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Behavioral alterations in mice lacking the translation repressor 4E-BP2

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Abstract

The requirement for *de novo* protein synthesis during multiple forms of learning, memory and behavior is well-established; however, we are only beginning to uncover the regulatory mechanisms that govern this process. In order to determine how translation initiation is regulated during neuroplasticity we engineered mutant C57Bl/6J mice that lack the translation repressor eukaryotic initiation factor 4E-binding protein 2 (4E-BP2) and have previously demonstrated that 4E-BP2 plays a critical role in hippocampus-dependent synaptic plasticity and memory. Herein, we examined the 4E-BP2 knockout mice in a battery of paradigms to address motor activity and motor skill learning, anxiety and social dominance behaviors, working memory and conditioned taste aversion. We found that the 4E-BP2 knockout mice demonstrated altered activity in the rotating rod test, light/dark exploration test, spontaneous alternation T-maze and conditioned taste aversion test. The information gained from these studies builds a solid foundation for future studies on the specific role of 4E-BP2 in various types of behavior, and for a broader, more detailed examination of the mechanisms of translational control in the brain.

Keywords: Protein synthesis; Behavior; Learning and memory; Taste aversion; Rota-rod; Working memory; eIF4E; Translation initiation; Hippocampus; Amygdala; Insular cortex; Cerebellum

1. Introduction

The cellular mechanisms underlying learning, memory and behavior are generally attributed to molecular events that impact synaptic physiology. *De novo* protein synthesis contributes to several forms of long-lasting synaptic plasticity and morphogenesis; therefore, it is not surprising that multiple forms of learning, memory and behavior are altered when protein synthesis is disrupted.

Protein synthesis is primarily regulated at the level of mRNA translation initiation (reviewed in Dever, 2002). The rate-limiting event is recruitment of the 43S ribosome to the mRNA. A critical step in this process is the binding of the eukaryotic initiation factor 4E (eIF4E) as part of a protein complex with eIF4G and eIF4A (collectively termed eIF4F) to the cap structure at the mRNA 5' terminus (Gingras, Raught, & Sonenberg, 1999). Inhibition of initiation is achieved via 4E-binding proteins (4E-BP), which compete with eIF4G for binding eIF4E (Pause et al., 1994; Poulin, Gingras, Olsen, Chevalier, & Sonenberg, 1998). Of the three known 4E-BP isoforms, 4E-BP2 is the predominant isoform expressed in the mouse brain (Banko et al., 2005; Tsukiyama-Kohara et al., 2001). We have recently begun to identify the specific contributions of 4E-BP2 to synaptic plasticity and learning and memory by examining these phenomena in mice engineered to lack 4E-BP2. Our analyses have disclosed that 4E-BP2 knockout mice have upregulated eIF4F complex formation, altered hippocampal long-term potentiation (LTP) and long-term

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depression (LTD) and hippocampus-dependent memory impairments (Banko, Hou, Poulin, Sonenberg, & Klann, 2006, 2005).

Protein synthesis regulates synaptic function in extra-hippocampal brain regions as well. For example, synaptic plasticity in the amygdala and behaviors attributed to this brain region rely on protein synthesis (Huang, Martin, & Kandel, 2000; Nader, Schafe, & LeDoux, 2000; Schafe & LeDoux, 2000). A similar case has been argued for the cerebellum (Bracha et al., 1998; Linden, 1996) and the insular cortex (Jones, French, Bliss, & Rosenblum, 1999; Rosenblum, Meiri, & Dudai, 1993) among others. We therefore sought to determine whether the lack of 4E-BP2 impacted behaviors that utilize these extra-hippocampal brain regions. Our examination included a battery of paradigms to address 4E-BP2 knockout motor activity and motor skill learning, anxiety and social dominance behaviors, working memory and conditioned taste aversion. Interestingly, we found that 4E-BP2 knockout animals demonstrated altered behavior in the rotating rod test, light/dark exploration, spontaneous alternation T-maze and conditioned test aversion test. This investigation extends our previous findings that 4E-BP2 is critical for hippocampus-dependent synaptic plasticity and memory to include a role for 4E-BP2 in behaviors that are governed by additional brain regions.

2. Methods

2.1. Animals

Behavioral testing was performed on one colony of male 4E-BP2 knockout mice and their wild-type (WT) littermates. Tests were performed in four blocks with each block containing a unique group of animals between the ages of three to five months old. In each block, tests were separated by at least one week where the animals were housed in their home cages under standard husbandry conditions. The first block consisted of testing performed in the following order: rotating rod task, open field test and elevated plus maze. The second block consisted of the spontaneous alternation T-maze test. The third block consisted of an additional open field test, light/dark and light/light tests and the tube test. Finally, the fourth block consisted of the conditioned taste aversion tests. All behavioral testing procedures were approved by the Baylor College of Medicine Animal Care and Use Committee and followed the National Institutes of Health Guidelines.

2.2. Open field test

Mice were placed in the center of an open field (40 × 40 × 30 cm) for 15 min and a computer-operated optical system (Acuscan) monitored the movement of mice as they explored in the open field. The apparatus was cleaned with 70% EtOH between subjects. The data were pooled according to genotype, and a mean value was determined for each group. The parameters tested were: distance traveled, ambulatory time, vertical beam breaks and the ratio of center to total time. Results are presented as means + SEM and were analyzed by Student's *t*-test with $p \leqslant .05$ used as significance criteria.

2.3. Rotating rod task

The rotating rod test was performed on an accelerating Rota-Rod Treadmill (Ugo Basile) using a 3.8-cm-diameter rubberized rod. Testing was done for three sequential days with four trials per day spaced 45 min apart. The apparatus was cleaned with 70% EtOH between subjects. The test protocol involved an accelerating protocol from 4 to 40 rpm over a 5 min period that ended whenever a test animal fell or when the protocol was completed. Two episodes of holding onto the rod rotating 360° also were scored as a fall, and the time of the second rotation was recorded as such. The time to fall was noted (latency), and a mean for the four trials was calculated for each day. The data were pooled according to genotype, and a mean value was determined for each group. Results are presented as means ± SEM and were analyzed by two-way ANOVA with $p \leqslant .05$ used as significance criteria.

2.4. Light/dark and light/light tests

Apparatus for these tests consisted of a polypropylene chamber divided into two either by a black (light/dark) or a white (light/light) partition containing a small opening. For the light/dark test, one chamber was open and illuminated while the other was closed and dark. Each subject was placed in the light chamber first. The latency to cross to the dark chamber, total number of light entries and total time spent in the light chamber were recorded over 10 min. For the light/light test each chamber was open and illuminated and each subject was placed in the left chamber. The latency to cross to the other chamber and total number of crosses were recorded over 10 min. Results are presented as means + SEM and were analyzed by Student's *t*-test with $p \leqslant .05$ used as significance criteria.

2.5. Passive avoidance

Step-through passive avoidance was performed by placing animals in a conditioning chamber separated into two compartments (one illuminated and one dark) by a partition containing a trap door. On the training day, mice were placed in the illuminated section and allowed to move freely in the chamber. The latency for the mouse to move into the dark compartment was recorded. When the mouse had completely entered the dark half of the chamber, the trap door was shut and a mild foot shock (1 s, 0.5 mA) was delivered. After 10 s in the dark section the animal was removed and returned to its home cage. Testing was conducted 24 h later and consisted of placing the animal back into the lit compartment. Step-through latencies were once again measured. Results are presented as means + SEM and were analyzed by Student's *t*-test with $p \leqslant .05$ used as significance criteria.

2.6. Elevated plus maze

The plus maze consisted of two open arms (30 cm × 5 cm) facing each other and two enclosed arms (30 cm × 5 cm × 15 cm) also facing each other. Each arm was attached to a common center platform (5 cm × 5 cm) and the apparatus was elevated 40 cm off the floor. Each mouse was placed in the center of the maze facing an open arm. During the testing period of 5 min, the total time spent in each arm was recorded. Results are presented as means + SEM and were analyzed by Student's *t*-test with $p \leqslant .05$ used as significance criteria.

2.7. Tube test

Two mice of different genotypes (one KO and one WT) that were approximately the same weight were placed in opposite ends of a plastic tube (40.6 cm long × 3.5 cm inner diameter). A win was achieved by the dominant mouse pushing the other mouse out of the other end of the tube. Each mouse was confronted with up to three different combatants with at least a 5 min inter-trial interval. Results are presented as percent wins and were analyzed by χ^2 analysis with $p \leqslant .05$ used as significance criteria.

2.8. Spontaneous alternation T-maze

Mice were placed in the stem arm of a T-maze. The mouse was allowed to travel down the stem into either the left or the right choice arm. Ten seconds after the choice, the mouse was returned to the stem arm. The mouse remained in the stem arm behind a Plexiglas barrier for 30 s. The barrier

then was removed and the mouse was allowed to travel down either choice arm. The choice was scored as either "same" or "alternate." The mouse then was returned to its home cage. Each mouse was given two choices on two consecutive days. Data were analyzed by χ^2 analysis with $p \leqslant .05$ used as significance criteria.

2.9. Conditioned taste aversion

During the first 4 days, mice were trained to drink from pipettes in limited time periods (mild water deprivation (W.D.)). Then the mice were allowed to drink ad libitum from two pipettes of 3 ml water each, at two drinking sessions per day. The first session was at 11 a.m. and persisted for 20 min and the second session was at 3 p.m. persisted for 10 min. Day five was the conditioning day. Either saccharin (0.5%) or NaCl (0.5%) was used as a novel taste (CS) and LiCl (0.14 M, 2% body wt) was used as a toxic agent (US). Mice were permitted to drink the CS during the first drinking session. One hour later, they were injected with LiCl. On day six, mice were permitted to drink water (W.D. as in days one to four). On the first session of day seven, a test was preformed. In this test mice received two pipettes simultaneously; one containing 3 ml of CS and the other containing 3 ml of water. The aversion index is defined as: [ml water/(ml water + ml CS)] * 100 consumed in the test, where 50% is chance level; therefore, the higher the aversion index, the more the mice prefer water to novel conditioned taste. The aversion index is defined to minimize the variability in fluid consumption on the results.

The control groups for the CTA experiments were subjected to a similar manipulation to the experiment group, but saline was employed as the US. Mice in control groups chose between water and the novel taste with no earlier exposure to the novel taste and without saline injection. This information will reveal whether there is a difference in the natural preference for the novel taste between knockout and wild-type mice. In order to address possible differences in sensory levels between the knockout and wild-type mice, we tested the natural aversion to quinine (bitter taste). Over three consecutive drinking sessions, mice received two pipettes simultaneously, one containing 3 ml of 0.04% quinine and the other containing 3 ml of water. The aversion index was defined as above. Statistical analysis was performed using one-way ANOVA and ANOVA repeated measures. For post hoc comparisons, the LSD contrast test was used with a level of 0.05 used as significance criteria. Results are presented as means + SEM.

3. Results

3.1. Motor activity

The most standardized general measure of motor function is spontaneous activity in the open field (Holland & Weldon, 1968). A 15-min session in an open field is sufficient to evaluate gross abnormalities in locomotion such as hyperactivity or behavioral sedation. When exposed to the novel open field chamber, the 4E-BP2 knockout mice were indistinguishable from their wild-type counterparts in total distance traveled, total ambulatory time and the number of vertical beam breaks (Fig. 1A–C). In addition to general locomotor activity, the open field test also can be used as a nominal assay for anxiety-related behavior phenotypes. The time spent in the center of the open field is inversely correlated with anxiety (Crawley, 1985). The ratio of the center to total time calculated for the 4E-BP2 knockout mice was comparable to that calculated for their wild-type counterparts (Fig. 1D). Taken together, these results indicate that the 4E-BP2 knockout mice have

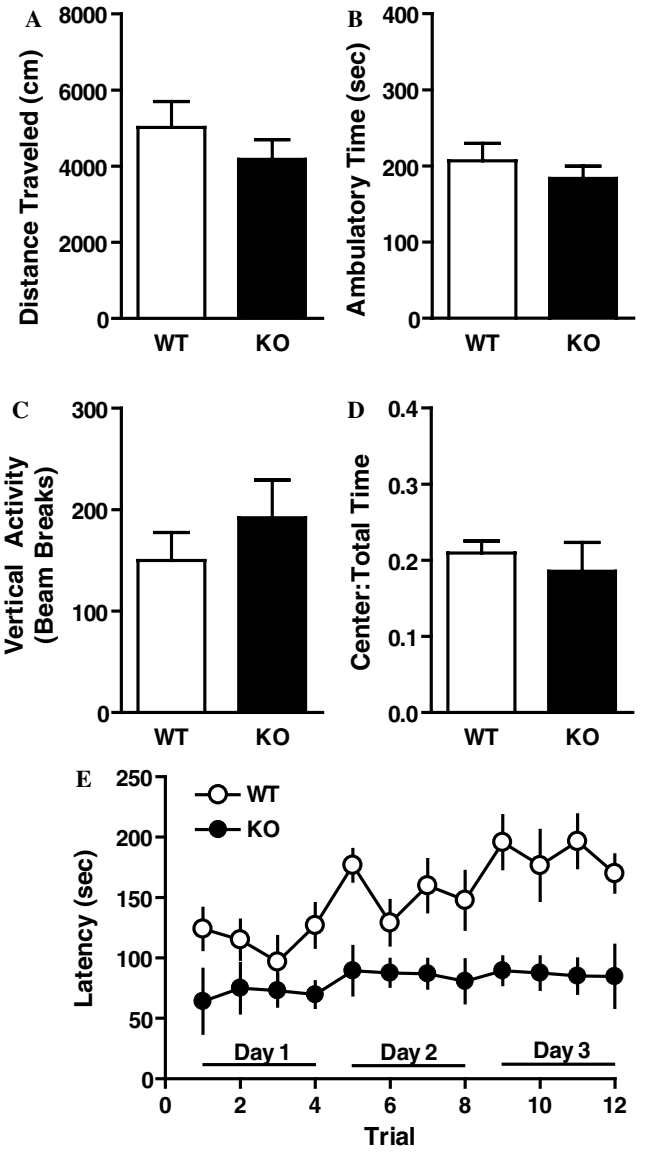

Fig. 1. Motor skill learning requires 4E-BP2. Motor activity and motor skill learning were evaluated with a 15-min open field test and the rotating rod task. Wild-type (WT) and 4E-BP2 knockout (KO) mice behaved similarly in the open field test for each parameter examined, but exhibited compromised motor coordination and learning on the rotating rod task. (A) Total distance traveled in the open field (WT = 5013 ± 685.1 cm, KO = 4180 ± 512.6 cm; $p = .34$, $n = 14$ animals per genotype). (B) Ambulatory time in the open field (WT = 206.7 ± 23.08 s, KO = 183.8 ± 15.78 s; $p = .42$, $n = 14$ animals per genotype). (C) Vertical activity in the open field (WT = 150 ± 27.5 beam breaks, KO = 192.1 ± 37.08 beam breaks; $p = .3793$, $n = 7$ animals per genotype). (D) Center: total time in the open field (WT = 0.2093 ± 0.02, KO = 0.1855 ± 0.04; $p = .57$, $n = 8$ animals per genotype). (E) Latency to fall off the rotating rod ($p < .001$, $n = 13$ animals per genotype).

normal spontaneous locomotor activity and that they do not exhibit altered anxiety when presented with a novel open field environment.

Motor coordination and balance were measured by performance on the rotating rod (Jones & Roberts, 1968). A feature of the rotating rod task is that it is largely independent of reward, of spatial and of instrumental/operand

features. Falling off the rod may represent a weak aversive stimulus. However, the distance to fall was small (40 cm), and we avoided additional aversive stimuli such as electric shocks, which are often used during the rotating rod test. Therefore, motor skill learning can be assumed as the major component responsible for improvements in rotating rod performance. Performance on the rotating rod task over days of repeated training is quantified as a measure of motor learning. Performance on the rotating rod task by the 4E-BP2 knockout mice was significantly decreased compared to wild-type controls on the first trial and failed to improve with the same rate as the wild type controls (Fig. 1E). These results indicate that the 4E-BP2 knockout mice suffer impaired motor learning and suggest that the 4E-BP2 knockout mice also have compromised motor coordination and balance. Interestingly, the overall activity of the 4E-BP2 knockout mice in the open field analysis parallels that of the wild-type mice, and their swimming ability is uncompromised (Banko et al., 2005), which suggests that the phenotype demonstrated by the 4E-BP2 knockout mice on the rotating rod represents a subtle deficit in motor coordination and balance that is only observed when the animals are presented with a more demanding motor task than either ambulation or swimming.

3.2. Anxiety and social dominance behavior

Several methods have been used to show that the amygdala, septohippocampal system, medial hypothalamus, central periaqueductal grey and frontal and cingulate cortices are important brain structures involved in the regulation of anxiety and fear (Camargo & Saad, 2001; LeDoux, 1998; McNaughton & Gray, 2000; Panksepp, Siviy, Normansell, White, & Bishop, 1982). Tests of rodent anxiety-related behaviors are generally based on conflicts. Anxiety-like behavior is thought to result from the conflict inherent in approach–avoidance situations (for review, Belzung & Griebel, 2001). A mouse may want to explore a new environment but may not want to venture out into the open, exposed space in the daylight, where it is an easy target for predators. The light/dark test is a nonshock, naturalistic conflict, optimized for mice, titrating the tendency of mice to explore a novel environment versus the aversive properties of a brightly lit open field. A reduced level of anxiety is correlated with a higher latency to initially cross into the dark chamber as well as a greater number of lit chamber entries. The 4E-BP2 knockout mice demonstrated a higher latency to initially cross into the dark chamber (Fig. 2A), suggesting a reduced level of anxiety. Unexpectedly, however, the 4E-BP2 knockout mice exhibited less number of light entries as well as

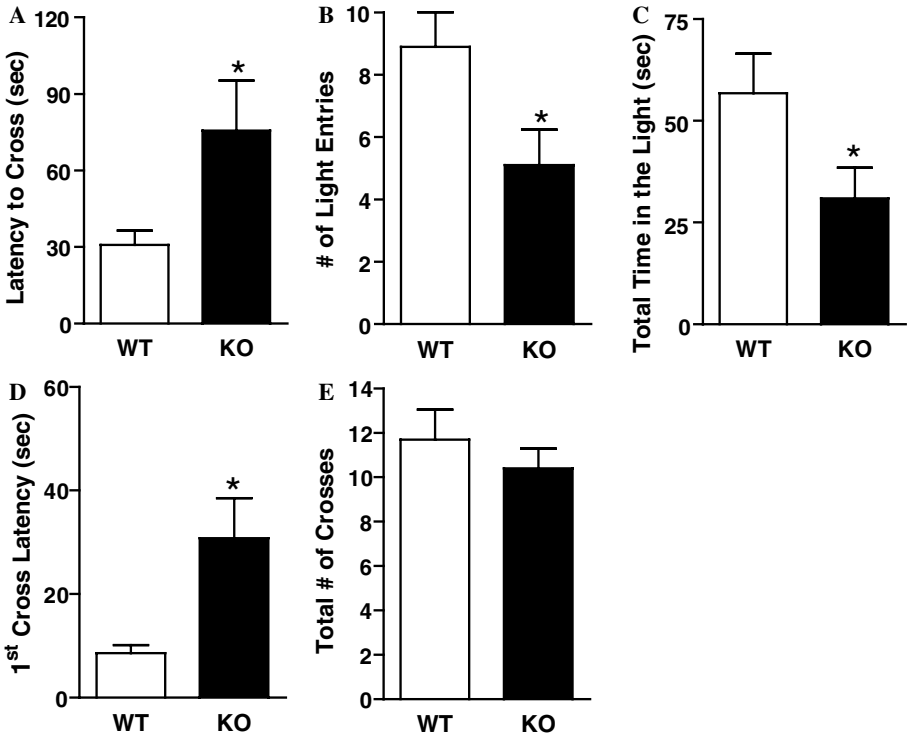

Fig. 2. 4E-BP2 knockout mice demonstrate altered exploratory behavior. The light/dark and the light/light tests were employed to examine exploratory behavior. The 4E-BP2 knockout mice favored exploration of a novel context based on first experience rather than light versus dark. (A) Latency to first cross from the light to the dark side during the light/dark test (WT = 30.78 ± 5.66 s, KO = 75.67 ± 19.57 s, $p = .04$, $n = 9$ animals per genotype). (B) Number of light entries during the light/dark test (WT = 8.9 ± 1.1, KO = 5.1 ± 1.1, $p = .03$, $n = 9$ animals per condition). (C) Total time spent in the light chamber during the light/dark test (WT = 56.78 ± 9.75 s, KO = 30.90 ± 7.61 s, $p = .04$, $n = 9$ animals per genotype). (D) First cross latency during the light/light test (WT = 8.6 ± 1.54 s, KO = 30.80 ± 7.72 s, $p = .01$, $n = 9$ animals per genotype). (E) Total number of crosses during the light/light test (WT = 11.7 ± 1.3, KO = 10.4 ± 0.9, $p = .4311$, $n = 9$ animals per genotype). * Denotes statistical significance as described in the Methods.

significantly reduced total time spent in the light chamber when compared to their wild-type counterparts (Fig. 2B and C). These findings are inconsistent with a reduced level of anxiety. A possible explanation for this discrepancy is that the 4E-BP2 knockout mice are not discriminating on the basis of light versus dark, but rather on the basis of their first experience. To further examine this phenotype, we conducted a similar exploration-based test in which the light/dark apparatus was modified such that both chambers were brightly lit. Again, the 4E-BP2 knockout mice demonstrated a higher latency to initially cross into the other chamber (Fig. 2D). There was no significant difference between wild-types and the 4E-BP2 knockout mice in the total number of crosses (Fig. 2E). Taken together, these results suggest that the 4E-BP2 knockout mice favored exploration of a novel context based on first experience rather than light versus dark.

Another example of an anxiety-related light/dark exploratory task is the step-through passive avoidance test. This task introduces a memory component that has been reported to involve the hippocampus (Belzung & Griebel, 2001). Avoidance tasks employ an aversive stimulus to train the rodent to avoid certain locations. The stimulus is usually a mild footshock; the response is avoidance of the location in which the footshock was received 24 h later. When subjected to the step-through passive avoidance task, the 4E-BP2 knockout mice again demonstrated a significantly longer latency to initially cross into the dark chamber on the training day (Fig. 3A). During the 24-h post-test, the 4E-BP2 knockout mice exhibited similar latencies to cross into the dark chamber as the wild-type mice, suggesting that the learning and memory mechanisms required for passive avoidance are not disrupted by the lack of 4E-BP2 protein expression.

The elevated plus maze rests on the same naturalistic conflict between the tendency of mice to explore a novel environment and the aversive properties of a brightly lit, open area (Handley & Mithani, 1984). This test improves on the light/dark exploration test by adding a new component to the test where the apparatus is raised one meter above the floor. Mice prefer the closed arms of the plus maze but will venture out into the open arms. The amount of time spent in the open arms is correlated with anxiety. The 4E-BP2 knockout mice did not spend significantly different amounts of time in the closed versus open arms than their wild-type counterparts (Fig. 3B). These results suggest that the 4E-BP2 knockout mice do not demonstrate altered anxiety-related behavior on the elevated plus maze.

Genetic components underlying aggressive behaviors have been demonstrated in many studies of inbred strains of mice (reviewed in Crusio, 1996). The social dominance tube test is a simple test to measure aggressive tendencies without allowing the mice to injure each other in a true fight. In this test, a mutant mouse is placed at one end of a tube and a wild-type mouse is placed at the other end. Both mice enter the tube and meet somewhere in the middle; the one who backs out "loses" the match, having ceded territory to the other mouse. Interestingly, when mice lacking the translation repressor fragile X mental retardation protein (FMRP) were subjected to the tube test they won significantly fewer matches than their wild-type littermates (Spencer, Alekseyenko, Serysheva, Yuva-Paylor, & Paylor, 2005), suggesting that translational regulation by FMRP is pertinent for normal social interaction. When we subjected the 4E-BP2 knockout mice to the tube test we found no differences in social dominance tendency between the 4E-BP2 knockout mice and their wild-type counterparts, each genotype won an equal number of matches (Fig. 3C). These results are consistent with the aforementioned assessment of 4E-BP2 knockout generalized anxiety and argue against a role for 4E-BP2 in social dominance patterning.

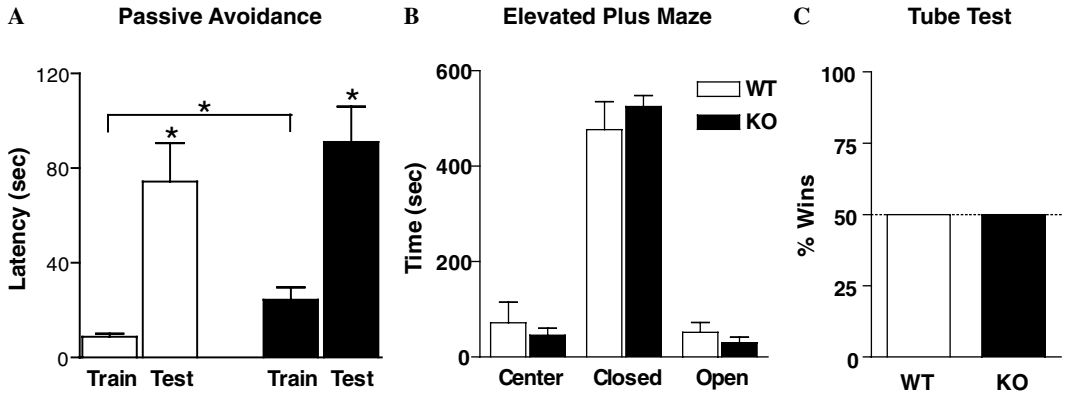

Fig. 3. General anxiety and social dominance behavior in 4E-BP2 knockout mice. General anxiety was evaluated via the step-through passive avoidance task and the elevated plus maze and social dominance was examined with the tube test. (A) The 4E-BP2 KO mice exhibited increased tolerance for the light during training for the passive avoidance test (latency during traing WT = 11.05 ± 2.55 s, KO = 24.83 ± 5.52 s, $p = .03$, $n = 20$ WT and 18 KO) confounding the results of the test for long-term memory of the task. (B) WT and KO mice exhibited similar behavior on the elevated plus maze (Center time WT = 71.80 ± 42.58 s, KO = 45.43 ± 15.09 s; $p = .52$. Closed time WT = 476.20 ± 59.02 s, KO 524.86 ± 22.56 s; $p = .40$. Open time WT = 52.00 ± 20.29 s, KO = 29.71 ± 11.44 s; $p = .33$, $n = 9$ animals per genotype). (C) WT and KO mice demonstrate similar social dominance tendency in the tube test (WT = 10/20 wins, KO = 10/20 wins, $p = 1.00$, $n = 20$ matches). * Denotes statistical significance as described in the Methods.

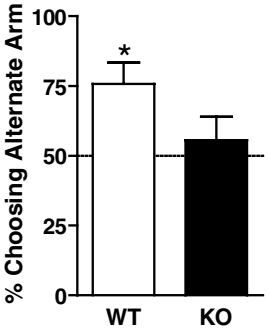

Fig. 4. Working memory requires 4E-BP2. The T-maze spontaneous alternation test was employed to assess spatial working memory. WT mice chose the alternate arm 75.76 ± 7.58% of the time during their second trial demonstrating normal working memory of the first trial ($p = .002$, $n = 33$ second trials). On the other hand, KO mice chose the alternate arm 55.56 ± 8.40% of the time during their second trial which is not significantly different from the hypothetical 50% ($p = .51$, $n = 36$ second trials) indicating that they had impaired working memory of the first trial. * Denotes statistical significance as described in the Methods.

3.3. Working memory

Spatially based exploratory behavior was further evaluated using a T-maze version of spontaneous alternation. The spontaneous alternation test is a nonrewarded exploration task that takes advantage of the propensity of rodents to explore a novel environment and has been used as an indicator of working memory for previously visited locations (Paylor, Zhao, Libbey, Westphal, & Crawley, 2001). An increased perseverance has been observed in mice with a variety of hippocampal dysfunction (for examples, see Gerlai, 1998). Given our previous demonstration of altered hippocampal synaptic plasticity and compromised hippocampus-dependent learning and memory exhibited by the 4E-BP2 knockout mice, we hypothesized that hippocampus-dependent working memory also would be disrupted in these animals. Indeed, we found that the 4E-BP2 knockout mice had a significantly reduced alternation rate compared to their wild-type counterparts; the 4E-BP2 knockouts chose left or right arms with equal frequency (Fig. 4). The role of protein synthesis and translational control has not been previously examined in this task. Our results suggest that the translation repressor 4E-BP2 is important for working memory in this type of working memory task. These results compliment our previous demonstration of the 4E-BP2 knockout mice exhibiting altered hippocampus-dependent long-term memory (Banko et al., 2005) and extend those findings to include a role for 4E-BP2 in online hippocampus-dependent memory processing.

3.4. Conditioned taste aversion

Conditioned taste aversion (CTA) represents a robust form of associative learning in which a normally appetizing taste becomes aversive following its association with gastric distress. Interestingly, translation has been shown to be regulated during both the initiation and elongation phases following novel taste learning in the insular cortex (Belelovsky, Elkobi, Kaphzan, Nairn, & Rosenblum, 2005). We found that 4E-BP2 knockout mice had the same natural aversion to quinine as did the wild-type mice, in three consecutive tests (Fig. 5A). In addition, natural preference among the respective control groups revealed that the

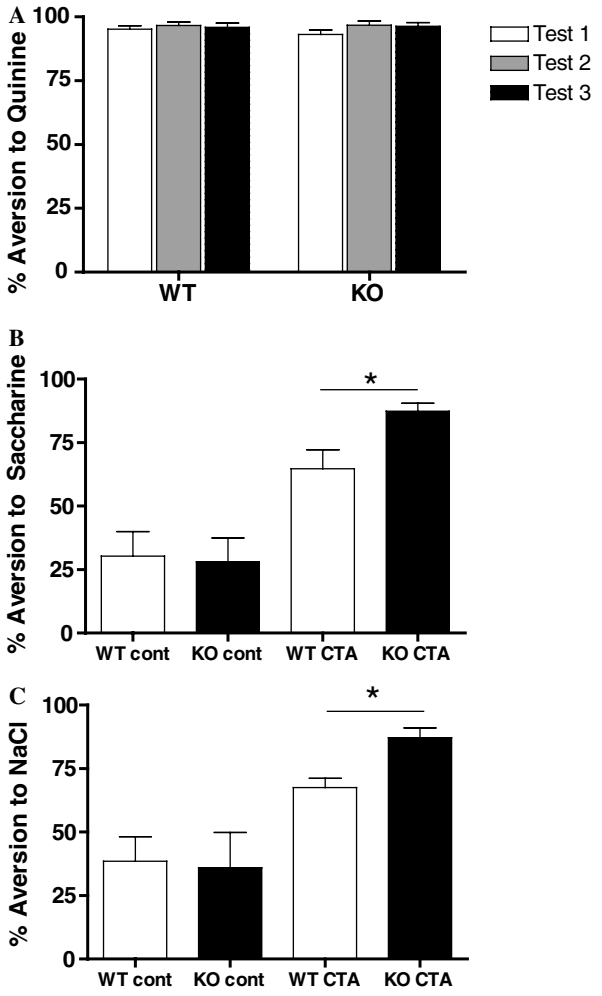

Fig. 5. 4E-BP2 knockout mice demonstrate enhanced memory for conditioned taste aversion. CTA was performed to examine the role of 4E-BP2 in cortical-dependent taste learning. The 4E-BP2 knockout mice were not different in recognition or in reaction for different novel tastes. However, their memory for the association between novel tastes and malaise was better. (A) WT and 4E-BP2 knockout mice exhibit a similar natural aversion to quinine in three tests. Aversion indices: Test-1 WT = 95 ± 1.3%, KO = 93 ± 1.8%. Test-2 WT = 97 ± 1.4%, KO = 97 ± 1.5%. Test-3 WT = 96 ± 1.7%, KO = 96 ± 1.5%. Repeated measures; *ANOVA*; $F_{1,21} = 0.258$ ($p = .135$, WT $n = 14$, KO $n = 11$). (B) WT and KO mice show similar preference to saccharin. Aversion indices: WT = 30 ± 9.7%, KO = 28 ± 9.5% ($p = .853$, $n = 4$ animals per genotype). On the other hand, KO mice exhibit an increased aversion following CTA to saccharin. Aversion indices: WT = 65 ± 7.4%, KO = 87 ± 3.1% ($p = .039$, WT $n = 7$, KO $n = 5$). (C) WT and KO mice exhibit similar preference to NaCl. Aversion indices: WT = 38 ± 9.7%, KO = 36 ± 14% ($p = .882$, $n = 4$ animals per genotype). As in B, 4E-BP2 knockout mice show a higher aversion following CTA. Aversion indices: WT = 68 ± 3.6%, KO = 87 ± 3.8% ($p = .0015$, $n = 10$ animals per genotype). * Denotes statistical significance as described in the Methods.

4EBP2 knockout and wild-type mice preferred saccharin in a similar way (Fig. 5B). In contrast to the similarity in taste recognition and preference, 4EBP2 knockout mice demonstrated a significantly enhanced memory for CTA by avoiding the saccharin and NaCl solutions to a higher degree than wild-types following a one trial pairing of saccharin or NaCl with LiCl (Fig. 5C). Taken together with our previous findings that translation regulatory pathways are activated following novel taste learning (Belelovsky et al., 2005), these results underscore the importance of translation regulatory mechanisms for CTA and represent the first example of extra-hippocampal cognitive enhancement due to the genetic alteration of a translation factor.

4. Discussion

The behavioral characterization presented in this study was undertaken to expand our previous findings demonstrating the requirement for endogenously expressed 4E-BP2 for hippocampus-mediated behavior. We performed a battery of tests aimed toward probing a broad range of behavioral phenotypes that employ multiple brain regions. There are several reports demonstrating that disruption in protein synthesis results in alteration of behaviors that rely on brain regions in addition to the hippocampus. We thought it pertinent to investigate the extra-hippocampal behavioral profile of the 4E-BP2 knockout mice to determine which additional brain regions require 4E-BP2 activity for normal behavior.

Motor skill learning circuits include the sensorimotor cortex, cerebellum and the basal ganglia (Hikosaka, Nakamura, Sakai, & Nakahara, 2002). Consolidation of rotating rod motor skill memory is sensitive to the disruption of *de novo* translation (Luft, Buitrago, Ringer, Dichgans, & Schulz, 2004); therefore, we examined the ability of the 4E-BP2 knockout mice to acquire and perform the motor skills associated with the rotating rod task. Although we observed no evidence for compromised locomotor activity with the open field test (Fig. 1A–C) or water maze (Banko et al., 2005), we found that not only did naïve 4E-BP2 knockout mice perform poorly on their initial rotating rod training day; they failed to demonstrate significant improvement over three consecutive days of training (Fig. 1E). These results are consistent with the notion that 4E-BP2 is important for complex motor skill performance.

The amygdala, septohippocampal system, medial hypothalamus, central periaqueductal grey and frontal and cingulate cortices are important brain structures involved in the regulation of anxiety and fear (Camargo & Saad, 2001; LeDoux, 1998; McNaughton & Gray, 2000; Panksepp et al., 1982). Concordant with our previous report that the 4E-BP2 knockout mice were normal with respect to the cued component of the conditioned fear test (Banko et al., 2005), additional anxiety-related analyses including light/dark exploration and elevated plus maze did not reveal consistent anti-anxiety phenotypes in the 4E-BP2 knockout mice (Figs. 1D, 2 and 3). Interestingly, the chamber-based exploratory tasks did suggest that the 4E-BP2 knockout mice favored exploration of a novel context based on first experience rather than light versus dark.

CTA requires a neural circuit that includes the brain stem, pons, insular cortex and amygdala (Gallo, Roldan, & Bures, 1992; Lamprecht, Hazvi, & Dudai, 1997; Schafe & Bernstein, 1998; Spector, 1995; Yasoshima & Yamamoto, 1997). However the role of the hippocampus in this task is not well defined. For example, activation of discrete signaling components involved in gene regulation are observed in the insular cortex and the hippocampus following taste learning (Yefet et al., 2006). Not surprisingly, general inhibition of protein synthesis in the insular cortex during novel taste learning disrupts long-term but not short-term taste memory (Houpt & Berlin, 1999; Rosenblum et al., 1993). In fact, the precise mechanisms responsible for the regulation of protein synthesis during taste learning are beginning to be revealed. For example, two kinases well-known for their ability to activate multiple translation factors, ERK2 and S6K1, as well as eukaryotic elongation factor 2 undergo increased phosphorylation in the insular cortex within 20 min of novel taste learning (Belelovsky et al., 2005). Our examination of CTA in the 4E-BP2 knockout mice provides further insight into the mechanisms of translational control involved in taste learning. We found that CTA memory was increased as a result of the genetic elimination of 4E-BP2 (Fig. 5B and C). Since amygdala-dependent learning was unaffected in the 4E-BP2 knockout mice, these results suggest that in contrast to the hippocampus, shifting the scale towards enhanced translation initiation in the insular cortex improves learning and memory. Interestingly, it was recently reported that hippocampal inactivation via the GABA-enhancing compound muscimol strongly enhanced CTA learning (Stone, Grimes, & Katz, 2005). Whether the enhanced CTA memory in the 4E-BP2 knockouts can be attributed to functional disruption of the hippocampus would be interesting to investigate.

We previously have demonstrated that genetic elimination of the translation repressor 4E-BP2 results in impaired hippocampus-dependent learning and long-term memory. Specifically, 4E-BP2 knockout mice exhibited reduced long-term memory for the context component but not the cued component of the conditioned fear test. Furthermore, 4E-BP2 knockout mice demonstrated compromised spatial learning and memory in the hidden platform water maze test (Banko et al., 2005). Herein, we extended these findings by demonstrating compromised hippocampus-dependent working memory with the spontaneous alternation T-maze (Fig. 4). The role of protein synthesis and translational control has not previously been examined in this task. Our results suggest that the translation repressor 4E-BP2 is important for working memory in this type of this task.

Interestingly, passive avoidance learning and memory is spared in the 4E-BP2 knockout mice. One possible explanation for why this particular hippocampus-dependent process is not disrupted while spatial learning in the water maze and associative contextual learning are impaired

(Banko et al., 2005) is that the passive avoidance paradigm is a less challenging test. Because of the constellation of cues that the mice must take in to navigate the water maze or learn a specific context, these learning tasks historically have been considered fairly challenging. In contrast, passive avoidance requires discrimination only on the basis of light versus dark. Overall, the hippocampus-dependent behavioral phenotypes, taken together with our previous observations of altered synaptic plasticity in the Schaffer collateral pathway of the 4E-BP2 knockout mice (Banko et al., 2006; Banko et al., 2005), strongly suggest that loss of 4E-BP2 results in reduced functional capacity of the hippocampus for learning and memory.

The requirement of *de novo* protein synthesis for multiple forms of learning, memory and behavior is well-established; however, the regulatory mechanisms that govern translation during these processes only recently have begun to be discovered (Kelleher, Govindarajan, & Tonegawa, 2004; Klann & Dever, 2004). The sheer complexity of the signaling cascades that govern the control of translation initiation suggest that there could be multiple pathways, converging and diverging, responsible for the regulation of translation initiation factors during each of type of learning and memory. Examination of the relative contribution of each individual translation factor involved in learning and memory, as well as memory-independent behaviors, is a necessary step toward elucidating these translation regulatory mechanisms. Moreover, the identification of the proteins synthesized during differ types of memory processes and behaviors will be required because not all proteins are translated via the same array of translation factors. Strikingly, we have found that the utility of 4E-BP2 appears to vary with different types of learning, memory and behavior. One possibility to explain these varied effects is the differential expression pattern of translational regulatory elements in discrete brain areas. Another possibility is that the different behavioral paradigms have different temporal constraints that 4E-BP2 participates in to various degrees. The results described herein provide a framework for future studies on the specific role of 4E-BP2 in various types of behavior, and for broader questions concerning mechanisms of translational control in the brain.

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