiocchetti AG, Coon H, Crawford EL, Crooks L, Curran SR, Dawson G, Duketis E, Fernandez BA, Gallagher L, Geller E, Guter SJ, Hill RS, Ionita-Laza I, Gonzalez PJ, Kilpinen H, Klauck SM, Klevzon A, Lee I, Lei J, Lehtimäki T, Lin C-F, Ma'ayan A, Marshall CR, McInnes AL, Neale B, Owen MJ, Ozaki N, Parellada M, Parr JR, Purcell S, Puura K, Rajagopalan D, Rehnström K, Reichenberg A, Sabo A, Sachse M, Sanders SJ, Schafer C, Schulte-Rüther M, Skuse D, Stevens C, Szatmari P, Tammimies K, Valladares O, Voran A, Wang L-S, Weiss LA, Willsey AJ, Yu TW, Yuen RKC, Cook EH, Freitag CM, Gill M, Hultman CM, Lehner T, Palotie A, Schellenberg GD, Sklar P, State MW, Sutcliffe JS, Walsh CA, Scherer SW, Zwick ME, Barrett JC, Cutler DJ, Roeder K, Devlin B, Daly MJ & Buxbaum JD (2014) Synaptic, transcriptional, and chromatin genes disrupted in autism. *Nature* 515, 209–215. [PubMed: 25363760]
- Dearborn JT, Harmon SK, Fowler SC, O'Malley KL, Taylor GT, Sands MS & Wozniak DF (2015) Comprehensive functional characterization of murine infantile Batten disease including Parkinson-like behavior and dopaminergic markers. *Sci Rep* 5, 12752. [PubMed: 26238334]
- Dougherty JD, Maloney SE, Wozniak DF, Rieger MA, Sonnenblick L, Coppola G, Mahieu NG, Zhang J, Cai J, Patti GJ, Abrahams BS, Geschwind DH & Heintz N (2013) The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors. *J Neurosci* 33, 2732–53. [PubMed: 23407934]
- Eisener-Dorman AF, Grabowski-Boase L & Tarantino LM (2011) Cocaine locomotor activation, sensitization and place preference in six inbred strains of mice. *Behav Brain Funct* 7, 29. [PubMed: 21806802]
- Engblom D, Bilbao A, Sanchis-Segura C, Dahan L, Perreau-Lenz S, Balland B, Parkitna JR, Luján R, Halbout B, Mameli M, Parlato R, Sprengel R, Lüscher C, Schütz G & Spanagel R (2008) Glutamate Receptors on Dopamine Neurons Control the Persistence of Cocaine Seeking. *Neuron* 59, 497–508. [PubMed: 18701074]
- Fadok JP, Dickerson TMK & Palmiter RD (2009) Dopamine Is Necessary for Cue-Dependent Fear Conditioning. *J Neurosci* 29, 11089–11097. [PubMed: 19741115]
- Gafford G, Jasnow AM & Ressler KJ (2014) Grin1 Receptor Deletion within CRF Neurons Enhances Fear Memory. *PLOS ONE* 9, e111009.
- Ghoshal N, Dearborn JT, Wozniak DF & Cairns NJ (2012) Core features of frontotemporal dementia recapitulated in progranulin knockout mice. *Neurobiol Dis* 45, 395–408. [PubMed: 21933710]

- Heath FC, Jurkus R, Bast T, Pezze MA, Lee JLC, Voigt JP & Stevenson CW (2015) Dopamine D1-like receptor signalling in the hippocampus and amygdala modulates the acquisition of contextual fear conditioning. *Psychopharmacology (Berl)* 232, 2619–2629. [PubMed: 25743759]
- Kang H & Schuman EM (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. *Science* 273, 1402–1406. [PubMed: 8703078]
- Kirkpatrick SL, Goldberg LR, Yazdani N, Babbs RK, Wu J, Reed ER, Jenkins DF, Bolgioni AF, Landaverde KI, Luttik KP, Mitchell KS, Kumar V, Johnson WE, Mulligan MK, Cottone P & Bryant CD (2017) Cytoplasmic FMR1-Interacting Protein 2 Is a Major Genetic Factor Underlying Binge Eating. *Biol Psychiatry* 81, 757–769. [PubMed: 27914629]
- Kohls G, Chevallier C, Troiani V & Schultz RT (2012) Social ‘wanting’ dysfunction in autism: neurobiological underpinnings and treatment implications. *J Neurodev Disord* 4, 10. [PubMed: 22958468]
- Kohls G, Schulte-Rüther M, Nehr Korn B, Müller K, Fink GR, Kamp-Becker I, Herpertz-Dahlmann B, Schultz RT & Konrad K (2013) Reward system dysfunction in autism spectrum disorders. *Soc Cogn Affect Neurosci* 8, 565–572. [PubMed: 22419119]
- Kosaki Y & Watanabe S (2016) Impaired Pavlovian predictive learning between temporally phasic but not static events in autism-model strain mice. *Neurobiol Learn Mem* 134, 304–316. [PubMed: 27521755]
- Kumar V, Kim K, Joseph C, Kourrich S, Yoo SH, Huang HC, Vitaterna MH, de Villena FP-M, Churchill G, Bonci A & Takahashi JS (2013) C57BL/6N mutation in Cytoplasmic FMR interacting protein 2 regulates cocaine response. *Science* 342, 1508–1512. [PubMed: 24357318]
- Ladd AN, Nguyen NH, Malhotra K & Cooper TA (2004) CELF6, a member of the CELF family of RNA-binding proteins, regulates muscle-specific splicing enhancer-dependent alternative splicing. *J Biol Chem* 279, 17756–17764. [PubMed: 14761971]
- MacPherson P, McGaffigan R, Wahlsten D & Nguyen PV (2008) Impaired fear memory, altered object memory and modified hippocampal synaptic plasticity in split-brain mice. *Brain Res* 1210, 179–188. [PubMed: 18417102]
- Makanjuola RO, Hill G, Dow RC, Campbell G & Ashcroft GW (1977a) The effects of psychotropic drugs on exploratory and stereotyped behaviour of rats studied on a hole-board. *Psychopharmacology (Berl)* 55, 67–74. [PubMed: 414263]
- Makanjuola RO, Hill G, Maben I, Dow RC & Ashcroft GW (1977b) An automated method for studying exploratory and stereotyped behaviour in rats. *Psychopharmacology (Berl)* 52, 271–277. [PubMed: 406630]
- Maloney SE, Akula S, Rieger MA, McCullough KB, Chandler K, Corbett AM, McGowin AE & Dougherty JD (2018) Examining the Reversibility of Long-Term Behavioral Disruptions in Progeny of Maternal SSRI Exposure. *eNeuro ENEURO*.0120–18.2018.
- Maloney SE, Khangura E & Dougherty JD (2016) The RNA-binding protein Celf6 is highly expressed in diencephalic nuclei and neuromodulatory cell populations of the mouse brain. *Brain Struct Funct*.
- Maloney SE, Yuede CM, Creeley CE, Williams SL, Huffman JN, Taylor GT, Noguchi KK & Wozniak DF (2019) Repeated neonatal isoflurane exposures in the mouse induce apoptotic degenerative changes in the brain with relatively mild long-term behavioral deficits. *Sci Rep*.
- Monteggia LM, Luikart B, Barrot M, Theobald D, Malkovska I, Nef S, Parada LF & Nestler EJ (2007) Brain-Derived Neurotrophic Factor Conditional Knockouts Show Gender Differences in Depression-Related Behaviors. *Biol Psychiatry* 61, 187–197. [PubMed: 16697351]
- Moy SS, Nadler JJ, Poe MD, Nonneman RJ, Young NB, Koller BH, Crawley JN, Duncan GE & Bodfish JW (2008) Development of a Mouse Test for Repetitive, Restricted Behaviors: Relevance to Autism. *Behav Brain Res* 188, 178–194. [PubMed: 18068825]
- Neuhaus E, Beauchaine TP & Bernier R (2010) Neurobiological correlates of social functioning in autism. *Clin Psychol Rev* 30, 733–748. [PubMed: 20570622]
- Oliver RJ, Brigman JL, Bolognani F, Allan AM, Neisewander JL & Perrone-Bizzozero NI (2017) Neuronal RNA-binding protein HuD regulates addiction-related gene expression and behavior. *Genes Brain Behav*.

- Perusini J, Cajigas Stephanie A., Cohensedgh Omid, Lim Sean C., Pavlova Ina P., Donaldson Zoe R. & Denny Christine A. (2017) Optogenetic stimulation of dentate gyrus engrams restores memory in Alzheimer's disease mice. *Hippocampus* 27, 1110–1122. [PubMed: 28667669]
- Pierce K & Courchesne E (2001) Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. *Biol Psychiatry* 49, 655–664. [PubMed: 11313033]
- Redondo RL & Morris RGM (2011) Making memories last: the synaptic tagging and capture hypothesis. *Nat Rev Neurosci* 12, 17–30. [PubMed: 21170072]
- Rieger MA, King DM, Cohen BA & Dougherty JD (2018) CLIP-Seq and massively parallel functional analysis of the CELF6 RNA binding protein reveals a role in destabilizing synaptic gene mRNAs through interaction with 3'UTR elements in vivo. *bioRxiv* 401604.
- Santos AR, Kanellopoulos AK & Bagni C (2014) Learning and behavioral deficits associated with the absence of the fragile X mental retardation protein: what a fly and mouse model can teach us. *Learn Mem* 21, 543–555. [PubMed: 25227249]
- Sisti HM, Glass AL & Shors TJ (2007) Neurogenesis and the spacing effect: Learning over time enhances memory and the survival of new neurons. *Learn Mem* 14, 368–375. [PubMed: 17522028]
- Smith LN, Jedynak JP, Fontenot MR, Hale CF, Dietz KC, Taniguchi M, Thomas FS, Zirlin BC, Birnbaum SG, Huber KM, Thomas MJ & Cowan CW (2014) Fragile X mental retardation protein regulates synaptic and behavioral plasticity to repeated cocaine administration. *Neuron* 82, 645–658. [PubMed: 24811383]
- Stein JM, Bergman W, Fang Y, Davison L, Brensinger C, Robinson MB, Hecht NB & Abel T (2006) Behavioral and Neurochemical Alterations in Mice Lacking the RNA-Binding Protein Translin. *J Neurosci* 26, 2184–2196. [PubMed: 16495445]
- Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA & Paylor R (2009) Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl)* 204, 361–373. [PubMed: 19189082]
- Thomas MJ, Beurrier C, Bonci A & Malenka RC (2001) Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. *Nat Neurosci* 4, 1217–1223. [PubMed: 11694884]
- Thomsen M & Caine SB (2011) False positive in the intravenous drug self-administration test in C57BL/6J mice. *Behav Pharmacol* 22, 239–247. [PubMed: 21522054]
- Tzschentke TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict Biol* 12, 227–462. [PubMed: 17678505]
- Wang DO, Kim SM, Zhao Y, Hwang H, Miura SK, Sossin WS & Martin KC (2009) Synapse- and stimulus-specific local translation during long-term neuronal plasticity. *Science* 324, 1536–1540. [PubMed: 19443737]
- Wang L, Shen M, Yu Y, Tao Y, Zheng P, Wang F & Ma L (2014) Optogenetic activation of GABAergic neurons in the nucleus accumbens decreases the activity of the ventral pallidum and the expression of cocaine-context-associated memory. *Int J Neuropsychopharmacol* 17, 753–763. [PubMed: 24456857]
- Young AMJ, Moran PM & Joseph MH (2005) The role of dopamine in conditioning and latent inhibition: what, when, where and how? *Neurosci Biobehav Rev* 29, 963–976. [PubMed: 16045987]
- Zweifel LS, Parker JG, Lobb CJ, Rainwater A, Wall VZ, Fadok JP, Darvas M, Kim MJ, Mizumori SJY, Paladini CA, Phillips PEM & Palmiter RD (2009) Disruption of NMDAR-dependent burst firing by dopamine neurons provides selective assessment of phasic dopamine-dependent behavior. *Proc Natl Acad Sci* 106, 7281–7288. [PubMed: 19342487]

Figure 1. Independent examination of resistance to change phenotype in *Celf6* mutant mice. (a) Schematic of example placement of odorant-containing and empty corner holes for the Holeboard Exploration/Olfactory Preference task. (b) Total holepokes exhibited by WT and *Celf6*^{-/-} littermates during test day 1 and, following sweetened milk familiarization, test day 2 in the Holeboard task (test day x genotype interaction, $p=.003$; Cohort 1). *Denotes significant difference in WT mice between Test Days 1 and 2 ($p=.024$). (c) Number of holepokes into each odorant-containing and empty corner hole during test days 1 and 2 for WT and *Celf6*^{-/-} littermates (test day x genotype interaction, $p=.017$). WT mice show a potentiation of poking behavior into the hole containing fresh familiar bedding on test day 2 ($p=.007$) and all mice demonstrate typical olfaction by poking more into the hole containing fresh familiar bedding compared to the empty corner hole ($p<.013$). (d) Mean number of consecutive dips into any single hole for WT and *Celf6*^{-/-} littermates. No difference between genotypes was observed. Data are means $\pm$ SEM, with each sample represented as filled circles (WT, black; *Celf6*^{-/-}, gray).

Figure 2. Across multiple tasks, *Celf6* mutants do not exhibit restricted patterns of behavior.

(a) Schematic of the daily trials for both learning set MWM and massed-trial reversal MWM. (b) Average path length to the escape platform during the second trial each day of the learning set MWM. No differences were observed between WT and *Celf6*^{-/-} littermates of Cohort 1 suggesting normal short-term working memory performance. (c) Both WT, $F(1,18)=39.433$, $p=.000006$, and *Celf6*^{-/-}, $F(1,18)=39.155$, $p=.000007$, littermates exhibited shorter path lengths to the escape platform during Trial 4 vs. Trial 1 for each test day with a new platform location during the learning set MWM (Trial 1, dark gray circles; Trial 4, light gray circles) *Denotes differences between Trial 1 and Trial 4, $p<.035$. (d) During the massed trial reversal MWM, no differences in escape path length were observed between WT and *Celf6*^{-/-} littermates during any blocks of acquisition (A) and reversal (R) trials. (e) Number of marbles buried as a proxy for compulsive digging behavior. While no difference between genotypes was observed in Cohort 1 male mice, *Celf6*^{-/-} females buried less marbles than WT females (genotype x sex interaction, $F(1,18)=4.926$, $p=.040$). (f) Number of marbles buried for the all-male Cohort 2. No differences between genotypes were observed. (g) Percent trials exhibiting an alternation in the spontaneous alternation T-maze for WT and *Celf6*^{-/-} littermates of Cohort 1. No difference between genotypes was observed. Data are means $\pm$ SEM, with each sample represented as filled circles (WT, black; *Celf6*^{+/-}, dark gray; *Celf6*^{-/-}, light gray).

Figure 3. *Celf6* mutants display normal response to natural reward yet altered activity and exploration.

(a) Schematic of sucrose preference bottle placement and designated zones for analysis within the apparatus and (b) sucrose preference procedure. (c) Amount of liquid consumed during sucrose preference habituation to pure water and to 1% sucrose water by *Celf6* mutants and WT littermates of Cohort 1. (d) The increased consumption of 1% sucrose water by both *Celf6* mutants and WT littermates during the 48-h sucrose preference tests. *Denotes significant increase from water ($p < .00003$). (e-f) During the two 1-h habituation sessions, WT mice demonstrated an increase in (e) time spent near the sucrose bottle compared to the water-only bottle ($p = .023$), with a marginal increase in time near the sucrose bottle compared to *Celf6* mutants ($p = .097$), despite no differences in entries made into this area (f). (g-h) Time spent in (g) and entries into (h) the area near the 1% sucrose and water-only bottles during the 48-h sucrose preference test. (i-j) *Celf6* mutants displayed fewer total ambulations and exploratory rearing compared to WT littermates during both the habituation (i) and preference test (j) sessions. Data are means $\pm$ SEM, with each sample represented as filled circles (WT, black; *Celf6*^{-/-}, gray).

Figure 4. *Celf6* null mice do not condition to the rewarding substance cocaine and show deficits to fear conditioning.

(a) Schematic of cocaine CPP chamber set up and (b) cocaine CPP procedure. (c) Exposure to cocaine did not increase conditioning score (time spent in drug-paired chamber post-conditioning relative to pre-conditioning) in *Celf6*^{-/-} mice compared to exposure to saline only of Cohort 3. Both WT and *Celf6*^{+/-} littermates condition to cocaine exposure, as shown by a significantly larger conditioning score compared to saline-exposed controls, $F(1,49)=6.798$, $p=.036$ and $F(1,49)=7.087$, $p=.030$. Conditioning score = time spent in drug-paired chamber post-conditioning relative to pre-conditioning. Filled circles: saline, dark gray; cocaine, red. (d) *Celf6* mutant mice administered cocaine failed to show an increase in activity levels compared to saline-exposed controls as assessed by distance traveled in the drug-paired chamber during conditioning trials. Only cocaine-exposed WT mice traveled a significantly greater distance on conditioning days 2 and 3 relative to saline-exposed mice of the same genotype, $F(1,147)=11.978$, $p=.002$ and $F(1,147)=31.763$, $p<.00005$, respectively. *Denotes significant difference from day 1 for cocaine-exposed mice at $p<.012$. Filled circles: saline, dark gray; cocaine, red. (e) During fear conditioning day 1, *Celf6*^{-/-} mice of Cohort 4 demonstrated decreased % time spent freezing compared to WT littermates during the last minute of the tone/shock training (genotype x minute interaction, $F(1,210, 21.799)=5.825$, $p=.017$). (f) During the contextual fear conditioning test, *Celf6*^{-/-} mice again showed decreased % time spent freezing compared to WT littermates during minutes 2–5 (minute x genotype interaction, $F(5,655, 101.790)=3.115$, $p=.009$). (g) No differences in freezing behavior was observed during auditory-cued fear conditioning testing. (h–j) In the separate, larger cohort, Cohort 2, the effect of *Celf6* mutation on (h) tone/shock training was not replicated, yet the decreased freezing behavior by *Celf6*^{-/-} mice was replicated during

both tests of **(i)** contextual (main effect of genotype, $F(2,50)=10.501$, $p=.0002$) and **(j)** auditory-cued conditioning (main effect of genotype, $F(2,50)=11.169$, $p=.0001$). **(k-m)** In Cohort 5, the *Celf6* mutant females did not show differences on **(k)** day 1 baseline or training or **(m)** day 3 auditory cued-conditioning, but replicated the **(l)** day 2 contextual conditioning deficits previously observed in males (main effect of genotype, $F(2,27)=3.506$, $p=.044$). Data are means $\pm$ SEM.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1.
Mouse cohorts used for behavioral testing.

Cohort sample sizes including distribution between sexes, order of behavioral testing and age at testing.

Mouse behavior cohorts	
Cohort 1	
WT (n=10, 6F & 4M)	
<i>Cellf6</i> ^{-/-} (n=12, 6F & 6M)	
Behavioral Tests	Age
1 -h Locomotor Activity/Exploration	3–5 mos.
Sensorimotor Battery	3–5 mos.
Typical MWM	3–5 mos.
Learning Set MWM	3.5–5.5 mos.
Holeboard	5.5–7.5 mos.
Massed-trial Reversal MWM	5.5–7.5 mos.
Sucrose Preference Test	6–8 mos.
Marble Burying	8–10 mos.
Spontaneous Alternation T-maze	8–10 mos.
Cohort 2	
WT (n=19, M)	
<i>Cellf6</i> ^{+/-} (n=20, M)	
<i>Cellf6</i> ^{-/-} (n=20, M)	
Behavioral Tests	Age
Holeboard	5–6 mos.
1-h Locomotor Activity/Exploration	5–6 mos.
Sensorimotor Battery	5–6 mos.
Marble Burying	5–6 mos.
Conditioned Fear	6–7 mos.
Cohort 3	
WT (n=16, 8F&8M)	
<i>Cellf6</i> ^{+/-} (n=21, 11F& 10M)	
<i>Cellf6</i> ^{-/-} (n=18, 10F & 8M)	
Behavioral Tests	Age
Cocaine CPP	2–3 mos.
Cohort 4	
WT (n=10, M)	
<i>Cellf6</i> ^{-/-} (n=10, M)	
Behavioral Tests	Age
Conditioned Fear	4–6 mos.
Cohort 5	
WT (n=15, 8F & 7M)	

Mouse behavior cohorts	
<i>Cellf6</i> ^{+/-} (n=22, 10F & 12M)	
<i>Cellf6</i> ^{-/-} (n=22, 12F & 10M)	
Behavioral Tests	Age
Conditioned Fear	2–6 mos.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript