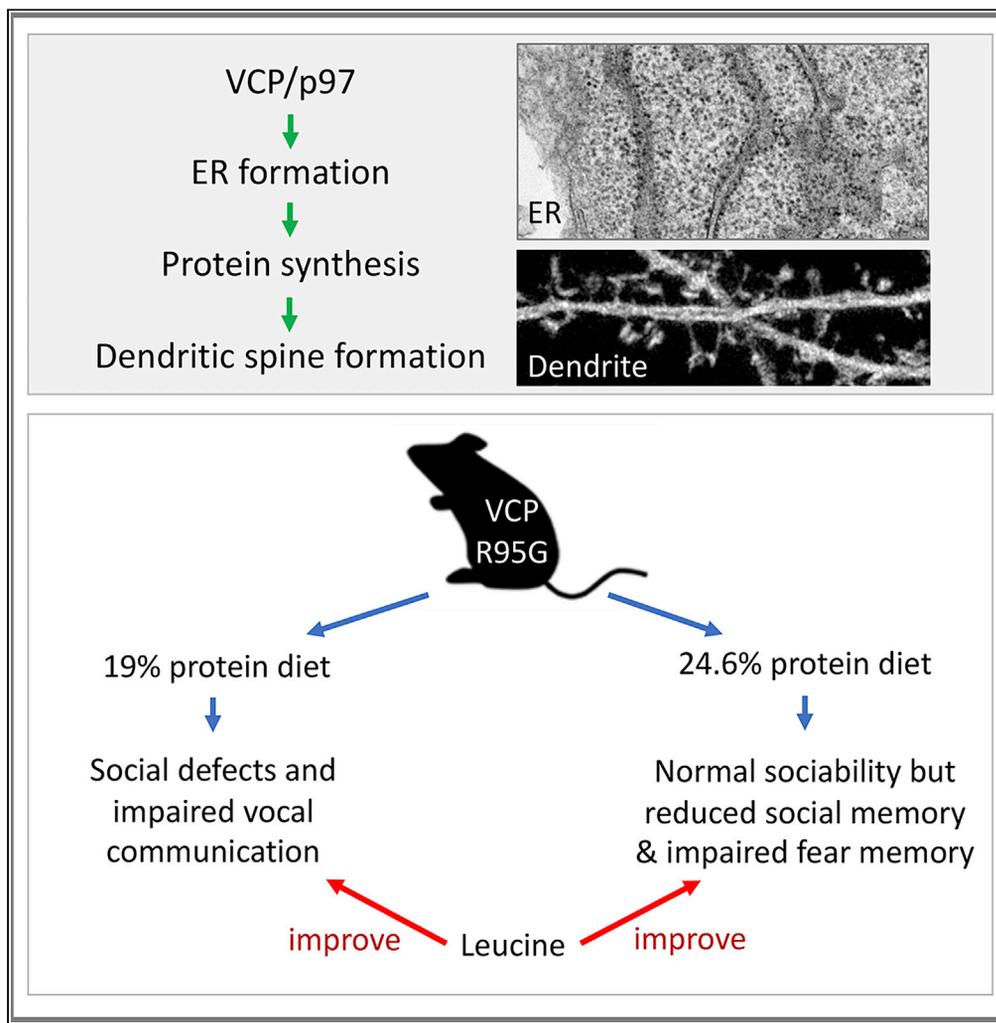


Article

Social behaviors and contextual memory of *Vcp* mutant mice are sensitive to nutrition and can be ameliorated by amino acid supplementation



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HIGHLIGHTS

Body weight and muscle strength of aged VCP R95G knockin mice are influenced by diet

Vcp deficiency results in social deficits and reduced vocal communications

Social behaviors of VCP R95G mice are sensitive to protein content in diet

Leucine supplementation improves social behaviors and fear memory of VCP R95G mice



Article

Social behaviors and contextual memory of *Vcp* mutant mice are sensitive to nutrition and can be ameliorated by amino acid supplementationTzzy-Nan Huang,^{1,2} Yu-Tzu Shih,^{1,2} Si-Cih Lin,^{1,2} and Yi-Ping Hsueh^{1,3,*}

Summary

Both genetic variations and nutritional deficiency are associated with autism spectrum disorders and other neurological disorders. However, it is less clear whether or how nutritional deficiency and genetic variations influence each other under pathogenic conditions. "Valosin-containing protein" (VCP, also known as p97) is associated with multiple neurological disorders and regulates dendritic spine formation by controlling endoplasmic reticulum formation and protein synthesis efficiency. Increased protein synthesis ameliorates the dendritic spine defects of *Vcp*-deficient neurons. Therefore, we investigated if *Vcp*-deficient mice are sensitive to nutritional conditions. Here, we show that social interaction and contextual memory of *Vcp*-deficient mice are indeed influenced by different dietary protein levels. Moreover, leucine supplementation ameliorates the behavioral deficits and dendritic spine density of *Vcp*-deficient mice, strengthening evidence for the role of protein synthesis in VCP function. Our study illustrates that genetic variation and nutrient factors cross-talk to influence neuronal and behavioral phenotypes.

Introduction

Autism spectrum disorders (ASDs) are frequently associated with nutritional deficiency and imbalance (Erickson et al., 2005; Kawicka and Regulska-Ilow, 2013; Srinivasan et al., 2016). Various nutritional elements such as vitamins, calcium, zinc, and branched chain amino acids (BCAAs, including leucine, isoleucine, and valine) have been linked to ASDs, together representing one type of contributory environmental factor (Bolte et al., 2019; Curtin et al., 2018; Lee et al., 2015; Novarino et al., 2012; Pfaender et al., 2017; Shih et al., 2020a, 2020b; Shih and Hsueh, 2016; Smith et al., 2019; Srinivasan et al., 2016; Tarlungeanu et al., 2016; Tirouvanziam et al., 2012; Yasuda et al., 2011). Food selectivity and gastrointestinal factors are thought to be at least partly responsible for nutritional imbalance in patients with ASDs (Berry et al., 2015; Erickson et al., 2005; Sharp et al., 2013). Rare mutations in genes controlling BCAA metabolism or transportation have also been reported in patients with ASDs (Novarino et al., 2012; Tarlungeanu et al., 2016). However, it remains unclear whether and how nutritional deficiency and gene mutations cross-talk to influence neuronal activity and function in ASD. Determining the specific function of critical nutrients for neurons and brains, as well as the interactions between nutrients and specific gene mutations, will improve the efficiency and minimize the side effects of dietary therapy.

Valosin-containing protein (VCP, also known as p97) is a hexameric ATPase of the AAA (ATPase with multiple cellular activity) protein family. Monoallelic mutations in the *VCP* gene were first identified as causing inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD) (Abrahao et al., 2016; Watts et al., 2004). Later, whole-exome sequencing indicated an association of VCP with ASD (Cortes and Wevrick, 2018; lossifov et al., 2012). Although it is unclear why mutations in the *VCP* gene result in such varied neurological disorders, *Vcp* deficiency results in reduced dendritic spine density (Hsueh, 2012; Shih and Hsueh, 2016; Wang et al., 2011), which may account for neurological impairment in patients. Our previous study further indicated that VCP acts downstream of neurofibromin, a protein encoded by the *neurofibromatosis type 1* (*NF1*) gene, to control dendritic spine formation (Wang et al., 2011). Since NF1 is highly relevant to ASD (Molosh and Shekhar, 2018), the interaction between neurofibromin and VCP strengthens evidence for the role of VCP in ASD. Although several studies have reported IBMPFD-related

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<https://doi.org/10.1016/j.isci.2020.101949>



phenotypes in *Vcp* mutant mice (Badadani et al., 2010; Custer et al., 2010; Weihl et al., 2007), the evidence for ASD-related phenotypes is still lacking.

As an AAA ATPase, VCP functions as a chaperon to regulate diverse cellular processes including endoplasmic reticulum (ER) and Golgi morphogenesis (Kondo et al., 1997; Latterich et al., 1995; Shih and Hsueh, 2016; Vedrenne and Hauri, 2006), ER-associated protein degradation (Jarosch et al., 2002; Ye et al., 2001), the ubiquitin-proteasome system (Dai et al., 1998; Meyer et al., 2012), chromatin remodeling (Meyer et al., 2012), and autophagy (Ju and Weihl, 2010; Tresse et al., 2010). Among these various cellular processes, ER formation is the key downstream pathway of VCP in controlling dendritic spine density (Shih and Hsueh, 2016, 2018; Shih et al., 2020b). Knockdown of endogenous *Vcp* or expression of VCP R95G mutant proteins, a mutation identified from patients with IBMPFD, impairs ER formation and ribosomal attachment on ER and consequently reduces the protein synthesis efficiency of neurons (Shih and Hsueh, 2016). Rescue of protein synthesis by means of leucine supplementation to activate the mTOR pathway increases the dendritic spine density of cultured neurons (Shih and Hsueh, 2016; Shih et al., 2020b), confirming the critical role of protein synthesis in VCP-regulated dendritic spine formation.

We previously generated VCP R95G knockin mice to study the effect of *Vcp* mutation on ER formation (Shih and Hsueh, 2016). In this report, we investigate if VCP R95G knockin mice exhibit autism-like behaviors and whether nutritional conditions can influence the neuronal and behavioral phenotypes of *Vcp* mutant mice. By comparing *Vcp* mutant mice provided with two different diets containing 19% or 24.6% protein, we found that the mutant mice are indeed sensitive to nutrition, though both 19% and 24.6% protein contents are within the regular range for mouse diets. To further confirm the effect of increased protein intake, we supplemented the drinking water of mutant mice with leucine. We found that synaptic defects and behavioral deficits of VCP R95G knockin mice were ameliorated by leucine supplementation. Our data suggest that *Vcp* mutation renders mice sensitive to nutritional conditions and that increasing protein intake may offset the behavioral deficits of *Vcp* mutant mice. This study eloquently illustrates the cross-talk between genetic variation and environmental factors.

Results

Body weight and muscle strength of aged VCP R95G mice are influenced by diet

To investigate if the phenotypes of VCP R95G mice are influenced by nutrient intake, we first fed VCP R95G mice and their wild-type (WT) littermates for entire lifespans with two different chows, i.e. LabDiet 5K54 and 5010, which contain 19% and 24.6% protein, respectively (Table S1). Though these chows differ in protein content by almost 6%, both 5K54 and 5010 represent standard chows for experimental rodents (<https://www.labdiet.com/Products/StandardDiets/index.html>). To emphasize the difference in protein content, hereafter we use “19% protein diet” and “24.6% protein diet” to represent LabDiet 5K54 and 5010, respectively. Since the VCP R95G mutation was originally identified in patients with IBMPFD, a neurodegenerative disorder that gives rise to muscle weakness and bone defects (Watts et al., 2004), we investigated if that mutation influences body weight and muscle strength particularly in aged mice. We found no difference in these two parameters between VCP R95G mice and WT littermates at the age of 12 months, regardless of diet (Figures 1A and 1B). However, at 18 months, VCP R95G mice fed on 19% protein diet exhibited reduced body weight and weaker muscle strength compared with their WT littermates (Figure 1A). In contrast, at 18 months, there was still no difference in the groups of mice fed with 24.6% protein diet (Figure 1B, note all statistical methods and results are summarized in Table S2). These results suggest that the phenotypes of VCP R95G mice can be influenced by nutrition.

VCP R95G mice fed on 19% protein diet exhibit impaired vocalization and reduced social interaction

In addition to body weight and muscle strength of aged mice, we investigated if VCP R95G mutation results in ASD-related phenotypes. To investigate that possibility, we further characterized VCP R95G mice typically at the age of 2–3 months. In Table 1, we summarize all examined features and corresponding ages of different groups of mice studied in this report. Compared with WT littermates, VCP R95G mice were comparatively normal in terms of mouse appearance, body weight, bony features, muscle strength, brain appearance, and anatomical features (Figures 2A–2F). We then applied a series of behavioral paradigms to further characterize VCP R95G mice. In an open field, locomotion activity, numbers of rearing and grooming events, and time spent in the corners and central area by VCP R95G mice were comparable to those of WT littermates (Figure 2G). In Y-maze, rotarod, light/dark box, and elevate plus maze assays, the

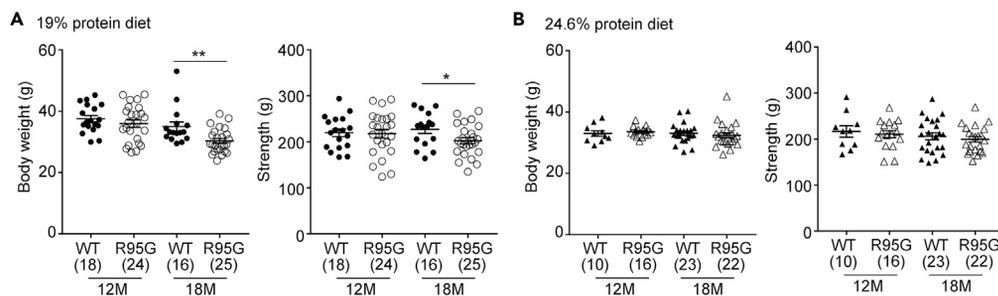


Figure 1. The effect of different diets on body weight and muscle strength of aged mice

Mice were fed on either (A) 19% or (B) 24.6% protein diet for their entire lifespan. Body weight and muscle strength were measured at 12 and 18 months. Compared with WT littermates, VCP R95G mice fed on 19% protein diet had lower body weight and muscle strength at 18 months but not at 12 months. Numbers of examined mice for each group are indicated in parentheses. Each dot represents the result of an individual mouse. Mean and standard error of the mean (error bars) are also presented. * $p < 0.05$; ** $p < 0.01$. All statistical methods and results are summarized in Table S2.

performance of VCP R95G mice was also comparable to that of WT littermates (Figures 2H–2K). These results suggest that although VCP R95G mice fed on 19% protein diet exhibit reduced body weight and weaker muscle strength at 18 months of age, their performance is indistinguishable from WT littermates in terms of general morphology, locomotion activity, movement balance, and anxiety at a younger age.

We then investigated if VCP R95G mice fed on 19% protein diet exhibit defects in vocal communication or social interaction, two core symptoms of ASD. Ultrasonic vocalization of isolated pups was recorded and analyzed at postnatal day (PND) 4, 6, 8, and 10. We found that the call numbers of ultrasonic vocalization of VCP R95G mice were always lower than those of WT mice, though only the results at PND 8 could be deemed statistically significant (Figure 3A and Table S2). In reciprocal social interaction, VCP R95G mice fed on 19% protein diet spent less time interacting with stranger mice (Figure 3B). We assessed two

Table 1. The age and grouping of mice for analyses in the current study (text in brackets reflect dietary provision)

Groups	Experiments	Age (weeks)	Housing (group or single)
I	Mouse appearance (including X-ray) (24.6%)	24	Group
II	Body weight (24.6%)	9 & 12	Group
III	Muscle strength (24.6%)	9 & 12	Group
IV	Open field (24.6%)	12	Group
V	Light-dark box (24.6%)	12	Group
	Y-maze task (24.6%)	12	Group
VI	Contextual fear conditioning (24.6% + Leu)	12	Single, at least one week
VII	Modified three-chamber test (24.6% + Leu)	10	Single, at least one week
VIII	Open field (24.6%) for Figure 7	8	Group
	Reciprocal social interaction (24.6%) for Figure 7	9	Single, at least one week
IX	Open field (19%)	8	Group
	Y-maze (19%)	8	Group
	Light-dark box (19%)	8	Group
	Reciprocal social interaction (19%)	10	Single, at least one week
	Rotarod test (19%)	10–12	Group
X	Open field (19% + Leu) for Figure 7	8	Group
	Reciprocal social interaction (19% + Leu) for Figure 7	9	Single, at least one week
XI	Dendritic spine density counting(19% + Leu)	9	Group
XII	Three-chamber test (19%)	9–10	Single, at least one week
XIII	Ultrasonic vocalization (19%)	1	Group

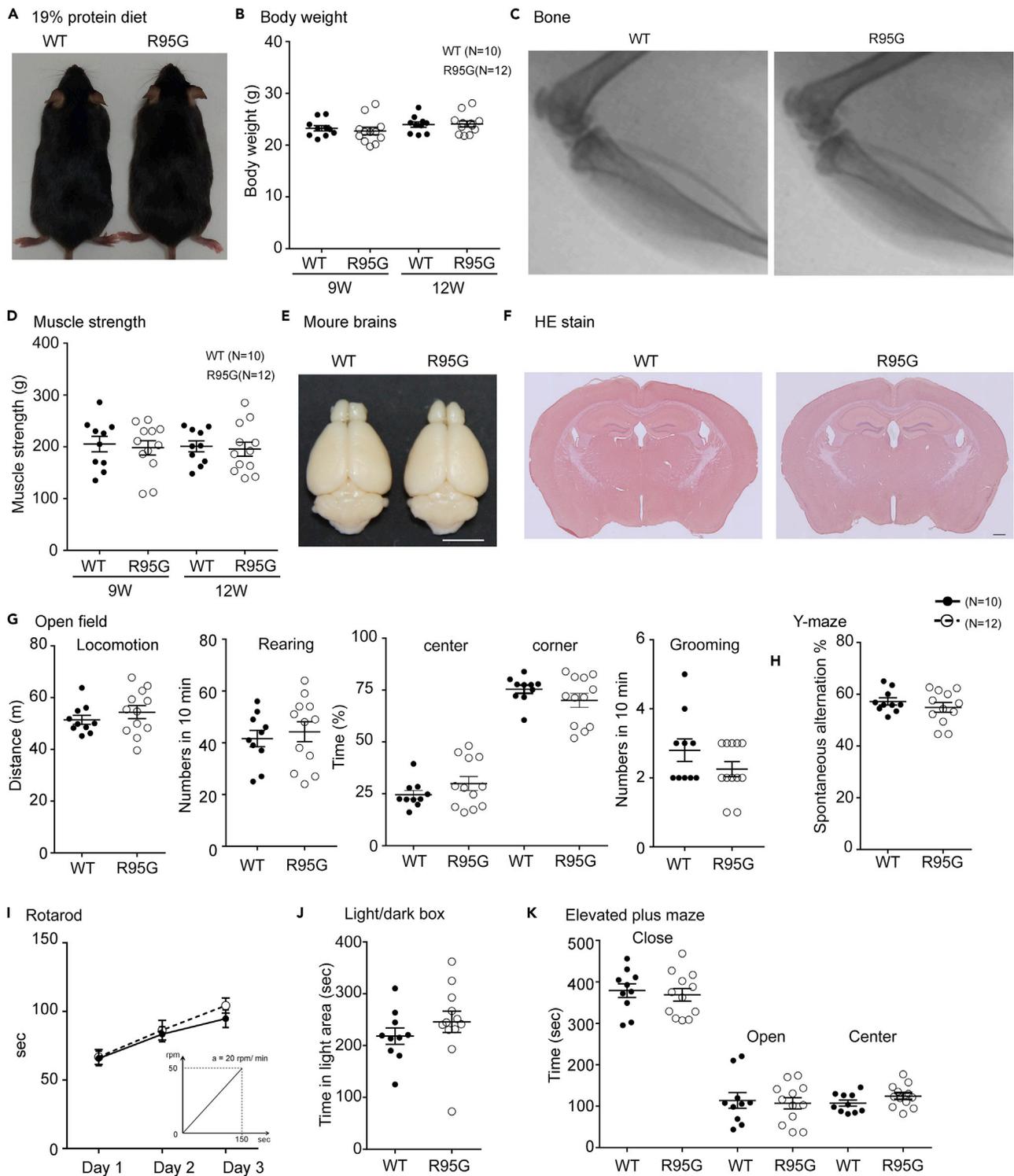


Figure 2. VCP R95G mice fed on 19% protein diet are generally healthy and normal

(A) Mouse appearance at 24 weeks.
(B) Body weight at 9 and 12 weeks.
(C) X-ray images of anterior tibia and posterior femur of 24-week-old mice.
(D) Muscle strength at 9 and 12 weeks.
(E) Brain appearance of mice at 24 months.

Figure 2. Continued

(F) Hematoxylin and eosin staining of mouse brains.

(G) Open field test. Total moving distance, time spent at the center and corners, and numbers of rearing and grooming events for VCP R95G mice were comparable to those of WT littermates.

(H) Y-maze test.

(I) Rotarod test.

(J) Light-dark box test.

(K) Elevated plus maze.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. Numbers of examined mice (N) are indicated. All statistical methods and results are summarized in Table S2. Scale bar, (E) 5 mm; (F) 0.5 mm.

parameters, i.e. sociability and social novelty, in the second and third sessions of our three-chamber test, respectively (Figure 3C, left panel). For sociability, we found that VCP R95G mice fed on 19% protein diet spent less time interacting with stranger 1 compared with their WT littermates (Figure 3C, middle). For social novelty, there was no apparent difference between VCP R95G mice and WT littermates (Figure 3C, right).

Taken together, these analyses suggest that though VCP R95G mice fed on 19% protein diet are generally healthy and normal before 12 months of age, they exhibit the core autism-like characteristics, including deficits in ultrasonic vocalization and sociability.

VCP R95G mice fed on 24.6% protein diet do not exhibit social deficits

We also analyzed the phenotypes of VCP R95G mice fed on 24.6% protein diet typically at the age of 2–3 months (Table 1). Similar to the results from mice fed on 19% protein diet, we did not find that VCP R95G mice fed on 24.6% protein diet exhibited defects in mouse appearance, body weight, bony features, brain appearance, or anatomical characteristics (Figures 4A–4E). Moreover, we observed no differences between VCP R95G knockin mice and wild-type littermates in open field, light/dark box, and Y-maze assays (Figures 4F–4H), suggesting that VCP R95G mice fed on 24.6% protein diet exhibit no obvious deficits in locomotion, anxiety, or working memory.

We further analyzed if our mutant mice fed on 24.6% protein diet present social deficits. In reciprocal social interaction, VCP R95G mice fed on 24.6% protein diet behaved similarly to their WT littermates fed on the same diet (Figure 5A), unlike the results from mice fed on 19% protein diet (Figure 3B). In the three-chamber test, VCP R95G mice fed on 24.6% protein diet also behaved comparably to their WT littermates in sociability and social novelty (Figures 5B–5D). Together, these results indicate that VCP R95G mice fed on 24.6% protein diet exhibit no obvious defect in social behaviors. Thus, similar to our results on body weight and muscle strength at 18 months of age, based on comparison of the social behaviors of VCP R95G mice fed different diets, these mutant mice are indeed sensitive to nutrition.

VCP R95G mice fed on 24.6% protein diet exhibit impaired long-term social novelty preference and contextual memory

Our previous study demonstrated that VCP forms a complex with neurofibromin encoded by the *NF1* gene to control dendritic spine formation (Wang et al., 2011). Similar to VCP R95G mice, *Nf1*^{+/-} mice fed on 24.6% protein diet did not exhibit deficits of sociability and social novelty in a three-chamber test (Shih et al., 2020b). However, in a modified three-chamber test (Figure 5B), *Nf1*^{+/-} mice fed on 24.6% protein diet did exhibit reduced long-term social novelty preference (Shih et al., 2020b). If VCP and neurofibromin do act together to control mouse behaviors, we anticipated that VCP R95G mice fed on 24.6% protein diet would also present a defect in long-term social novelty preference. Therefore, we extended the classical three-chamber test to assess long-term social novelty on the second day (Figure 5B). Indeed, similar to *Nf1*^{+/-} mice, VCP R95G mice fed on 24.6% protein diet also exhibited much lower interaction durations with stranger 3 (Figures 5C and 5D), implying a defect in long-term social novelty preference, despite consuming a 24.6% protein diet.

Our recent study further indicated that providing 1.8% leucine in drinking water for 7 days ameliorates the long-term social novelty preference deficit of *Nf1*^{+/-} mice fed on 24.6% protein diet (Shih et al., 2020b). We have also shown previously that the beneficial effects of 0.45% or 0.9% leucine water on behaviors were not as obvious as for 1.8% leucine water provisioning (Shih et al., 2020b). In addition, 1.8% leucine water treatment for 7 days was previously shown to be sufficient to increase leucine concentration and to enhance

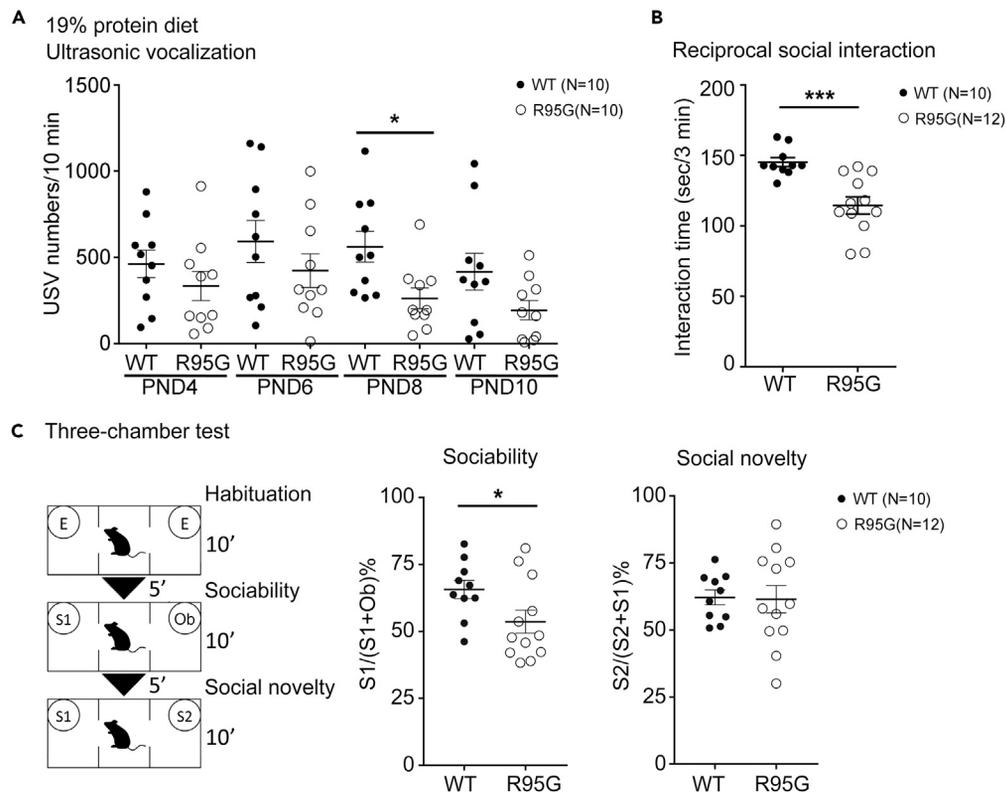


Figure 3. VCP R95G mice fed on 19% protein diet exhibit deficits in vocal communication and social interaction

(A) Ultrasonic vocalization (USV). VCP R95G and WT pups were isolated for 10 min at postnatal day (PND) 4, 6, 8 and 10. Total call numbers were measured to represent vocal communication of mice. The result at PND 8 reveals a difference between VCP R95G mice and WT littermates.

(B) Reciprocal social interaction. Compared with WT littermates, VCP R95G mice spent less time approaching and interacting with strangers.

(C) Three-chamber test. A flowchart of the experiment is shown at left. The test comprises three 10-min sessions, i.e. habituation, sociability, and social novelty, with 5-min intervals between them. E, wire cage without any mouse; Ob, wire cage with inanimate object; S1, cage with stranger mouse 1; S2, cage with stranger mouse 2. Time difference over total interaction time (as percentages) represents indexes for sociability or social novelty as indicated at middle or right, respectively.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. Numbers of examined mice (N) are indicated. * $p < 0.05$; *** $p < 0.001$. All statistical methods and results are summarized in Table S2.

protein synthesis in the mouse brain (Shih et al., 2020b). Based on these published studies, we applied the same leucine supplementation treatment to our VCP R95G mice. Indeed, 1.8% leucine in drinking water effectively improved the long-term social novelty preference of VCP R95G mice fed on the 24.6% protein diet (Figures 5C and 5D). Together, these results suggest that although VCP R95G mice fed on 24.6% protein diet do not show defects in sociability, they still exhibit deficits in long-term social novelty preference, which can be ameliorated by leucine supplementation.

In addition to long-term social novelty preference, *Nf1*^{+/-} mice fed on 24.6% protein diet exhibit defective contextual fear memory, which is also improved by leucine supplementation (Cui et al., 2008; Shih et al., 2020b). We applied the same behavioral paradigm and leucine supplementation (Figure 6A) to analyze contextual fear memory of VCP R95G mice fed on 24.6% protein diet. The assay involved one trial per day for five consecutive days, with contextual fear memory being assessed on day 6 (Figure 6A). WT littermates exhibited freezing responses on day 6 of >40% (Figure 6B). In contrast, for VCP R95G mice, the freezing response was only ~20%. However, leucine supplementation enhanced the freezing response of mutant mice to >40%, i.e. a level comparable to that of WT littermates (Figure 6B). Leucine supplementation did not influence the performance of WT mice in contextual fear conditioning (Shih et al., 2020b). The

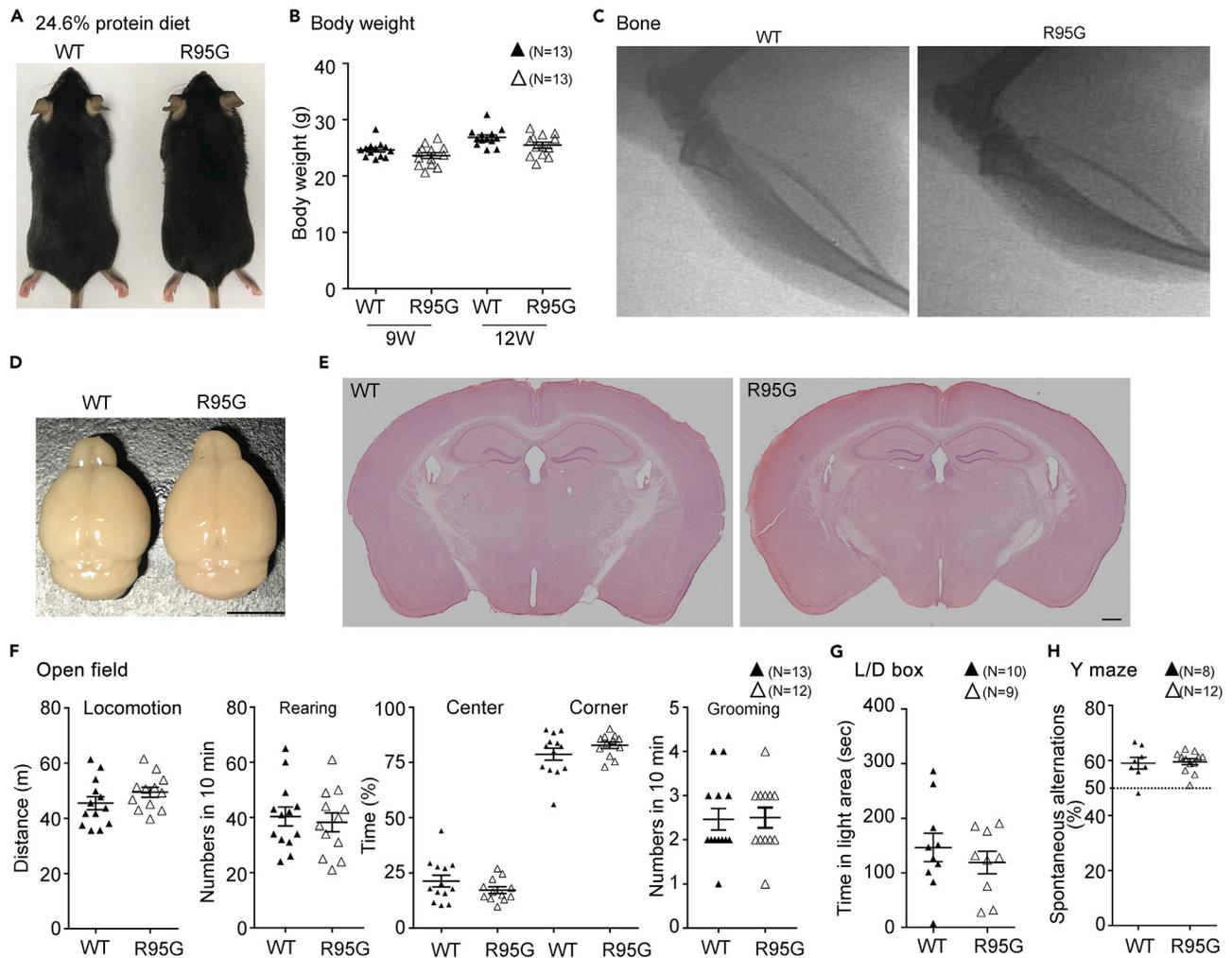


Figure 4. VCP R95G mice fed on 24.6% protein diet are generally healthy and normal

(A) Mouse appearance at 24 weeks of age.

(B) Body weight at 9 and 12 weeks.

(C) X-ray images suggest that R95G knockin mice at the age of 24 weeks do not show defects in the anterior tibia or posterior femur.

(D) Images of mouse brains at 24 weeks of age.

(E) Hematoxylin and eosin staining of 6-month-old mouse brains.

(F) Open field test. Total moving distance, time spent at the center and corners and the numbers of rearing and grooming events for VCP R95G knockin mice were comparable to those of WT mice.

(G) Light-dark box test. Times spent and moving distances in the light area and the numbers of transitions between light and dark areas were comparable between WT and mutant mice. VCP R95G knockin mice exhibit no obvious anxious behavior.

(H) Y-maze test for working memory was also comparable between VCP R95G mice and WT littermates. Spontaneous alternations in three arms by sequential choice and number of entries were analyzed. Numbers of mice examined (N) are indicated.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. All statistical methods and results are summarized in Table S2. Scale bar, (D) 0.5 cm; (E) 0.5 mm.

reduced memory performance of VCP R95G mice was unlikely due to differential immediate freezing responses right after foot shock stimulation because the freezing percentage right after foot shock on day 1 for VCP R95G mice was actually slightly higher and not lower than that of wild-type littermates, though the difference is not statistically significant (Figures 6C and Table S2).

In conclusion, though VCP R95G mice fed on 24.6% protein diet present no defects in sociability, they do exhibit deficits in long-term social novelty preference and contextual fear memory, both of which were ameliorated by leucine supplementation.

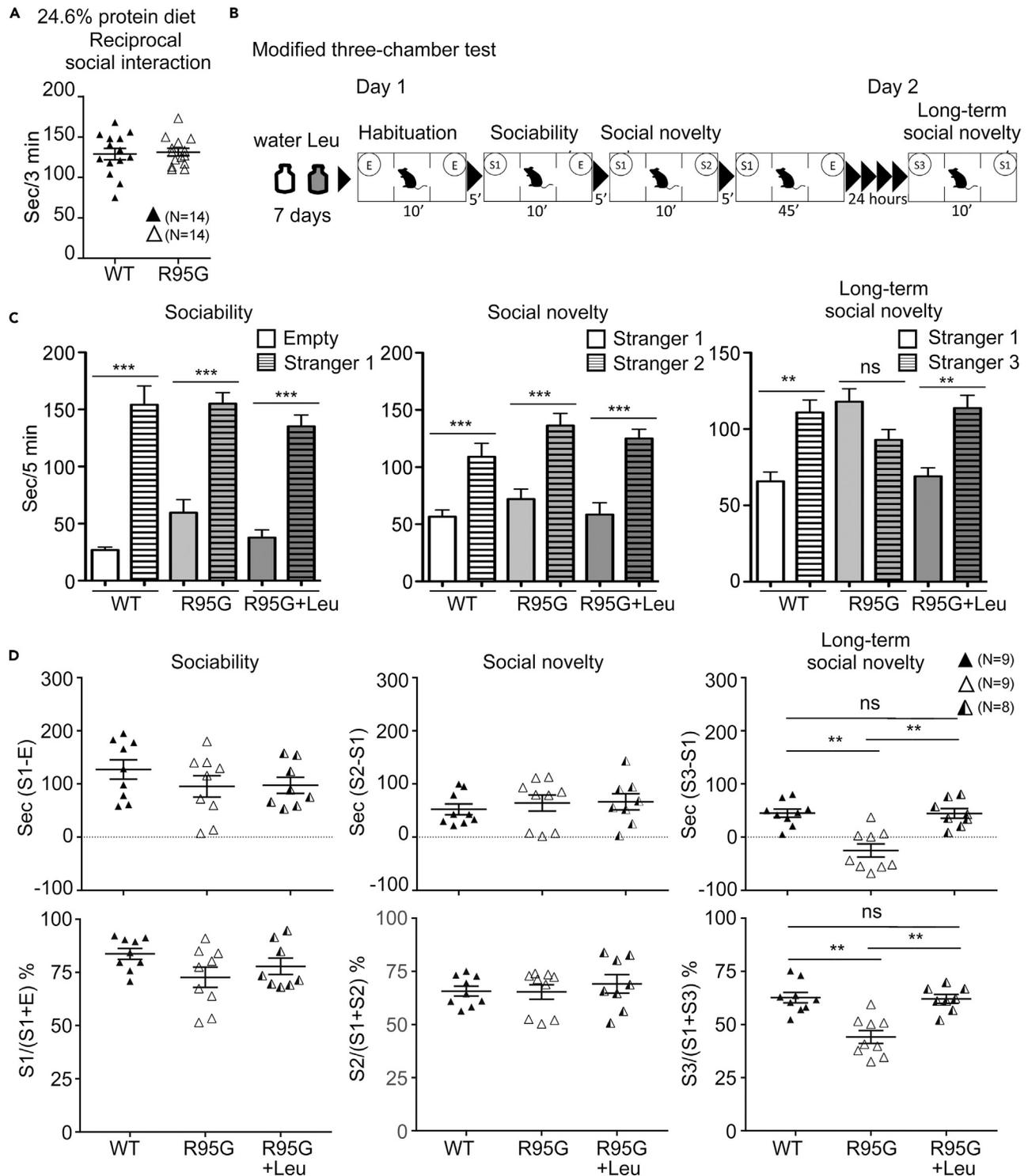


Figure 5. VCP R95G mice fed on 24.6% protein diet exhibit normal social interaction but impaired long-term social novelty preference

(A) Reciprocal social interaction. The interaction times with stranger mice were comparable between VCP R95G and WT mice.

(B) Schematic of the modified three-chamber social test. The first three sessions are identical to a classical three-chamber test. Two additional sessions were incorporated to investigate long-term social novelty preference. Three groups of mice were tested. One group drank water supplemented with 1.8% leucine for 7 days before testing and then continuously over the entire experimental period. The other two groups drank regular water. E, wire cage without any mouse; S1, cage with stranger mouse 1; S2, cage with stranger mouse 2; S3, cage with stranger mouse 3.

Figure 5. Continued

(C) Time taken to approach different cages in different sessions of the modified three-chamber test.

(D) Time difference and index of each session in the modified three-chamber test. Differences between S1 and E (S1-E) represent sociability; S2-S1 indicates novelty preference; S3-S1 represents long-term novelty preference. Numbers of examined mice (N) are indicated.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. All statistical methods and results are summarized in Table S2. ***, $p < 0.001$. ns, non-significant.

Social deficits of VCP R95G mice fed on 19% protein diet are also improved by leucine supplementation

Apart from protein levels, the concentrations of fats, carbohydrates, minerals, and vitamins also differ between the 19% and 24.6% protein diets (Table 1). Since our recent study suggested that leucine supplementation increases protein synthesis in *Nf1*^{+/-} mouse brains to ameliorate behavioral deficits and since VCP and neurofibromin act together to regulate neuronal functions, we speculated that the protein element of dietary nutrition is the critical factor involved in controlling the social interactions of VCP R95G mice. To investigate that possibility, we provided the same leucine supplementation to VCP R95G mice fed on the 19% protein diet. We found that leucine supplementation slightly reduced locomotion activity of both WT and VCP R95G mice in an open field (Figure 7A, left). Importantly, leucine supplementation increased social interaction of VCP R95G mice fed on 19% protein diet (Figure 7A, right). By comparing the results from mice fed on the 19% or 24.6% protein diets, we also observed that the social deficits of VCP R95G mice fed on 19% protein diet were reduced compared with the mutant mice fed 24.6% protein diet and that both WT and VCP R95G mice fed on 24.6% protein diet have slightly reduced locomotion activity in an open field compared with mice fed on the 19% protein diet (Figure 7B). The rescue effect of nutrition on social behavior may not be relevant to the difference in locomotion because there was a dietary effect on locomotion but a genetic effect on social behavior in response to different diet (Figures 7A and 7B). Taken together, these comparisons also support that the protein element in chow is indeed critical to regulating the behaviors of VCP R95G mice.

Leucine supplementation increases dendritic spine density in VCP R95G knockin mice

Our previous study suggests that leucine supplementation increases dendritic spine density of cultured VCP R95G mutant neurons (Shih and Hsueh, 2016) and CA1 and prefrontal cortical neurons of *Nf1*^{+/-} mice *in vivo* (Shih et al., 2020b). Here, we further investigated if leucine supplementation for 7 days could rescue these dendritic spine deficits *in vivo*. To monitor neuronal morphology *in vivo*, we crossed VCP R95G knockin mice with Thy1-eYFP transgenic mice (Sun et al., 2010). We used eYFP signals to outline entire dendrites, including the dendritic spines. We found that VCP R95G mutation indeed resulted in a reduced dendritic spine density (Figures 8A and 8B). Although leucine supplementation did not affect the dendritic spine density of wild-type littermates, we found that leucine supplementation for 7 days is sufficient to increase the dendritic spine density of VCP R95G mutant mice to levels comparable to their wild-type littermates (Figures 8A and 8B).

Thus, our results demonstrate that leucine supplementation for 7 days is sufficient to improve the defects of dendritic spine density and behavioral interaction caused by VCP R95G mutation.

Discussion

In this report, our study suggests that *Vcp* mutation makes mice particularly sensitive to nutrient intake, even under nutritional conditions sufficient for normal mice. The effects of two standard diets for rodents, i.e. LabDiet 5K54 (19% protein diet) and 5010 (24.6% protein diet), are compared here. Reduced body weight and weaker muscle strength, both characteristics of IBMPFD (Watts et al., 2004), were observed in 18-month-old VCP R95G mice fed on 19% protein diet but not mice provisioned with 24.6% protein diet. For younger mice, i.e. at 9 weeks, 12 weeks, or even 12 months of age, there was no difference in body weight or muscle strength between VCP R95G mice and WT littermates. Importantly, we found that VCP R95G mice fed on 19% protein diet but not 24.6% protein diet exhibit social deficits in reciprocal social interaction and three-chamber test. Interestingly, VCP R95G mice fed on 24.6% protein diet still possess deficits in long-term social novelty preference and contextual fear memory, which can be improved by providing extra leucine in their drinking water. Our study provides an example of dietary protein content influencing the outcome of genetic variation. Since different diets result in differential phenotypes in terms of mouse behaviors and since different animal facilities provide different diets to their mice, importantly our

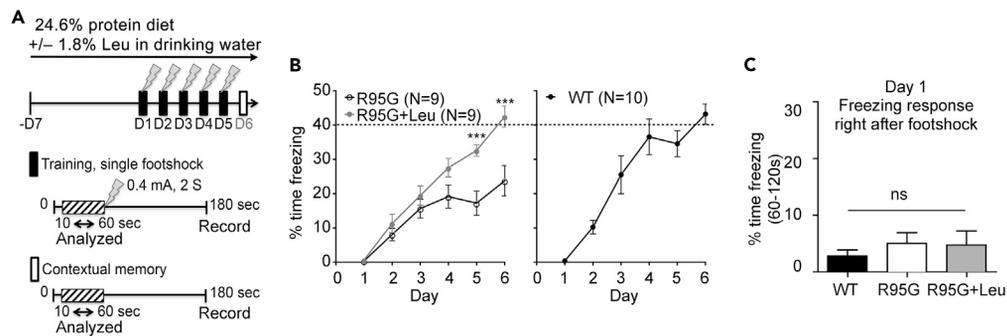


Figure 6. VCP R95G mice fed on 24.6% protein diet have impaired contextual memory

(A) Schematic flowchart of the modified contextual fear conditioning assay. A single foot shock was applied daily on training days 1–5 (D1–D5). The freezing response before foot shock was analyzed to indicate learning/memory performance. At D6, contextual memory in the absence of foot shock was measured. Drinking water supplemented with 1.8% leucine was provided to one group of mice 7 days before being subjected to contextual fear conditioning and then continuously over the entire assay period.

(B) Acquisition of daily freezing responses. VCP R95G mice drinking regular water have a much slower learning curve. In contrast, leucine supplementation improves learning and memory performance, as reflected in higher freezing responses, particularly at days 5 and 6.

(C) Freezing responses right after foot shock (60–120 s) on the first day of contextual fear conditioning. There was no significant difference among groups for immediate freezing responses, though VCP R95G mice tended to freeze more. Numbers of mice examined (N) are indicated.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. ***, $p < 0.001$; ns, non-significant. All statistical methods and results are summarized in [Table S2](#).

study also suggests that mouse diets are likely a factor contributing to discrepancies in studies on mice subjected to the same assay but raised in different facilities.

BCAAs, particularly leucine, have been widely used to induce protein synthesis through activation of the mTOR pathway (Blomstrand et al., 2006; Ishizuka et al., 2008; Ventrucchi et al., 2004). Our previous study demonstrated that VCP controls ER formation and protein synthesis efficiency to regulate dendritic spine density and that leucine supplementation increases protein synthesis via the mTOR pathway to ameliorate the dendritic spine defects observed in cultured neurons (Shih and Hsueh, 2016). In this report, we further suggest that the behavioral defects of VCP R95G knockin mice can also be rescued by leucine supplementation. Apart from our own study, recent studies from other laboratories have also indicated an essential role of BCAAs in brain functions (Novarino et al., 2012; Tarlungeanu et al., 2016). An aberrant BCAA metabolic pathway, caused by mutations in “branched chain ketoacid dehydrogenase kinase”, leads to ASD, intellectual disability, and epilepsy (Novarino et al., 2012). Similarly, mutations in *solute carrier transporter 7a5* (*SLC7A5*)—an amino acid transporter localized in brain capillaries—have also been identified in patients with ASD (Tarlungeanu et al., 2016). Local administration of BCAA solution into cerebroventricles of *Slc7A5*-knockout mice ameliorated abnormal mouse behaviors (Tarlungeanu et al., 2016). Together with our studies, these findings evidence that availability of BCAA is critical for neuronal activity and brain function, which are at least partially mediated by mTOR-regulated protein synthesis.

Previous studies have shown that high protein diets, such as protein contents greater than 33% or even 50–60%, result in lower food consumption and reduced body weight of WT mice (Solon-Biet et al., 2014). Doubling BCAA intake may also result in obesity and a shorter lifespan of WT mice (Solon-Biet et al., 2019). In our study, the 19% protein diet (LabDiet 5k54) and 24.6% protein diet (LabDiet 5010) are standard rodent chows. Details of the chemical composition and calories provided by the protein, carbohydrate, and fat of these two diets are available in [Table S1](#). Although differences in component concentrations are not as large as those reported in the aforementioned studies (Solon-Biet et al., 2014, 2019), we did observe a beneficial effect of the enhanced protein content of LabDiet 5010 relative to LabDiet 5k54. Collectively, these studies indicate that nutritional balance is critical for mouse health and cognitive performance and that genetic variation might shift or reset nutritional balance. For instance, under *Nf1*- or *Vcp*-deficient conditions, mutant neurons suffer from reduced protein synthesis efficiency (Shih and Hsueh, 2016; Shih et al., 2020b), consequently negatively impacting dendritic spine formation and behavioral performance. The

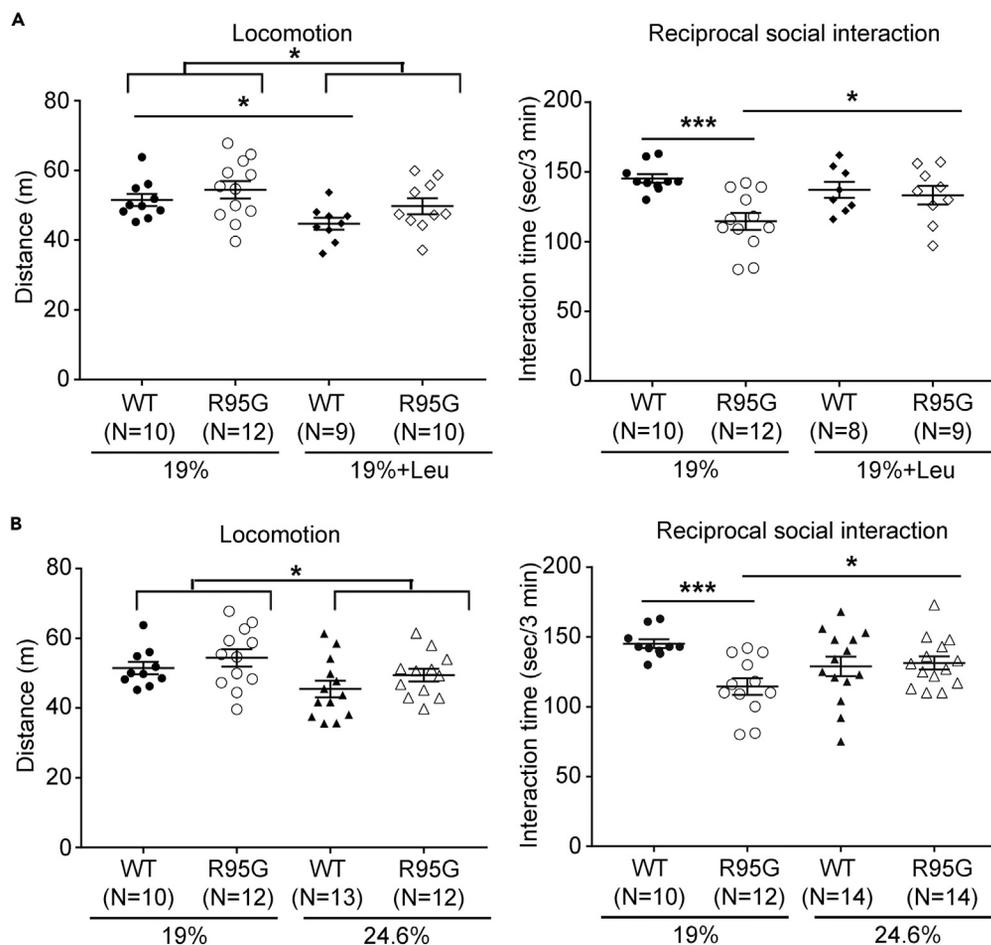


Figure 7. Nutrition influences the behavioral outcome of VCP R95G mice

(A) The effect of leucine supplementation on VCP R95G mice and WT littermates fed on 19% protein diet. Locomotion activity in an open field (left) and interaction time of reciprocal social interaction assay (right) are shown.

(B) Comparison of the effects of 19% and 24.6% protein diets. Numbers of examined mice (N) are indicated.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. *, $p < 0.05$; ***, $p < 0.001$. All statistical methods and results are summarized in Table S2.

behavioral deficits of VCP R95G mice can be offset by increasing total protein intake (such as by changing the diet from 19% to 24.6% protein) or using leucine supplementation to enhance protein synthesis under the condition of having normal protein intake. Note, for WT mice, reducing dietary protein content has been shown to have a beneficial effect on dendritic spine density and cognitive performance in old mice (Wahl et al., 2018). Thus, it is necessary to account for nutritional balance in dietary therapy. In particular, long-term high-dose BCAA supplementation may alter the uptake of other amino acids, resulting in nutritional imbalance. Accordingly, long-term BCAA supplementation has to be closely monitored to ensure that amino acid concentrations and those of other metabolic parameters in blood are not adversely impacted, thereby minimizing potential negative side effects.

Apart from our VCP R95G mice, several other genetic mouse models have been generated to study VCP. Transgenic mice expressing the human VCP gene harboring R155H or A232E mutations have been produced (Custer et al., 2010; Wehl et al., 2007). Another VCP R155H knockin mutant line has also been studied (Badadani et al., 2010; Nalbandian et al., 2013). Those studies focused on bony defects, myopathy, and brain function at the age of 9 months or older and they confirmed that these mutations of the *Vcp* gene cause IBMPFD phenotypes in mice. In our case, the difference in body weight and muscular defects were only observed in 18-month-old mice fed on the 19% protein diet. Since different VCP mutants exhibit

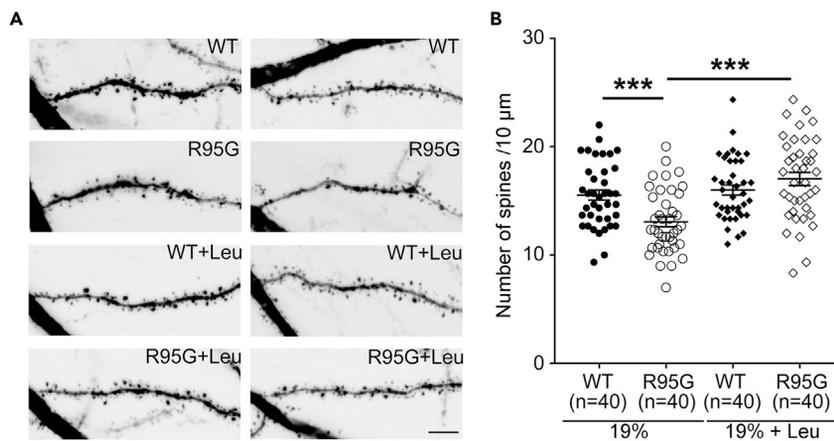


Figure 8. Leucine supplementation increases dendritic spine density in the brains of VCP R95G mice

VCP R95G mice and wild-type littermates carrying the Thy1-eYFP transgene were given leucine-supplemented drinking water or regular water for 7 days and subjected to dendritic spine analysis. (A) Representative images of dendrites of hippocampal CA1 neurons.

(B) Quantification of dendritic spine density. A total of 40 neurons collected from four mice were examined for each group. Each dot indicates the average of two dendrites from each neuron.

Data represented as mean \pm standard error of the mean (error bars) and the results of individual animals are shown. ***, $p < 0.001$. Scale bar, 5 μm . All statistical methods and results are summarized in Table S2.

differential cofactor binding and abnormal ATPase activity (Buchberger et al., 2015; Zhang et al., 2015), the R95G and R155H mutations may have differential cofactor-binding preferences, resulting in variable ATPase activity. In addition, genetic modifiers have been suggested to modulate VCP activity and function (Weihl, 2011), and the various VCP-interacting proteins can bias VCP functions (Hsueh, 2012; Shih and Hsueh, 2016), potentially explaining why our VCP R95G knockin mice exhibit different phenotypes to these other mutant mice. It will be interesting to further compare the biochemical properties of VCP R95G knockin mice with other VCP mutants in the future. Furthermore, we found that VCP R95G mutation results in impaired contextual and social memory, as well as altered social interaction, in mice. These defects may be relevant to symptoms of autism and dementia associated with human patients carrying VCP mutations. Other VCP mutant mice should be analyzed using the same paradigms applied here to assess if impaired contextual learning and memory and social interaction are general defects caused by diverse VCP mutations.

In addition to impaired social behaviors and vocal communication, abnormal sensation is another common feature of ASD, though manifestations of this abnormality vary considerably and patients with ASD may not always present with symptoms of altered sensation. Nevertheless, abnormal pain sensation may influence the results of contextual fear memory assay, which relies on the response of mice to foot shock. In our analysis, we used immediate freezing percentage to evaluate the sensation and immediate response of mice to foot shock. The immediate freezing percentage represents the net response to pain sensation and the danger awareness of mice. Since the net effect was not different among experimental groups, the differences in contextual fear memory of our mutant mice relative to WT is unlikely caused by different immediate freezing responses. However, to truly exclude a contribution of abnormal pain sensation, other paradigms independent of foot shock, such as water maze or Barnes maze, would need to be applied. In addition, specific paradigms to measure pain sensation are also required to conclude if VCP R95G mice exhibit any defect in pain sensation. Since pain sensation is a complex neuronal response involving many different stimuli and neuronal pathways (Kumazaki et al., 2018), a variety of assays would have to be carried out to truly address the issue. In addition, it would be interesting to also investigate olfaction and tactile and auditory senses of our mutant mice as the sensory abnormality is one of the features of ASD. Such analyses would provide a more comprehensive understanding of the sensory profile of our VCP R95G mice. Certainly, mice fed on 19% protein diet would be the most appropriate model for such future experiments, given that they are more likely to manifest phenotypic differences, and amino acid supplementation can also be considered appropriate for rescue experiments.

In conclusion, our study provides strong primary evidence that increased protein intake or amino acid supplementation ameliorates the behavioral and dendritic spine defects of VCP mutant mice. These results confirm the important role of protein synthesis in VCP-regulated brain functions and further suggest a potential therapeutic approach by increasing amino acid and/or protein intake for patients harboring VCP mutations.

Limitations of the study

First of all, we here investigated the effect of VCP R95G mutation identified from patients. It would be interesting to include different VCP mutations and other genetic variations and explore if the conclusion can also be applied to other VCP mutations or other genes. Secondly, leucine supplementation was performed for 7 days before behavioral test. We have not yet investigated whether long-term treatment have similar beneficial effect or any other side effect. Thirdly, it is unclear if Vcp mutation would result in altered sensation, another feature of ASD. Finally, a p value of <0.05 was considered significant in this report.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yi-Ping Hsueh (yph@gate.sinica.edu.tw)

Material availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze data sets or code.

Methods

All methods can be found in the accompanying [Transparent methods supplemental file](#).

Supplemental information

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2020.101949>.

Acknowledgments

We thank the Transgenic Core Facility (funded by AS-CFII-108-104) and the Animal Facility of the Institute of Molecular Biology, Academia Sinica, for excellent technical assistance, Dr. John O'Brien for English editing, and members of Y.-P.H.'s laboratory for relabeling samples for blind experiments. This work was supported by grants from Academia Sinica (AS-IA-106-L04) and the Ministry of Science and Technology (MOST 108-2321-B-001-002 and 108-2311-B-001-008-MY3) to Y.-P.H.

Author contributions

T.-N.H., Y.-T.S., and S.-C.L. designed and performed experiments, analyzed data, and wrote the manuscript. Y.-P.H. performed the project planning, experimental design, writing of the manuscript, and secured funding support.

Declaration of interests

Y.-T.S., S.-C.L., and T.-N.H. declare that the research was conducted in the absence of any potential conflict of interest. Y.-P.H. reported the patent on "Increase of protein synthesis ameliorates synaptopathy-related neurological disorders".

Received: April 14, 2020

Revised: November 1, 2020

Accepted: December 11, 2020

Published: January 22, 2021

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iScience, Volume 24

Supplemental Information

**Social behaviors and contextual memory of *Vcp*
mutant mice are sensitive to nutrition
and can be ameliorated by amino acid supplementation**

Tzyy-Nan Huang, Yu-Tzu Shih, Si-Cih Lin, and Yi-Ping Hsueh

Supplemental Table S1. Chemical composition of LabDiet 5K54 (19%) and 5010 (24.6%), Related to Figure 1.

	5K54	5010	Total Digestible Nutrients, %	73.8	74.8
Nutrients			Gross Energy, kcal/ gm	4.02	4.17
Protein, %	19	24.6	Physiological Fuel Value, kcal/ gm	3.4	3.43
Arginine, %	0.99	1.55	Metabolizable Energy, kcal/ gm	3.07	3.02
Cystine, %	0.25	0.42	Minerals		
Glycine, %	0.92	1.21	Ash, %	6.3	6.1
Histidine, %	0.43	0.63	Calcium, %	1.17	1
Isoleucine, %	0.83	1.02	Phosphorus, %	0.92	0.79
Leucine, %	1.52	1.86	Phosphorus (non-phytate), %	0.68	0.46
Lysine, %	0.93	1.45	Potassium, %	0.63	0.99
Methionine, %	0.73	0.56	Magnesium, %	0.22	0.22
Phenylalanine, %	0.83	1.08	Sulfur, %	0.33	0.31
Tyrosine, %	0.54	0.73	Sodium, %	0.26	0.29
Threonine, %	0.66	0.93	Chloride, %	0.45	0.48
Tryptophan, %	0.22	0.28	Fluorine, ppm	38	15
Valine, %	0.88	1.14	Iron, ppm	370	250
Serine, %	0.93	1.17	Zinc, ppm	84	130
Aspartic acid, %	1.77	2.64	Manganese, ppm	160	120
Glutamic acid, %	4.35	4.8	Copper, ppm	10	19
Alanine, %	1.12	1.48	Cobalt, ppm	0.8	0.6
Proline, %	1.51	1.52	Iodine, ppm	2.2	1.6
Taurine, %	0.03	0.05	Chromium (added), ppm	2	0.01
Fat			Selenium, ppm	0.32	0.47
Fat (ether extract), %	4.6	5	Vitamins		
Fat (acid hydrolysis), %	5.6	6.4	Carotene, ppm	1.5	1.3
Cholesterol, ppm	240	283	Vitamin K (as menadione), ppm	20	3.4
Linoleic Acid, %	2.07	1.42	Thiamin Hydrochloride, ppm	78	81
Linolenic Acid, %	0.23	0.14	Riboflavin, ppm	9	16
Arachidonic Acid, %	0.01	0.02	Niacin, ppm	90	120
Omega-3 Fatty Acids, %	0.33	0.42	Pantothenic Acid, ppm	37	26
Total Saturated Fatty Acids, %	1.01	1.17	Choline chloride, ppm	2000	2200
Total Monounsaturated Fatty Acids, %	1.04	1.29	Folic Acid, ppm	1.9	6.1
Fiber			Pyridoxine, ppm	15	17
Fiber (Crude), %	4.2	4.2	Biotin, ppm	0.3	0.3
Neutral Detergent Fiber, %	15.4	14.6	B ₁₂ , mcg/ kg	50	50
Acid Detergent Fiber, %	5.2	5.3	Vitamin A, IU/ gm (IU= 0.3mcg)	20	24
Nitrogen-Free Extract (by difference), %	55.5	50	Vitamin D ₃ (added), IU/ gm	4.3	4.6
Carbohydrate			Vitamin E, IU/ kg (IU= 1mg)	66	61
Starch, %	40.7	29.4	Ascorbic Acid, mg/ gm	-	-
Glucose, %	0.16	0.23	Calories provided by:		
Fructose, %	0.19	0.27	Protein, %	22.382	28.668
Sucrose, %	0.58	1.16	Fat (ether extract), %	12.192	13.111
Lactose, %	0	0	Carbohydrates, %	65.426	58.221

Transparent Methods

Animals

All animals were housed and bred in the animal facility of the Institute of Molecular Biology, Academia Sinica, under controlled humidity and temperature and a 12 h light/dark cycle (light off at 20:00). Animals accessed water and food *ad libitum*. Two mouse diets, LabDiet 5010 and 5K54, were compared. Protein content in 5K54 is 19%, whereas 5010 contains 24.6% protein. The complete chemical composition of these two diets is presented in **Table S1**. Our mouse facility routinely uses LabDiet 5010 for breeding and maintaining all wild-type and mutant mouse lines. To investigate if mice fed with different diets exhibit differential behaviors, we compared mice fed from embryonic stage with 19% or 24.6% protein diets, which did not affect offspring number. VCP R95G knockin mice were generated as previously described (Shih and Hsueh, 2016) and had been backcrossed to WT C57BL/6 mice (National Laboratory Animal Center, Taiwan) for more than six generations to minimize off-target effects. Thy1-eYFP-H (Sun et al., 2010) mice were purchased from Jackson laboratory. Male VCP R95G mice were mated with wild-type female C57BL/6 mice to generate breeders and mice (both +/+ and +/R95G) for behavioral experiments. To monitor dendritic spine morphology, female Thy1-eYFP transgenic mice were mated with male VCP R95G knockin mice. The signals of eYFP were used to outline neuronal morphology. Adult male mice were used for behavior analyses and littermates comprised different genotypes (3-5 mice/cage on average) housed together after weaning at 3-4 weeks without selection for behavioral assays. Before behavior experiments (summarized in **Table 1**), the mice were acclimatized in the experimental area for at least one week. Only littermates were used for comparisons. Wild-type male C57BL/6 mice of similar age to test mice (for the three-chamber test) or 2-3 weeks younger than test mice (for reciprocal social interaction) were purchased from the National Laboratory Animal Center, Taiwan, as strangers for social interaction tests. To avoid potential personal bias, all behavioral analyses were performed blind, i.e., without knowing the genotype and/or treatment during experiments and quantification. All animal experiments were performed with the approval of the Academia Sinica Institutional Animal Care and Utilization Committee (Protocol No. 14-11-759), and in strict accordance with its guidelines and those of the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals, Taiwan.

Dendritic spine analyses using Thy1-eYFP transgenic mice

The morphology of hippocampal CA1 neurons was visualized by labeling with the Thy1-eYFP transgene and then recorded using a confocal microscope (LSM700, Zeiss) equipped with a

Plan-Apochromat 63× NA 1.4 oil objective lens (Zeiss) and captured with Zen acquisition and analysis software (Zeiss) at 20–22 °C as a Z-series of 5–25 sections spaced 0.2 μm apart. The Z-series was then projected into single images for quantification and publication. For publication, the images were processed with Photoshop (Adobe). To analyze dendritic spine density, the numbers of spines for dendrite fragments of 20 μm in length, starting from a point 5 μm away from the base of the first branch, were manually counted using ImageJ software. To account for variation among different mice, data from four mice for each group were pooled for statistical analysis. To minimize potential personal bias, experiments were performed blind by relabeling the samples with the assistance of other laboratory colleagues. Thus, quantification was performed without knowing genotype or treatment.

X-ray imaging

Bone anatomies of 6-month old mice were examined using a GTI-5000 Real-Time Image Workstation (Glenbrook Technologies Inc., Randolph, NJ, USA).

Hematoxylin and eosin staining

Fifty-μm-thick sections mounted on slides were sequentially hydrated with 70%, 95% and 100% ethanol and then put into hematoxylin solution. After washing with ddH₂O, sections were put into acid ethanol (3% HCl in 70% ethanol) to remove excess stain. After incubating with Scott's tap water solution (Sigma-Aldrich), sections were incubated with eosin solution. After rinsing with distilled water, the sections were dehydrated by 70%, 95%, and 100% ethanol and then by xylene, before being mounted on slides with Permount (Electron Microscopy Sciences).

Mouse behavioral tasks

Male mice were habituated in the behavior room for at least one week prior to undertaking tasks. Measurements of body weight and muscle strength, as well as behavioral assays, were performed at the ages summarized in **Table 1**. Housing condition for each group of mice was also summarized in **Table 1** of main text. Muscle strength was measured using a force gauge (FG-5005, Lutron Electronic). Before social interaction tests, mice were individually isolated for at least one week.

Open-field tests were performed based on a previous study (Chung et al., 2011). Briefly, mice were individually placed into an open box (transparent plastic box 40 x 40 x 30 cm) and allowed

to freely explore it for 10 min. The area of the central region was equal to the total area of the four corners, and the regions were marked on the bottom of the box. The movement distance of the test mouse was recorded from above by videotaping for 10 min. The rearing number was counted manually. The total moving distance and the time spent in the four corners and the central area were quantified with the Smart Video Tracking System (Panlab). Total travel distance indicates horizontal locomotor activity. The time spent in the corners indicates anxiety.

Light-dark box assays were carried out as described (Lin and Hsueh, 2014). Briefly, the apparatus for this test was modified from the open field box. An open black box (19 x 39 x 45 cm) was inverted and put into the open field box to divide it into two compartments of equal size. A small opening (5 cm in diameter) at the bottom of the black box facilitated mouse access between the two compartments. An individual mouse was placed into the light compartment and then allowed to explore the apparatus for 10 min. Its movement was recorded by videotaping and analyzed using the Smart Video Tracking System (Panlab, Barcelona, Spain). The time spent in the light box, number of light-to-dark transitions, and total moving distance in the light box were measured.

Y-maze was performed as described (Chung et al., 2011). Briefly, the Y-maze apparatus consisted of three identical arms of enclosed Plexiglas (40 x 4.5 x 12 cm) spaced 120° apart. Each mouse was placed at the end of one arm facing the center and allowed to freely explore the apparatus for 8 or 10 min. Movement in the Y maze was videotaped from above. The series of arm entries was analyzed with the Smart Video Tracking System. Actual alternation was defined as consecutive entries into each of the three arms without repetition (for example, ABC, BCA, CAB). Spontaneous alternation was defined by dividing the number of actual alternations by the number of possible alternations (total arm entries - 2).

Rotarod test was performed using a rotarod apparatus (RT-01, SINGA Diagnostic & Research Instruments Co., Taiwan) as described (Hung et al., 2018; Shiotsuki et al., 2010), except that two different speed settings were used. Briefly, mice were first placed on the resting drum for 30 sec to ensure that they could stably remain on the drum (habituation). To test motor coordination, rotation was initiated and continuously accelerated stepwise from 0-30 rpm or at a fixed accelerated rate of 0-50 rpm for 3 min. For each setting, mice were subjected to three trials per day with 10–15 min intervals between trials for three consecutive days. The apparatus

was cleaned with 70% ethanol and air-dried between usages by different mice. Latency to fall in each trial was recorded and the longest latency for each day represented the rotarod performance of mice.

Modified contextual fear conditioning: Since VCP works together with neurofibromin to control dendritic spine formation, we employed a modified contextual fear conditioning assay developed for *Nf1*-deficient mice (Cui et al., 2008) to analyze our VCP R95G knockin mice. Briefly, the entire task was carried out over six days, with one trial per day. For the first five days, mice were placed into a conditioning chamber for 60 sec before receiving a single foot shock (0.4 mA intensity for 2 sec). After foot shock, mice stayed in the chamber for another 120 sec before returning to their home cage. On the sixth day, mice were placed into the conditioning chamber for 180 sec without foot shock. Mouse behaviors during the entire 180-sec period were videotaped. Freezing responses were measured with the FreezeScan 2.0 system (CleverSys) (Huang et al., 2014). Freezing responses during the initial 10-60 sec of the first trial indicates basal habituation and responses for the 60-120 sec of the same trial reflects pain sensation of mice. The freezing percentage of the trials on the subsequent second to fifth days were used to indicate learning performance in response to the cumulative stimulation. The response on the sixth day represented fear memory.

Modified three-chamber test for social memory: The social memory of animals was assessed using the three-chamber test also adapted for *Nf1*-deficient mice (Huang et al., 2014; Molosh et al., 2014) (**Fig. 2D**). Briefly, mice were housed individually for at least 1 week before being assayed. Stranger mice of similar age to test mice were habituated and familiarized with a wire cage in a three-chambered apparatus for 10 min at least one day before acting as strangers. For our modified three-chamber assay, a test mouse (testee) was always placed in the middle chamber to initiate exploration for each session. Mouse behaviors were recorded from above. The test comprised five consecutive sessions. In the first session (for habituation), two empty wire cages were placed in the two side chambers. After freely exploring all three chambers for 10 min, the testee was returned to its home cage for 5 min. Of the two side chambers, the one the testee spent most time in during the first session was designated the “preferred chamber”. In the second session (to test sociability), stranger mouse 1 (S1) was placed in a wire cage at the opposite side of the testee’s preferred chamber and then the testee was placed in the middle chamber again to explore all three chambers for 10 min. After being returned to its home cage for 5 min, stranger mouse 2 (S2) was placed in the empty wire cage and the testee was again

allowed to explore all three chambers for another 10 min (third session for novelty preference). S2 was then returned to its home cage, but the testee was allowed to remain interacting with S1 for a further 45 min before being returned to its home cage (fourth session). Twenty-four hours later, stranger mouse 3 (S3) was placed in the cage originally occupied by S1 and S1 was switched to the opposite end of the apparatus. In this last session to test long-term novelty preference, the testee was placed back in the chamber and allowed to freely explore all three chambers for 10 min. Chambers were cleaned with 75% EtOH and air-dried for 10 min before starting a test with a new mouse. All wire cages were washed with 75% EtOH and water after each session. The time spent interacting or sniffing each wire cage of the left and right side chambers was measured using the Smart Video Tracking System (Panlab). Three parameters were analyzed in each session to represent sociability, novelty preference, and long-term novelty preference. The first parameter was approach time to a wire cage without any mouse (E), or a cage with stranger 1 (S1), S2 or S3 in different sessions. The second parameter was the difference in approach times to E, S1, S2 or S3. The third parameter was a social index representing the ratio of the difference in approach time to the total approach time for each session.

Reciprocal social interaction was performed as described previously (Chung et al., 2011; Lin et al., 2013). Briefly, an unfamiliar 4-5-week-old mouse was put into the home cage of the test mouse for 10 min, though we only analyzed the first 3 min of the assay. The lid of the cage was removed for the entire session to minimize aggressive behaviors. Mouse behaviors were recorded by videotaping from above. The total time a test mouse spent interacting with and sniffing the unfamiliar mouse was manually recorded to represent social interaction. By keeping the lid open, we found that aggressive behavior was very limited and so was not assessed.

Ultrasonic vocalization was performed as described (Huang et al., 2014; Shih et al., 2020a). At postnatal days 4, 6, 8 and 10, pup was individually transferred into a clean beaker with new bedding in a soundproof chamber for recording. Ultrasonic vocalizations of isolated pup were recorded by Avisoft Bioacoustics ultrasonic recording system (Model 116H) for 10 min. Before analysis, the spectrograms of vocalization were set within the range between 500 kHz and 30 kHz. The squirming noise of pup was manually removed. Mouse calling whistles with a duration ≥ 5 ms and a hold time of 30 ms were identified by “automatic” (whistle tracking) of

the Avisoft-SASLab Pro software. These experiments were performed and analyzed without knowing the genotypes of mice.

Leucine supplementation

Since VCP and neurofibromin work together to control dendritic spine density (Wang et al., 2011), leucine supplementation designed for rescuing the behavioral deficits of *Nfl*^{+/-} mice (Shih et al., 2020b) was applied to VCP R95G mice. Mice were provided with normal water or leucine-supplemented water (1.8%) for 7 days prior to testing and then continuously for the duration of the entire behavioral task. Leucine solutions were replaced daily throughout the entire assay period. We measured the daily amounts of water drunk to be ~5 mL per mouse. Since the body weight of mice at the time of experimentation was ~25 g, additional leucine uptake was estimated to be 1.8g leucine/100 mL x 5 mL water/25 g body weight = 3.6 mg/g mouse body weight/day.

Statistical analysis

All quantitative data in this report are presented as means plus SEM. Graphs were plotted using GraphPad Prism 5.0 or 7.0 (GraphPad software). No statistical method was applied to evaluate the sample size, but our sample sizes are similar to those of previous publications (Huang et al., 2014; Sananbenesi et al., 2007; Shih and Hsueh, 2016). Basically, 10 neurons/mouse were collected from a total of four mice for dendritic spine analysis. Dendritic spine density of each neuron was the average of two dendrite fragments of one neuron. Data collection and analysis were conducted randomly and blind. Statistical analysis was performed using the two-tailed unpaired or paired Student's *t* test for two-group comparisons. For nonparametric data, Kruskal-Wallis test with Dunn's multiple comparisons was conducted in Prism 7.0. Two-way ANOVA or two-way ANOVA with repeat measures and Bonferroni's correction was performed in SigmaStat 3.5 to analyze the effects of two genetic factors combined with two different treatments. *P* values of less than 0.05 were considered significant. All statistical methods and results are summarized in **Table S2**

Supplemental References

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