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14. ABSTRACT

We hypothesize that excessive cap-dependent translation is a causative factor in autism spectrum disorder (ASD). To test this hypothesis, we have been studying transgenic mice that overexpress eIF4E have been testing the following specific aims: 1) to determine whether eIF4E transgenic mice display behaviors consistent with ASD, 2) to determine whether ASD-like behaviors displayed by eIF4E transgenic mice can be reversed by novel cap-dependent translation inhibitors, and 3) to determine whether eIF4E transgenic mice display cellular and molecular abnormalities due to excessive cap-dependent translation. mice. Our studies will provide information concerning whether overexpression of eIF4E is a *biological risk factor* for ASD. Our studies also will provide important information concerning the role of upregulated cap-dependent translation in ASD, and could link ASD mechanistically at the level of cap-dependent translational control to fragile X syndrome (FXS), tuberous sclerosis complex (TSC), and autistic patients with *PTEN* and *EIF4E* mutations. Moreover, the results of these studies would provide information for the design and use of compounds to *therapeutically target* eIF4E-eIF4G interactions and eIF4A for treating patients with ASD.

15. SUBJECT TERMS

autism spectrum disorder, cap-dependent translaiton, eIF4E, repetitive behaviors, perseverative behaviors, social behaviors

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Introduction

We hypothesize that excessive cap-dependent translation is a causative factor in autism spectrum disorder (ASD). To test this hypothesis, we have been studying transgenic mice that overexpress eIF4E have been testing the following specific aims: **1) to determine whether eIF4E transgenic mice display behaviors consistent with ASD, 2) to determine whether ASD-like behaviors displayed by eIF4E transgenic mice can be reversed by novel cap-dependent translation inhibitors, and 3) to determine whether eIF4E transgenic mice display cellular and molecular abnormalities due to excessive cap-dependent translation.** We have been determining whether eIF4E transgenic mice display behaviors consistent with ASD by examining their social behaviors, anxiety-related behaviors, perseverative/repetitive behaviors, and their responses to sensory stimuli. In previous studies we have used a novel compound termed 4EGI-1 that selectively disrupts interactions between translation factors eIF4E and eIF4G to inhibit translation in the brain. Therefore, we have been determining whether 4EGI-1 reverses ASD-like behaviors displayed by eIF4E transgenic mice. Moreover, because eIF4E/eIF4G interactions promote the helicase activity of eIF4A to stimulate cap-dependent translation, we will determine whether the eIF4A inhibitor hippuristanol can reverse ASD-like behaviors displayed by the eIF4E transgenic mice. Finally, we have been conducting studies to determine whether eIF4E transgenic mice have increased translation, altered neuronal morphology, and altered synaptic plasticity due to excessive cap-dependent translation. Our studies will provide information concerning whether overexpression of eIF4E is a ***biological risk factor*** for ASD. Our studies also will provide important information concerning the role of upregulated cap-dependent translation in ASD, and could link ASD mechanistically at the level of cap-dependent translational control to fragile X syndrome (FXS), tuberous sclerosis complex (TSC), and autistic patients with *PTEN* and *EIF4E* mutations. Moreover, the results of these studies would provide information for the design and use of compounds to ***therapeutically target*** eIF4E-eIF4G interactions and eIF4A for treating patients with ASD.

Body

Herein I will describe the research accomplishments associated with each task that was outlined in the approved Statement of Work.

The first task in the Statement of Work was to obtain regulatory approval for the use of mice by New York University (NYU) IACUC Committee and USAMRMC Office of Research Protections. The animal protocol was approved the NYU IACUC and Committee as well as the USAMRMC Office of Research Protections.

The second task in the Statement of Work was to determine whether the eIF4E transgenic mice exhibit behaviors consistent with ASD. This included subtask 1, which was to measure the social behaviors of the eIF4E transgenic mice and subtask 2, which was to measure anxiety-related behaviors, perseverative/ repetitive behaviors, and sensorimotor gating of the eIF4E transgenic mice. We have completed these studies, which are included in a manuscript that was published in the prestigous journal *Nature* (Santini et al., 2013).

Perseverative and repetitive behaviors are one of the domains required for the diagnosis of ASD (Lewis et al., 2007). These behaviors include stereotypies (purposeless repetitive movements and activities) as well as cognitive inflexibility (inability to disengage from a previously learned behavior and adopt a new behavioral strategy). We employed a marble burying test to quantify repetitive digging behavior (Thomas et al., 2009) and found that the eIF4E transgenic mice buried significantly more

marbles compared to their wild-type littermates (Figure 2a in Santini et al., 2013). We also examined self-grooming, which is a stereotypic repetitive behavior that has been observed in other mouse models of ASD (Peca et al., 2011; McFarlane et al., 2008) and discovered that the eIF4E transgenic mice exhibited increased self-grooming (Figure 2b in Santini et al., 2013). We next evaluated whether eIF4E transgenic mice exhibited cognitive inflexibility by examining choice arm reversal in a water-based Y-maze task (Hoeffer et al., 2008). eIF4E transgenic mice showed intact learning abilities during the acquisition phase of the task and normal memory for the escape arm when tested 24 hours later. However, when the position of the escape arm was changed in the reversal phase of the task, eIF4E transgenic mice required significantly more trials to satisfy the same success criterion compared to their wild-type littermates (Figure 2c in Santini et al., 2013). Taken together, these experiments suggest that increased eIF4E expression and consequently, exaggerated cap-dependent protein synthesis, results in repetitive and perseverative behaviors.

Abnormalities in social interaction skills are another behavioral defect displayed by individuals with ASD (Rapin and Tuchman, 2008). Thus, we also tested social behavior in eIF4E transgenic mice with two well-established behavioral paradigms, the three-chamber arena and the reciprocal social interaction task (Moy et al., 2004). eIF4E transgenic mice exhibited reduced preference for a nonspecific stranger as indicated by an equal amount of time spent interacting with the mouse stranger and a novel object (Figure 2f in Santini et al., 2013). Similarly, the eIF4E transgenic mice displayed a reduced preference for the chamber where the stranger mouse was located (Figure 2g in Santini et al., 2013). Moreover, eIF4E transgenic mice also exhibited diminished reciprocal interactions with a freely moving stranger mouse (Figure 2h in Santini et al., 2013), further supporting deficits in social behavior. The deficits in social behavior of the eIF4E transgenic mice are unlikely to be caused by a generalized increased anxiety since the mice did not display anxiety-like traits when tested in other paradigms (i.e. center/total distance ratio in the open field test (Supplementary Figure 2c in Santini et al., 2013) and elevated plus maze (Supplementary Figure 2j in Santini et al., 2013). Moreover, the eIF4E transgenic mice exhibited a mild hyperactivity (first 10 min of novelty and open field tests (Supplemenatry Figure 2a and 2b in Santini et al., 2013) but no impairments in motor coordination, motor learning and sensorimotor gating abilities (Supplementary Figure 2i and 2k in Santini et al., 2013). All together, the behavioral analysis of the eIF4E transgenic mice indicates that increased cap-dependent protein synthesis in the brain results in a distinct pattern of behavioral abnormalities consistent with ASD.

The third task in the Statement of Work was to determine whether the ASD-like behaviors displayed eIF4E transgenic mice could be reversed by novel cap-dependent translation inhibitors. Subtask 1 was to determine whether ICV infusion of 4EGI-1 could reverse ASD-like behaviors exhibited by eIF4E transgenic mice. Subtask 2 was to determine whether ICV infusion of hippuristanol can reverse ASD-like behaviors by eIF4E transgenic mice.

We largely completed the experiments in first subtask and these studies were published in the Nature manuscript (Santini et al., 2013). We employed a subthreshold dose of 4EGI-1 previously described in our laboratory (Hoeffer et al., 2011) to normalize the behavioral abnormalities in eIF4E transgenic mice without impairing their wild-type littermates. We infused either 4EGI-1 or vehicle directly into the lateral ventricle of cannulated eIF4E transgenic mice and their wild-type littermates. eIF4E transgenic mice treated with 4EGI-1 exhibited a decrease in repetitive behavior in the marble burying task starting on day four and persisted throughout day five, whereas the wild-type mice treated with 4EGI-1 behaved similarly to the vehicle-treated wild-type mice (Figure 4a in Santini et al., 2013). We then tested the ability of 4EGI-1 to correct the behavioral inflexibility displayed by eIF4E transgenic mice in the Y-maze. We found that blockade of eIF4E/eIF4G interactions with 4EGI-1 significantly improved the performance of eIF4E transgenic mice by decreasing the number of trials required to reach the success criterion in the reversal phase of the task (Figure 4b in Santini et al., 2013). These findings indicate that chronic treatment of eIF4E transgenic mice with 4EGI-1 reverses their repetitive and perseverative behaviors.

Finally, we determined whether infusions of 4EGI-1 also rescued the social behavior deficits displayed by the eIF4E transgenic mice. We found that eIF4E transgenic mice infused with 4EGI-1 for four days exhibited a preference for a non-specific stranger as indicated by an increased amount of time spent in interacting with the stranger mouse over the novel object (Figure 4c in Santini et al., 2013). This result suggests that chronic treatment with 4EGI-1 also corrects social behavior deficits displayed by eIF4E transgenic mice.

We are continuing studies on second subtask to determine whether ICV infusion of hippuristanol can reverse ASD-like behaviors by eIF4E transgenic mice. We have had trouble obtaining enough hippuristanol from our collaborators at McGill University to complete this task. We attempting to obtain a second pharmacolgoical inhibitor of eIF4A and have begun making siRNAs for eIF4A so that we can knockdown its expression in the brains of eIF4E transgenic mice. We plan on finishing these experiments in the coming years.

The fourth task in the Statement of Work was to determine whether the eIF4E transgenic mice display cellular and molecular abnormalities due to excessive cap-dependent translation. Subtask 1 was to determine whether cap-dependent protein synthesis is increased in the brains of eIF4E transgenic mice. Indeed, we found that protein synthesis was elevated in the brains of the eIF4E transgenic mice (Figure 1d in Santini et al., 2013). Moreover, we found that ICV infusion of 4EGI-1 blocked the increase in protein synthesis in the prefrontal cortex of the eIF4E transgenic mice (Supplementary Figure 4f and 4g in Santini et al., 2013). Subtask 2 was to determine whether eIF4E transgenic mice exhibit altered dendritic spine morphology and if so, whether 4EGI-1 and hippuristanol reversed the alterations. We found that there was an increase in spine density in pyramidal neurons in layer 2/3 of the prefrontal cortex (Figure 3c and 3d in Santini et al., 2013). We currently are doing the studies to determin whether ICV infusion of 4EGI-1 and eIF4A inhibitors can reverse the increased spine density in the pyramidal neurons in layer 2/3 of the prefrontal cortex in the eIF4E transgenic mice. We will plan to complete these studies in the near future. Subtask 3 was to determine whether eIF4E transgenic exhibit abnormal protein synthesis-dependent synaptic plasticity and if so, whether 4EGI-1 and hippuristanol reverse the abnormalities. We found that the eIF4E transgenic mice exhibited enhanced metabotropic glutamate receptor-dependent long-term depression (LTD) in the striatum and the hippocampus (Figure 3e and 3f in Santini et al., 2013). Moreover, we found that 4EGI-1 reversed the enhanced striatal LTD (Figure 3h in Santini et al., 2013). As mentioned above, it has been difficult for us to obtain enough hippuristanol from our collaborators at McGill University to complete this task. We have been attempting to obtain a second pharmacolgoical inhibitor of eIF4A and have begun making siRNAs for eIF4A so that we can knockdown its expression in the brains of eIF4E transgenic mice. We plan on finishing these experiments in the coming years.

The fifth task is data analysis and reporting. As evidenced in this report, we have been analyzing the data throughout the performance of the experiments.

Please note that a detailed description of the behavioral tests is provided in the Appendix.

Key Words

autism spectrum disorder (ASD), protein synthesis, cap-dependent translation, repetitive behaviors, social behaviors, striatum

Research Accomplishments

- Demonstration that eIF4E transgenic mice display repetitive and perseverative behaviors that are consistent with ASD.

- Demonstration that eIF4E transgenic mice display impaired social behaviors that are consistent with ASD.

- Demonstration that repetitive/perseverative behaviors and impaired social behaviors exhibited by eIF4E transgenic mice can be reversed by the 4EGI-1, which inhibits eIF4E-eIF4G interactions and cap-dependent translation.

- Demonstration that the eIF4E transgenic mice exhibit increased cap-dependent synthesis, altered dendritic spine morphology, and altered synaptic plasticity in the striatum and hippocampus.

- The key findings described above were published in the prestigious journal *Nature*.

Reportable Outcomes

(Year 1 1 August 2011 - 31 July 2012)

1) Poster presentation at 2011 Society for Neuroscience meeting in Washington, DC by Dr. Emanula Santini. Abstract and poster included in appendices.

2) Invited seminar, Department of Neuroscience, University of Wisconsin School of Medicine and Public Health, Madison, WI

3) Invited speaker at Society for Neuroscience meeting, Mini-symposium entitled "Translational Control at the Synapse and in Disease", Washington, DC

4) Invited speaker at Joint Meeting of the Haifa Forum for Brain and Behavior and the Molecular and Cellular Cognition Society - Europe, University of Haifa, Haifa, Israel

5) Invited speaker at Weizmann Institute of Science - New York University Science Days: Frontiers in Brain and Cognition, Weizmann Institute of Science, Rehovot, Israel

6) Invited seminar, Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel

7) Invited seminar, Intellectual and Developmental Disabilities Research Center, Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA

8) Invited seminar, Department of Neuroscience, Case Western Reserve University School of Medicine, Cleveland, OH

9) Invited seminar entitled ""Department of Neuroscience, Baylor College of Medicine, Houston, TX

10) Invited speaker at Gordon Research Conference entitled "Fragile X and Autism-related Disorders: From Basic Neuroscience to Improved Clinical Care", Stonehill College, Easton, MA

11) Invited speaker at Institute for Genomics & Systems Biology, Conte Center Brain Awareness Day public presentation, University of Chicago, Chicago, IL

12) Invited speaker at Gordon Research Conference entitled "Neurobiology of Brain Disorders: Synaptic Dysfunction and Neurodegeneration", Stonehill College, Easton, MA

(Year 2, 1 August 2012 - 31 July 2013)

1) Invited speaker at Conference entitled "Frontiers in Stress and Cognition: From Molecules to Behavior", Ascono, Ticino, Switzerland

2) Poster presentation at 2012 Society for Neuroscience meeting in Washington, DC by Dr. Emanula Santini. Abstract and poster included in appendices.

3) Invited seminar, Riken Brain Science Institute, Wako-shi, Japan

4) Invited seminar, Department of Neurochemistry, Tokyo University Graduate School of Medicine, Tokyo, Japan

5) Invited seminar, Biotechnology Research Institute and Molecular Neuroscience Center, Division of Life Sciences, The Hong Kong University of Science and Technology, Clearwater Bay, Kowloon, Hong Kong

6) Invited seminar, Department of Bioscience, Tokyo University of Agriculture, Tokyo, Japan

7) Invited seminar, Department of Biological Sciences, Seoul National University, Seoul, South Korea

8) Invited speaker, Symposium entitled "Recent Advances in Biosciences", Sponsored by Institute of Biomedical Sciences, Academia Sinica and College of Medicine, Tzu Chi University, Hualien, Taiwan

9) Invited seminar, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

10) Invited seminar, Solomon H. Snyder Department of Neuroscience, Johns Hopkins University, Baltimore, MD

11) Invited speaker, The International Meeting for Autism Research, Symposium entitled "Reversibility", San Sebastian, Spain

12) Invited seminar, Department of Biological Sciences, Korea Advanced Institute for Science and Technology, Daejeon, South Korea

(Year 3, 1 August 2013 - 31 July 2014)

1) Invited seminar, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT

2) Abstract submitted for Molecular and Cellular Cognition Society Meeting, San Diego, CA, chosen for oral presentation by Dr. Emanuela Santini. Abstract and presentation included in appendices.

3) American College of Neuropsychopharmacology Meeting, Speaker for session entitled "Autism Spectrum Disorders: From Rare Chromosomal Abnormalities to Common Molecular Targets", Hollywood, FL

4) Invited seminar, Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA

5) Invited seminar, International Max Planck Research School, Max Planck Institute for Brain Research,

Frankfurt, Germany

6) Adler Lecture, 30th Annual Retreat, Mahoney Institute for Neurosciences, University of Pennsylvania, Philadelphia, PA

7) Invited seminar, Biozentrum, University of Basel and Friedrich Miescher Institute for Biomedical Sciences, Basel, Switzerland

8) Invited seminar, Patrick Wild Centre, University of Edinburgh, Edinburgh, Scotland, United Kingdom

9) Invited speaker, Wellcome Trust meeting entitled "Translational Control of Brain Function in Health and Disease", London, England, United Kingdom

10) Invited speaker, Gordon Research Conference entitled "Fragile X and Autism-related Disorders: Progress and Struggles in Translating Scientific Advances into Human Therapy", Mt. Snow Resort, West Dover, VT

11) Invited speaker, Ramon Areces Foundation Symposium entitled "Found in Translation: Exploring the Role of Protein Synthesis in Stress and Disease", Madrid, Spain

Impact

Our results have had an impact in the ASD field. The findings from our studies were published in the prestigious journal *Nature.* We have been invited to present these findings at a number of universities and research institutes, and at national and international conferences. We were asked to write a review for the prestigious journal *Science Signaling*, which is included in the appendix. Our work has been featured in the popular press, so it has been communicated to the general public. Finally and most importantly, our studies have resulted in a collaboration with Egenix, a biotechnology company that is developing compounds that cross the blood brain barrier to target eIF4E and other components of the eIF4F initiation complex as potential therapeutics for treatment of individuals with ASD.

Conclusion

We have demonstrated that increased eIF4E expression and consequently, exaggerated cap-dependent protein synthesis results in the appearance of ASD-like behaviors in mice. We speculate that exaggerated cap-dependent protein synthesis, which is sufficient for the generation of synaptic alterations leading to ASD-like behaviors, results in enhanced translation of a specific subset of mRNAs. Importantly, our results also indicate that a pharmacological intervention that targets the formation of the eIF4F initiation complex (eIF4E+eIF4G+eIF4A) is sufficient to correct ASD-like endophenotypes displayed by the eIF4E transgenic mice. These experiments directly demonstrate that dysegulated translational control at the initiation phase of protein synthesis causes behavioral abnormalities in several domains consistent with ASD. Moreover, these results suggest that the eIF4F initiation complex is a viable therapeutic target for the treatment of individuals with ASD.

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Santini, E. and Klann, E. (2014) Reciprocal signaling between translational control pathways and synaptic proteins in autism spectrum disorders. *Sci. Signal.* **7:** re10.

Thomas, A. *et al.* Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology* **204**, 361–373 (2009).

Personnel Supported by this Award During the Funding Period

Eric Klann, Ph.D. – Principal Investigator
Emanula Santini, Ph.D. – Postdoctoral Fellow
Thu Huynh, M.S. – Graduate Student
Linnaea Ostroff, Ph.D. – Postdoctoral Fellow
Nicole Zeak, B.S. – Research Technician

Appendices

List of items in appendices:

1) Detailed description of behavioral paradigms.
2) 2011 Society for Neuroscience abstract
3) 2011 Society for Neuroscience poster
4) 2012 Society for Neuroscience abstract
5) 2012 Society for Neuroscience poster
6) Santini, E., Huynh, T.N., MacAskill, A.F., Carter, A.G., Pierre, P., Ruggero, D., Kaphzan, H., and Klann, E. (2013) Exaggerated translation causes synaptic and behavioural aberrations associated with autism. *Nature* **493:** 411-415.
7) 2013 Molecular and Cellular Cognition Society abstract
8) 2013 Molecular and Cellular Cognition Society presenation
9) Santini, E. and Klann, E. (2014) Reciprocal signaling between translational control pathways and

synaptic proteins in autism spectrum disorders. *Sci. Signal.* **7:** re10.

Social Interaction Tests:

Social Approach: Mice will be placed in a three-chambered testing arena, where two areas are novel and the central area familiar to the mouse. One novel chamber will contain a caged stranger mouse (social target), the middle section the neutral starting location, and the other novel chamber identical to the first except that it does not contain a mouse (inanimate target). Time spent in each chamber will be measured and mice tested for propensity to approach and engage with a stranger mouse. ASD-like phenotypes would manifest as low social approach scores to the caged stranger mouse.

Social Novelty: This test will be conducted in the same testing apparatus as the social approach test, except that a familiar mouse will be added to the previously empty novelty chamber. In this case, preference for interaction between the familiar mouse and the stranger mouse will be tested. Under normal conditions, mice habituate to the conspecific mouse and rapidly move to investigate a novel conspecific mouse. Mice exhibiting ASD-like phenotypes may demonstrate the opposite behavior and spend more time with the familiar conspecific mouse.

Anxiety-related Behaviors:

Open Field Analysis: Mice normally remain low to the surface and near the edges of novel environments and venture to more exposed areas, i.e. the center of a space or upward by rearing, with less frequency. Measuring these parameters gives a reflection of the anxiety a mouse exhibits when exposed to a novel environment. Mice will be placed in a bright open testing arena, enclosed by transparent material. Mice will be placed in the center of the arena and allowed to explore the novel environment for 15 min. Mice with an ASD-related anxiety phenotype would be expected to spend less time in the center of the arena and show a reduced frequency to engage in rearing exploratory behaviors. Additionally, stereotypic (repetitive) movements can be captured by the tracking system used for this task, thereby allowing the measurement of this phenotype with general anxiety testing.

Elevated Plus Maze: The mice will be tested in a plus-shaped maze. Two arms are enclosed on the sides by non-transparent materials (closed) while two arms are completely exposed (open). Mice will be placed in the center of the maze and the time spent in each arm recorded. This test of anxiety and exploratory behavior (novelty) will measure the tendency of the mouse to leave the “protected” closed arms and venture into the exposed area in the open arms of the maze. ASD individuals generally display enhanced anxiety. If the eIF4E transgenic mice display phenotypes similar to those in ASD, then they would display reduced time in the open arms of the maze.

Repetitive/Perseverative Behaviors:

Marble Burying: Mice often “bury” or otherwise conceal glass marbles placed in their home cages. Burying behavior is thought to involve reward pathways associated with either anxiety relief or compulsiveness, and is considered an animal model of anxiety and obsessive-compulsive disorder (OCD), but also has been employed in the examination of ASD mouse models. Increased number of marbles buried by eIF4 transgenic mice might reflect enhanced anxiety or a tendency to exhibit repetitive behavior.

Arm Reversal in Y-Maze: Mice will be trained in a simple Y-water maze based escape task. Visual cues are located at the either arm of the Y-maze. Mice will be trained to locate a submerged escape platform in one arm denoted by a specific visual cue. This version of the Y-maze does not require food restriction to enforce appetitive acquisition. Then the location of the platform will be changed. The ability of the mice to change their arm choice will be measured. In this way, resistance to change

(perseveration) can be observed by measuring the latency to choose the new arm compared to the original escape location. This task will permit us to more specifically separate perseverance phenotypes from phenotypes derived from search strategies. This task models the propensity for ritualistic and repetitive behaviors exhibited by ASD-afflicted individuals. Mice with an ASD-like phenotype would be expected to display an increase in the time spent investigating previous escape locations.

Prepulse Inhibition: This test measures the hearing and reflex startle response of the animals. The animal will be removed from its home cage and is place into a sound proof chamber. The startle response tone (120 decibels for 20 ms) will be given and startle response scored by an automated system. Then a series of mild prepulse tones will be paired with the 120 decibel tone and the response scored. The prepulse tones will be 74, 78, 82, 86, and 90 decibels and 20 ms in duration. Each prepulse tone will be paired with the 120 decibel tone, with the prepulse tone increasing with each pairing. Four trials of each pairing will be performed. The startle response decreases with increasing prepulse tone in wild-type mice.

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Presentation Abstract

Program#/Poster#: 150.02/R10

Presentation Title: eIF4E transgenic mice as a novel animal model of autism spectrum disorders.

Location: Hall A-C

Presentation time: Sunday, Nov 13, 2011, 9:00 AM -10:00 AM

Authors: ***E. SANTINI**, E. KLANN;
Ctr. for Neural Sci., New York Univ., New York, NY

Abstract: Autism spectrum disorders (ASDs) are a heterogeneous group of heritable neuropsychiatric disorders whose symptoms, which include abnormal social interaction, impaired communication, and repetitive/perseverative behaviors, appear in early childhood and continue throughout life. One of the leading hypotheses for a common molecular mechanism underlying ASDs is alteration in the translational control machinery. In particular, studies of humans and evidence obtained from animal models have revealed that upregulated mTORC1 signaling is a molecular abnormality that may contribute to ASDs-like behaviors. Consistent with this notion, it recently was found that at least two autistic patients carry a mutation in the promoter region of the EIF4E gene, which encodes the cap-binding translation factor eIF4E, a downstream effector of the mTORC1-signaling pathway. Importantly, in vitro studies have demonstrated that this mutation increases eIF4E promoter activity, suggesting that some these autistic patients may have increased expression of eIF4E.
eIF4E mediates cap-dependent translation initiation by binding eIF4G, thereby forming the eIF4F initiation complex. Activation of the mTORC1 signaling pathway promotes protein synthesis by releasing eIF4E from its repressor eIF4E-binding protein (4E-BP), thus increasing the availability of eIF4E to interact with eIF4G.
We recently have began studies of a transgenic mouse line overexpressing eIF4E to test the hypothesis that increased protein synthesis is a causative factor in ASDs-like behaviors. We have found that eIF4E transgenic mice exhibit increased eIF4F complex formation as measured by the interaction between eIF4E with

eIF4G, suggesting increased protein synthesis. The eIF4E transgenic mice also exhibit ASDs-like behaviors, including repetitive and stereotypical behaviors and impairments in reversal learning, suggesting behavioral inflexibility. In addition, the eIF4E transgenic exhibit alterations in several forms of synaptic plasticity that are due to excessive cap-dependent protein synthesis. Thus, eIF4E mice are a new resource that can be utilized to specifically address the involvement of dysregulated translational control in ASDs.

Disclosures: **E. Santini:** None. **E. Klann:** None.

Keyword(s): PROTEIN SYNTHESIS

AUTISM

ANIMAL MODEL

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eIF4E transgenic mice as a novel animal model of autism spectrum disorders

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150.02/R10

Introduction

Autism spectrum disorders (ASDs) are a heterogeneous group of heritable neuropsychiatric disorders whose symptoms, which include abnormal social interaction, impaired communication, and repetitive/perseverative behaviors, appear in early childhood and continue throughout life. One of the leading hypotheses for a common molecular mechanism underlying ASDs is alteration in the translational control machinery. In particular, it seems that upregulated mTORC1 signaling is a molecular abnormality that may contribute to ASDs-like behaviors. Consistent with this notion, it was recently found in autistic patients a mutation increasing *eIF4E* promoter activity.

eIF4E mediates cap-dependent translation initiation by binding eIF4G, thereby forming the eIF4F initiation complex. Activation of the mTORC1 signaling pathway promotes protein synthesis by releasing eIF4E from its repressor eIF4E-binding protein (4E-BP), thus increasing the availability of eIF4E to interact with eIF4G.

Here we employed a transgenic mouse line, in which the expression of eIF4E is augmented, to test the hypothesis that increased cap-dependent protein synthesis play a causative role in the etiology of ASDs.

Materials and Methods

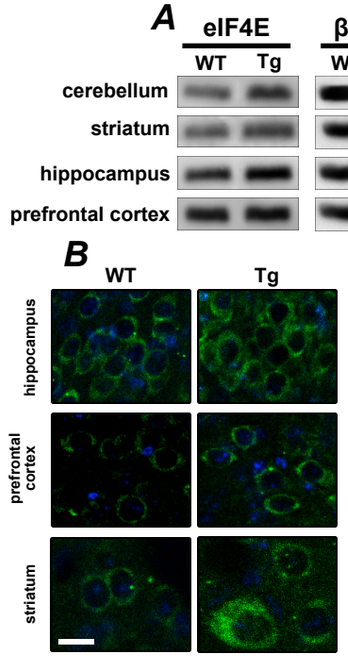
Animals. eIF4E transgenic and wild-type mice were generated as previously described (Ruggero et al., 2004).
Western Blotting. Mice were killed by decapitation and hippocampal slices were prepared as described in Banko et al., 2006. Levels of total proteins were assessed as described in Svenningsson et al., 2000.
Immunofluorescence. Mice were perfused and immunofluorescent staining was performed as described in Santini et al., 2009 .
Electrophysiology. Hippocampal slices were incubated in an interface chamber and fEPSP were evoked and recorded as described previously (Banko et al., 2005) .
Marble Burying test. In this test the innate tendency of mice to bury object is used to study repetitive behavior. The duration of test is 30 min and the parameter used for statistical analysis is the number of marbles buried.
Y maze test. Mice are trained to locate a submerged platform in one of the arms of a Y-shaped maze for 20 trials. Once the mice achieve a 90% success criterion, the platform is moved into a new arm. The number of trials needed to find for ten times consecutively the platform in the new location is the parameter used for statistical analysis.
Morris Water Maze and reversal learning. Morris water maze paradigm is performed as previously described (Hoeffer et al., 2008). The reversal learning protocol was adapted from previous studies (Hoeffer et al., 2008) The parameter used for statistical analysis is the time employed by the mice to locate the platform

Conclusions

We show that eIF4E is overexpressed in the brain of the transgenic mice without compensation by other translational control proteins. We have also found that eIF4E transgenic mice exhibit increased eIF4F complex formation as measured by the interaction between eIF4E with eIF4G, suggesting increased protein synthesis. Importantly, eIF4E transgenic mice exhibit some specific ASDs-like behaviors, such as repetitive and stereotypical behaviors and impairments in reversal learning, suggesting behavioral inflexibility. In addition, eIF4E transgenic exhibit alterations in several forms of synaptic plasticity that are due to excessive cap-dependent protein synthesis. Thus, eIF4E mice are a new resource that can be utilized to specifically address the involvement of dysregulated translational control in ASDs.

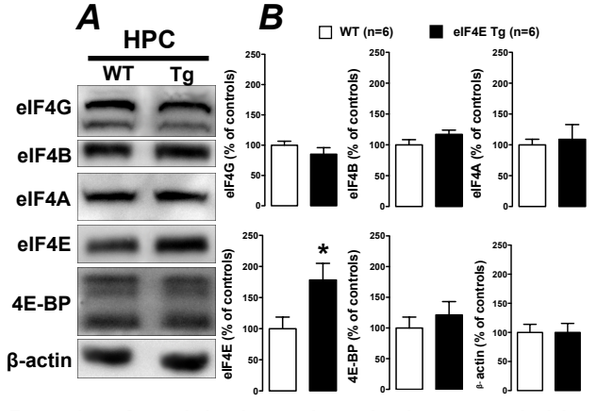
Results

1 eIF4E is ubiquitously overexpressed in the brain of eIF4E transgenic mice



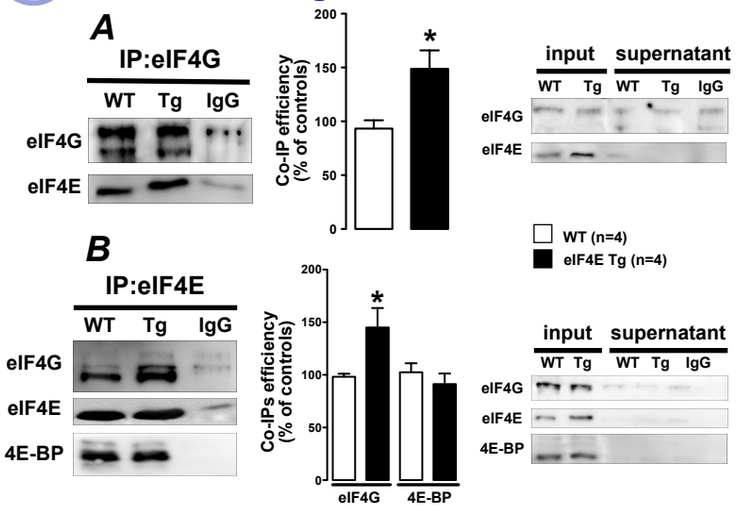
eIF4E transgenic mice show increased expression of eIF4E. (***A***) representative autoradiograms showing the levels of eIF4E. β-actin is used as loading control; (***B***) immunofluorescence detection of eIF4E *(green)* in hippocampus (CA1 area), prefrontal cortex (layer 2/3) and striatum (dorsolateral portion). DAPI *(blue)* was used to stain nuclei. Scale = 20 μm

2 Basal expression of translational control proteins is not altered in eIF4E transgenic mice



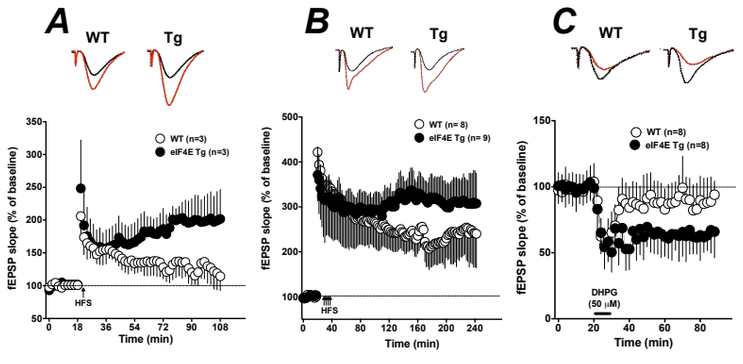
Expression of translational control proteins is not perturbed by increased eIF4E levels in eIF4E transgenic mice. (***A***) representative autoradiograms showing the total levels of translational control proteins. β-actin is used as loading control; (***B***) Data are expressed as percentage of wild-type and represent means ± SEM. Levels of eIF4E are significantly higher in eIF4E transgenic mice as compared to wild type littermates. t-test, *p<0.05.

3 eIF4E is preferentially bound to eIF4G in eIF4E transgenic mice



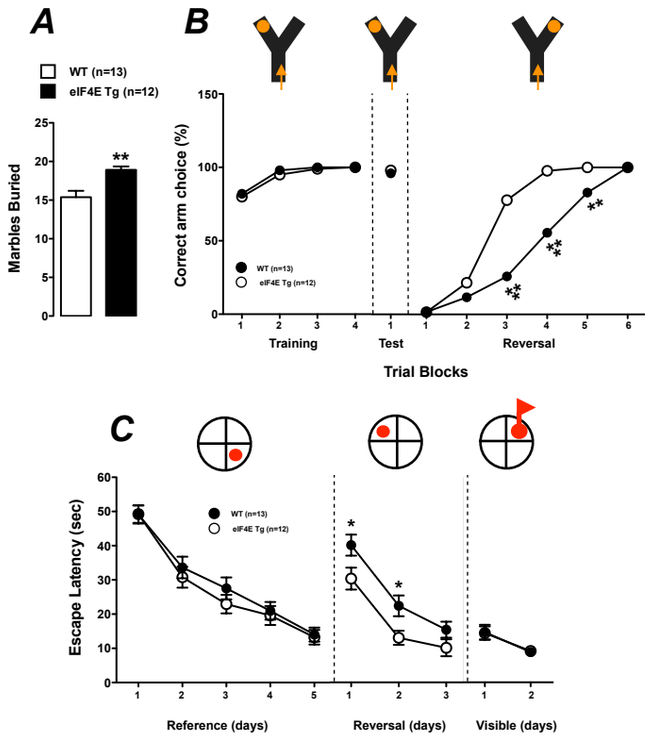
Overexpression of eIF4E leads to increased formation of the initiation complex eIF4F. eIF4G (***A***) and eIF4E (***B***) were immunoprecipitated *(IP)* using hippocampal protein extracts of wild-type and eIF4E transgenic mice. Representative autoradiograms showing eIF4G (***A***, right) and eIF4E (***B***, right) co-immunoprecipitated *(co-IP)* with theirs binding partners; Bar graphs of data expressed as percentage of the ratio eIF4E/eIF4G (***A***, center) or eIF4G/eIF4E and 4E-BP/eIF4E (***B***, center) and represent means ± SEM. *t-test, *p<0.05.* Representative autoradiograms showing control parameters of IP experiments (***A***, ***B***, left).

4 eIF4E transgenic mice display altered hippocampal synaptic plasticity



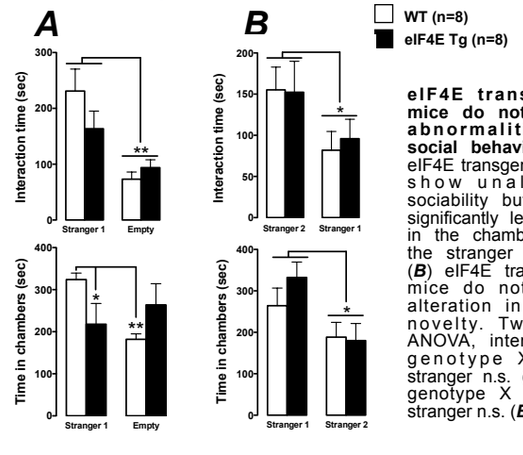
eIF4E transgenic mice exhibit increased E-LTP, augmented L-LTP and enhanced mGluR-LTD. Representative fEPSP of hippocampal slices of eIF4E transgenic mice and their wild-type littermates that receive one train of 100 Hz HFS for 1 sec (***A***, upper), four trains (5min intertrain interval) of 100 Hz HFS for 1 sec (***B***, upper) or application of DHPG (50 μM, 10 min; ***C***, upper). Black traces: baseline, red traces: poststimulation; (***A***) E-LTP induced by one train HFS is increased in Tg mice, p<0.05 RM-ANOVA; (***B***) L-LTP induced by four trains of HFS is enhanced in Tg mice, p<0.05 RM-ANOVA; (***C***) mGluR-LTD induced by bath application of DHPG is increased in eIF4E Tg mice, p<0.005 RM-ANOVA.

5 eIF4E transgenic mice show perseverative and repetitive behavior



eIF4E transgenic mice exhibit enhanced repetitive behavior in three different paradigms. (***A***) eIF4E transgenic mice showed increased marble burying behavior, *t-test,**p<0.01;* (***B***) eIF4E transgenic mice exhibit impaired reversal Y-maze test; ***p<0.001 and **p<0.01; (***C***) eIF4E transgenic mice show deficits in MWM's reversal learning; *p< 0.05. Repeated measure ANOVA followed by Bonferroni-Dunn test. A significant interaction was found between genotype and time; [(***B***) $F_{(5,96)}$=43.27, p<0.0001], [(***C***) DAY1: $F_{(3,69)}$ =40.34; p<0.05; DAY2: $F_{(3,69)}$=45.78, p<0.05; DAY3: n.s.]

6 Social behavior of eIF4E transgenic mice



eIF4E transgenic mice do not show abnormalities in social behavior. (***A***) eIF4E transgenic mice show unaltered sociability but spent significantly less time in the chamber with the stranger mouse; (***B***) eIF4E transgenic mice do not show alteration in social novelty. Two way ANOVA, interactions genotype X first stranger n.s. (***A***) and genotype X second stranger n.s. (***B***).

Acknowledgments

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eIF4E transgenic mice as a novel animal model of autism spectrum disorders.

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Autism spectrum disorders (ASDs) are a heterogeneous group of heritable neuropsychiatric disorders whose symptoms, which include abnormal social interaction, impaired communication, and repetitive/perseverative behaviors, appear in early childhood and continue throughout life. One of the leading hypotheses for a common molecular mechanism underlying ASDs is alteration in the translational control machinery. In particular, studies of humans and evidence obtained from animal models have revealed that upregulated mTORC1 signaling is a molecular abnormality that may contribute to ASDs-like behaviors. Consistent with this notion, it recently was found that at least two autistic patients carry a mutation in the promoter region of the *EIF4E* gene, which encodes the cap-binding translation factor eIF4E, a downstream effector of the mTORC1-signaling pathway. Importantly, in vitro studies have demonstrated that this mutation increases *eIF4E* promoter activity, suggesting that some these autistic patients may have increased expression of eIF4E.

eIF4E mediates cap-dependent translation initiation by binding eIF4G, thereby forming the eIF4F initiation complex. Activation of the mTORC1 signaling pathway promotes protein synthesis by releasing eIF4E from its repressor eIF4E-binding protein (4E-BP), thus increasing the availability of eIF4E to interact with eIF4G.

We studied a transgenic mouse line overexpressing eIF4E to test the hypothesis that increased protein synthesis is a causative factor in ASDs-like behaviors. We have found that eIF4E transgenic mice exhibit increased brain protein synthesis as measured by SUnSET method. The eIF4E transgenic mice also exhibit ASDs-like behaviors, including repetitive and stereotypical behaviors, behavioral inflexibility and deficit in social interaction. In addition, the eIF4E transgenic exhibit alterations in several forms of synaptic plasticity and in excitatory vs. inhibitory brain synaptic transmission, which correlates with changes in spine density. Importantly, the behavioral, biochemical and electrophysiological abnormalities of eIF4E transgenic mice were corrected by 4EGI-1, a specific inhibitor of eIF4E/eIF4G interaction. Thus, eIF4E mice are a new resource that can be utilized to specifically address the involvement of dysregulated translational control in ASDs.

eIF4E transgenic mice as a novel animal model of autism spectrum disorder

Emanuela Santini[1], Thu N. Huynh[1], Andrew F. MacAskill[1], Adam G. Carter[1], Phillipe Pierre[2], Davide Ruggero[3], Hanoch Kaphzan[1] and Eric Klann[1]

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Introduction

Autism spectrum disorder (ASD) are a heterogeneous group of heritable neuropsychiatric disorders whose symptoms, which include abnormal social interaction, impaired communication, and repetitive/perseverative behaviors, appear in early childhood and continue throughout life. One of the leading hypotheses for a common molecular mechanism underlying ASD is alteration in the translational control machinery. In particular, it seems that upregulated mTORC1 signaling is a molecular abnormality that may contribute to ASD-like behaviors. Consistent with this notion, it was recently found in autistic patients a mutation increasing *eIF4E* promoter activity.

eIF4E mediates cap-dependent translation initiation by binding eIF4G, thereby forming the eIF4F initiation complex. Activation of the mTORC1 signaling pathway promotes protein synthesis by releasing eIF4E from its repressor eIF4E-binding protein (4E-BP), thus increasing the availability of eIF4E to interact with eIF4G.

Here we employed a transgenic mouse line, in which the expression of eIF4E is augmented, to test the hypothesis that increased cap-dependent protein synthesis play a causative role in the etiology of ASD.

Materials and Methods

Animals. eIF4E transgenic and wild-type mice were generated as previously described (Ruggero et al., 2004).

Western Blotting. Mice were killed by decapitation and hippocampal slices were prepared as described in Banko et al., 2006. Levels of total proteins were assessed as described in Svenningsson et al., 2000.

Immunofluorescence. Mice were perfused and immunofluorescent staining was performed as described in Santini et al., 2009.

Electrophysiology. mPFC and striatal slices were incubated in an interface chamber and fEPSP were evoked and recorded as described previously (Banko et al., 2005; Calabresi et al., 1992) .

Dendritic spine density. Dendritic spine density experiments were performed as previously described (Dumitriu et al., 2011).

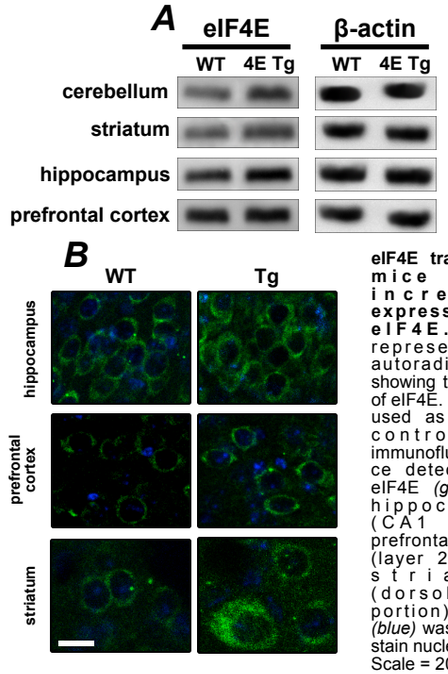
Behavioral tests. Marble burying task, self-grooming test, Y-maze reversal task, social behavior test and reciprocal social interaction task were performed as described in Banko et al., 2007, Hoeffer et al., 2008 and Moy et al., 2004.

Conclusions

We show that eIF4E is overexpressed in the brain of the transgenic mice. We have also found that eIF4E transgenic mice exhibit increased eIF4F complex formation and enhanced cap-dependent protein synthesis. Importantly, eIF4E transgenic mice exhibit specific ASD-like behaviors. In addition, eIF4E transgenic show changes in synaptic function, dendritic spine density and synaptic plasticity. FInally, treatment with 4EGI-1, which inhibits cap-dependent protein synthesis, normalizes the ASD-like behaviors displayed by the eIF4E transgenic mice. Thus, eIF4E mice are a new resource that can be utilized to specifically address the involvement of dysregulated translational control in ASD.

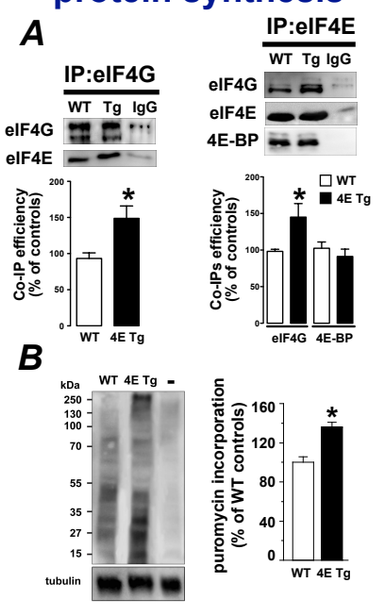
Results

1 eIF4E is ubiquitously overexpressed in the brain of eIF4E transgenic mice



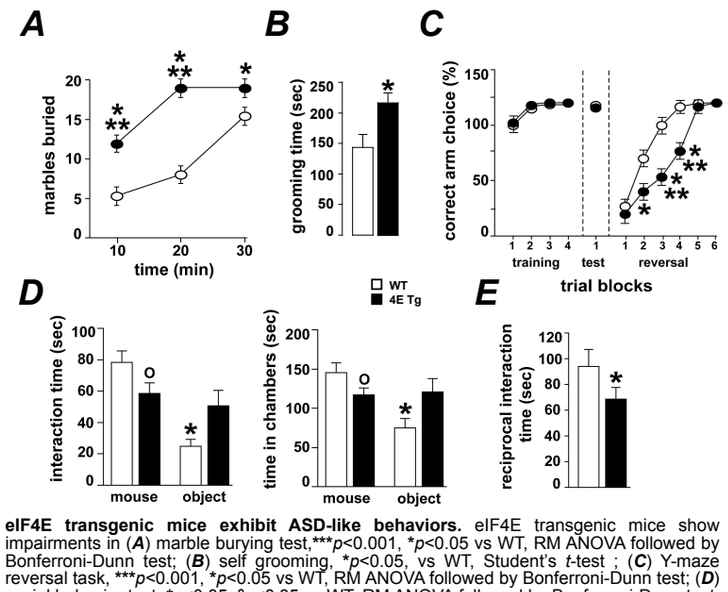
eIF4E transgenic mice show increased expression of eIF4E. (***A***) representative autoradiograms showing the levels of eIF4E. β-actin is used as loading control; (***B***) immunofluorescence detection of eIF4E *(green)* in hippocampus (CA1 area), prefrontal cortex (layer 2/3) and striatum (dorsolateral portion). DAPI *(blue)* was used to stain nuclei. Scale = 20 µm

2 eIF4E transgenic mice show increased cap-dependent protein synthesis



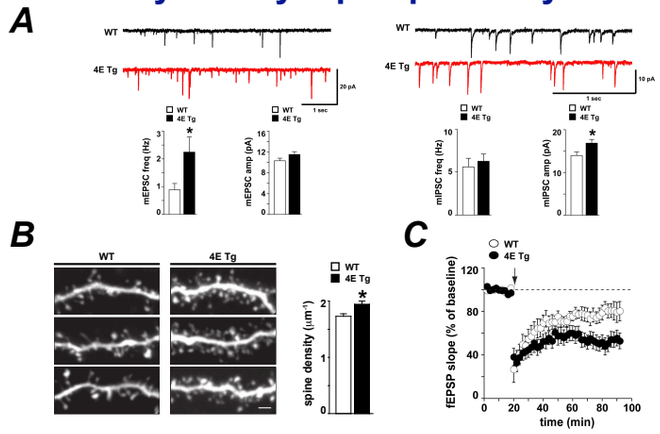
Increased eIF4E/eIF4G interactions and cap-dependent protein synthesis in the brain of eIF4E transgenic mice. Representative Western blots and quantification of (***A***) proteins recovered after immunoprecipitation (IP) of eIF4E (right) and eIF4G (left); (***B***) newly synthesized proteins labeled with puromycin using the SUnSET method. *p<0.05 vs WT, Student's *t*-test.

3 eIF4E transgenic mice exhibit ASD-like behaviors



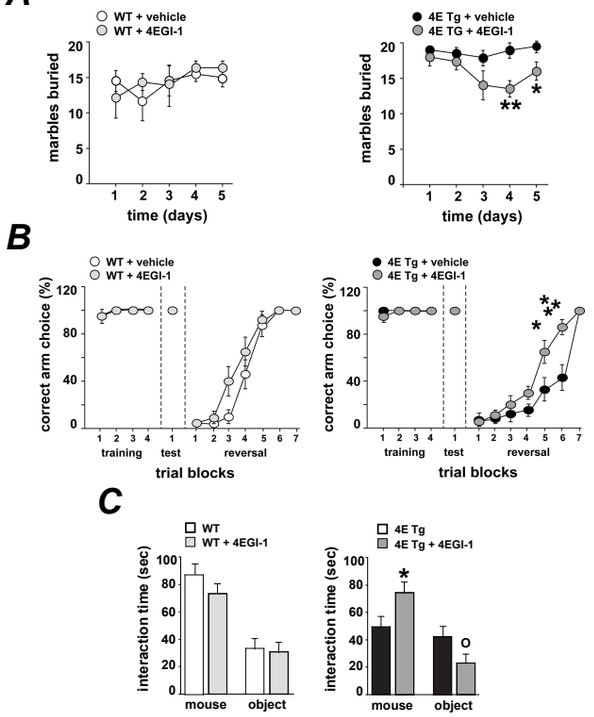
eIF4E transgenic mice exhibit ASD-like behaviors. eIF4E transgenic mice show impairments in (***A***) marble burying test,***p<0.001, *p<0.05 vs WT, RM ANOVA followed by Bonferroni-Dunn test; (***B***) self grooming, *p<0.05, vs WT, Student's *t*-test ; (***C***) Y-maze reversal task, ***p<0.001, *p<0.05 vs WT, RM ANOVA followed by Bonferroni-Dunn test; (***D***) social behavior test, *p<0.05, °p<0.05 vs WT, RM ANOVA followed by Bonferroni-Dunn test; (***E***) reciprocal social interaction task, *p<0.05, vs WT, Student's *t*-test.

4 eIF4E transgenic mice show changes in synaptic function, dendritic spine density and synaptic plasticity



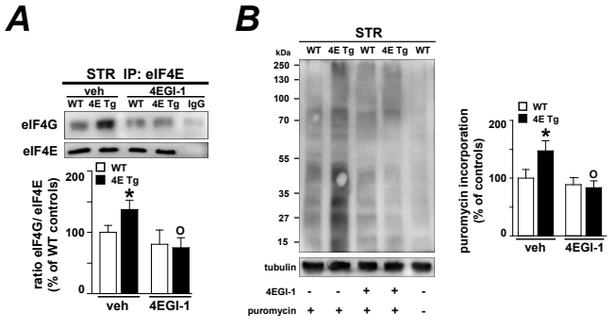
eIF4E transgenic mice exhibit changes in mEPSC and mIPSC, dendritic spine density and synaptic plasticity. mEPSC (***A***, left) and mIPSC (***A***, right) traces and quantifications obtained from layer 2/3 mPFC pyramidal neurons; (***B***) high-magnification images and quantification of spiny dendrites; (***C***) striatal LTD, arrow indicate time of high-frequency stimulation (HFS). *p<0.05 vs WT, Student's *t*-test.

5 4EGI-1 reverses the ASD-like behaviors



The cap-dependent protein synthesis inhibitor 4EGI-1 reverses ASD-like behaviors displayed by eIF4E transgenic mice. (***A***) marble burying behavior, **p<0.01, *p<0.05 vs 4E Tg, two-way RM ANOVA followed by Bonferroni-Dunn test; (***B***) Y-maze reversal task, **p<0.01, *p<0.05 vs 4E Tg, two-way RM ANOVA followed by Bonferroni-Dunn test; (***C***) social behavior, *p<0.05, °p<0.05 vs 4E Tg, two-way RM ANOVA followed by Bonferroni-Dunn test.

6 4EGI-1 normalizes eIF4E/eIF4G interactions and cap-dependent protein synthesis



Enhanced eIF4E/eIF4G interactions and cap-dependent protein synthesis is normalized in the eIF4E transgenic mice treated with 4EGI-1. Representative Western blots and quantification of (***A***) proteins recovered after immunoprecipitation (IP) of eIF4E; (***B***) newly synthesized proteins labeled with puromycin using the SUnSET methods. *p<0.05, °p<0.05 vs 4E Tg, two-way ANOVA followed by Bonferroni-Dunn test.

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LETTER

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Exaggerated translation causes synaptic and behavioural aberrations associated with autism

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Autism spectrum disorders (ASDs) are an early onset, heterogeneous group of heritable neuropsychiatric disorders with symptoms that include deficits in social interaction skills, impaired communication abilities, and ritualistic-like repetitive behaviours[1,2]. One of the hypotheses for a common molecular mechanism underlying ASDs is altered translational control resulting in exaggerated protein synthesis[3]. Genetic variants in chromosome 4q, which contains the *EIF4E* locus, have been described in patients with autism[4,5]. Importantly, a rare single nucleotide polymorphism has been identified in autism that is associated with increased promoter activity in the *EIF4E* gene[6]. Here we show that genetically increasing the levels of eukaryotic translation initiation factor 4E (eIF4E) in mice[7] results in exaggerated cap-dependent translation and aberrant behaviours reminiscent of autism, including repetitive and perseverative behaviours and social interaction deficits. Moreover, these autistic-like behaviours are accompanied by synaptic pathophysiology in the medial prefrontal cortex, striatum and hippocampus. The autistic-like behaviours displayed by the eIF4E-transgenic mice are corrected by intracerebroventricular infusions of the cap-dependent translation inhibitor 4EGI-1. Our findings demonstrate a causal relationship between exaggerated cap-dependent translation, synaptic dysfunction and aberrant behaviours associated with autism.

eIF4E-transgenic mice (βT-*Eif4e*)[7] exhibited increased levels of eIF4E across brain regions (Fig. 1a) without compensatory changes in levels of other translational control proteins (Fig. 1b). We investigated whether eIF4E was bound preferentially to either eIF4E-binding protein (4E-BP) or eIF4G, which repress and promote, respectively, the initiation of cap-dependent translation[8,9]. We found significantly higher levels of eIF4E–eIF4G interactions in the brains of eIF4E-transgenic mice (Fig. 1c and Supplementary Fig. 1a), with no alterations in the interaction between eIF4E and 4E-BP (Fig. 1c, left, and Supplementary Fig. 1a). To confirm that the increased eIF4E–eIF4G interactions resulted in increased protein synthesis, we infused puromycin into the lateral ventricle of cannulated mice and labelled newly synthesized proteins using SUnSET[10,11], and observed increased *de novo* cap-dependent translation (Fig. 1d and Supplementary Fig. 1b–g). Overall, our results indicate that overexpression of eIF4E results in exaggerated cap-dependent translation in the brains of eIF4E-transgenic mice.

We then determined whether eIF4E-transgenic mice display repetitive and perseverative behaviours, which are behavioural domains required for ASD diagnosis[2]. eIF4E-transgenic mice exhibited repetitive digging behaviour in the marble-burying test[12] and increased self-grooming[13] compared with wild-type littermate controls (Fig. 2a, b). eIF4E-transgenic mice also displayed cognitive inflexibility in both a water-based Y-maze task and a modified version of the Morris water maze[14,15]. Learning ability in the acquisition and memory phases of these tasks was intact; however, in the reversal phases, eIF4E-transgenic mice were impaired in locating the new platform positions (Fig. 2c, d and Supplementary Fig. 2e–h). We tested an additional form of behavioural inflexibility by examining the eIF4E-transgenic mice for extinction of cued fear conditioning and found that they did not exhibit a significant reduction in freezing responses after extinction training (Fig. 2e). These experiments suggest that excessive cap-dependent translation in the brain affects the ability to suppress previously codified response patterns and the ability to form new behavioural strategies in response to changed environmental circumstances.

Abnormalities in social interaction skills are another behavioural defect displayed by individuals with ASDs[2]. In tests to examine social behaviour[16–18], the eIF4E-transgenic mice did not show a preference for a nonspecific stranger versus a new, inanimate object (Fig. 2f, g). Moreover, eIF4E-transgenic mice exhibited diminished reciprocal interactions with a freely moving stranger mouse (Fig. 2h), further evidence of deficits in social behaviour. The deficits in social behaviour of the eIF4E-transgenic mice are unlikely to be caused by a generalized increase in anxiety (Supplementary Fig. 2c, d, j). Moreover, the eIF4E-transgenic mice exhibited mild hyperactivity (Supplementary Fig. 2a, b), but no impairments in motor coordination, motor learning and sensorimotor gating (Supplementary Fig. 2i, k, l). Taken together, our behavioural analysis of the eIF4E-transgenic mice indicates that increased cap-dependent translation in the brain results in a distinct pattern of behavioural abnormalities consistent with ASDs.

Previous studies suggest that ASD symptoms such as cognitive inflexibility and deficits in social behaviour are generated by abnormalities in prefrontal and/or striatal circuits[19]. Consistent with this idea, the medial prefrontal cortex (PFC) is implicated in the modulation of social behaviours and social skills[20], whereas motor, social and communication impairments in boys with ASDs are associated with anatomical abnormalities in the striatum[21]. Therefore, we next examined whether the eIF4E-transgenic mice exhibited specific synaptic pathophysiologies in the medial PFC and striatum.

In the eIF4E-transgenic mice, examination of spontaneous synaptic 'mini' events in layers 2/3 of acute medial PFC slices revealed an increase in the frequency but not amplitude of excitatory events (miniature excitatory postsynaptic currents (mEPSCs); Fig. 3a), and an increase in the amplitude, but not frequency, of inhibitory events (miniature inhibitory postsynaptic currents (mIPSCs); Fig. 3b). No changes were observed in layer 5 (Supplementary Fig. 3a, b). Thus, our data suggest an enhancement of excitatory input and postsynaptic sensitivity for inhibitory events onto layer 2/3 pyramidal neurons, consistent with the hypothesis that autism may arise from an imbalance between excitatory and inhibitory synaptic transmission[22].

To determine whether the increased frequency of spontaneous mEPSCs might result from an enhanced number of synaptic contacts, we imaged dendritic spines using two-photon laser-scanning

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*These authors contributed equally to this work.

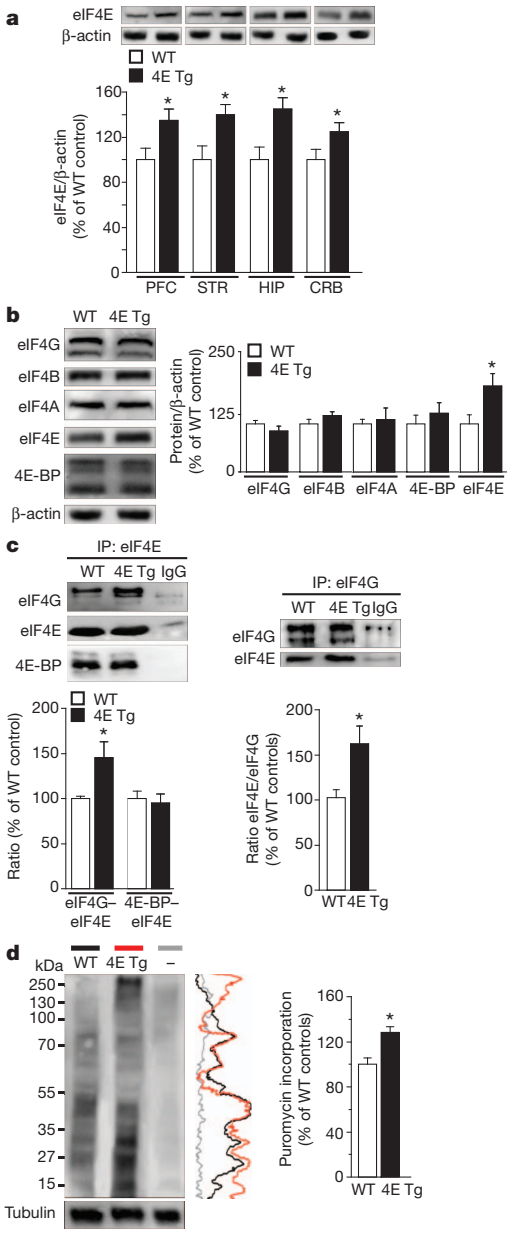

Figure 1 | eIF4E-transgenic mice exhibit increased eIF4E/eIF4G interactions and exaggerated cap-dependent translation. **a**, eIF4E-transgenic mice (4E Tg) exhibit increased eIF4E expression in multiple brain regions. $n = 4$ mice per genotype; $^*P < 0.05$ versus wild type (WT), Student's *t*-test. CRB, cerebellum; HIP, hippocampus; PFC, prefrontal cortex; STR, striatum. **b**, eIF4E-transgenic mice exhibit normal expression of other translational control proteins. $n = 4$ mice per genotype; $^*P < 0.05$, Student's *t*-test. **c**, eIF4E-transgenic mice exhibit increased eIF4E–eIF4G interactions. Immunoprecipitation (IP) of eIF4E (left) and eIF4G (right). $n = 3$ mice per genotype; $^*P < 0.05$, Student's *t*-test. **d**, eIF4E-transgenic mice exhibit increased translation as measured with SUnSET (see Methods). Vertical line traces of each autoradiogram are shown on the right. $n = 3$ mice per genotype; $^*P < 0.05$, Student's *t*-test. '–' represents a control sample without puromycin. All data are shown as mean and s.e.m.

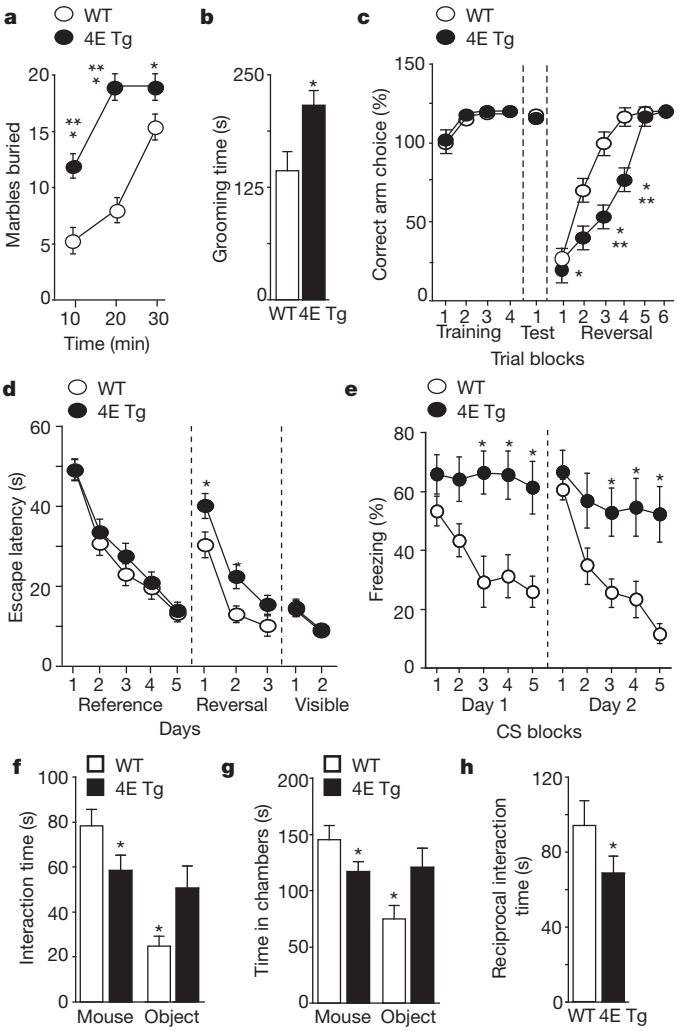

Figure 2 | eIF4E-transgenic mice exhibit ASD-like behaviours. eIF4E-transgenic mice were compared to wild-type littermates. **a**, Marble-burying test. $n = 21$–22 mice per genotype; $^*P < 0.05$, $^{***}P < 0.001$, repeated-measures analysis of variance (ANOVA) (time × genotype, $F_{(2,46)} = 31.62$, $P < 0.001$) followed by Bonferroni–Dunn post-hoc test. **b**, Self-grooming test. $n = 12$ mice per genotype; $^*P < 0.05$, Student's *t*-test. **c**, Y-maze reversal task. $n = 21$–22 mice per genotype; $^*P < 0.05$, $^{***}P < 0.001$, repeated-measures ANOVA (time × genotype, $F_{(5,138)} = 16.74$, $P < 0.001$) followed by Bonferroni–Dunn post-hoc test. **d**, Morris water maze reversal learning. $n = 12$–13 mice per genotype; $^*P < 0.05$, repeated-measures ANOVA (time × genotype, $F_{(3,92)} = 6.1$, $P < 0.001$) followed by Bonferroni–Dunn post-hoc test. **e**, Extinction of cued fear memory (15 conditioned stimuli (CS) per day represented as three CS blocks). $n = 6$ mice per genotype; $^*P < 0.05$, repeated-measures ANOVA (day 1: time × genotype, $F_{(4,40)} = 5.73$, $P < 0.001$; day 2: time × genotype, $F_{(4,40)} = 4.81$, $P < 0.01$) followed by Bonferroni–Dunn post-hoc test. **f**, **g**, Social behaviour test. The time spent either interacting with a stranger mouse (**f**) or in the chambers (**g**). $n = 6$ mice per genotype; $^*P < 0.05$, repeated-measures ANOVA (stimulus × genotype, $F_{(1,10)} = 6.04$, $P < 0.05$ (**f**); stimulus × genotype, $F_{(1,10)} = 6.12$, $P < 0.05$ (**g**)) followed by Bonferroni–Dunn post-hoc test. **h**, Reciprocal social interaction task. $n = 6$ mice per genotype; $^*P < 0.05$, Student's *t*-test. All data are shown as mean and s.e.m.

microscopy (Fig. 3c, d and Supplementary Fig. 3c, d). We found a significant increase (~12%) in spine density and observed a significantly smaller spine volume in the eIF4E-transgenic mice than in wild-type littermates (wild type = $0.123 \pm 0.004\ \mu m^3$ (mean ± s.e.m.) and eIF4E-transgenic = $0.110 \pm 0.004\ \mu m^3$, $P = 0.01$ versus wild type, Student's *t*-test).

Next, we examined whether increased expression of eIF4E also resulted in synaptic pathophysiology in the striatum. We used high-frequency stimulation to induce long-term depression (LTD) in acute striatal slices[23], and found that eIF4E-transgenic mice exhibited enhanced LTD compared to wild-type littermates (Fig. 3e and Supplementary Fig. 3e, f). We propose that the enhanced LTD in eIF4E-transgenic mice results in altered efficiency of striatal information storage and processing, culminating in the inability to form new motor patterns and/or to disengage from previously learned motor behaviours.

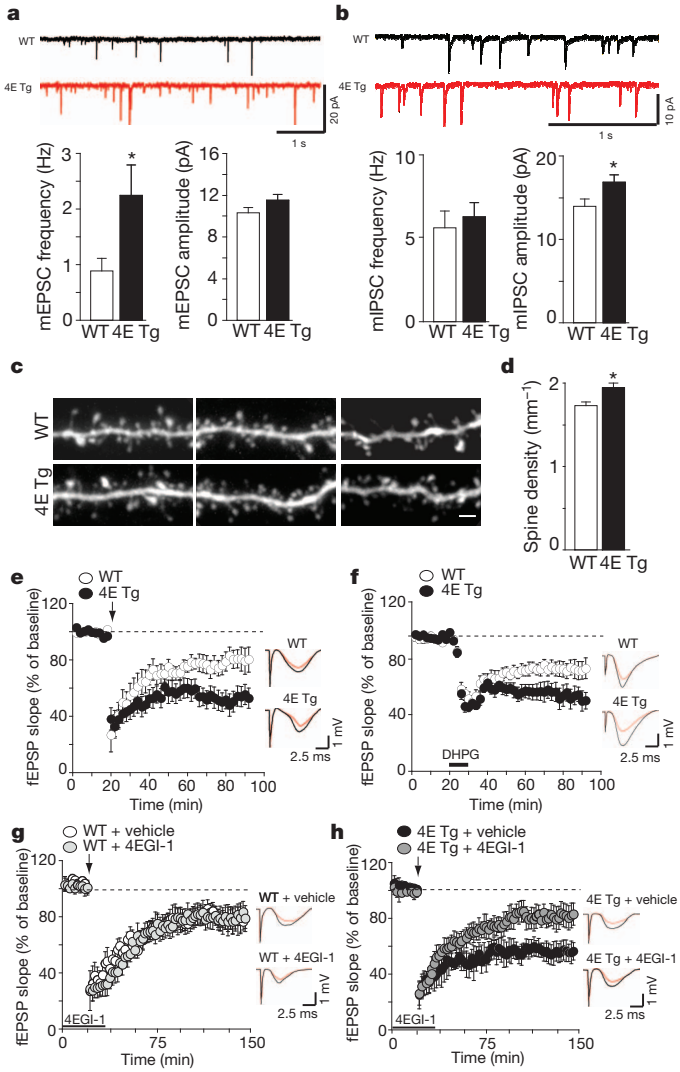

Figure 3 | eIF4E-transgenic mice exhibit alterations in synaptic function, dendritic spine density and synaptic plasticity. **a**, **b**, eIF4E-transgenic mice exhibit increased mEPSC frequency (**a**) and increased mIPSC amplitude (**b**) in layer 2/3 medial PFC pyramidal neurons. $n = 27–30$ neurons per genotype; $^*P < 0.05$, Student's t-test. **c**, **d**, eIF4E-transgenic mice exhibit increased dendritic spine density in layer 2/3 medial PFC pyramidal neurons. High-magnification images (**c**) and quantification (**d**) of spiny dendrites. $n = 12$ neurons per genotype; $^*P < 0.05$, Student's t-test. Scale bar, 2 μm. **e**, eIF4E-transgenic mice exhibit enhanced striatal LTD. $n = 13$ slices from 8 mice per genotype. **f**, eIF4E-transgenic mice exhibit enhanced hippocampal mGluR-LTD. DHPG denotes the mGluR agonist 3,5-dihydroxyphenylglycine. $n = 15$ slices from 8 mice per genotype. **g**, **h**, 4EGI-1 normalizes enhanced striatal LTD shown by eIF4E-transgenic mice (**h**), without affecting LTD in wild-type mice (**g**). $n = 18$ slices from 9 mice per genotype and treatment. All field recordings were analysed with repeated-measures ANOVA. Arrows indicate delivery of high-frequency stimulation. Solid bars indicate the duration of bath application of DHPG (10 μM, 10 min) and 4EGI-1 (100 μM, 45 min). Representative traces (right) showing field excitatory postsynaptic potentials (fEPSPs) before (black) and 60 min after (red) high-frequency stimulation. All data are shown as mean and s.e.m.

To determine whether the synaptic alterations described in the eIF4E-transgenic mice were selective for the frontostriatal circuit, we examined synaptic plasticity in the hippocampus[24]. We found that eIF4E-transgenic mice exhibited enhanced metabotropic glutamate receptor-dependent LTD (mGluR-LTD) compared to wild-type littermates (Fig. 3f and Supplementary Fig. 3g, h), consistent with previous studies showing that changes in brain protein synthesis are accompanied by altered (enhanced or reduced) hippocampal mGluR-LTD[25,26]. Thus, consistent with the ubiquitous increase in brain expression of eIF4E, the eIF4E-transgenic mice display altered synaptic function and plasticity in several brain regions (medial PFC, striatum and hippocampus) implicated in behavioural abnormalities associated with ASDs.

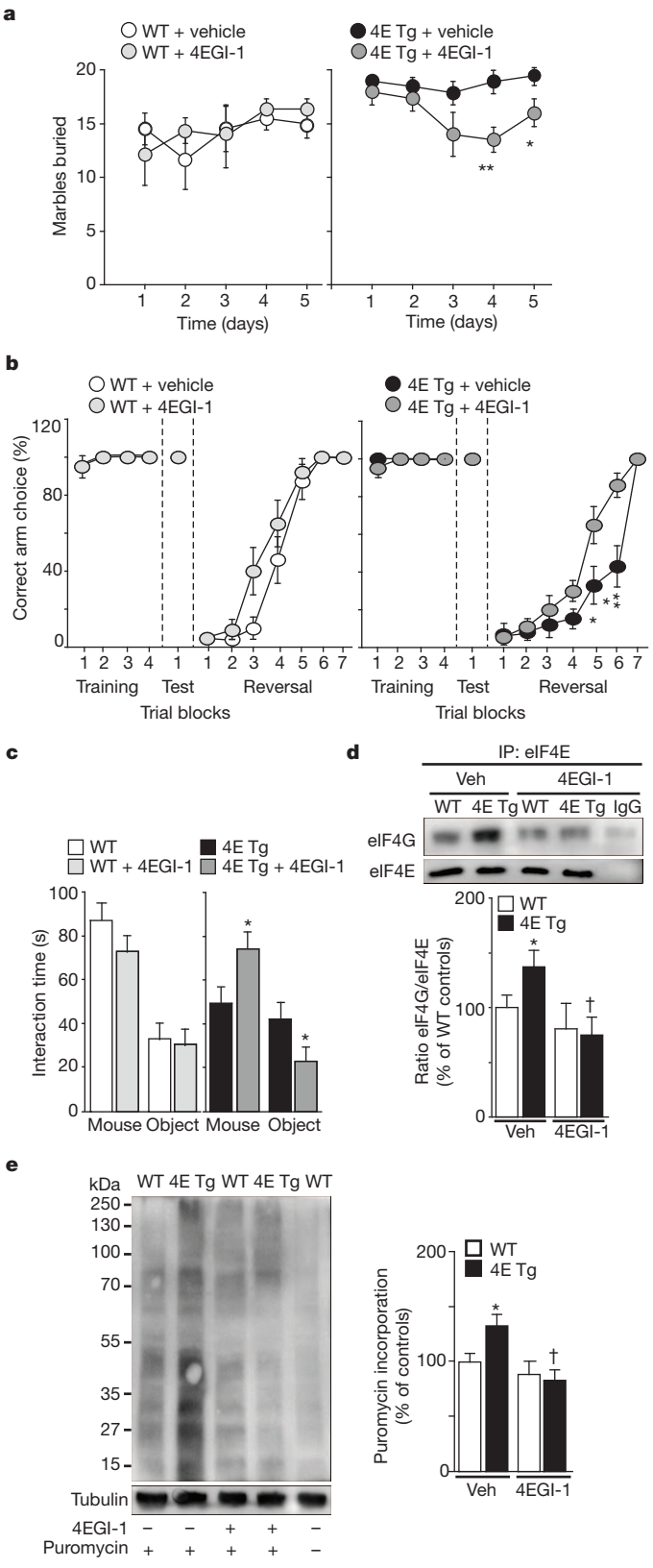

Figure 4 | The cap-dependent translation inhibitor 4EGI-1 reverses ASD-like behaviours shown by eIF4E-transgenic mice. **a**, Treatment of eIF4E-transgenic mice with 4EGI-1 reduces the marble-burying behaviour. $n = 6$ mice per genotype and treatment; $^{*}P < 0.05$, $^{**}P < 0.01$, two-way repeated-measures ANOVA (treatment × genotype, $F_{(1,20)} = 4.21$, $P < 0.05$) followed by Bonferroni–Dunn post-hoc test. **b**, 4EGI-1 improves the cognitive flexibility of eIF4E-transgenic mice in the Y-maze test. $n = 6$–7 mice per genotype and treatment; $^{*}P < 0.05$, $^{**}P < 0.01$, two-way repeated-measures ANOVA (treatment × genotype, $F_{(1,21)} = 4.61$, $P < 0.05$) followed by Bonferroni–Dunn post-hoc test. **c**, 4EGI-1 improves social behaviour of eIF4E-transgenic mice in the three-chamber arena test. $n = 6$ mice per genotype and treatment; $^{*}P < 0.05$, two-way repeated-measures ANOVA (treatment × genotype, $F_{(1,20)} = 6.26$, $P < 0.05$) followed by Bonferroni–Dunn post-hoc test. **d**, 4EGI-1 decreases the enhanced eIF4E–eIF4G interactions in eIF4E-transgenic mice. Immunoprecipitation of eIF4E in the striatum. $n = 4$ mice per genotype; $^{*}P < 0.05$ (versus vehicle-treated wild type), $^{\dagger}P < 0.05$ (versus 4EGI-1-treated wild type), two-way ANOVA, followed by Bonferroni–Dunn post-hoc test. **e**, 4EGI-1 normalizes the exaggerated cap-dependent translation in eIF4E-transgenic mice as measured with SUnSET. The last wild-type sample represents a control without puromycin. $^{*}P < 0.05$, $^{\dagger}P < 0.05$, two-way ANOVA followed by Bonferroni–Dunn post-hoc test. All data are shown as mean and s.e.m.

Finally, we asked whether exaggerated cap-dependent translation was responsible for the synaptic alterations and ASD-like behaviours shown by the eIF4E-transgenic mice. We took advantage of 4EGI-1, an inhibitor of eIF4E–eIF4G interactions[8,11], to block the synaptic and behavioural consequences of increased eIF4E expression. Bath application of 4EGI-1 normalized the enhanced striatal LTD observed in the eIF4E-transgenic mice (Fig. 3g, h), suggesting that exaggerated striatal LTD (Fig. 3h) is a direct consequence of increased binding of eIF4E to eIF4G (Supplementary Fig. 3i–k).

Next, we used a subthreshold dose of 4EGI-1 (ref. 11) to normalize the behavioural abnormalities in eIF4E-transgenic mice without impairing their wild-type littermates. eIF4E-transgenic mice treated with 4EGI-1 exhibited a decrease in repetitive behaviour during the marble-burying task, which started on day four and persisted throughout day five (Fig. 4a). Moreover, we found that 4EGI-1 maintained the behavioural effects observed in the marble-burying task (Supplementary Fig. 4a, b). We also found that blockade of eIF4E–eIF4G interactions with 4EGI-1 significantly improved the performance of eIF4E-transgenic mice in the reversal phase of the Y-maze test (Fig. 4b). These findings indicate that chronic treatment of eIF4E-transgenic mice with 4EGI-1 reverses their repetitive and perseverative behaviours. We also found that infusions of 4EGI-1 rescued the social behaviour deficits shown by the eIF4E-transgenic mice in the three-chamber arena test, as they exhibited an increased preference for a nonspecific stranger compared to a new object (Fig. 4c).

At the completion of the behavioural studies with 4EGI-1, we performed co-immunoprecipitation experiments, confirming that 4EGI-1 reduced the increased eIF4E–eIF4G interactions exhibited by the eIF4E-transgenic mice (Fig. 4d and Supplementary Fig. 4c–e). Furthermore, puromycin-labelling of newly synthesized proteins was reduced to wild-type levels, indicating that 4EGI-1 was effective in attenuating the increased cap-dependent translation in the eIF4E-transgenic mice (Fig. 4e and Supplementary Fig. 4f, g). Together, these results indicate that repeated treatment of eIF4E-transgenic mice with 4EGI-1 reverses the increased binding of eIF4E to eIF4G, exaggerated cap-dependent translation, and reversal of ASD-like behaviours.

Here we have demonstrated that increased eIF4E expression and, consequently, dysregulated translational control at the initiation phase of protein synthesis in mice results in the appearance of synaptic dysfunction and aberrant behaviours consistent with ASDs. On the basis of our observations, we propose that exaggerated cap-dependent protein synthesis in the eIF4E-transgenic mice and fragile X syndrome model mice[27,28] results in enhanced translation of a specific subset of messenger RNAs. Thus, the identity of both these mRNAs and the *cis*-acting elements in the 5′ untranslated region responsible for eIF4E-dependent protein synthesis and their possible overlap with fragile X mental retardation protein target mRNAs will be important investigations in future studies.

Our studies with eIF4E-transgenic mice indicate that ASD-like behaviours can be induced by exaggerated cap-dependent translation in the brain. Moreover, we demonstrated that aberrant repetitive, perseverative and social behaviours shown by eIF4E-transgenic mice are reversed by reducing eIF4E–eIF4G interactions, thereby restoring translational homeostasis. Thus, our findings establish a causal link between exaggerated cap-dependent translation and behaviours associated with autism. Finally, our findings indicate that behavioural defects caused by exaggerated cap-dependent translation, which also occurs in fragile X syndrome[29,30], a disorder with a high incidence of autism, are not irrevocable and can be corrected well into adulthood.

METHODS SUMMARY

All procedures involving animals were approved by the New York University Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the use of animals in research. For a detailed description of all the techniques used in this study, please see the Methods. All the experiments were performed with the examiners blinded to genotype.

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

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Supplementary Information is available in the online version of the paper.

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Author Contributions The study was directed by E.K. and conceived and designed by E.S. and E.K. E.S. performed the molecular, behavioural and electrophysiological experiments. T.N.H. performed behavioural experiments. A.F.M. and A.G.C. performed the dendritic spine-density experiments. P.P. contributed the anti-puromycin (12D10) antibody. D.R. contributed with reagents and expertise concerning translation control by eIF4E. H.K. performed the cortical whole-cell electrophysiological experiments. The manuscript was written by E.S. and E.K. and edited by all of the authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.K. (eklann@cns.nyu.edu).

METHODS

Housing. Generation of βT-*Eif4e* transgenic mice (eIF4E-transgenic mice) has been described previously[7].

For all the experiments, we made use of littermates derived from crossing heterozygotes. Mice were backcrossed to the N10 generation in C57BL/6J mice. Overall, eIF4E-transgenic mice were viable, fertile and showed no gross anatomical abnormalities in the age range used for this study. eIF4E-transgenic mice and their wild-type littermates were housed in groups of 3–4 animals per cage and kept on a regular 12 h light/dark cycle (7:00–19:00 light period). Food and water were available ad libitum.

Surgery and drug infusion. Mice were anaesthetized (ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1})) and mounted onto a stereotaxic apparatus. Cannulae (26-gauge) were implanted unilaterally at the following coordinates: −0.22 mm anterioposterior, +1 mm mediolateral, and −2.4 mm dorsoventral[31]. Mice were allowed 1 week to recover after the surgery.

The infusions of the eIF4E–eIF4G inhibitor 4EGI-1 were performed as described previously[11]. In brief, 4EGI-1 dissolved in 100% dimethylsulphoxide (DMSO) was diluted in vehicle (0.5% (2-hydroxypropyl)-β-cyclodextrin and 1% DMSO in artificial cerebrospinal fluid (ACSF)). Vehicle or 4EGI-1 (20 μM) was infused over 1 min (0.5 μl min^{-1}; Harvard Apparatus). On the last day of treatment, mice received infusion of 4EGI-1 alone or puromycin (25 μg in 0.5 μl) before 4EGI-1 infusions. All behaviour and tissue dissection occurred 1 h after 4EGI-1 infusions.

Behaviour. The following behavioural tests were performed on male eIF4E-transgenic mice and their wild-type littermates (2–6 months of age) as described previously: novelty induced locomotor activity[32], open field[33], elevated plus maze[33], rotarod[34], prepulse inhibition[33], marble[14], social behaviour[16], direct social interaction[35,36], Y-maze and the Morris water maze[7,35].

For all experiments, mice were acclimated to the testing room 30 min before behavioural training and all behaviour apparatuses were cleaned between each trial with 30% ethanol. The experimenter was blinded to genotype and drug treatment while performing and scoring all behavioural tasks. All behavioural tests were performed starting with the least aversive task first (locomotor activity) and ending with the most aversive (either water-based mazes or extinction of fear memory).

Western blots. Mice were killed by decapitation 1 h after the infusion with either 4EGI-1 alone or 4EGI-1 plus puromycin. The striatum and prefrontal cortex were rapidly dissected, placed on an ice-cold surface, and sonicated in 1% SDS and boiled for 10 min. Aliquots (2 μl) of the homogenate were used for protein determination with a BCA (bicinchoninic acid) assay kit (Pierce, Thermo Scientific). Equal amounts of protein (20 μg) for each sample were loaded onto 10% polyacrylamide gels. Proteins were separated by SDS–PAGE and transferred overnight to polyvinylidene difluoride membranes (Immobilon-Psq, Millipore Corporation). The membranes were immunoblotted with antibodies against eIF4E (1:1,000), eIF4G (1:1,000), eIF4B (1:1,000), eIF4A (1:1,000) and 4E-BP (1:1,000) (Cell Signaling Technology). Antibodies against β-actin and tubulin (1:5,000, Cell Signaling Technology) were used to estimate the total amount of proteins. Detection was based on a horseradish peroxidase (HRP)-conjugated secondary antibody (Promega) and chemiluminescence reagent (ECL or ECL plus; GEHealthcare), and visualized using a Kodak 4000MM imager to obtain pixel density values for the band of interest (Carestream). All images were obtained using maximum sensitivity settings with no binning (0–65 K signal range). No images analysed presented saturating signals for the bands of interest (>65 K greyscale value). The amount of each protein was normalized for the amount of the corresponding β-actin or tubulin detected in the sample.

Immunoprecipitation. Tissue was homogenized in ice-cold lysis immunoprecipitation buffer containing (in mM): 40 HEPES, pH 7.5, 150 NaCl, 10 pyrophosphate, 10 glycerophosphate, 1 EDTA and 0.1% CHAPS, protease inhibitor II, phosphatase inhibitor mixture I, II (Sigma-Aldrich). Cleared homogenate (500 μg) was incubated with either anti-eIF4G (2.5 μg) or anti-eIF4E (2.5 μg) (Bethyl Laboratories) and gently shaken overnight at 4 °C. The antibody–lysate mix was incubated with 75 μl IgG bound to agarose beads (Thermo Scientific). The bead–sample slurry was incubated while rocking at 4 °C overnight. Supernatant was removed and saved, and immunoprecipitates were washed three times in lysis buffer, and once in wash buffer (50 mM HEPES, pH 7.5, 40 mM NaCl, 2 mM EDTA). SDS–PAGE buffer was added to the washed immunoprecipitates, which then were resolved on 4 to 12% gradient gels. Efficiency of the immunoprecipitation was determined by examining the supernatant and wash fractions obtained from the procedure on images obtained from Kodak 4000MM imager (see western blots section). Band density values for coimmunoprecipitated eFI4E, eIF4G and 4E-BP were normalized to immunoprecipitated eIF4G or eIF4E.

SUnSET. A protein synthesis assay was performed as previously described using the SUnSET method[11]. Puromycin-treated samples were identified on blots using the mouse monoclonal antibody 12D10 (1:5,000 from a 5 mg ml^{-2} stock). Because only a small fraction of the brain proteins were labelled, signal from blots was identified using ECL-Advance (GEHealthcare).

Electrophysiology. Hippocampal (400 μm), prefrontal and striatal slices (300 μm) for electrophysiology were prepared as described previously[24].

Solution to maintain slices. Cutting solution (in mM): 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose and 0.6 ascorbate. ACSF (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose, 2 $CaCl_2$ and 1 $MgCl_2$. Slices were incubated at room temperature and then were placed in the recording chamber for additional recovery time of 60 min at 33 °C.

Extracellular recordings. Extracellular fEPSPs were recorded as described previously[23,24]. In all the experiments, baseline synaptic transmission was monitored for at least 20 min before LTD induction. Three trains of high-frequency stimulation (3 s duration, 100 Hz frequency at 20 s intervals) were used to induce LTD in striatal slices[23], and 10 min of incubation with DHPG (50 μM) was used to induce mGluR-dependent LTD in hippocampal slices[24]. The slope of fEPSPs was expressed as a percentage of the baseline average before LTD induction.

Intracellular recordings. Medial prefrontal pyramidal cells were illuminated and visualized using a ×60 water-immersion objective mounted on a fixed-stage microscope (BX61-WI, Olympus), and the image was displayed on a video monitor using a charge-coupled device camera (Hamamatsu). Recordings were amplified by multiclamp 700B and digitized by Digidata 1440 (Molecular Devices). The recording electrode was pulled from a borosilicate glass pipette (3–5 MΩ) using an electrode puller (P-97, Sutter Instruments), filled with an internal solution according to the specific experimental requirement, and patched onto the soma. The series resistance of the patch pipette was compensated ~70% and re-adjusted before each experiment. A measured liquid junction potential was corrected by adjusting the pipette offset. All voltage–clamp recordings were low-pass filtered at 10 kHz and sampled at 50 kHz.

Internal solution for mEPSCs (in mM): 120 caesium-methane-sulphonate, 10 HEPES, 10 EGTA, 4 $MgCl_2$, 0.4 NaGTP, 4 MgATP, 10 phosphocreatine and 5 QX-314 (pH adjusted to 7.3 with CsOH, 290 mOsm). Bicuculline 50 μM and tetrodotoxin 1 μM (Tocris) were added to the external ACSF bath solution.

Internal solution for mIPSCs (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 2 $MgCl_2$, 2.0 Mg-ATP, 4 Na_2-ATP, 0.4 Na_2-GTP and 5 QX-314 (pH adjusted to 7.3 with CsOH, 290 mOsm), thus yielding a chloride reversal potential of around 2 mV for the chloride currents. Tetrodotoxin (1 μM), 6,7-dinitroquinoxaline-2,3-dione (DNQX) (40 μM) and D-2-amino-5-phosphonopentanoate (AP5) (50 μM) were added to the ACSF bath solution.

In these conditions, mEPSCs and mIPSCs were recorded in voltage clamp at −70 mV and measured for 120 s and 60 s, respectively.

Dendritic spine morphology. Dendritic spine density experiments were performed as previously described[37,38]. In brief, two-photon imaging was accomplished with a custom microscope and high-resolution stacks ($x = 0.13$ μm, $y = 0.13$ μm, $z = 0.2$ μm per voxel) of dendritic segments throughout the entire cell were taken for morphological analysis in NeuronStudio. Spine-head volume was calculated using a rayburst algorithm. Images were deconvolved before volume measurements using custom routines written in MATLAB (Mathworks).

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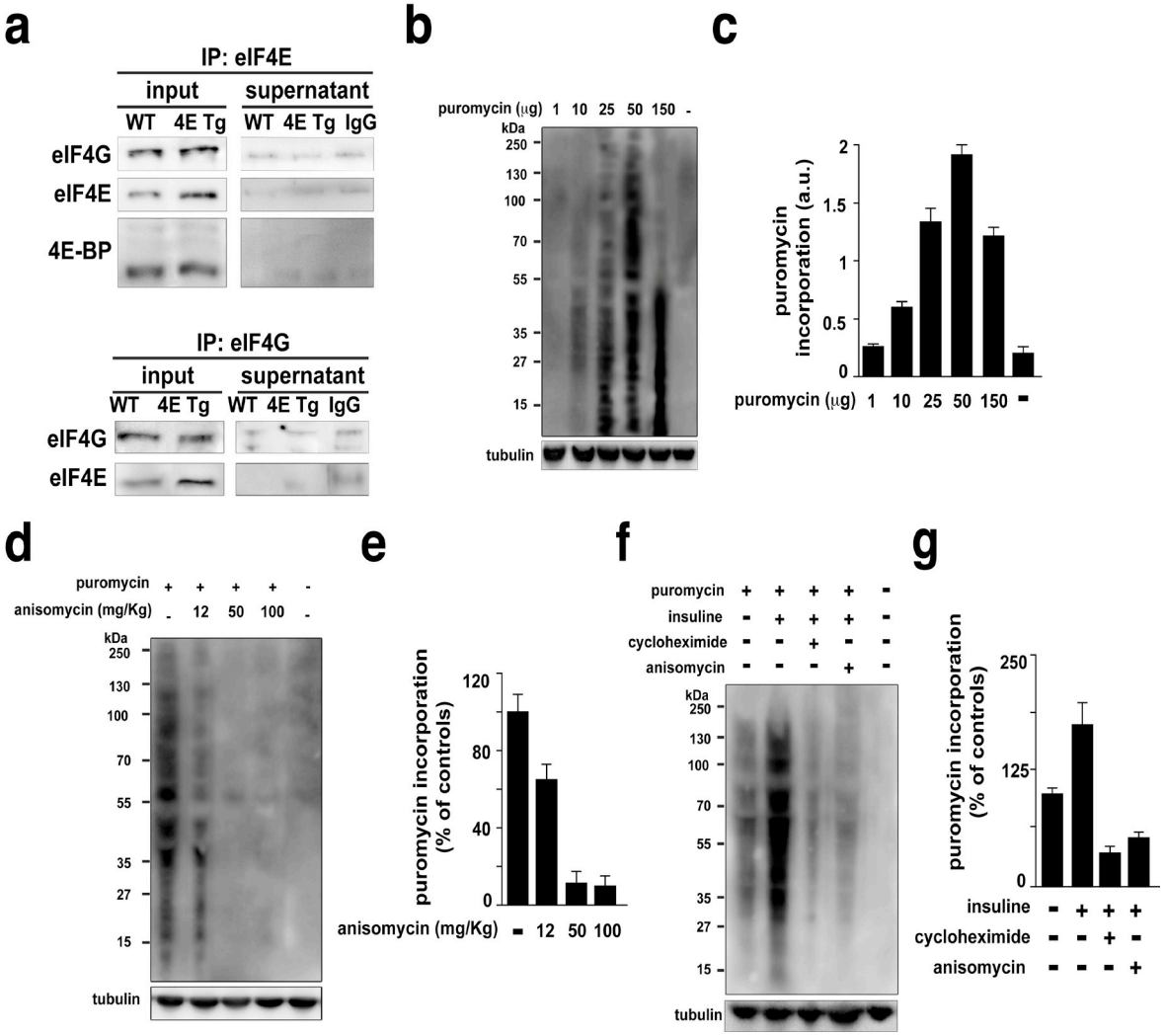
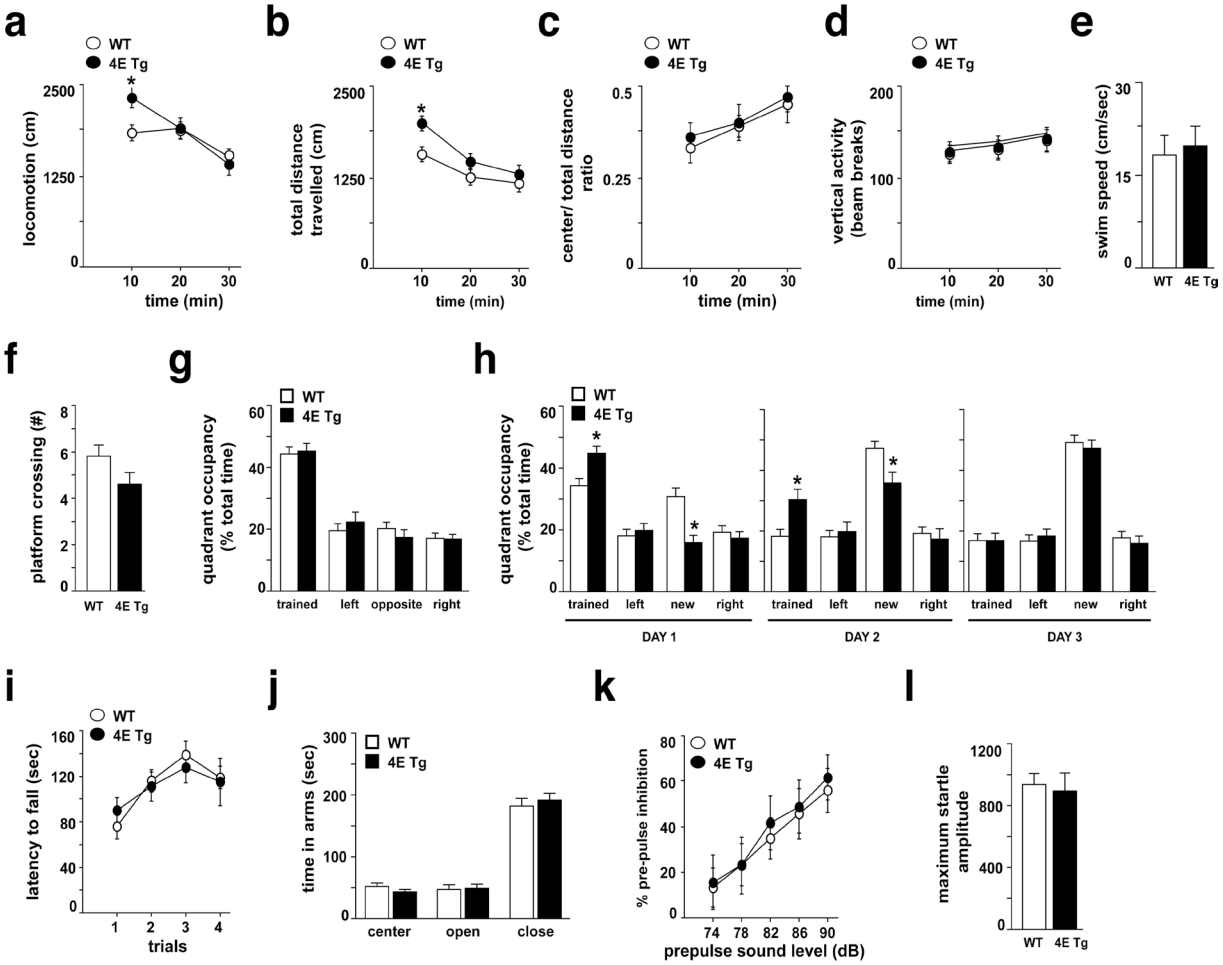
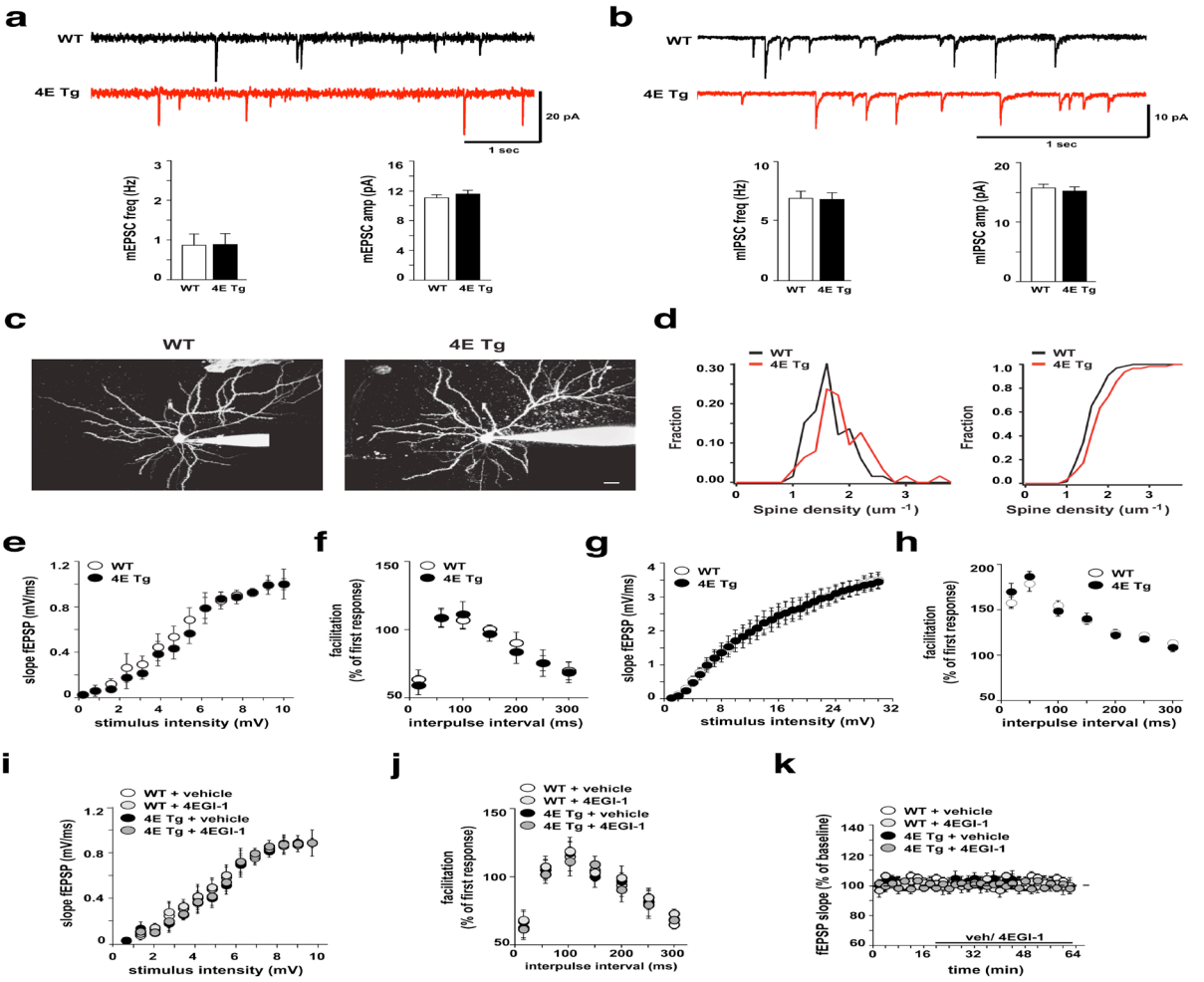


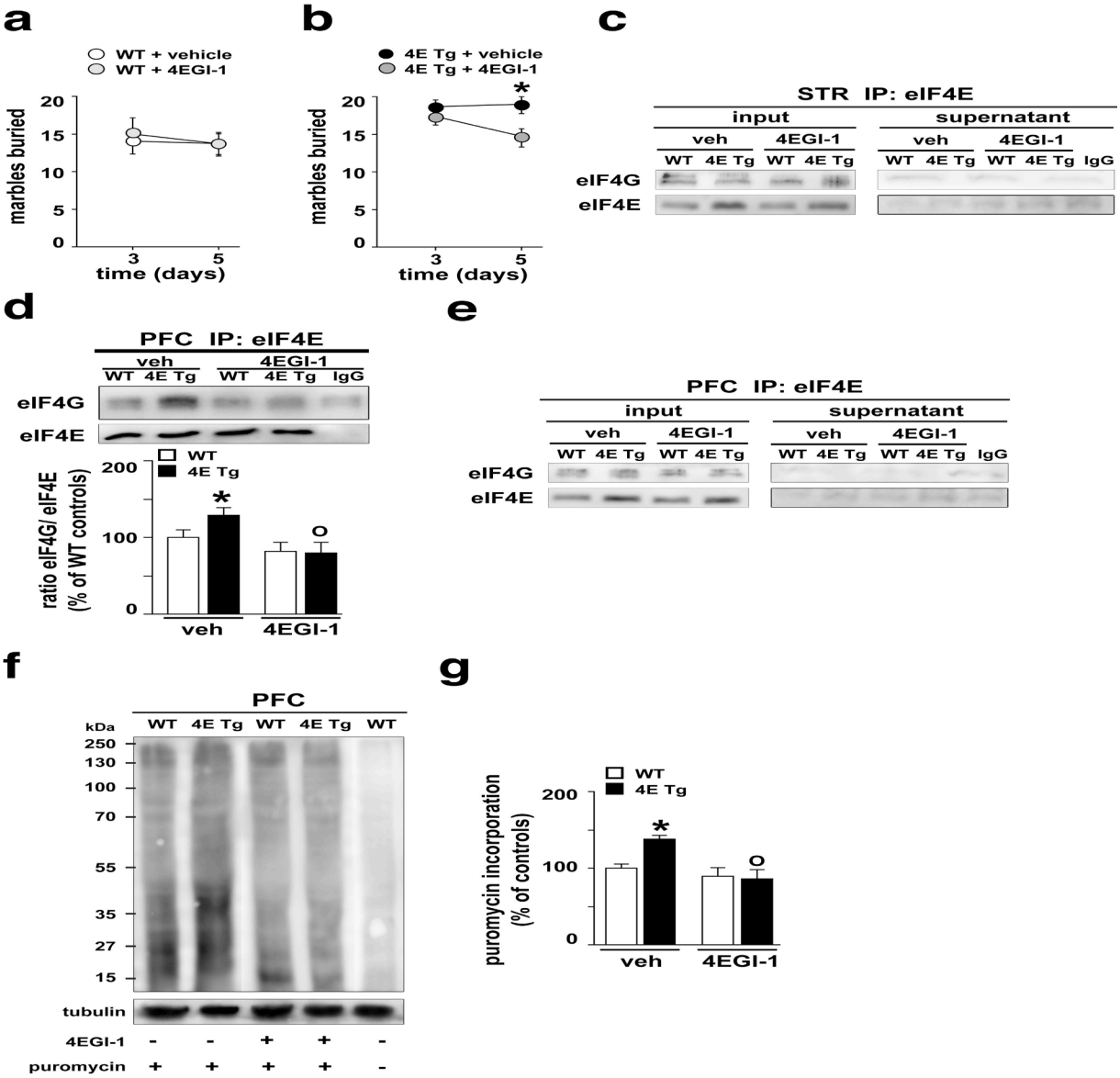
Supplementary Figure 1. (**a**) Representative Western blots showing brain levels of eIF4E, eIF4G and 4E-BP (input) and the supernatant after immunoprecipitation with eIF4E and eIF4G. All data are shown as mean ± SEM. (**b, c**) Dose response of puromycin infused ICV. Representative Western blots (**b**) and quantification (**c**) of newly synthesized brain proteins labeled with increasing doses of puromycin using the SUnSET method (see Supplementary Methods). n=3 mice/dose. One-way ANOVA [treatment effect, $F_{(5,17)}$=78.07, p<0.001] (**d, e**) Anisomycin blocks the incorporation of puromycin into newly synthesized brain proteins in a dose-dependent manner. Puromycin (25 μg in 0.5 μl; IVC) was infused either with or without anisomycin (12, 50, 100 mg/Kg; i.p.). Representative Western blots (**d**) and quantification (**e**) of proteins labeled with puromycin using the SUnSET method (see Supplementary Methods). n=3 mice/treatments. One-way ANOVA [treatment effect, $F_{(3,12)}$=35.52, p<0.001]. (**f, g**) Protein labeling with puromycin using the SUnSET is sensitive to stimulation and inhibition of protein synthesis. Hippocampal slices incubated with puromycin (10 mg/ml) and stimulated with either insulin (1 μM) alone or in combination with either cycloheximide (300 μM) or anisomycin (40 μM). Representative Western blots (**f**) and quantification (**g**) of proteins labeled puromycin using SUnSET (see Supplementary Methods). n=3 slices from 3 mice/treatments. One-way ANOVA [treatment effect, $F_{(3,11)}$=16.62, p<0.001] .In all the SUnSET experiments (panels **b-g**): – represents a control sample without puromycin. Lower panel shows the levels of tubulin, which was used as loading control. All data are shown as mean ± SEM.



Supplementary Figure 2. (**a**) Novelty-induced locomotor activity shown in 10 min intervals. *p<0.05 vs WT, repeated measures ANOVA [genotype X time, $F_{(5,100)}$=3.69, p<0.01] followed by Bonferroni-Dunn test. (**b, c, d**) Open field test. Total distance (**b**), ratio center/total distance (**c**) and vertical activity (**d**) shown in 10 min intervals. *p<0.05 vs WT, repeated measures ANOVA [genotype X time, $F_{(5,100)}$=3.82, p<0.01] followed by Bonferroni-Dunn test. (**e**) Swim speed (cm/sec) in the MWM. n.s., Student's t-test. (**f, g**) Number of platform crossings (**f**) and quadrant occupancy (**g**) during the probe test of the MWM. . n.s., repeated measure ANOVA. (**h**) Quadrant occupancy for each day of the reversal learning phase of the MWM. *p<0.05 vs WT, repeated measures ANOVA [DAY1: genotype X time, $F_{(3,92)}$=6.12, p<0.001; DAY2: genotype X time, $F_{(3,92)}$=5.93, p<0.01; DAY3: n.s.] followed by Bonferroni-Dunn test. (**i**) Accelerating rotarod test. Latency to fall from the accelerating rod (sec) is shown for four test trials. n.s., repeated measure ANOVA. (**j**) Elevated plus maze test. Time spent in the arms and in the center of the maze. n.s., two-way ANOVA. (**k, l**) Prepulse inhibition (PPI) of the acoustic startle response is represented as % of PPI of the startle response (**k**). n.s., repeated measures ANOVA. Acoustic startle response is expressed as maximum startle amplitude to the 120-dB stimulus (**l**). n.s., Student's t-test. In all the experiments n=12-13 mice/genotype. All data are shown as mean ± SEM.



Supplementary Figure 3. (**a**) mEPSC traces obtained from layer 5 mPFC pyramidal neurons (upper panel). n=22-25 neurons/genotype. n.s., Student's *t*-test. (**b**) mIPSC traces obtained from layer 5 mPFC pyramidal neurons (upper panel). n=34-35 neurons/genotype. n.s., Student's *t*-test. (**c**) Two-photon images showing layer 2/3 mPFC pyramidal neuron filled with Alexa Fluor-594 through the whole-cell recording pipettes. Scale bar= 20 µm. (**d**) Histograms (left) and cumulative histograms (right) of spine density in WT (black) and Tg (red) mice, showing a significant difference between the two populations. (**e**) Input versus output plot representing the slope of the striatal fEPSP in response to increasing stimulus intensity. n=10 slices from 8 mice/genotype. n.s., repeated measures ANOVA. (**f**) Percent facilitation in the striatum, determined by the ratio of the second fEPSP to the first fEPSP shown at interpulse intervals from 10 to 300 ms (right panel). n=13 slices from 8 mice/genotype. n.s., repeated measures ANOVA. (**g**) Input versus output plot representing the slope of the hippocampal fEPSP in response to increasing stimulus intensity (left panel). n=12 slices from 9 mice/genotype. n.s., repeated measures ANOVA. (**h**) Percent facilitation in the hippocampus, determined by the ratio of the second fEPSP to the first fEPSP is shown at interpulse intervals from 10 to 300 ms (right panel). n=13 slices from 8 mice/genotype. n.s., repeated measures ANOVA. (**i**) Input versus output plot representing the slope of the striatal fEPSP in response to increasing stimulus intensity. n=15 slices from 9 mice/genotype/treatment. n.s., repeated measures ANOVA. (**j**) Percent facilitation in the striatum, determined by the ratio of the second fEPSP to the first fEPSP is shown at interpulse intervals from 10 to 300 ms (right panel). n=13 slices from 9 mice/genotype/treatment. n.s., repeated measures ANOVA. 4EGI-1 (100 µM) was bath applied for 45 min prior and during the duration of the experiments. (**k**) 4EGI-1 does not alter basal synaptic transmission in striatal slices. Baseline fEPSP was recorded for 20 min prior and during the application of 4EGI-1 (100 µM). n=13 slices from 9 mice/genotype/treatment. n.s., repeated measures ANOVA. All data are shown as mean ± SEM.



Supplementary Figure 4. (**a, b**) Marble-burying behavior of WT (**a**) and eIF4E transgenic mice (**b**) tested five hours after the infusion of 4EGI-1. Data are shown as mean ± SEM (n=6 mice/genotype/treatment). *p<0.05 vs vehicle-treated 4E Tg mice, two-way repeated measures ANOVA [genotype X treatment, $F_{(1,20)}$=6.16, p<0.05] followed by Bonferroni-Dunn test. (**c**) Representative Western blots showing the levels of eIF4E and eIF4G in the striatum (**c**, STR, input,) and in the supernatant after immunoprecipitation with eIF4E also is shown (**c,** STR, supernatant). (**d**) Representative Western blots and quantification of proteins recovered after immunoprecipitation (IP) of eIF4E in the prefrontal cortex (PFC). n=4 mice/genotype. *p<0.05 and °p<0.05 vs vehicle-treated WT and 4E Tg, respectively, two-way ANOVA, followed by Bonferroni-Dunn test. (**e**) Representative Western blots showing the levels of eIF4E and eIF4G in the striatum (**e**, STR, input,) and in the supernatant after immunoprecipitation with eIF4E also is shown (**e,** STR, supernatant). (**f, g**) Representative Western blots and quantification of newly synthesized proteins labeled with puromycin using the SUnSET method in WT and 4E Tg mice infused with either vehicle or 4EGI-1 and puromycin in prefrontal cortex (PFC). The last sample represents a control without puromycin. Lower panel shows the levels of tubulin, used as loading control. *p<0.05 and °p<0.05 vs vehicle-treated WT and 4E Tg, respectively, two-way ANOVA, followed by Bonferroni-Dunn test. All data are shown as mean ± SEM.

Exaggerated translation causes synaptic and behavioral endophenotypes associated with autism.

Santini E., Huynh N. T., Macaskill A., Ruggero D., Pierre P., Carter A., Kaphzan H. and Klann E.

Autism spectrum disorder (ASD) are a heterogeneous group of heritable neuropsychiatric disorders whose symptoms, which include abnormal social interaction, impaired communication, and repetitive/perseverative behaviors, appear in early childhood and continue throughout life. One of the leading hypothesis for a common molecular mechanism underlying ASD is alteration in the translational control machinery. Indeed recent studies on humans patients and animal models have suggested that upregulated mTORC1 signaling is a molecular abnormality that contributes to ASD-like behaviors. The mTORC1 signaling pathway is involved in the initiation of cap-dependent translation, a process required for long-lasting synaptic plasticity, as well as consolidation and long-term memories. Dysfunction in mTORC1 signaling pathway may therefore, cause dysregulation of brain protein synthesis, leading to aberrant forms of synaptic plasticity, which ultimately results in cognitive impairments associated with ASD. Consistent with this notion, it was recently found in ASD patients a mutation increasing the expression of eIF4E gene, which encodes the cap-binding translation factor eIF4E, a downstream effector protein of the mTORC1 signaling pathway. eIF4E mediates cap-dependent translation initiation by binding eIF4G, thereby forming the eIF4F pre-initiation complex. Activation of the mTORC1 signaling pathway promotes protein synthesis by releasing eIF4E from its repressor eIF4E-binding protein (4E-BP), thus increasing the availability of eIF4E to interact with eIF4G and consequently cap-dependent protein synthesis. Thus, suggesting that some ASD may be caused by increased expression of the mTORC1 target eIF4E. We employed a transgenic mouse line (eIF4E Tg), in which the expression of eIF4E is augmented, to test the hypothesis that increased cap-dependent protein synthesis play a causative role in the etiology of ASD. We found that overexpression of eIF4E leads to increased cap-dependent protein synthesis in the brain of the eIF4E Tg. Importantly, eIF4E Tg mice exhibit specific behavioral abnormalities analogous to those observed in ASD patients. In addition, eIF4E Tg showed changes in synaptic function, dendritic spine density and synaptic plasticity, which recapitulate physiological dysfunctions reported in ASD animal models. Finally, we employed 4EGI-1, a new compound that inhibits eIF4E/eIF4G interaction to block the enhanced cap-dependent protein synthesis observed in eIF4E Tg mice. Treatment with 4EGI-1 normalizes ASD-like behaviors and corrects synaptic and molecular abnormalities displayed by the eIF4E Tg mice. Thus, eIF4E Tg mice are a new invaluable resource to specifically address the involvement of dysregulated translational control in ASD. Importantly, novel drugs designed to block eIF4E/eIF4G interaction may represent a new therapeutic strategy in patients affected by ASD.

NYU/CNS
Center for Neural Science

Exaggerated translation causes synaptic and behavioral endophenotypes associated with autism

Emanuela Santini

- **Autism is classified as pervasive developmental disorder**
- **Autism diagnosis is based on behavioral features and age of onset (before the age of 3)**
- **Behavioral features: abnormal social interactions, altered communication and repetitive behaviors**
- **Symptoms vary greatly between individuals**
- **The syndrome is more frequent in boys than in girls with a ratio 4:1**

Hypothesis

Excessive cap-dependent protein synthesis is involved in the etiology of ASD

- **Autism is classified as pervasive developmental disorder**
- **Autism diagnosis is based on behavioral features and age of onset (before the age of 3)**
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- **Symptoms vary greatly between individuals**
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Rationale

1) Animal models

TSC

FMRP

PTEN

FKBP12

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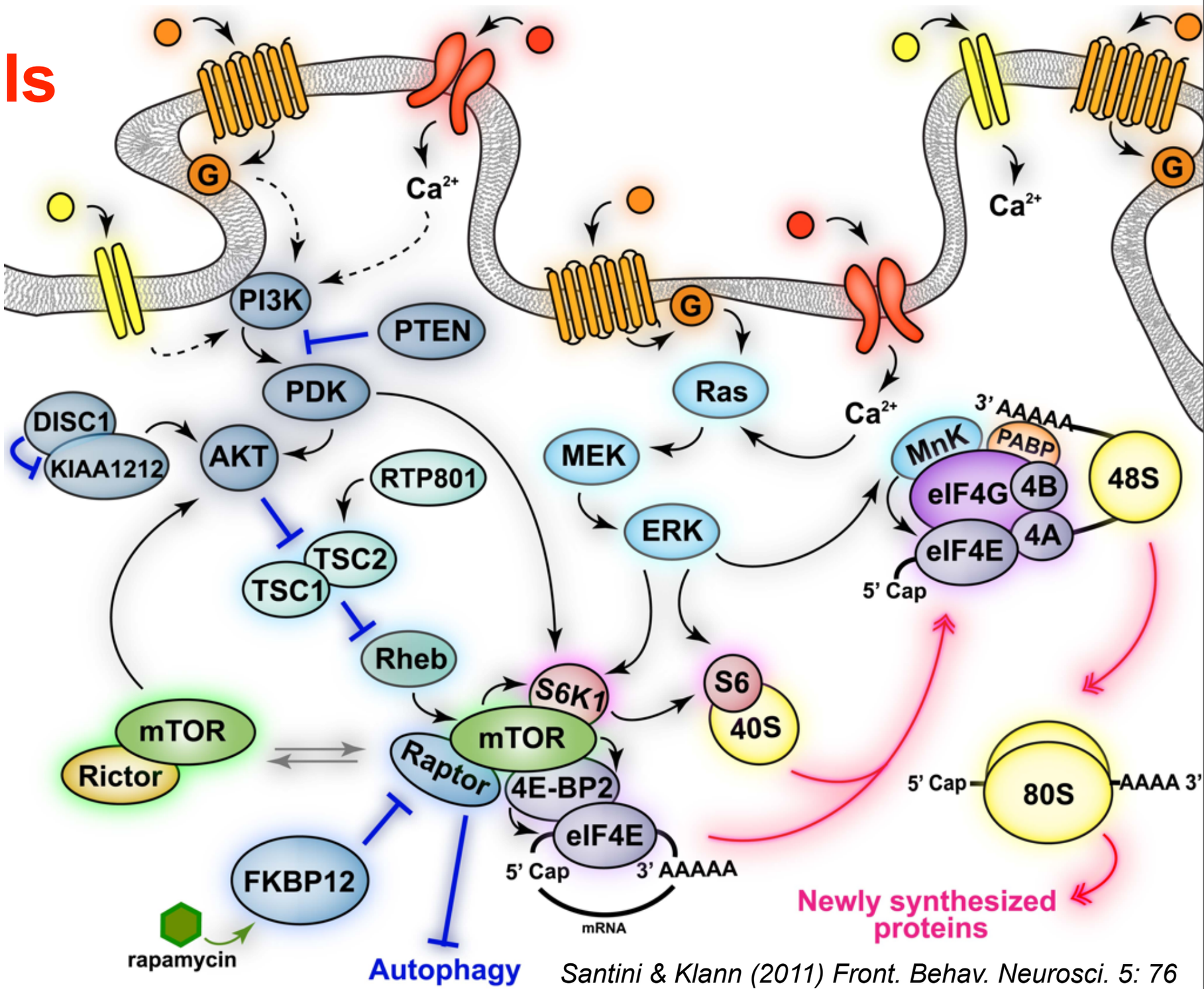

Santini & Klann (2011) Front. Behav. Neurosci. 5: 76

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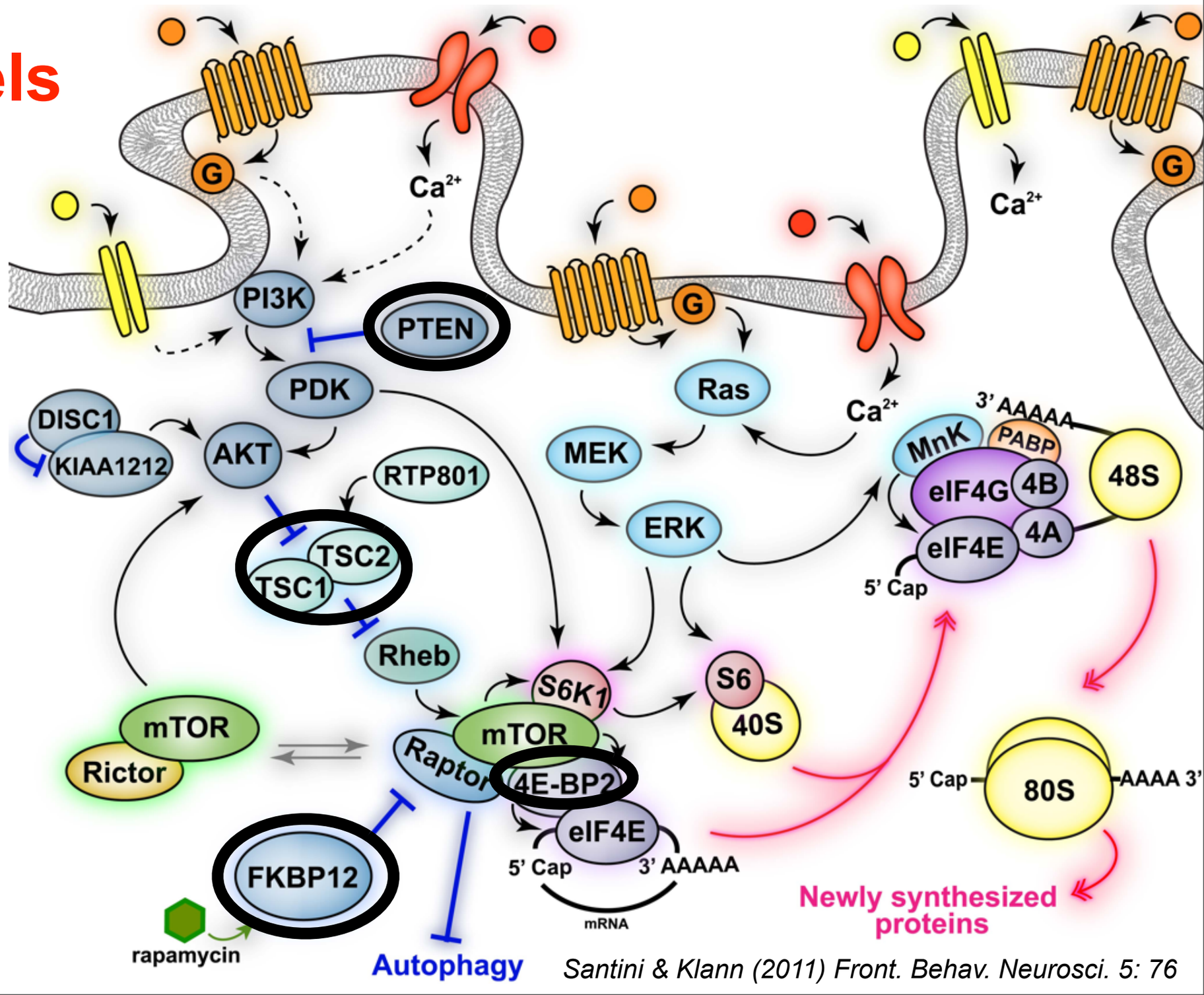

Santini & Klann (2011) Front. Behav. Neurosci. 5: 76

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2) A chromosomal translocation involving *eIF4E* gene is linked to autism in human

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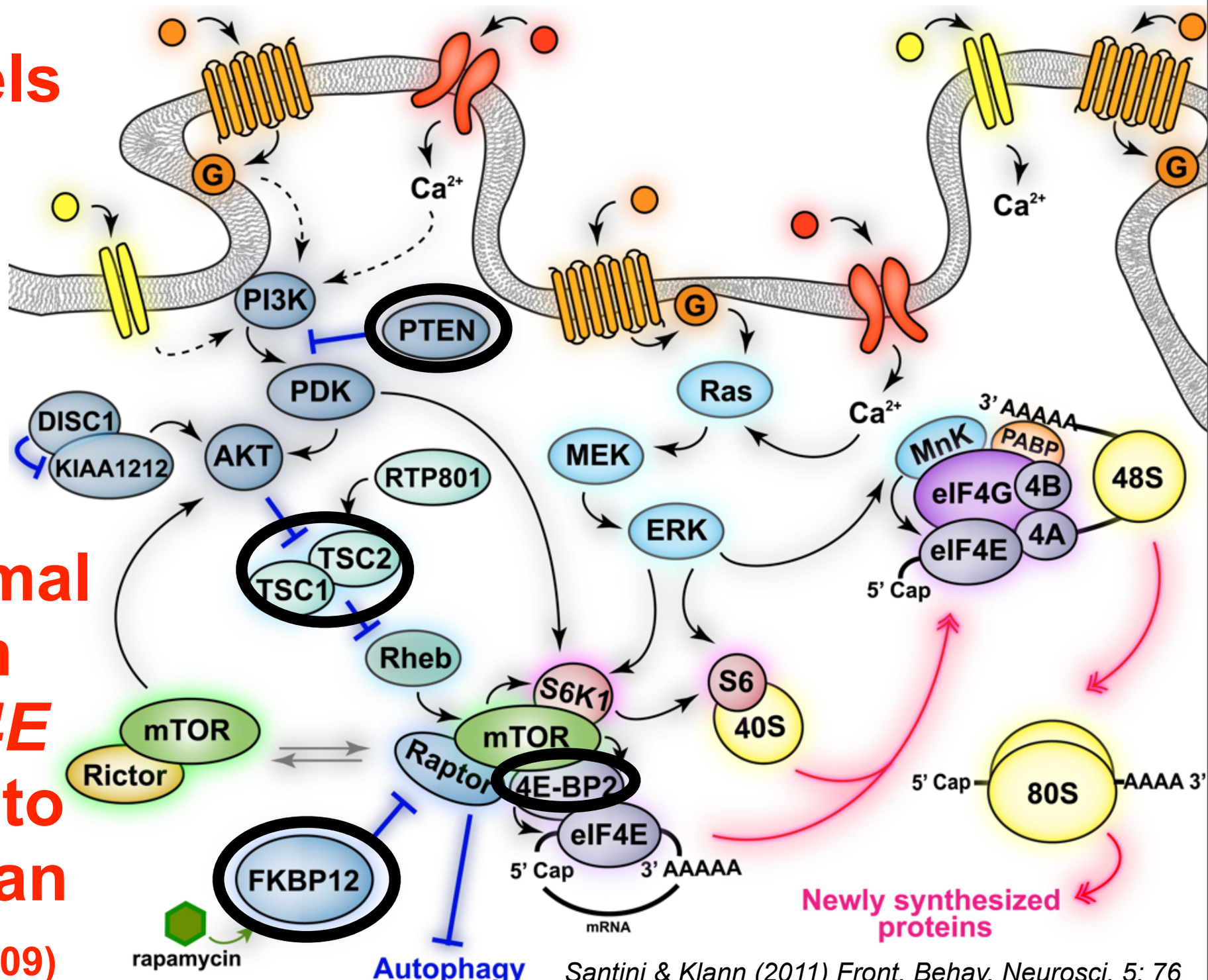

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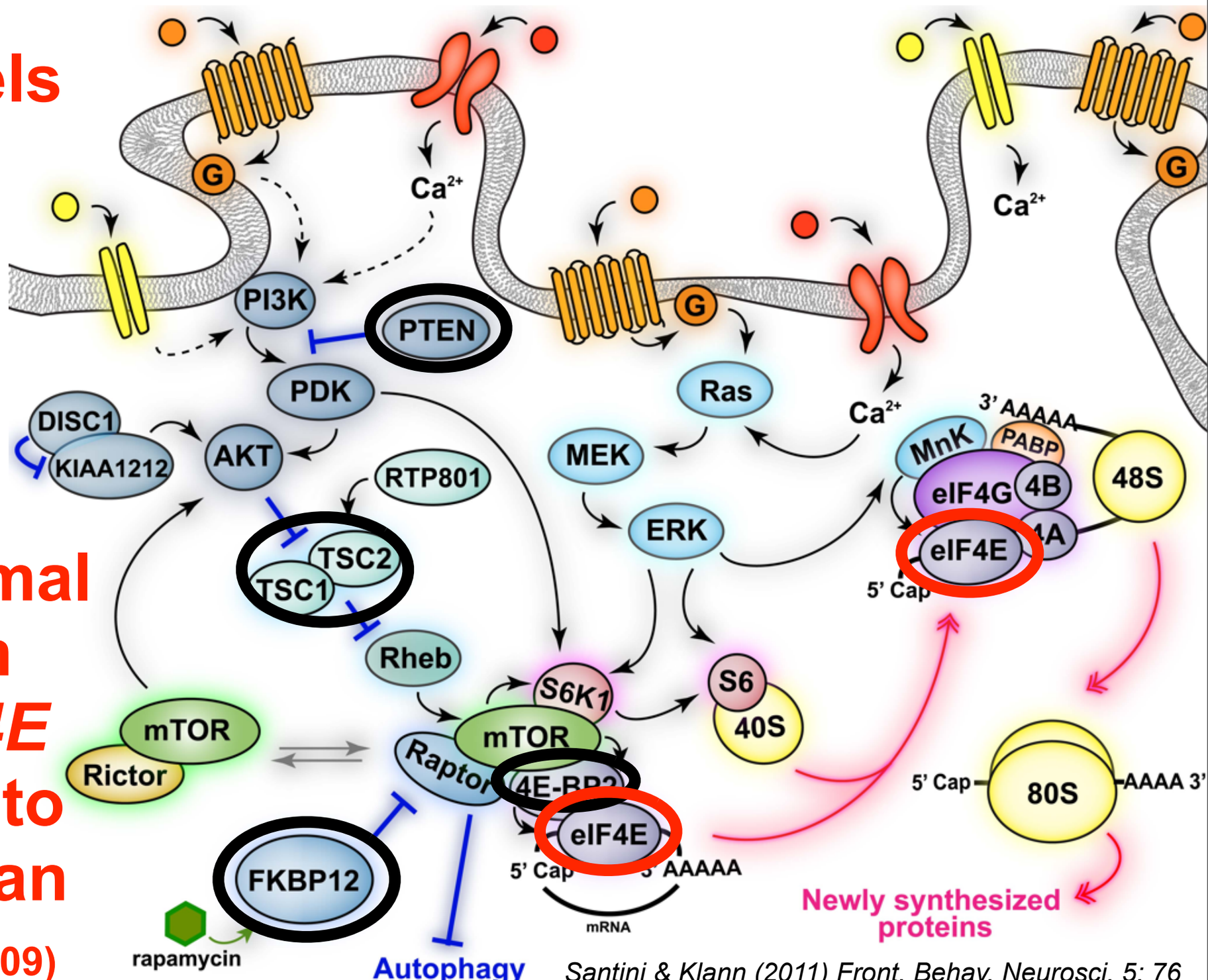

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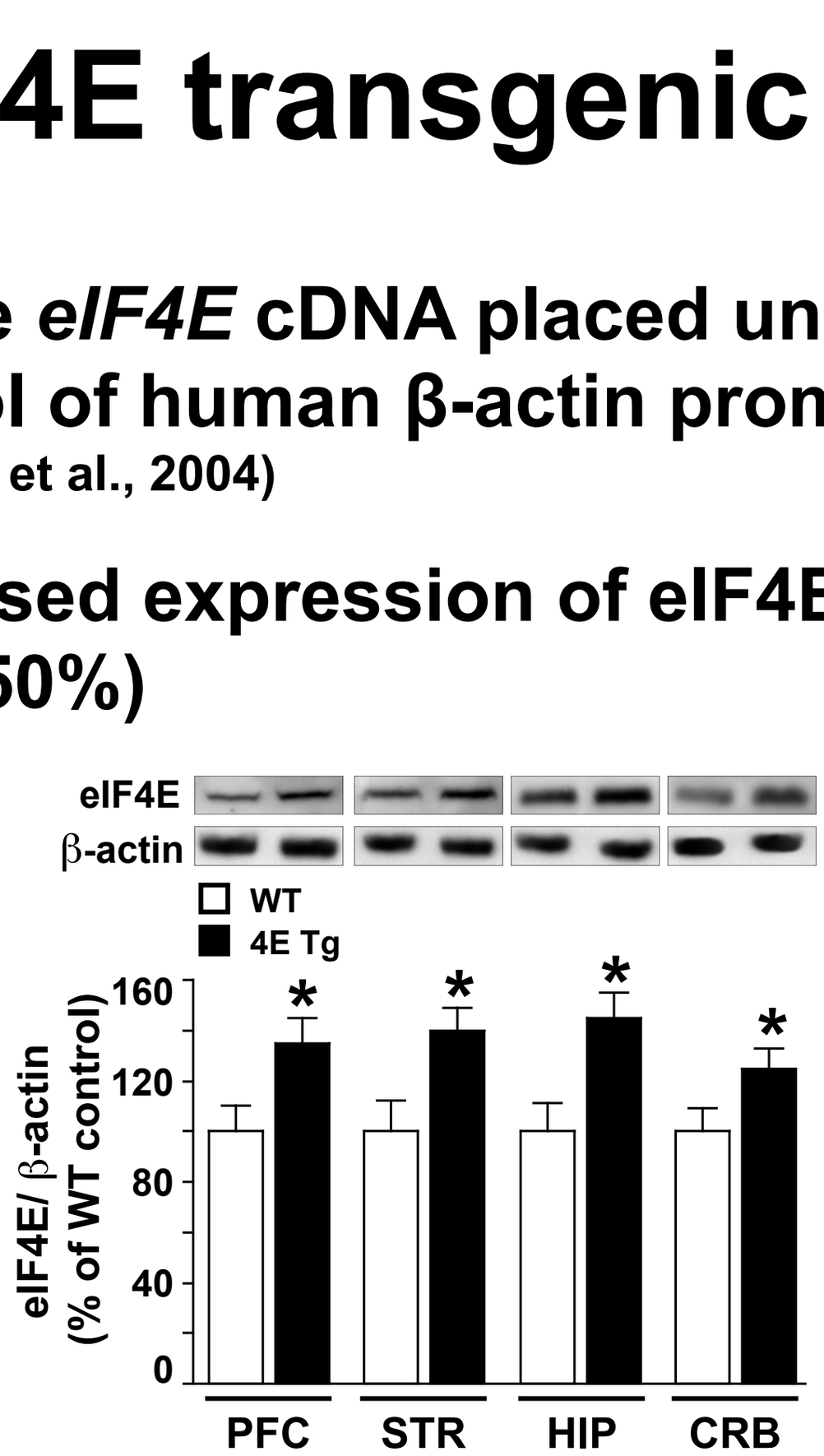
eIF4E transgenic mice

- **mouse *eIF4E* cDNA placed under the control of human β-actin promoter**
  (Ruggero et al., 2004)

- **increased expression of eIF4E in the brain (20%-50%)**



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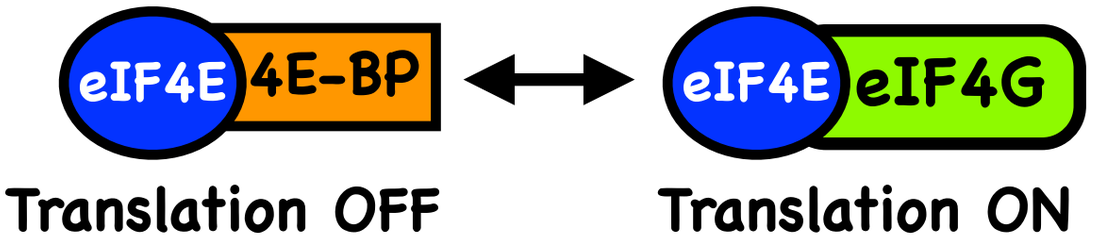
Overview

- Biochemical evidence that overexpression of eIF4E leads to increased cap-dependent protein synthesis
- Behavioral consequences of altered brain cap-dependent protein synthesis
- Synaptic consequences of altered brain cap-dependent protein synthesis
- Inhibition of cap-dependent protein synthesis reverses ASD-like behaviors of eIF4E transgenic mice

eIF4E transgenic mice have increased protein synthesis



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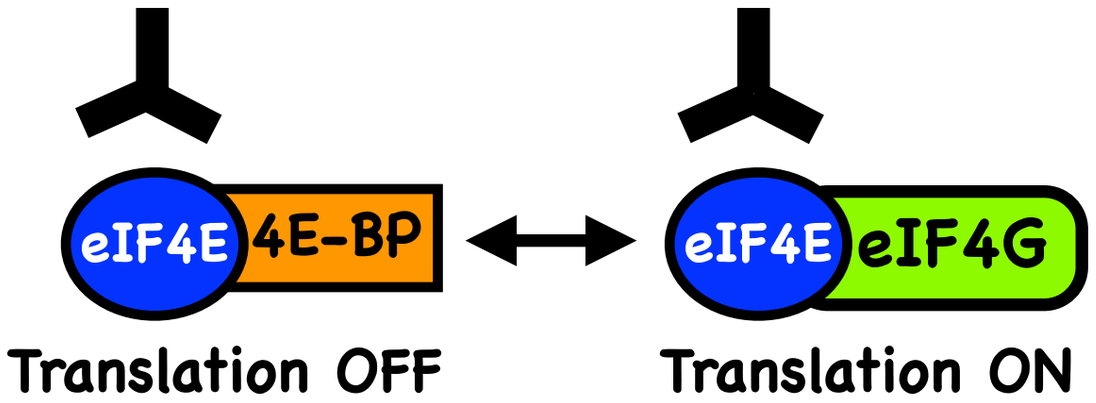

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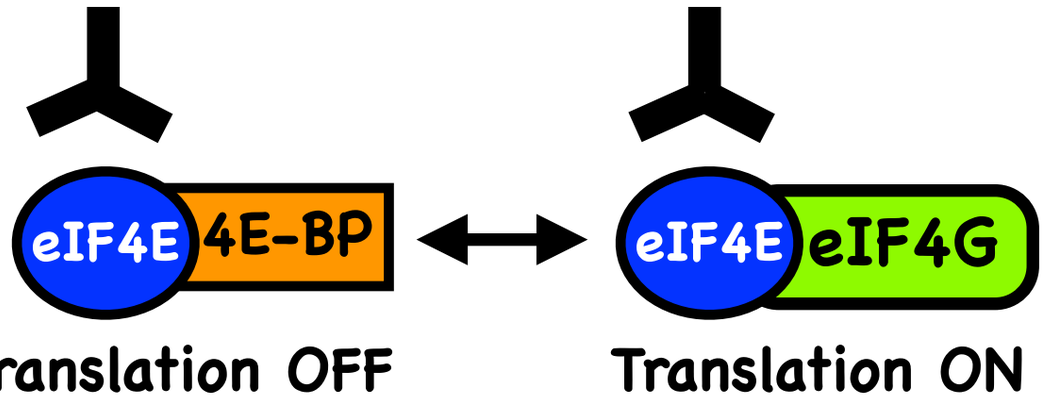

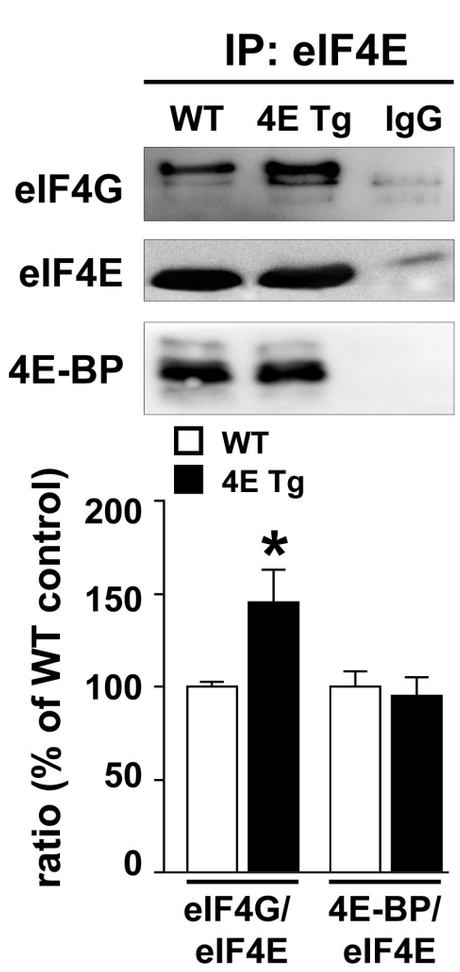

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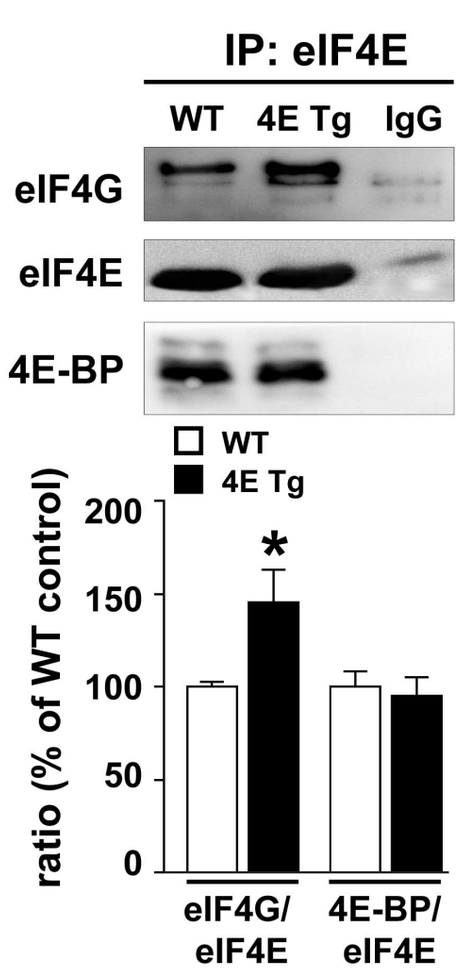

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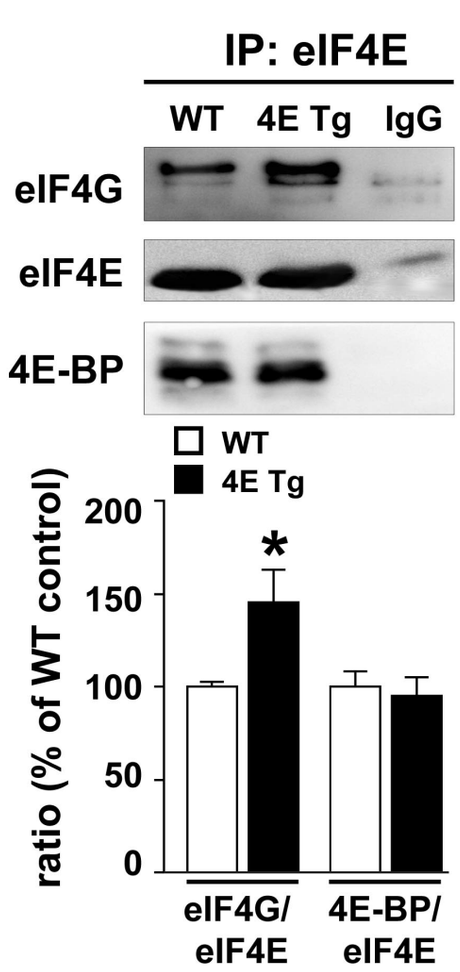

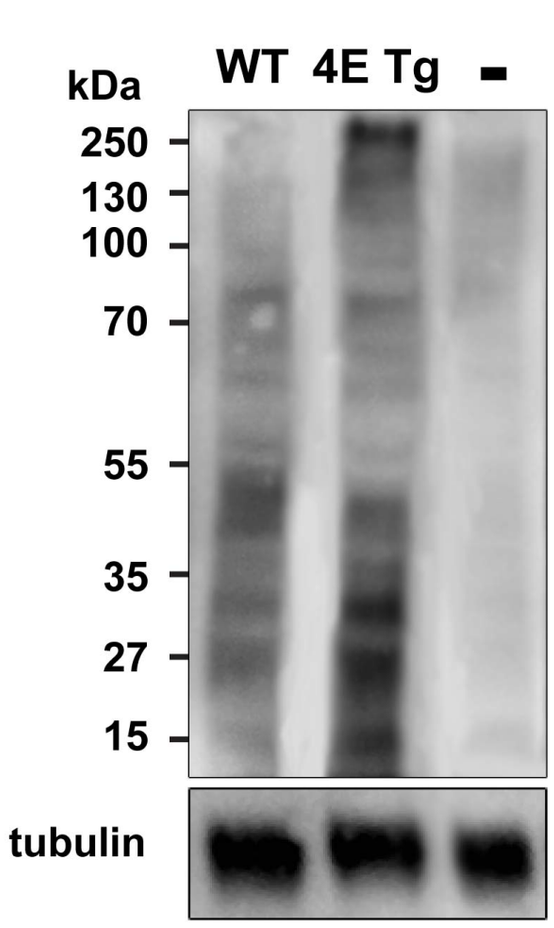

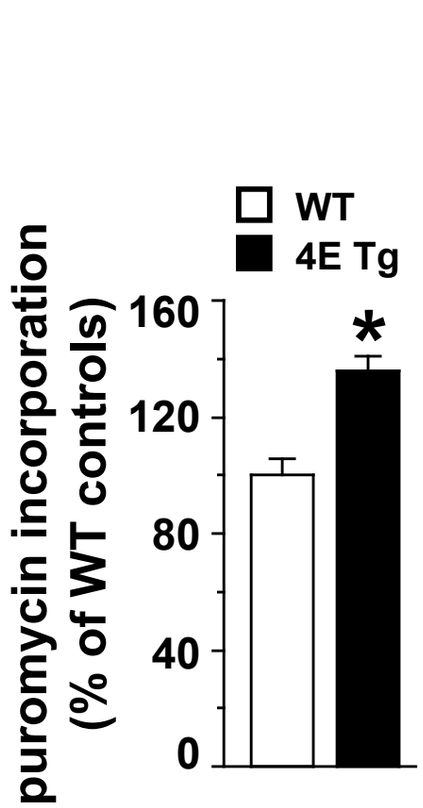

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eIF4E transgenic mice exhibit ASD-like behaviors

- repetitive behaviors
- abnormal social interactions
- impaired communication

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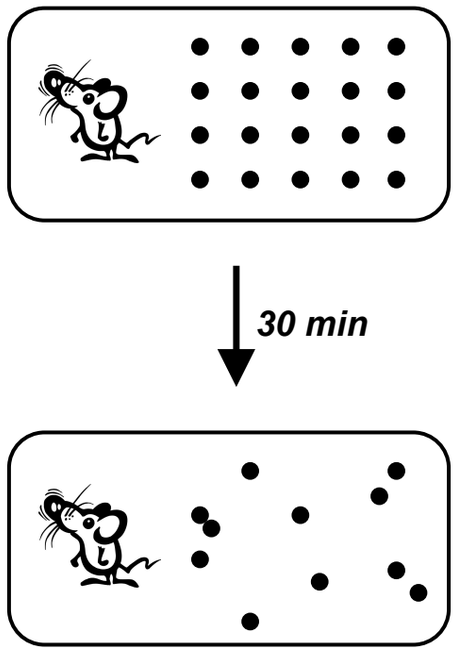

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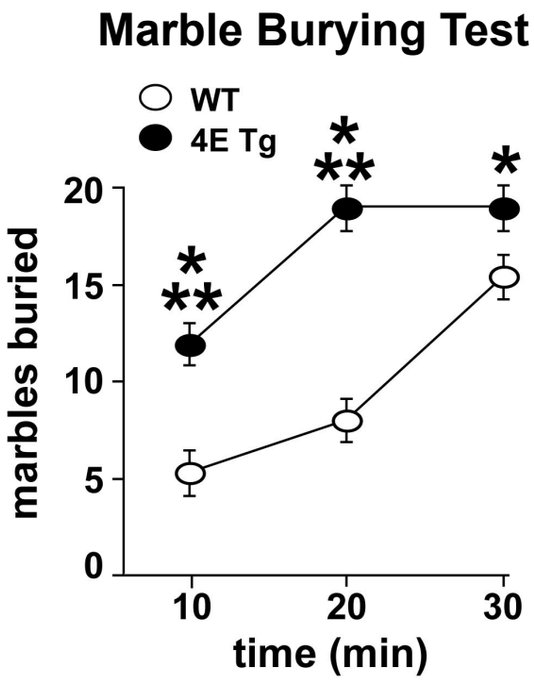

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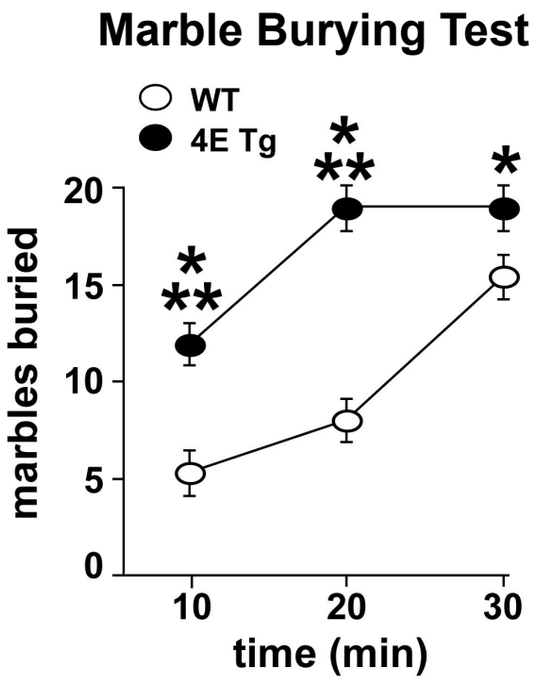

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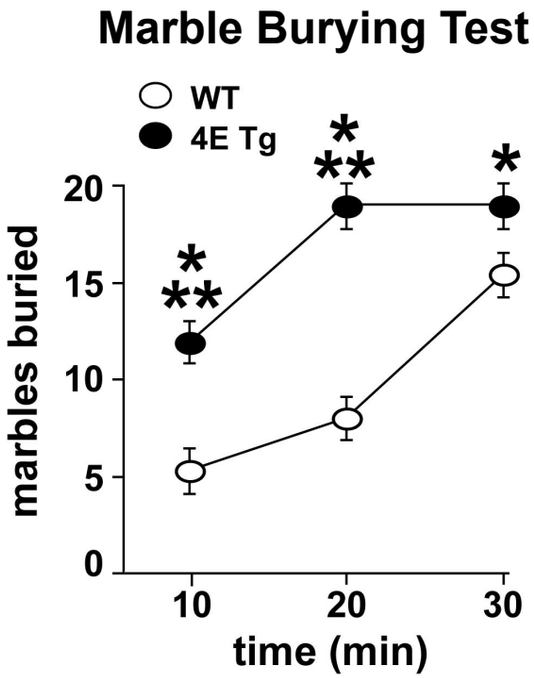

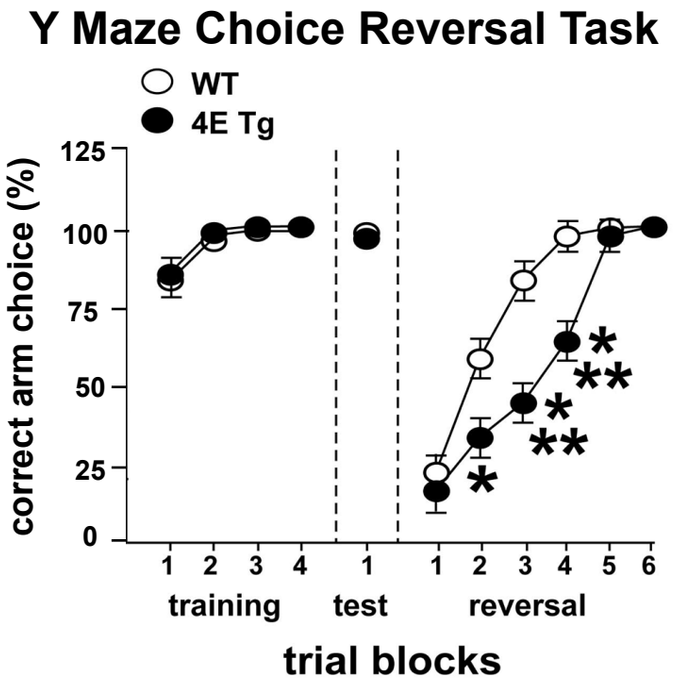

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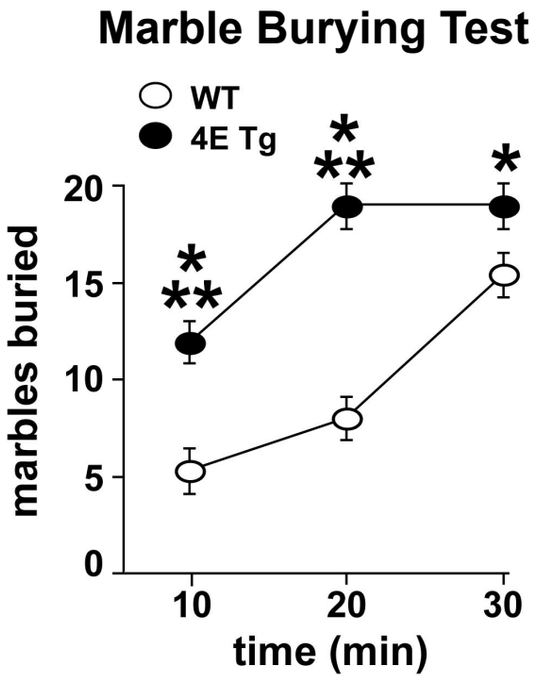

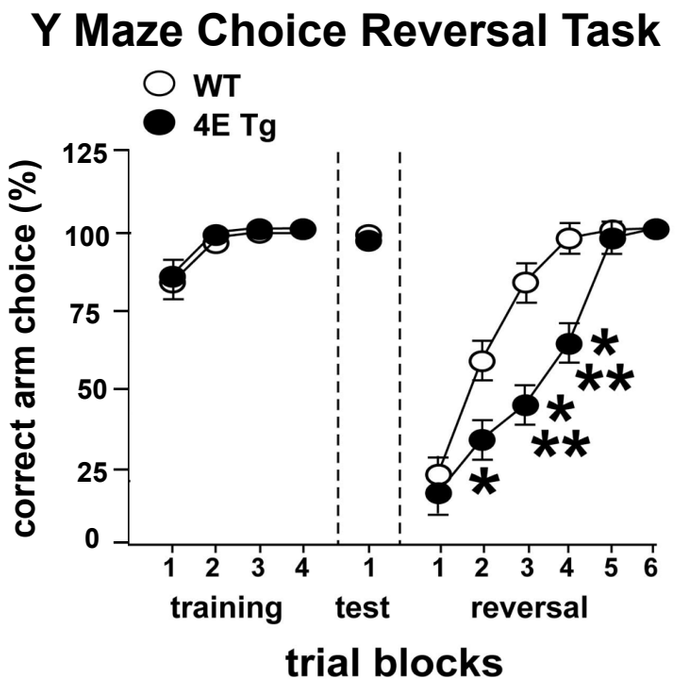

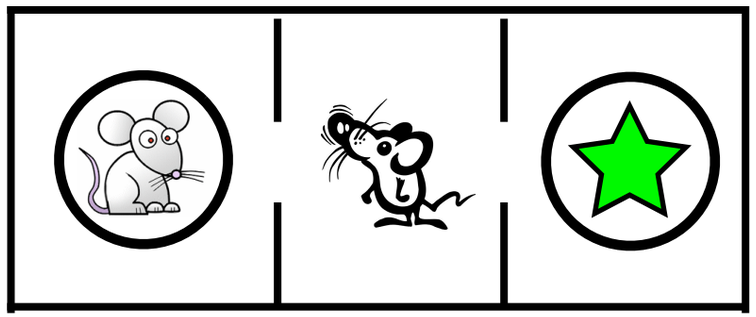

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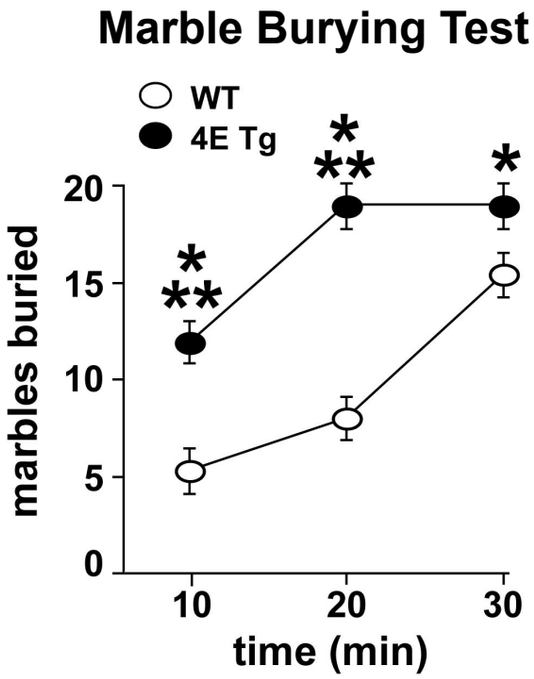

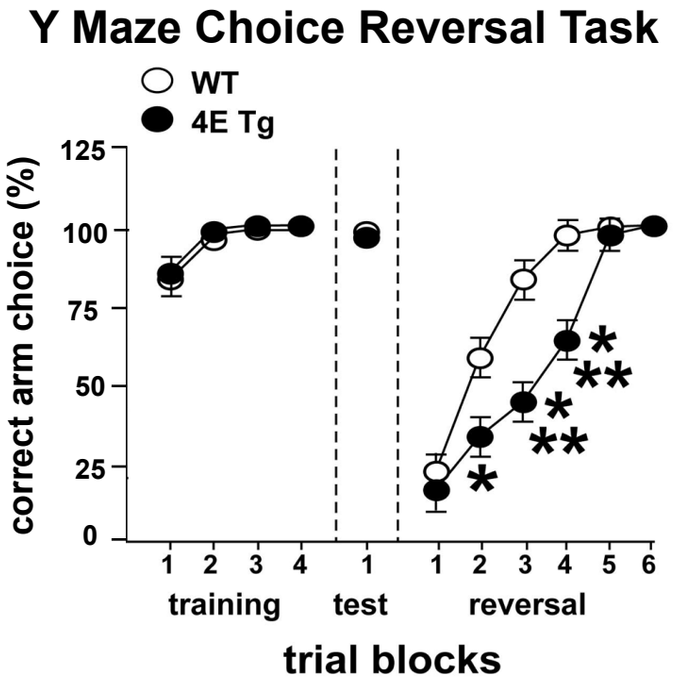

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eIF4E transgenic mice show changes in synaptic function

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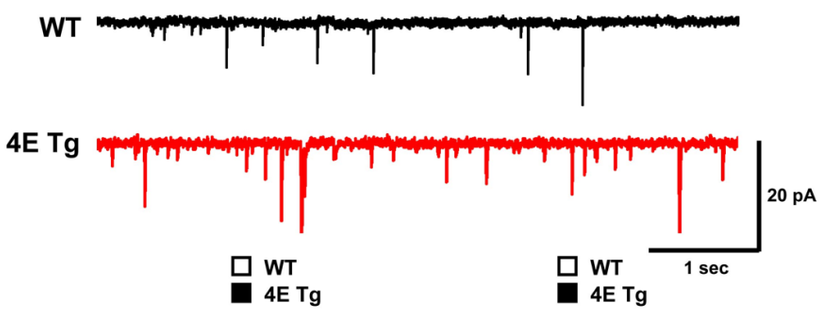
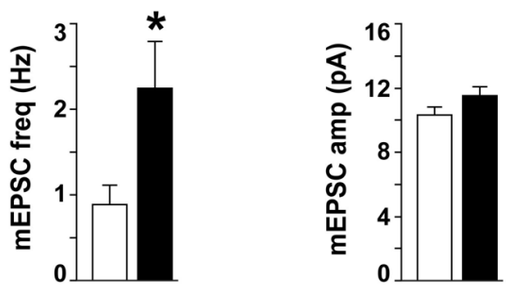
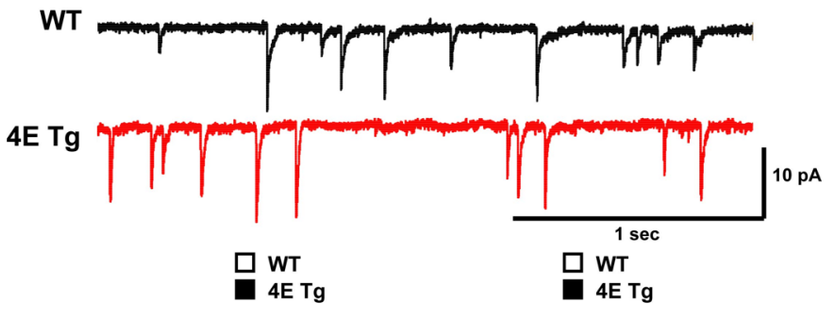
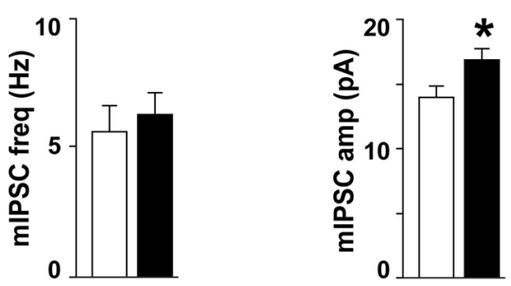
eIF4E transgenic mice show changes in synaptic function

Layer 2/3 mPFC









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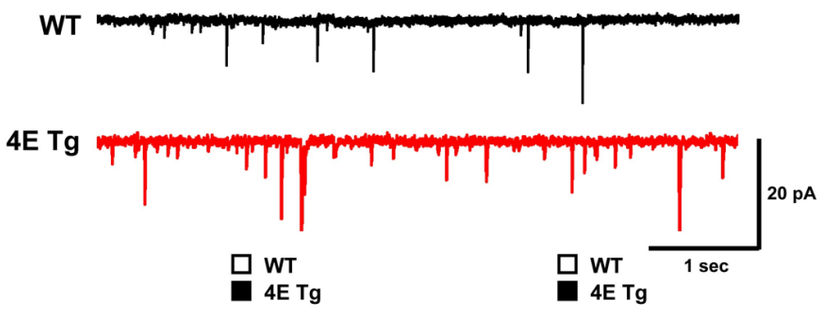
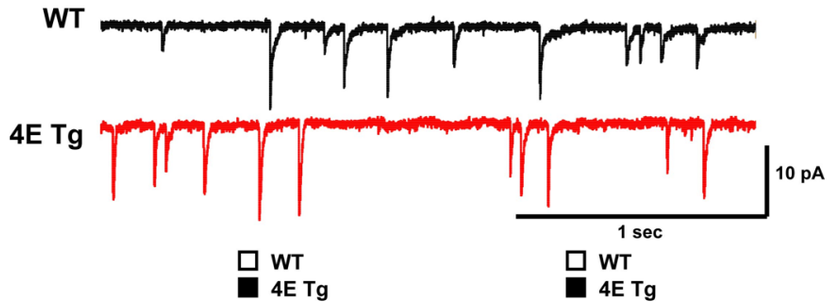
Layer 2/3 mPFC





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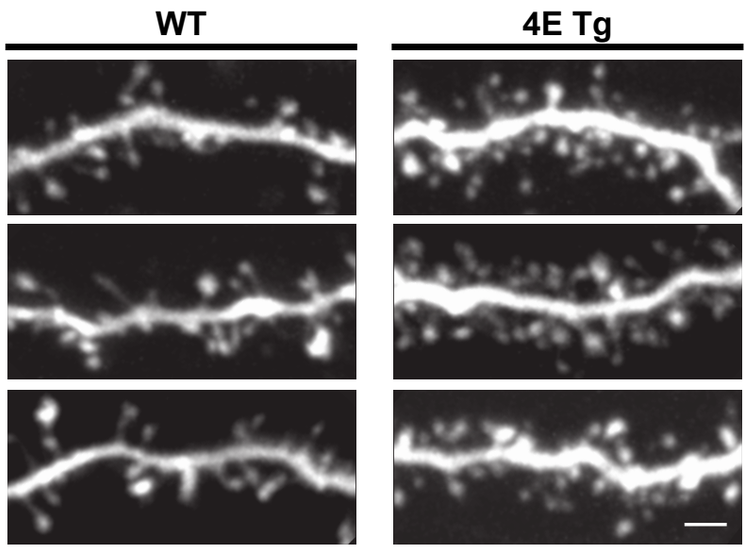

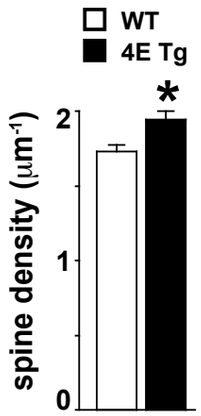

Andrew MacAskill
Adam Carter

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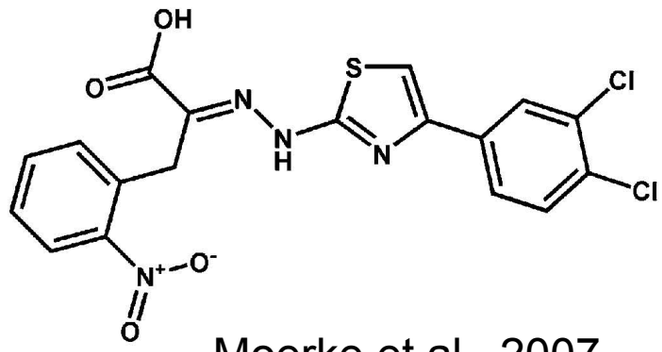
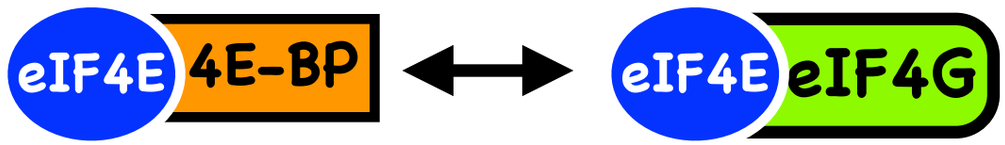
4EGI-1

4EGI-1



Moerke et al., 2007

- Inhibitor of eIF4E/eIF4G interactions
- Displaces eIF4G from eIF4E and enhances eIF4E/4E-BP association
- Blocks cap-dependent translation



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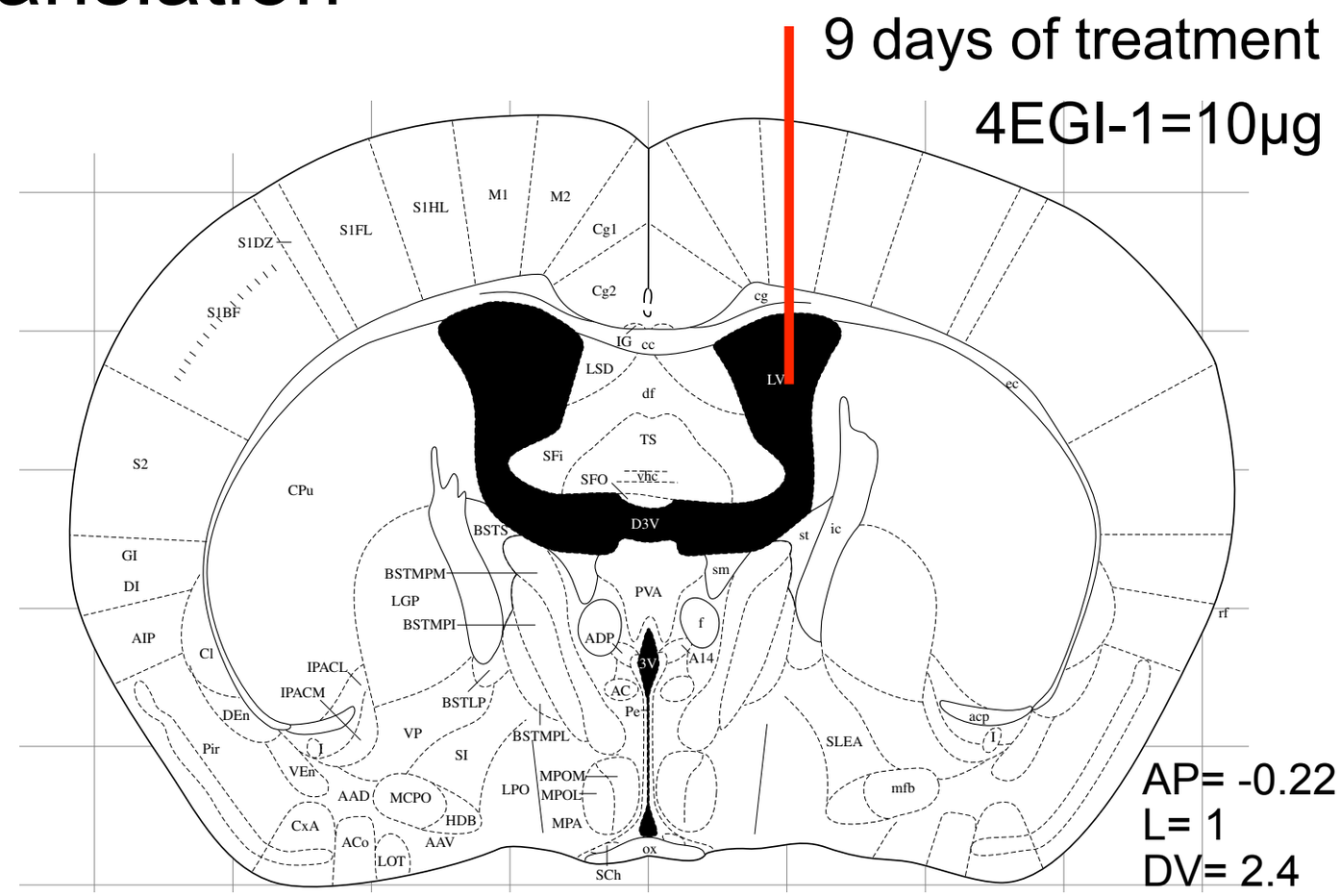
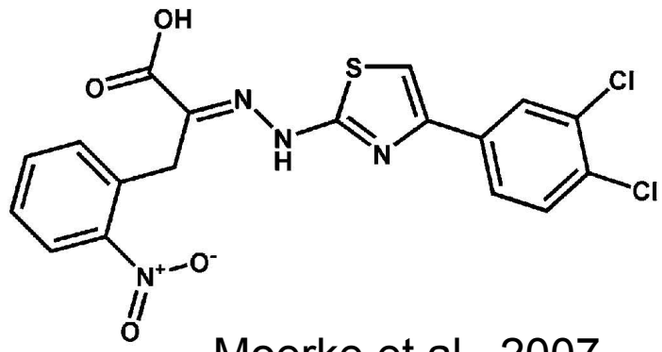


Hoeffer et al (2011) PNAS 108: 3383-3388

4EGI-1

4EGI-1



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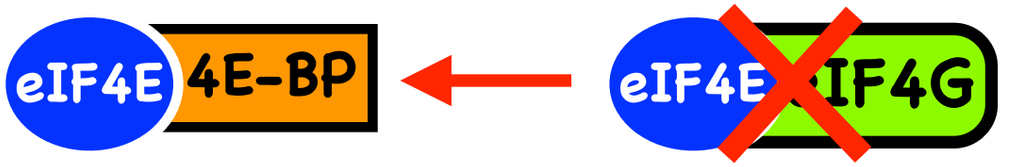

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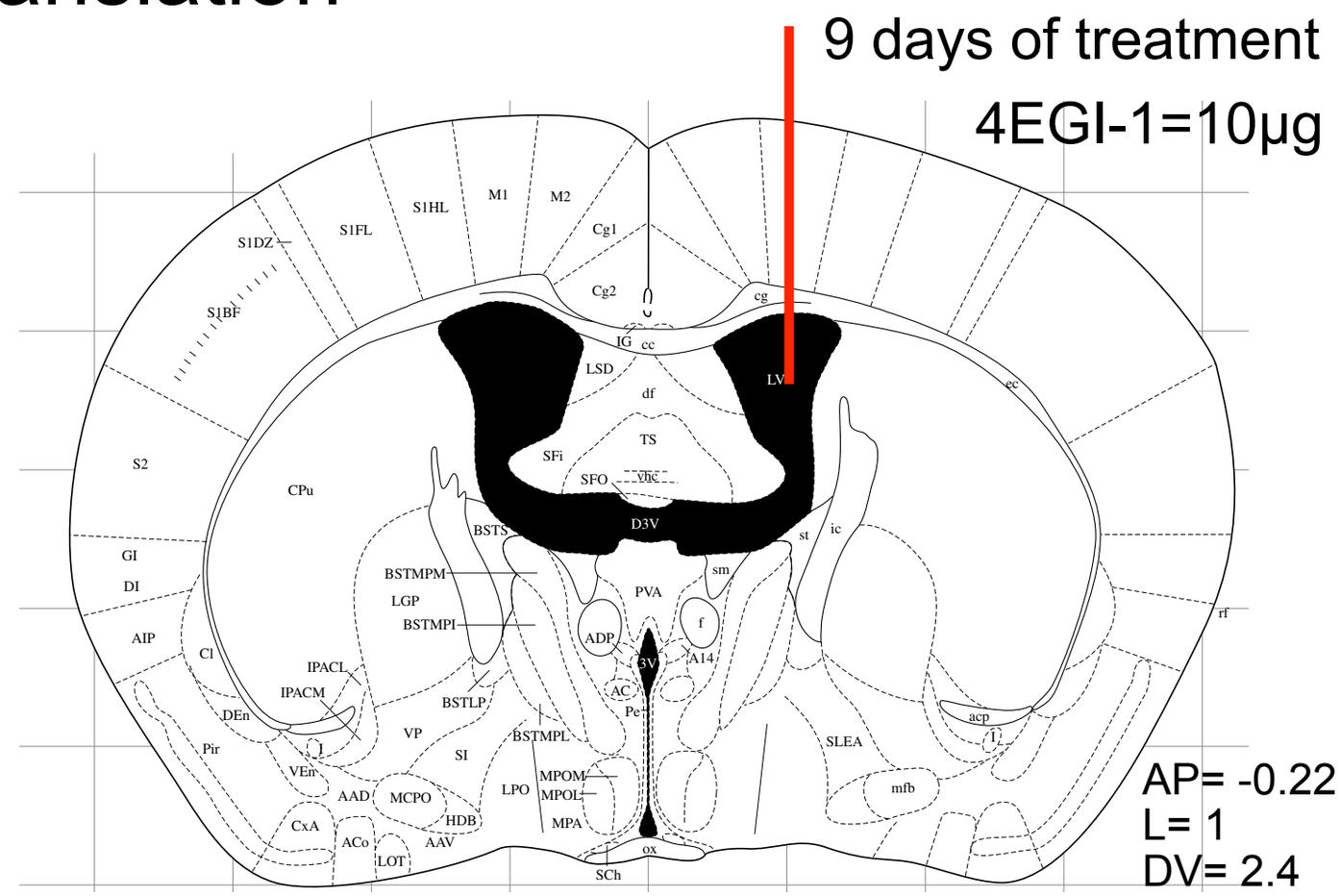
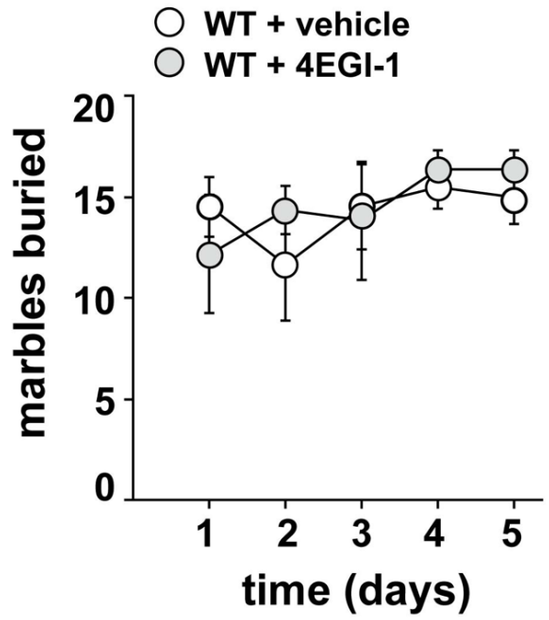
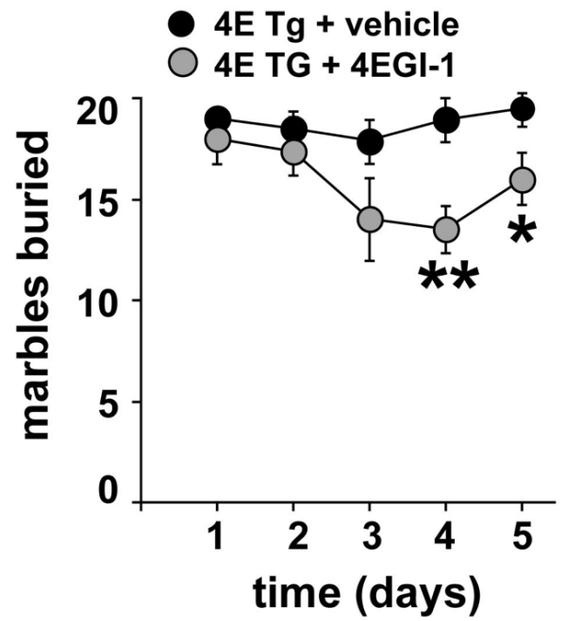


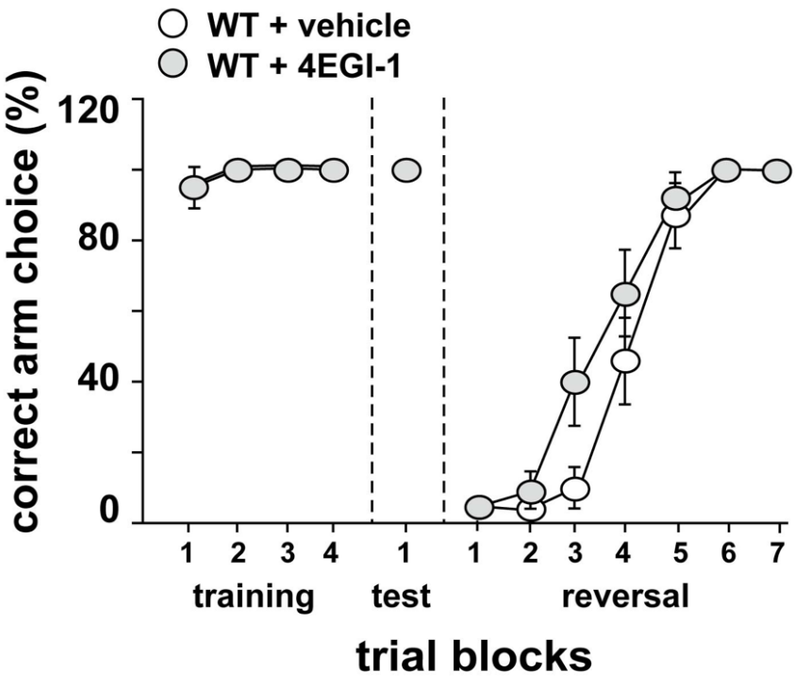
4EGI-1 reverses the ASD-like behaviors

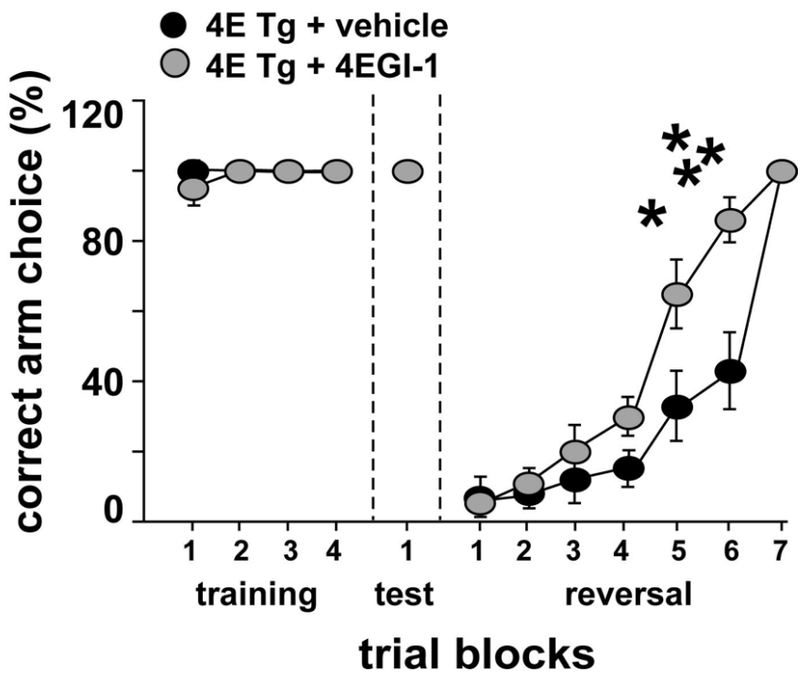
Marble Burying Test (days 1-5)





Y Maze Task (days 7-9)





Thu Huynh

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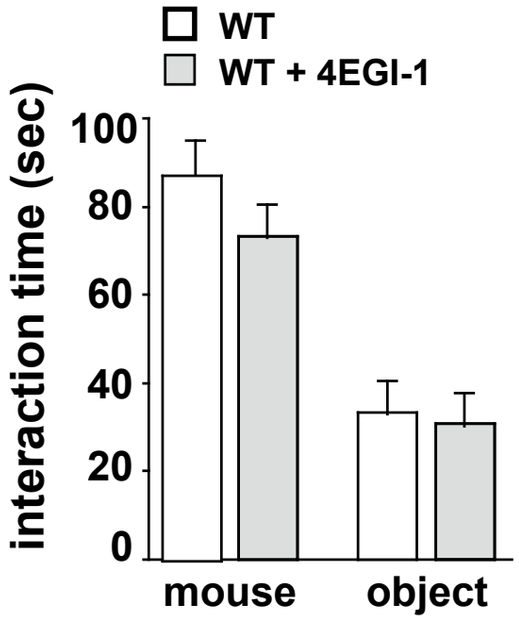
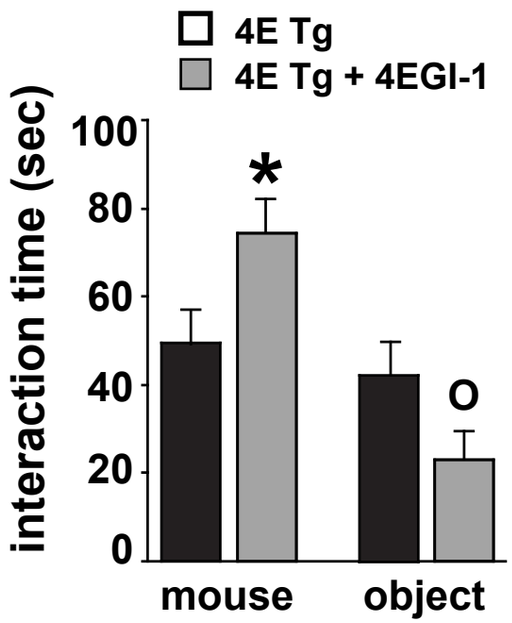
4EGI-1 reverses the ASD-like behaviors

Social behavior test (days 5)





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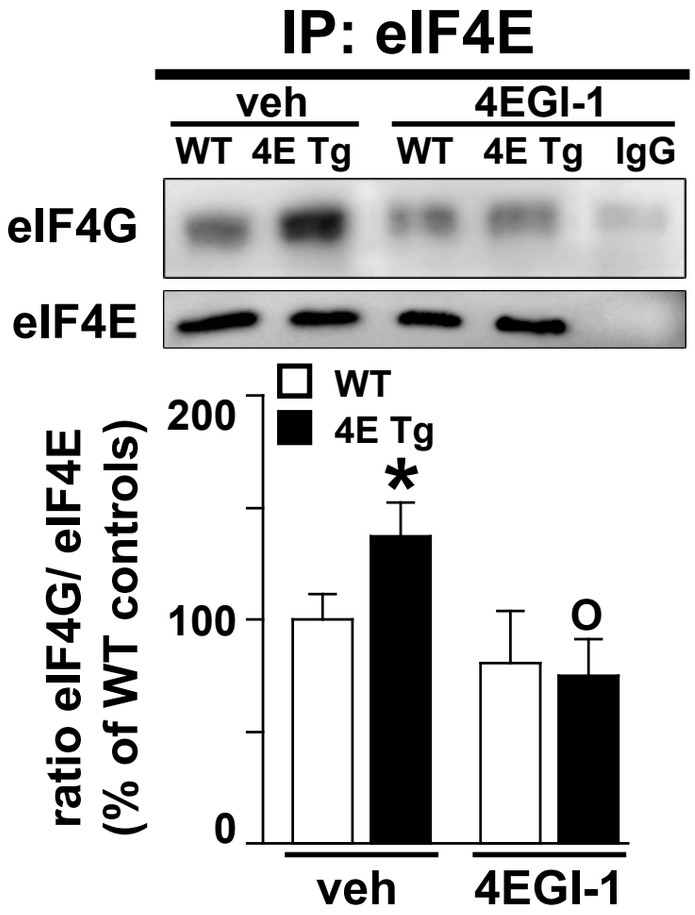
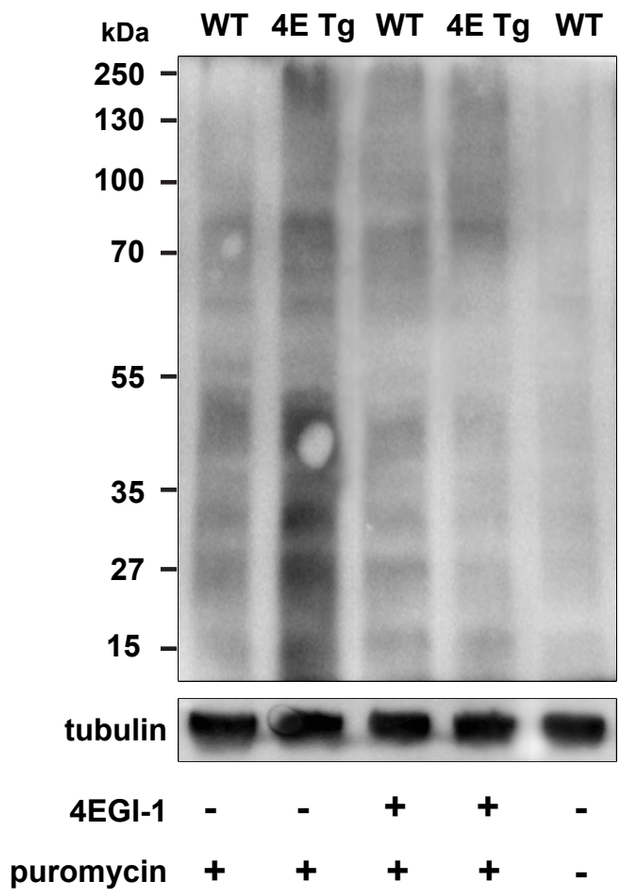
4EGI-1 normalizes eIF4E/eIF4G interactions and protein synthesis

10 days infusions

1 hours after last infusion





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Summary

eIF4E transgenic mice:

- Exhibit increased eIF4E/eIF4G interactions and exaggerated brain cap-dependent protein synthesis
- Exhibit ASD-like behaviors
- Display altered synaptic function (mPFC, striatum, hippocampus)
- Inhibition of cap-dependent protein synthesis reverses ASD-like behaviors and altered synaptic functions (striatum)

Future plans

- Determine whether perseverative/repetitive behaviors are caused by altered synaptic plasticity and morphology in the striatum

- Determine the identity of striatal proteins whose synthesis is dysregulated

NYU/CNS
Center for Neural Science

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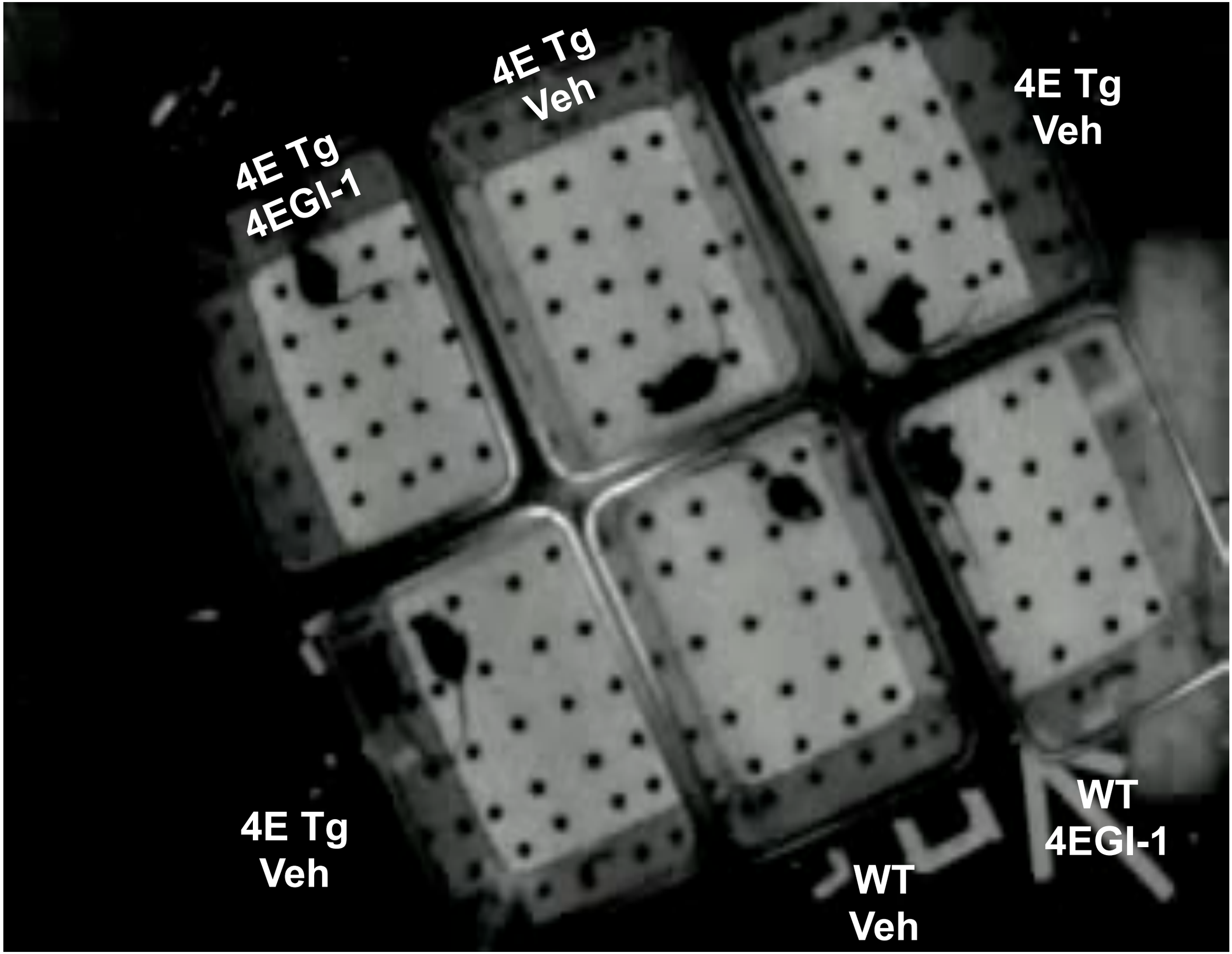
4E Tg
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Reciprocal signaling between translational control pathways and synaptic proteins in autism spectrum disorders

Emanuela Santini and Eric Klann (October 28, 2014)

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NEUROSCIENCE

Reciprocal signaling between translational control pathways and synaptic proteins in autism spectrum disorders

Emanuela Santini and Eric Klann*

Autism spectrum disorder (ASD) is a heterogeneous group of heritable neurodevelopmental disorders. Symptoms of ASD, which include deficits in social interaction skills, impaired communication ability, and ritualistic-like repetitive behaviors, appear in early childhood and continue throughout life. Genetic studies have revealed at least two clusters of genes frequently associated with ASD and intellectual disability: those encoding proteins involved in translational control and those encoding proteins involved in synaptic function. We hypothesize that mutations occurring in these two clusters of genes interfere with interconnected downstream signaling pathways in neuronal cells to cause ASD symptomatology. In this review, we discuss the monogenic forms of ASD caused by mutations in genes encoding for proteins that regulate translation and synaptic function. Specifically, we describe the function of these proteins, the intracellular signaling pathways that they regulate, and the current mouse models used to characterize the synaptic and behavioral features associated with their mutation. Finally, we summarize recent studies that have established a connection between mRNA translation and synaptic function in models of ASD and propose that dysregulation of one has a detrimental impact on the other.

Autism spectrum disorder (ASD) is a complex group of heterogeneous neurodevelopmental disorders categorized by three key behavioral abnormalities: restricted interests accompanied by repetitive behavior, deficits in language and communication skills, and inability to engage in reciprocal social interactions (*1*–*3*). These core symptoms often are comorbid with intellectual disability, epilepsy, motor impairment, anxiety, sleep disorder, attention-deficit hyperactivity disorder, and tics (*4*–*6*). The behavioral symptomatology of ASD encompasses a spectrum of wide-ranging phenotypes, which span from mild behavioral and personality traits to severe and debilitating impairments (*7*).

The remarkable clinical heterogeneity that characterizes ASD is paralleled by an equally multifaceted etiological heterogeneity. ASD is recognized to have a genetic component (twin concordance rate of 73 to 95%) and is extraordinarily heritable (>90%) (*8*, *9*). Recently, linkage and association studies have identified numerous susceptibility genes located on multiple chromosomes, especially 2q, 7q, 15q, and the X chromosome. Thus, ASD is considered a polygenic disorder caused by the additive effect of multiple common genetic variants in combination with unidentified environmental factors (*10*). These forms of ASD are referred to as nonsyndromic ASD (*11*).

In contrast to nonsyndromic ASD, in about 10% of cases, ASD appears as part of a syndrome with a known genetic cause (*12*–*14*). These monogenic forms of ASD can result from genomic DNA mutations, de novo copy number variants (CNVs), and chromosomal rearrangements (such as deletions and translocations) (*11*). Even when ASD is genetically defined, the clinical symptoms are highly heterogeneous, likely due to differences in the genetic background of the patient and epigenetic effects (*11*). Nevertheless, monogenic forms of ASD have been paramount for understanding key neurobiological processes and complex physiological pathways that when perturbed increase the risk for ASD.

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Recent studies of monogenic forms of ASD have focused on at least two different clusters of genes frequently associated with ASD and intellectual disability: genes encoding for structural synaptic proteins or proteins involved in regulating protein synthesis (reviewed in (*11*, *15*) (Figs. 1 and 2). These studies also suggest that mutations in the same clusters of genes may be pathogenic in nonsyndromic forms of ASD. We hypothesize that mutations in these two clusters of genes interfere with interconnected downstream signaling pathways in neuronal cells resulting in ASD symptomatology.

Here, we present an overview of the monogenic forms of ASD caused by mutations in these two clusters of genes, the molecular function of their protein products, and the current mouse models used to characterize the neurobiological features of these mutations. Altered dendritic morphology and synaptic pathophysiology in ASD mouse models have been intensely investigated and extensively described. However, for the most part, the relationship of these synaptic phenotypes to ASD-associated behaviors is still correlative, and thus, we do not discuss them in detail. Rather, we speculate and discuss the functional molecular signaling connections between the protein products of these two gene clusters in ASD (Figs. 1 and 2), which almost certainly affects synaptic and circuit pathophysiology.

ASD-Associated Genes Encoding Proteins That Regulate Translation

Fragile X mental retardation protein

Nearly all individuals with fragile X syndrome (FXS) have a trinucleotide (CGG) repeat expansion adjacent to the *fragile X mental retardation 1* (*FMR1*) promoter that leads to the transcriptional silencing and subsequent loss of its protein product, fragile X mental retardation protein (FMRP) (*16*, *17*). Recently, it was discovered that the silencing of *FMR1* is mediated by the formation of a DNA-mRNA duplex between the promoter and the trinucleotide repeat region of the mRNA (*18*). Epidemiological studies show that FXS is the most common disorder that is associated with

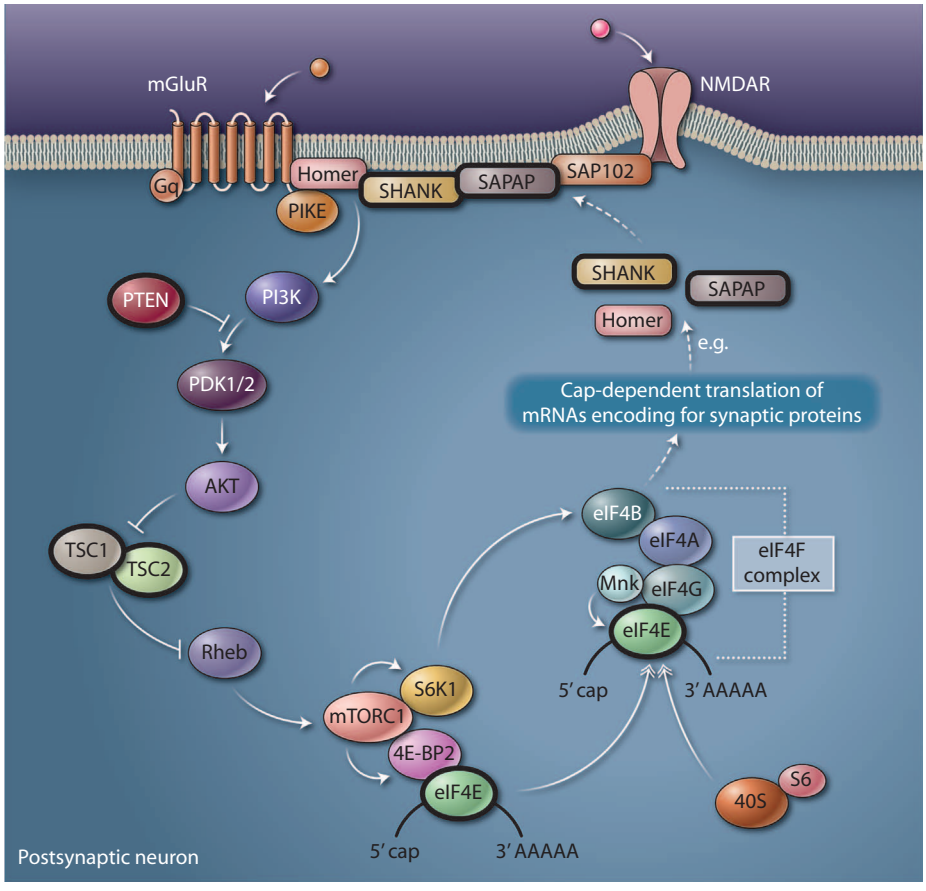

Fig. 1. Schematic of the hypothetical connection between protein synthesis and synaptic proteins. Activation of group I mGluRs results in the activation of mTORC1 signaling, which increases protein synthesis. mTORC1 phosphorylates p70 S6 kinase 1 (S6K1) and 4E-BP2; phosphorylation of 4E-BP2 releases eIF4E and results in the association of eIF4E with eIF4G to form the active eIF4F (eIF4E-eIF4G-eIF4A) complex. eIF4F promotes the binding of mRNAs to ribosomes and recruits MNK, which phosphorylates eIF4E, and eIF4B, which is phosphorylated by S6K1. The eIF4F complex and the polyadenylated tail act synergistically with Mnk-dependent phosphorylation of eIF4E to stimulate cap-dependent translation initiation. Cap-dependent protein synthesis translates some mRNAs that encode for synaptic proteins located in the PSD. It is possible that mutations in genes encoding for proteins involved in the regulation of the mTORC1 pathway result in aberrant synthesis of synaptic proteins such as neuroligins, SHANK, SAPAP, NMDA receptors. The altered synthesis of these proteins would generate changes in molecular, structural, and synaptic plasticity, contributing to ASD pathophysiology and ASD-like behaviors. The protein products of genes associated with ASD are circled in bold.

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inherited intellectual disability and ASD (*19*, *20*), occurring in about 1:5000 males and roughly half as many females. Affected males with FXS usually have other neurological and psychiatric conditions in addition to ASD and intellectual disability, including motor abnormalities, speech delay, hyperactivity, and anxiety. Postmortem neuropathological studies have revealed an increase in spine-like protrusions on apical and basal dendrites in the cerebral cortex of individuals with FXS (*21*, *22*).

FMRP is an RNA-binding protein that is involved in many aspects of the posttranscriptional regulation of mRNA, such as stability, dendritic transport, and translational control. In particular, its function as a repressor of protein synthesis has been intensively studied, but the molecular mechanism responsible for this repression remains controversial. Experimental evidence indicates a role for FMRP in the initiation (*23*–*28*) and elongation (*29*–*33*) steps of protein synthesis. In the initiation model, FMRP inhibits translation initiation by interacting with the eukaryotic initiation factor 4E (eIF4E)–binding protein (4E-BP)–like protein CYFIP1, which is associated with eIF4E, the cap-binding translation factor for mRNAs (*27*). On the other hand, there is experimental evidence indicating that the function of FMRP as a translation repressor is at the level of the elongation (*29*–*33*). Moreover, ribosomal runoff of these mRNAs demonstrated that FMRP is associated with mRNAs bound to stalled ribosomes (*34*), and ribosome transit assays indicate that elongation is enhanced in mice that lack FMRP (*35*). It is possible that FMRP acts by inhibiting both the initiation and elongation steps of translation that depends on mRNA identity and/or neuronal stimuli.

Fmr1 null mice exhibit high amounts of basal protein synthesis in the brain (*36*), and using high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP), at least 842 FMRP target mRNAs encoding for pre- and postsynaptic proteins have been identified (*34*). The postsynaptic proteins include Src homology 3 (SH3) and multiple ankyrin repeat domains protein 1 to 3 (SHANK1 to SHANK3), SAP90/PSD-95–associated protein 1 to 4 (SAPAP1 to SAPAP4), synaptic Ras guanosine triphosphatase (GTPase)–activating protein 1 (SynGAP1), and neuroligins; the presynaptic proteins include the neurexins, among others. These findings suggest that synaptic proteins and regulators of protein synthesis may functionally cooperate to generate the FXS phenotype (Figs. 1 and 2). In addition to directly repressing translation, FMRP affects protein synthesis by acting indirectly on signaling pathways involved in translational control. Increased signaling by the mammalian target of rapamycin complex 1 (mTORC1) is seen in *Fmr1* null mice (*37*) that likely is induced by the increased abundance of the GTPase PIKE, which connects the activation of metabotropic glutamate receptor 5 (mGluR5) to the phosphatidylinositol 3-kinase (PI3K)–mTORC1 signaling pathway in the hippocampus (*37*, *38*). Moreover, several mRNA targets of FMRP encode repressors of the mTORC1 signaling pathway, including tuberin (TSC2) and phosphatase and tensin homolog (PTEN) (*34*). Thus, it is possible that FMRP silencing may have an indirect, secondary effect on protein synthesis by repressing the translation of components of the mTORC1 signaling pathway.

Fmr1 null mice exhibit enhanced mGluR-dependent synaptic plasticity (*39*), increased density of dendritic spines, and numerous filopodia-like spines in the cortex, recapitulating a pathological feature observed in FXS patients (*21*, *40*–*42*). Moreover, *Fmr1* null mice display a range of phenotypes that mimic many of the symptoms observed in individuals with

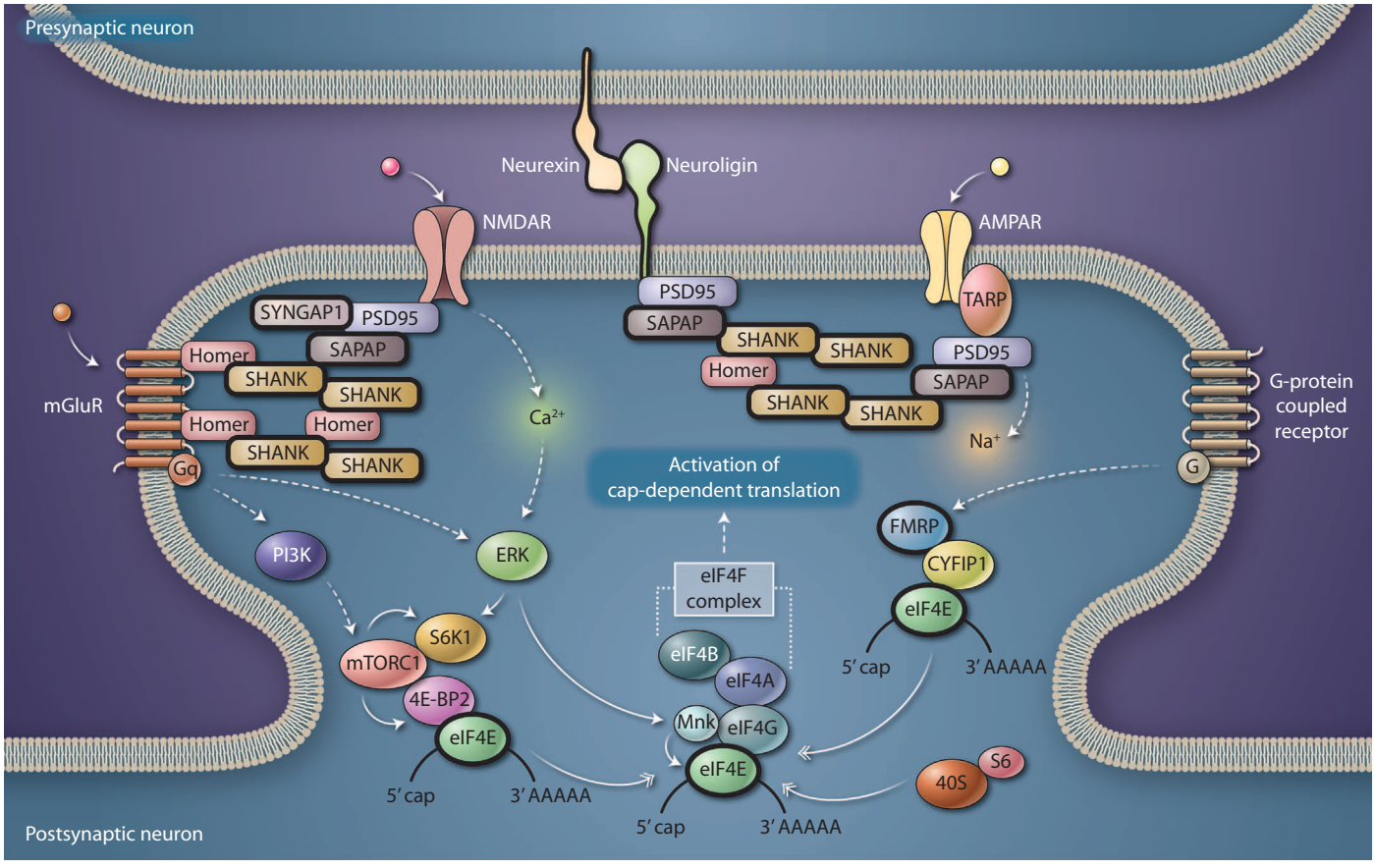

Fig. 2. Schematic of the hypothetical connection between synaptic proteins and protein synthesis. Intracellular signal transduction is initiated by the activation of neurotransmitter receptors that are organized with scaffolding proteins and adhesion molecules in the PSD. Receptor stimulation triggers the activation of intracellular signaling cascades including the mTORC1 and ERK pathways, which results in increased translation (see also Fig. 1). Given the importance of synaptic proteins in this type of signal transduction, mutations affecting genes encoding for these proteins could result in abnormal signaling that ultimately results in aberrant protein synthesis. The protein products of genes associated with ASD are circled in bold.

FXS. For example, these mice display hyperactivity, altered sensorimotor gating (filtering out unnecessary stimuli), deficits in learning and memory, increased susceptibility to audiogenic seizures, increased body growth rate, and macroorchidism (*43*).

Eukaryotic initiation factor 4E

Several studies suggest an association between ASD and mutations in *EIF4E*. Genetic variants in chromosome 4q, which contains the *EIF4E* locus, have been described in patients with ASD (*44*, *45*). Notably, in ASD subjects, several of these common genetic variants in the *EIF4E* gene are associated with a clinical phenotype characterized by repetitive and stereotyped behaviors, but not intellectual disability (*46*). A de novo chromosomal translocation involving the promoter region of the *EIF4E* gene in a boy with classic nonsyndromic ASD has been described (*47*). In addition, a nucleotide insertion in the promoter region of the *EIF4E* gene that increases promoter activity was discovered in two unrelated families with autistic siblings. These genetic studies link mutations in *EIF4E* to ASD; however, further investigations are needed to clearly establish a causal connection.

eIF4E binds to the cap structure at the 5′ terminus of mRNA and regulates the initiation step of cap-dependent translation (*48*, *49*). The main role of eIF4E in translation initiation is in the formation of the eIF4F initiation complex, which brings mRNAs to the ribosome. The critical step in the formation of the complex is the direct association of eIF4E with eIF4G (*50*), an mRNA-ribosome bridging factor, and the indirect association of eIF4E with the RNA helicase eIF4A (*51*). The interaction of eIF4E with eIF4G is regulated by 4E-BPs, which repress translation by blocking the interaction of eIF4E with eIF4G (*52*). Upon stimulation, 4E-BP is phosphorylated and inactivated by mTORC1, thereby enabling eIF4E to associate with eIF4G to form the eIF4F complex (*53*). As well, eIF4E is phosphorylated by mitogen-activated protein kinase (MAPK)–interacting kinases 1 and 2 (Mnk1/2), a substrate of extracellular signal–regulated kinase (ERK). In some experimental conditions, the phosphorylation of eIF4E is correlated with the rate of protein synthesis (*54*). Thus, eIF4E and cap-dependent protein synthesis can be regulated by both mTORC1 and ERK signaling (*55*) (Figs. 1 and 2).

The relationship between eIF4E, cap-dependent translation, and ASD has been recently studied by overexpressing eIF4E in a transgenic mouse (*56*). These mice show increased protein synthesis in the brain and aberrant behaviors reminiscent of ASD, including impairments in social interactions and repetitive or perseverative behaviors. The ASD-like behaviors were corrected by blocking the interaction between eIF4E and eIF4G with

the cap-dependent translation inhibitor 4EGI-1. Notably, mice with genetic deletion of 4E-BP2, the predominant 4E-BP isoform in the brain, exhibit ASD-like behaviors that mimic those displayed by eIF4E transgenic mice (*57*). Thus, mice with increased eIF4E-dependent translation display ASD-like behaviors, strongly suggesting a link between exaggerated protein synthesis and ASD.

TSC1 and TSC2

Tuberous sclerosis complex (TSC) is a multisystem disorder characterized by the presence of benign tumor-like lesions (hamartomas) in many organs, such as the brain, skin, eyes, kidneys, and heart (*58*). TSC is an autosomal dominant inherited disorder caused by loss-of-function mutations in either *TSC1* (encoding hamartin, also referred to as TSC1) or *TSC2* (encoding tuberin, also referred to as TSC2). These mutations include missense or nonsense mutations, insertions, and deletions involving nearly all exons in *TSC1* and *TSC2* (*4*, *5*, *59*). The impact of the different mutations on clinical phenotypes is extremely variable with respect to symptoms and disease severity, and in part is dependent on which *TSC* gene is affected (*60*). Seizures are the most common neurological symptom, occurring in up to 90% of the patients, whereas intellectual disability and ASD occur in about 50% of patients (*58*).

TSC1 and TSC2 form a heterodimeric complex that can regulate protein synthesis by controlling mTORC1 activity. TSC1 and TSC2 are phosphorylated by many kinases and factors, including Akt, ERK, glycogen synthase kinase–3β, adenosine monophosphate–activated kinase, and cyclin-dependent kinase 1 (*61*–*64*). The active TSC1-TSC2 complex inhibits mTORC1 through activation of the small GTPase Ras homolog enriched in the brain (Rheb). Rheb activates mTORC1 when it is bound to guanosine triphosphate (GTP). The TSC1-TSC2 complex has GTPase activity localized in the GAP domain of TSC2. When phosphorylated by Akt, the GAP activity of TSC1/TSC2 is increased, which in turn hydrolyzes GTP bound to Rheb, thereby inhibiting mTORC1 (*61*–*64*) (Fig. 1). Therefore, in the absence of either TSC1 or TSC2, high amounts of Rheb-GTP lead to constitutive activation of mTORC1 signaling, thereby resulting in dysregulated protein synthesis and cell growth (*64*). Several mouse models of TSC have been used to understand the etiology of this disorder. For example, heterozygous genetic deletion of either *Tsc1* or *Tsc2* in mice results in cognitive and synaptic impairments consistent with ASD (*65*–*67*). *Tsc1* and *Tsc2* mutant mice display ASD-like phenotypes in the absence of neuropathological brain tumors and epilepsy, suggesting that cognitive dysfunction in TSC arises independently. However, it should be noted that specific genetic ablation of *Tsc1* in either astrocytes (*68*) or neurons (*66*, *69*) results in epilepsy and lethality. Either genetic reduction or complete deletion of *Tsc1* in cerebellar Purkinje cells (PCs) results in ASD-like behaviors, including impaired social interaction, altered ultrasonic vocalizations, and increased repetitive behaviors that are correlated with decreased PC excitability and changes in the number and morphology of PCs (*70*).

In the aforementioned mouse models, postnatal and postdevelopment treatment with the mTORC1 inhibitor rapamycin ameliorates multiple behavioral and synaptic phenotypes (*66*, *69*–*72*). Thus, inhibition of mTORC1 activity in adult TSC mice is sufficient to correct ASD-like phenotypes, suggesting that these behaviors are caused by persistently increased mTORC1 signaling rather than irreversible pathophysiological changes that occur during brain development.

Phosphatase and tensin homolog

The gene encoding phosphate and tensin homolog (*PTEN*), located on chromosome 10q23, is a candidate risk gene for ASD and macrocephaly (*73*–*76*). Different studies have suggested a causal role for *PTEN* mutations in a subset of individuals with ASD. Recently, a frameshift variant of *PTEN* was identified in a patient with extreme macrocephaly, ASD, intellectual disability, and epilepsy, supporting the theory that mutations in this gene are involved in the etiology of ASD and macrocephaly (*77*). In general, *PTEN* mutations are more frequent in ASD children that develop macrocephaly than in those that do not (*78*, *79*).

PTEN is a phosphatase that removes the 3′ phosphate from phosphatidylinositol 3,4,5-trisphosphate (*80*) and thus inhibits PI3K signaling, thereby inactivating Akt and mTORC1. In contrast, deletion of *PTEN* results in a constitutively active Akt-mTORC1 signaling pathway (*81*) (Fig. 1). Given the importance of PI3K-Akt-mTORC1 signaling in the control of cell growth, survival, and proliferation, it is not surprising that PTEN inactivation leads to human cancers and neurological disorders (*81*).

Mice with *Pten* deletions have been studied mostly to clarify the role of PTEN in neuronal hypertrophy and number because the most obvious phenotype in human patients is macrocephaly. Overall, the effect of genetic deletion of *Pten* during development is dramatic, resulting in brain enlargement and gross anatomical abnormalities that are often accompanied by the development of seizures and premature death (*82*, *83*). Several studies have directly addressed the role of *PTEN* mutations in ASD. These studies bypassed the severe developmental phenotype by deleting *Pten* in mice in either a cell-specific or inducible manner using conditional genetic technology. For example, mice in which *Pten* is ablated in a subset of postmitotic cortical and hippocampal neurons develop macrocephaly and display ASD-like behaviors, including impaired social interactions, seizures, anxiety, and cognitive deficits (*84*). Treatment with the mTORC1 inhibitor rapamycin reverses the neuronal hypertrophy and ameliorates the seizures and social impairments (*85*). Moreover, mice with germline *Pten* haploinsufficiency ($Pten^{+/-}$) exhibit increased total brain mass and ASD-like behavioral impairments, such as abnormal social behavior and sensorimotor gating (*86*), increased repetitive behaviors, and depressive-like behaviors (*87*). These ASD-like behaviors are exacerbated in $Pten^{+/-}$ mice crossed with serotonin transporter heterozygote mice ($Slc6a4^{+/-}$). *SLC6A4* is also an ASD susceptibility gene (*86*). These findings demonstrate that deficiencies in PTEN and SLC6A4 can cooperate to contribute to ASD-like behavioral phenotypes.

ASD-Associated Genes Encoding Proteins Involved in Synaptic Function

SHANK

Phelan-McDermid syndrome (PMS) is a genetic disorder characterized by ASD and intellectual disability. Patients exhibit impairments in communication skills that are often accompanied by reduced socialization and stereotypical movements, as well as aggressive behavior and seizures (*88*–*90*). The disorder is caused by variable length deletions in the terminal region of the long arm of chromosome 22, which contains the ASD- and PMS-associated gene *SHANK3* (*90*–*92*). Duplications, CNVs, microdeletions, and mutations in *SHANK3* are also found in patients with ASD and intellectual disability (*93*–*98*).

SHANK3 is a member of the SH3 and multiple ankyrin repeat domains protein family, also known as proline-rich synapse-associated proteins (ProSAPs). The SHANK proteins are expressed abundantly in the central nervous system, are enriched in the postsynaptic density (PSD) of excitatory synapses (*99*–*101*), and interact with cytoskeleton and scaffolding proteins, which in turn bind to receptors to create a matrix for the stabilization and organization of the PSD. Indeed, SHANK proteins bind to SAPAPs, which interact with PSD-95 proteins associated with glutamate receptors (*102*). Moreover, SHANK proteins bind to the Homer family of scaffolding proteins, which are associated with mGluRs (*103*) (Fig. 2).

Also, SHANK proteins are involved in regulating the cytoskeleton by binding cortactin (*104*), inositol 1,4,5-trisphosphate (IP3) receptors, and F-actin (*105*, *106*).

Multiple mouse models with deletions of the *Shank* genes have been intensively studied. Four different lines of *Shank3* mice, each with a specific deletion of exons encoding for the functional interaction domains, exhibit behavioral deficits consistent with ASD, including social deficits, communication alterations, repetitive and stereotyped behaviors, and abnormal learning and memory that are accompanied generally by changes in synaptic function and molecular composition of the PSD (*107*–*109*). Notably, *Shank3B* mutant mice, which lack the PDZ domain of the protein, exhibit a particularly severe phenotype. Consistent with the marked expression of *Shank3* in the striatum, the *Shank3B* null mice groomed so excessively that they exhibited self-inflicted skin lesions and displayed anxiety-like behaviors and impaired social interactions. Genetic deletion of *Shank1* results in abnormal grooming behavior and impairments in ultrasonic vocalization, but normal social interactions (*110*, *111*), contextual fear, and long-term spatial memory (*112*). Overall, these PMS mouse models suggest that molecular changes perturbing synaptic and structural functions at the PSD of excitatory synapses are likely to generate ASD-like phenotypes.

Neuroligins and neurexins

Several mutations and deletions in genes encoding for neuroligin-3 (*NLGN3*), neuroligin-4 (*NLGN4*), and neurexin-1 (*NRXN1*) are associated with ASD and intellectual disability (*44*, *113*–*125*). A base pair substitution (A335G) in the *NLGN4* promoter was observed in a boy with autism and intellectual disability. This mutation causes increased activity of promoter and, subsequently, increased *NLGN4* expression (*126*), curiously suggesting that increased or decreased abundance of neuroligin-4 may be similarly detrimental to neuronal function and result in ASD-like phenotypes.

Neuroligins and neurexins are synaptic cell adhesion molecules that are critical for synaptic efficacy and plasticity (*127*–*132*). Neurexins are type 1 membrane proteins encoded by three genes (*NRXN1*, *NRXN2*, and *NRXN3*), which generate larger α-neurexins and shorter β-neurexins from independent promoters (*133*). Furthermore, each gene undergoes extensive alternative splicing that is capable of generating thousands of neurexin isoforms (*134*). Neuroligins are endogenous ligands for neurexins (*127*) and are encoded by four genes (*NLGN1*, *NLGN2*, *NLGN3*, and *NLGN4*) located on the X chromosome (*135*). Neuroligins are type 1 membrane proteins like neurexins, but have a simpler domain structure and less diversity. All neuroligins are enriched in PSD, but neuroligin-1 and neuroligin-2 are exclusively localized to excitatory and inhibitory synapses, respectively, whereas neuroligin-3 may be present in both (*130*, *136*–*138*) (Fig. 2).

Mouse models recapitulating the genetic mutations or deletions of *Nrxn*s and *Nlgns* described in ASD patients have been important for understanding their association with the clinical manifestation of the disorder. For example, mice with either a genetic deletion of *Nlgn3* (*139*) or a knock-in allele containing an R451C substitution in *Nlgn3* (*140*) display ASD-like behaviors that were mostly restricted to social and communication domains, such as impairments in ultrasonic vocalization, social interaction, and memory. Similarly, mice with a deletion of the *Nlgn4* ortholog exhibited impaired social interactions and ultrasonic vocalization (*141*). This indicates that neuroligins are important in the generation of normal social skills and vocalization.

The studies performed on mice with genetic ablation of the genes that encode the neurexins are more difficult to interpret, given the high degree of genetic redundancy. Mice with a genetic deletion that results in a lack of all neurexin α-isoforms die 1 day after birth, whereas mice with ablation of a single gene are viable but show compromised respiratory function and die prematurely (*128*). It will be interesting to study the role of neurexins in a specific neuron type (or postdevelopmental time frame) to avoid the lethal phenotype and establish a link with ASD.

SAPAP

Rare genetic variants in *DLGAP2*, which encodes SAPAP2, occur in some ASD patients (*142*). Although not clearly established, a possible involvement of the proteins of the SAPAP family in ASD is intriguing, given their demonstrated interaction with the proteins of the SHANK family, which as discussed above are more clearly described in ASD (*94*–*96*). However, the involvement of SAPAP3 in obsessive-compulsive spectrum disorders (OCD), trichotillomania, and Tourette syndrome is fairly well established (*143*–*146*).

The members of the SAPAP family, also referred to as guanylate kinase–associated proteins (GKAPs), are postsynaptic scaffold proteins that are localized to the PSD uniquely at excitatory synapses (*147*). SAPAP proteins are encoded by a family of four genes that are widely, but differentially, expressed throughout the nervous system. SAPAP proteins may link the PSD-95 family proteins with the actin cytoskeleton by interacting with SHANK proteins, which in turn bind the actin-binding protein cortactin (Fig. 2). Therefore, in the current model of PSD organization, PSD-95-SAPAP-SHANK interactions play an important role in the constitution of the large postsynaptic signaling complex at glutamatergic synapses (*148*).

The most studied member of the SAPAP family is SAPAP3, which is highly expressed in the striatum (*147*, *149*). Genetic ablation of *Sapap3* caused behavioral abnormalities consisting of extremely frequent and aggressive self-grooming accompanied by self-inflicted snout lesions and anxiety-like behaviors. Consistently, *Sapap3* null mice display synaptic, morphological, and molecular defects at striatal glutamatergic synapses. The behavioral and synaptic phenotypes of *Sapap3* null mice are similar to those generated by genetic ablation of *Shank3*, indicating that genetic changes perturbing these synaptic proteins in the striatum result in specific phenotypes that are consistent with ASD.

Similar to SAPAP3, genetic ablation of SAPAP2 (*DLGAP2*) in mice increases aggressive behavior and results in impaired social interactions. Moreover, the *DLGAP2* null mice exhibit reduced dendritic spine density, changes in receptor composition, and decreased PSD length and thickness (*150*). Overall, these results suggest that deletion of SAPAP2 may reduce synaptic and postsynaptic responses.

SYNGAP1

Deletion of *SYNGAP1* or a mutation introducing a premature stop codon in *SYNGAP1* is found in a few patients with intellectual disability with or without ASD (*121*, *142*). *SYNGAP1* encodes a Ras GTPase–activating protein (RasGAP) called synaptic GAP (SynGAP). *SYNGAP1* has several alternative start sites, and transcripts can be spliced extensively to generate multiple SynGAP1 isoforms (*151*, *152*). SynGAP1 is a brain-specific protein that is highly enriched at excitatory synapses and colocalizes and interacts with *N*-methyl-D-aspartate (NMDA) receptors and the PDZ domains of PSD-95 via its C-terminal amino acids (*151*, *152*) (Fig. 2). It inhibits signaling pathways linked to NMDA receptor–mediated synaptic plasticity and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor membrane insertion (*153*–*155*). It was shown that SynGAP1 connects Ca^{2+} influx to activation of the ERK pathway downstream of NMDA receptors (*156*). Given the multiple isoforms and the possible high degree of redundancy, the impact of deleting *SynGAP1* in neurons is not clear. In fact, deletion of *SynGAP1* in hippocampal neurons in culture has been reported to both enhance (*153*, *155*) and suppress (*154*) dendritic spine formation. *Syngap1*$^{-/-}$ but not *Syngap*$^{+/-}$ mice die within 2 days after birth (*157*). Heterozygous mice exhibit hyperactivity, diminished sensorimotor gating, and enhanced startle response. Moreover, they display a reduction in social

memory, a tendency toward social isolation, enhanced ERK activation, and impaired hippocampal synaptic plasticity (*156*). Hippocampal neurons in these mice have an accelerated rate of glutamatergic synapse maturation, consequently disrupting the excitation/inhibition balance (*158*). These studies indicate that changes in synaptic maturation during the development result in enduring behavioral abnormalities.

Reciprocal Signaling Links Two Clusters of ASD Genes

The studies summarized above are consistent with at least two defined clusters of genes that are involved in ASD and intellectual disability. One cluster encodes for proteins that regulate protein synthesis, a fundamental process for long-lasting changes in synaptic strength and dendritic spine plasticity underlying cognition. The second cluster of genes produces proteins involved in the regulation of synaptic transmission and structure, which are important in the establishment and remodeling of neuronal networks. Currently, there is limited experimental evidence suggesting a direct interaction between the protein products of these two gene clusters. However, their critical and central biological functions strongly suggest that an anomaly in one of these pathways would almost necessarily perturb the other (Figs. 1 and 2).

Activity-dependent changes in PSD composition and/or structure represent molecular mechanisms that drive complex brain functions, including learning and memory. These long-term synaptic and structural changes are critically dependent on dendritic protein synthesis (Fig. 1). Indeed, it recently was shown that aberrant protein synthesis driven by overexpression of the cap-binding translation factor eIF4E causes synaptic impairments and ASD-like behaviors (*56*), indicating that exaggerated translation directly influences synaptic and structural plasticity. Consistent with this idea, a related study revealed that overexpression of neuroligins is likely responsible for the generation of certain ASD-like phenotypes in eIF4E transgenic mice (*57*). Moreover, the synaptic, structural, and behavioral abnormalities in mice with exaggerated eIF4E-dependent translation were corrected by reducing protein synthesis and/or diminishing the expression of neuroligins with small interfering RNAs (siRNAs) (*56*, *57*).

The examination of FMRP-regulated target mRNAs—encoding both synaptic proteins and regulators of translation—also supports this idea and demonstrates that both pre- and postsynaptic proteins are part of the transcripts dysregulated in FXS (*34*), which include SHANK3, SynGAP1, neuroligin-3, and neurexin-1 (*34*) and SHANK1, SAPAP1, and SAPAP3 (*159*). This suggests that changes in synaptic and PSD proteins driven by dysregulated protein synthesis may contribute to enduring changes in synaptic plasticity, dendritic morphology, and ASD-like behavioral abnormalities. Another set of mRNA targets of FMRP are proteins directly involved in the regulation of translation, such as TSC2 and PTEN (*34*, *160*), suggesting that synaptic proteins and regulators of mTORC1 activity may interact to give rise to the FXS phenotype.

Conversely, it is possible that the ASD-associated mutations that result in changes in the level and function of synaptic and PSD proteins alter protein synthesis and contribute to the generation of ASD (Fig. 2). Unfortunately, there is limited information regarding the activity of translational control pathways in human patients and mouse models of ASD caused by mutations in genes encoding for synaptic proteins, as discussed above. However, a recent study investigating mGluR signaling in mice with a genetic deletion of *Fmr1* reveals a fundamental role of the PSD scaffolding protein Homer1a (*36*, *161*, *162*). Altered mGluR5-Homer interactions contribute to abnormal mGluR signaling, altered protein synthesis, and other ASD-like phenotypes in FXS model mice. Genetic deletion of Homer1a restores the normal mGluR5-Homer association and corrects several phenotypes in *Fmr1* null mice, including enhanced global protein synthesis (*161*, *162*). Although the effect of mGluR5-Homer interactions on protein synthesis is secondary to the direct role of FMRP in translation, this study indicates the possibility that alterations in synaptic proteins result in aberrant translational control. It is tempting to speculate that ASD linked to *SHANK* mutations is also associated with alterations in protein synthesis because SHANK directly interacts with Homer (*103*). Therefore, defects in synaptic protein function could result in aberrant protein synthesis, resulting in abnormal synaptic plasticity and ASD-like behaviors. Future studies are necessary to conclusively address this hypothesis.

Several lines of evidence indicate that loss-of-function mutations, deletions, and overexpression of synaptic and PSD proteins are detrimental and result in ASD-like behavioral phenotypes, as discussed above. This is in agreement with our hypothesis that increased expression of synaptic proteins generated by alteration in protein synthesis could trigger synaptic abnormalities and behaviors associated with ASD. In contrast, investigations concerning the proteins that regulate translation hint at a connection between exaggerated protein synthesis and ASD phenotypes in humans and animal models (*56*, *57*, *163*). However, inhibition of de novo protein synthesis impairs long-lasting plasticity and cognition [reviewed in (*48*, *164*)] and likely contributes to cognitive deficits in TSC model mice (*165*). An intriguing possibility is that excessive translation contributes to aberrant behaviors associated with ASD, whereas insufficient translation contributes to impaired cognition associated with the intellectual disability that often accompanies ASD.

Thus, recent data support the hypothesis that proteins involved in the regulation of translation and synaptic function may be interconnected and act in concert to give rise to synaptic and behavioral aberrations associated with ASD. Future genetic studies are necessary to reveal the molecular players that link these two pathways and to understand whether it is possible to intervene therapeutically at the level of these molecular crossroads.

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Supporting Data

All figures and figure legends are contained in the Santini et al. manuscript, which is attached in the preceding pages.