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Loss of *Tsc2* in Purkinje cells is associated with autistic-like behavior in a mouse model of tuberous sclerosis complex

R. Michelle Reith [a], James McKenna [b], Henry Wu [c], S. Shahrukh Hashmi [d], Seo-Hee Cho [e], Pramod K. Dash [f], Michael J. Gambello [b,*]

[a] *Program in Human and Molecular Genetics, UT Health, Houston, TX, USA*
[b] *Department of Human Genetics, Emory University, Atlanta, GA, USA*
[c] *Department of Biochemistry, Division of Cardiology, UT Health, Houston, TX, USA*
[d] *Department of Pediatrics, Division of Medical Genetics, UT Health, Houston, TX, USA*
[e] *Department of Anatomy and Cell Biology, Shriners Hospital's Pediatric Research Center, Temple University School of Medicine, Philadelphia, PA, USA*
[f] *Department of Neurobiology and Anatomy, UT Health, Houston, TX, USA*

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ABSTRACT

Tuberous sclerosis complex (TSC) is a dominant tumor suppressor disorder caused by mutations in either *TSC1* or *TSC2*. TSC causes substantial neuropathology, often leading to autism spectrum disorders (ASDs) in up to 60% of patients. The anatomic and neurophysiologic links between these two disorders are not well understood. We have generated and characterized a novel TSC mouse model with Purkinje cell specific *Tsc2* loss. These Tsc2f/−;Cre mice exhibit progressive Purkinje cell degeneration. Since loss of Purkinje cells is a well reported postmortem finding in patients with ASD, we conducted a series of behavior tests to asses if Tsc2f/−;Cre mice displayed autistic-like deficits. Tsc2f/−;Cre mice demonstrated increased repetitive behavior as assessed with marble burying activity. Using the three chambered apparatus to asses social behavior, we found that Tsc2f/−;Cre mice showed behavioral deficits, exhibiting no preference between a stranger mouse and an inanimate object, or between a novel and a familiar mouse. We also detected social deficits in Tsc2f/f;Cre mice, suggesting that Purkinje cell pathology is sufficient to induce ASD-like behavior. Importantly, social behavior deficits were prevented with rapamycin treatment. Altogether, these results demonstrate that loss of *Tsc2* in Purkinje cells in a *Tsc2*-haploinsufficient background leads to autistic-like behavioral deficits. These studies provide compelling evidence that Purkinje cell loss and/or dysfunction may be an important link between TSC and ASD as well as a general anatomic phenomenon that contributes to the ASD phenotype.

Introduction

Tuberous sclerosis complex (TSC) is a dominant neurogenetic disorder affecting about 1 in 6000 people (Osborne et al., 1991). The brain pathology is the most debilitating aspect of TSC and is often associated with autism spectrum disorders (ASD) (Gillberg et al., 1994; Hunt and Dennis, 1987; Hunt and Shepherd, 1993; Smalley et al., 1992). Typical brain lesions include: cortical tubers, subependymal nodules, white matter defects, and cerebellar lesions (Asano et al., 2001; Crino et al., 2006; DiMario, 2004; Eluvathingal et al., 2006). TSC is caused by heterozygous loss of function mutations of either the *TSC1* or *TSC2* gene, encoding the protein hamartin or tuberin, respectively (Consortium, 1993; van Slegtenhorst et al., 1997). Many lesions in TSC patients demonstrate loss of both alleles of either *TSC1* or *TSC2*, suggesting that the two-hit mechanism is operative (Au et al., 1999; Green et al., 1994a, 1994b; Henske et al., 1997). However, there is also evidence that haploinsufficiency is another important mechanism of pathogenesis (Henske et al., 1996; Niida et al., 2001). Cortical tubers also show evidence that both loss of heterozygosity (LOH) and haploinsufficiency play a role in pathogenesis (Crino et al., 2010; Qin et al., 2010). Hamartin and tuberin form a complex that inhibits the mammalian target of rapamycin complex 1 (mTORC1), a kinase that controls translation and cell growth (Inoki et al., 2002). The mTORC1 kinase is inhibited by tuberin's GTPase activating domain on the Ras-like protein Rheb (Inoki et al., 2003; Zhang et al., 2003). Thus, the loss of function of *TSC1* or *TSC2* leads to increased activity of mTORC1 (Bhaskar and Hay, 2007; Huang and Manning, 2008; Sarbassov et al., 2005). Accordingly, increased mTORC1 activity has been demonstrated in many TSC lesions (Chan et al., 2004; Crino et al., 2006). Interestingly, mTORC1 activation is seen in several other monogenetic disorders associated with ASD such as neurofibromatosis type 1, PTEN associated macrocephaly, and Fragile X syndrome (Bailey et al., 1998b; Butler et al., 2005; Ehninger

Abbreviations: TSC, tuberous sclerosis complex; ASD, autism spectrum disorder; mTORC1, mammalian target of rapamycin complex 1.

* Corresponding author. Fax: +1 11 1 404 727 3949.
E-mail address: Michael.j.gambello@emory.edu (M.J. Gambello).

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and Silva, 2011; Lee et al., 2012; Marui et al., 2004). Therefore, dysregulation of mTORC1 appears to be an important pathway leading to the autistic-phenotype. Since TSC is the prototypical mTORopathy, and about 25%–60% of children with TSC have ASD (Gillberg et al., 1994; Hunt and Dennis, 1987; Hunt and Shepherd, 1993; Smalley et al., 1992), an understanding of this association might uncover general principles applicable to idiopathic autism.

Autism spectrum disorders (ASDs) are developmental disabilities with abnormalities of varying severity in three modalities: social interactions, communication, and stereotypical repetitive movements (Cappon, 1953; Rutter, 1978; Wing, 1981). The incidence of ASD is about 1 in every 110 births, with a higher occurrence in boys than in girls (4.5:1) (Prevention, 2006), and an estimated heritability of about 90% based upon monozygotic twin studies (Folstein and Rutter, 1977; Muhle et al., 2004). Traditionally, the neurological basis of ASD has been thought to lie mainly in the cerebral cortex (Abell et al., 1999; Aylward et al., 1999; Courchesne and Pierce, 2005; Dawson et al., 1998). Recent evidence suggests that the cerebellum may also be an important determinant in ASD (Fatemi et al., 2012; Palmen et al., 2004; Vargas et al., 2005; Yip et al., 2009). The cerebellum is well known to coordinate motor function, but also has important roles in higher order cognitive functions (Gordon, 2007; Tavano et al., 2007). Patients with diseases confined to the cerebellum often demonstrate impaired executive functions including: planning, abstract reasoning, and language deficits—abnormalities often seen in ASD (Exner et al., 2004; Paulus et al., 2004; Schmahmann and Sherman, 1998; Tavano et al., 2007). Cerebellar abnormalities including: Purkinje cell loss, general cerebellar hypoplasia, vermal hypoplasia and hyperplasia, reduced gray matter, GABA dysfunction, and decreased attention-related cerebellar activation: were found in about 90% of autistic patients in both MRI and autopsy studies, further supporting a role for the cerebellum in ASD (Allen and Courchesne, 2003; Courchesne, 1997; Courchesne et al., 1994; Fatemi et al., 2012; Hashimoto et al., 1995). Phenotypic evaluations of syndromic autism also implicate cerebellar abnormalities (Fatemi et al., 2012). Specifically, abnormalities of the cerebellar vermis lobes VI–VII are seen in patients with Fragile X syndrome specific to the autistic subpopulation (Kaufmann et al., 2003). In TSC, the severity of the autistic phenotype is associated with number and severity of cerebellar lesions (Weber et al., 2000). While there is mounting evidence of a link between the cerebellum and ASD, the anatomical and physiological links remain poorly defined.

The cerebellum communicates with the cerebral cortex via the inhibitory GABAergic axons of the Purkinje cell that project to the deep cerebellar nuclei (Saab and Willis, 2003). The deep cerebellar nuclei then send projections to the thalamus and cerebral cortex (Gonzalo-Ruiz and Leichnetz, 1990; Middleton and Strick, 2001; Yamamoto et al., 1992). Purkinje cell loss is one of the most common anatomical abnormalities seen in autopsy studies of autistic patients (Bailey et al., 1998a; Fatemi et al., 2002; Palmen et al., 2004). How the loss of Purkinje cells, as either a direct or indirect effect, affects the autistic phenotype remains obscure. Previously characterized animal models provide some insight and a mechanism to further study this association. For example, heterozygous Lurcher mice, containing a naturally occurring gain of function mutation in the glutamate receptor delta 2 (GluRδ2) (Zuo et al., 1997), lose 100% of their Purkinje cells (Caddy and Biscoe, 1979). Studies of Lurcher mice revealed decreased anxiety-related behaviors, increased activity levels, and increased repetitive behaviors (Hilber et al., 2004; Martin et al., 2012). As repetitive behaviors are a hallmark of ASD (Association, 2000), the Lurcher mice provide one good model to study the mechanisms of Purkinje cell loss and ASD.

Given the comorbidity of TSC and ASD, we hypothesized that abnormalities in the cerebellum of TSC patients might contribute to the autistic phenotype. In support of this hypothesis, Purkinje cell loss has been reported in some patients with TSC (Boer et al., 2008). A recent report linked a Purkinje cell specific mutation in *Tsc1* to autistic-like behaviors in mice (Tsai et al., 2012). It is known, however, that patients with mutations in *TSC2* have a more severe neurological phenotype including an increased risk for autism (Au et al., 2007; Dabora et al., 2001). In an effort to study the relationship between the cerebellum, *TSC2*, and ASD, we created and characterized a mouse model with Purkinje cell loss due to Purkinje cell specific *Tsc2* deletion. These $Tsc2^{flox/ko}$;*Pcp2-Cre* (Tsc2f/−;Cre) mice model a patient with *TSC2* haploinsufficiency ($Tsc2^{ko}$ or Tsc2− allele) and subsequent loss of heterozygosity only in Purkinje cells due to Cre recombinase-mediated loss of the $Tsc2^{flox}$ (Tsc2f) allele. The Purkinje cells of Tsc2f/−;Cre mice have increased mTORC1 activity and progressively die beginning at one month of age. In the current study, we examined the behavioral phenotype of these mice between one and three months of age to asses if the cerebellar pathology is associated with autistic-like behavior. We show that Tsc2f/−;Cre mice exhibit intact gross motor-function, reflexes, vision, and olfaction. Nevertheless, we demonstrate that Tsc2f/−;Cre mice have impaired social interactions and increased repetitive behavior, suggestive of an autistic-like phenotype. Furthermore, we demonstrate that treatment with the mTORC1 inhibitor, rapamycin, prevents social behavior deficits. These results highlight the importance of Purkinje cells outside of the motor circuit, implicate a function for Purkinje cells in TSC-associated ASD, and provide a mouse model of a *Tsc2* mutation in which to study the relationship between the cerebellum and ASD.

Materials and methods

Animals

All animal experimentation was approved by the UTHSC Animal Welfare Committee. Mice were on a combined 129X1/SvJ and C57BL/6J background. Generation of the $Tsc2^{+/flox}$ and $Tsc2^{+/KO}$ mice has been previously described (Way et al., 2009). The expression of Cre recombinase was controlled by the Pcp2 Purkinje cell protein specific promoter as previously described (Barski et al., 2000).

Behavioral Testing

The order of testing was done from the least stressful to the most stressful. The timeline is shown in Table 1. The experimenter was blind to the genotypes of the mice for all behavior testing.

Home cage behavioral video recording

Each cage used in behavior testing was videotaped for 20 min at 8:00 AM, 12:00 PM, and 4:00 PM for a total of 1 h of video.

Table 1
Timeline of testing. Testing began at one month of age and continued to three months of age. The tests were ordered from the least stressful to the most stressful test. In general, two tests a week were conducted.

Age	Test
P30–31	General observation and reflexes
P37	Nest building
P40	Response to social cues
P44	Marble burying
P47	Open-field
P49–54	Buried food
P58	Social behavior
P61	Inkblot
P65	RotaRod
P68	Light/dark box
P72–77	Water maze
P85–89	Reverse water maze
P90	Vision water maze

Reflexes

Before behavior testing, mice were tested for intact neurological reflexes. Reflex testing included eye blink, ear twitch, forepaw extension, grasping, and whisker twitch (Crawley, 2008). Mice were also examined to make sure that hind limb clasping, indicating a significant neurological/motor impairment, was not observed.

Marble burying

Repetitive behaviors were assessed by marble burying activity. Mice were placed in a clean cage with 4.5 cm corncob bedding with 20 black glass marbles (15 mm diameter) arranged in a grid on top of the bedding. Mice were allowed to explore the cage for 30 min. At the end of the experiment, the number of marbles buried (>50% of the marble covered by the bedding) was recorded (Thomas et al., 2009).

Open-field activity

Exploratory locomotor activity was measured in an open field (16×16 in.) plexiglass chamber with photobeams (Photobeam Activity System, San Diego Instruments). Mice were placed in the chamber for 30 min. Total distance traveled as well as average speed was measured. To assess for anxiety related behaviors, the percent of time in the center of the chamber was also recorded. Mice spending more time in the center are generally described as less anxious (Crawley, 2008).

Buried food

To assess olfaction, a buried food test was performed (Allan et al., 2008). Two days prior to testing, mice were placed on a food restricted diet (0.5 g of mouse chow/mouse/day). On each of the four days of testing, mice were placed in a standard housing cage with 3 cm of bedding. Latency to find a buried 0.5 g pellet in the bedding was recorded. Food pellet location was changed for each trial.

Social vs. inanimate preference

The social test apparatus consisted of a 60×40×35(h) cm plywood chamber lined with white contact paper and a plexiglass bottom. The chamber was evenly divided into three sections by plexiglass partitions with a 5×8 cm opening in the center. On one side of the chamber, a non-familiar female mouse was placed in an inverted wire mesh cage (stranger mouse). An empty inverted wire mesh cage (inanimate object) was on the opposite side of the chamber. A weight was placed on the top of each of the cages to prevent the test mice from tipping the cage over. The test mouse was placed in the center chamber with the partitions closed off to the other chambers and allowed to acclimatize for 10 min. At the initiation of the test, the partitions were removed and the mouse was allowed to freely explore all three chambers. Mice were video-recorded and the time spent in each chamber was recorded using ANY-maze software (Stoelting Wood Dale, IL).

Preference for social novelty

The preference for social novelty test immediately followed the social vs. inanimate preference test. In the chamber with the empty wire mesh cage (inanimate), a novel unfamiliar female mouse was placed in the mesh cage (novel). The previous stranger mouse remained in the opposite chamber (familiar). The test lasted for 10 min and was video-recorded. The time spent in each chamber was recorded using ANY-maze software (Stoelting Wood Dale, IL). The chamber was wiped down with 95% ethanol between each test mouse.

Inkblot

Gait was evaluated by using inkblot analysis. Non-toxic ink was placed on the fore (red) and hind (black) paws of the mouse. The mouse was made to walk down a dark tunnel. The average length and width of the steps were measured.

RotaRod

Motor deficits were evaluated by measuring latency to fall (180 s max) on an accelerating (4–40 rpm over 200 s) ENV-576M RotaRod (Med Associates, Georgia, VT). Two trials were conducted on one day with approximately 2 h between trials. The average of the two trials was used in the analysis.

Light/dark box

The light/dark box was a 60×40×35(h) cm plywood chamber with a plexiglass bottom and lined with contact paper. The chamber was divided by a plexiglass partition with a 5×8 cm opening in the center. The light side was 40×40 cm and lined with white contact paper. The dark side was 20×40 and was lined with black contact paper and covered. Mice were placed in the light side and allowed to freely explore for 10 min. ANY-maze software (Stoelting Wood Dale, IL) tracked the mice.

Morris water maze

Spatial memory was assessed using the standard hidden platform Morris water maze. Mice were given four trials a day for five days. Each trial began from each of four random starting positions. Mice were given a maximum of 60 s to find the platform. If a mouse failed to find the platform after 60 s, it was lead there. Mice were allowed to remain on the platform for 10 s before being placed in a 37 °C warming cage between trials. The intertrial interval was 4 min. 24 h following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 s. Latency to first platform location and total number of platform crossings were recorded using tracking software (Ethovision, Noldus Information Technology, Leesbury, VA, USA).

Reversal water maze

To measure resistance to change, the reverse Morris water maze was performed one week after the Morris water maze. The location of the platform was changed with respect to the original Morris water maze. Mice were given four trials a day for four days to learn the new location of the platform. 24 h following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 s. Latency to first platform location and total number of platform crossings were recorded.

Visual water maze

Vision was assessed using a visual Morris water maze. Upon completion of the reverse water maze, a white brick was placed on the platform to make it visible. Mice were given three trials to find the visible platform.

Immunohistochemistry

Mice were anesthetized with 2.5% avertin and transcardially perfused with PBS and then 4% paraformaldehyde (PFA). Brains and eyes were extracted, post fixed overnight in 4% PFA, dehydrated, embedded in paraffin, and sectioned at 5 μm. Sections were rehydrated and subjected to antigen retrieval in a microwave with 10 mM

sodium citrate buffer, pH 6. Sections were blocked with 10% goat serum and 0.5% Triton X-100 in 1× PBS for 20 min. Slides were incubated in primary antibody solution overnight at 4 °C. Sections were then washed in 1× PBS and incubated with secondary antibody for 1 h at room temperature. Sections were then washed in 1× PBS and incubated with Hoechst 33258 (Invitrogen, Carlsbad, CA) and coverslipped with Fluoromount G (Southern Biotech, Birmingham, AL). Imaging was performed with an Olympus IX81 microscope. Images were obtained with a Qimaging RETIGA-2000RV camera and processed with Adobe Photoshop (San Jose, CA). Confocal images of the retina were obtained using a TCS SP5 confocal laser microscope (Leica, Wetzlar, Germany).

Antibodies

The primary antibodies used were: Calbindin (1:250; Sigma-Aldrich, St. Louis, MO), pax6 (1:200; Covance, Emery Ville, CA), GS (1:300; BD Biosciences, Franklin Lakes, NJ), PKCa (1:500; Millipore, Billerica, MA), R4D2 (1:200; Molday, 1983), and Cone Arrestin (1:200; Connie Cepko, Harvard Medical School, Boston, MA). Secondary antibodies (1:250; Invitrogen, Carlsbad, CA) were: Alexa Fluor 488 (anti-rabbit IgG) (anti-mouse IgG_1), and Alexa Fluor 555 (anti-rabbit IgG) (anti-mouse IgG_1) (anti-mouse IgG_{2a}).

X-gal staining

For X-gal staining, *Rosa26* reporter mice were crossed with *Pcp2-Cre* mice. Mice were first anesthetized with 2.5% avertin and then transcardially perfused with PBS and then 4% PFA. Brains were extracted, post fixed for 1 h in 4%PFA, then washed in 1× PBS overnight at 4 °C. Brains were then transferred to 30% sucrose overnight at 4 °C and then embedded in OCT and stored at −80 °C. Sagittal sections (18 μm, 3 per slide) were made using a cryostat. Slides were washed in Rinse Buffer (100 mM sodium phosphate, pH7.3, 2 mM $MgCl_2$) and then incubated in Rinse Buffer Plus (Rinse buffer plus 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal) for 2 h at 37 °C. Slides were then rinsed in 1× PBS, counterstained with eosin, dehydrated, and coverslipped.

Rapamycin treatment

Rapamycin (MP Biomedicals, Solon, OH) was dissolved in ethanol and stored in a 1 mg/ml stock solution at −20 °C. A working solution was diluted in PBS before each use. Beginning at postnatal day 10 (P10), male mice were given IP (intraperitoneal) injections three times per week with 2 mg/kg rapamycin. Injections continued until completion of behavior testing.

Statistical analysis

Statistical analyses were conducted using analysis of variance (ANOVA) followed by Tukey post-hoc comparisons to compare the results of the Tsc2f/+, Tsc2f/−, and Tsc2f/−;Cre genotypes. For social preference and social novelty, a t-test was conducted to examine the difference between time spent in the social and inanimate object chambers. Statistical significance is claimed when $p<0.05$. However, data reported with a $p<0.1$ is also reported as possibly relevant. Error bars are shown as standard error of the mean.

Results

Purkinje cell loss

Purkinje cell specific homozygous deletion of *Tsc2* causes Purkinje cell loss as previously described (Reith et al., 2011) (Figs. 1A–D). Behavioral testing was conducted on Tsc2f/+, Tsc2f/−, and Tsc2f/−;Cre mice. All behavior testing was performed between one and three months of age, the timeline is shown in Table 1. At one month of age, the mice were given a general physical examination (Crawley, 2008). All mice were healthy and had normal reflexes (eye blink, ear twitch, whisker twitch, grasping, and forepaw reach).

Since autism has also been associated with dysfunction in the frontal cortex and thalamus (Binder, 1997; Binder et al., 1997; Cheon et al., 2011; Egawa et al., 2011; Fletcher et al., 1995; Frith and Frith, 1999; Tamura et al., 2010; Tsatsanis et al., 2003), we performed X-gal staining on PCP-ROSA mice at two months of age. Examination of the frontal cortex and thalamus revealed no ectopic Cre expression. There were some Cre positive cells in the dentate gyrus consistent with previous studies (Barski et al., 2000). As a positive control, X-gal staining was also detected in the Purkinje cells (Supplemental Fig. 1). Since Pcp2-Cre expression also occurs in retinal bipolar cells (Barski et al., 2000), it was important to assess vision and retinal histology. Furthermore, retinal cell type-specific staining at five months of age detected no difference across the seven cell types of the retina including the bipolar cells in Tsc2f/f;Cre mice (Supplemental Fig. 2).

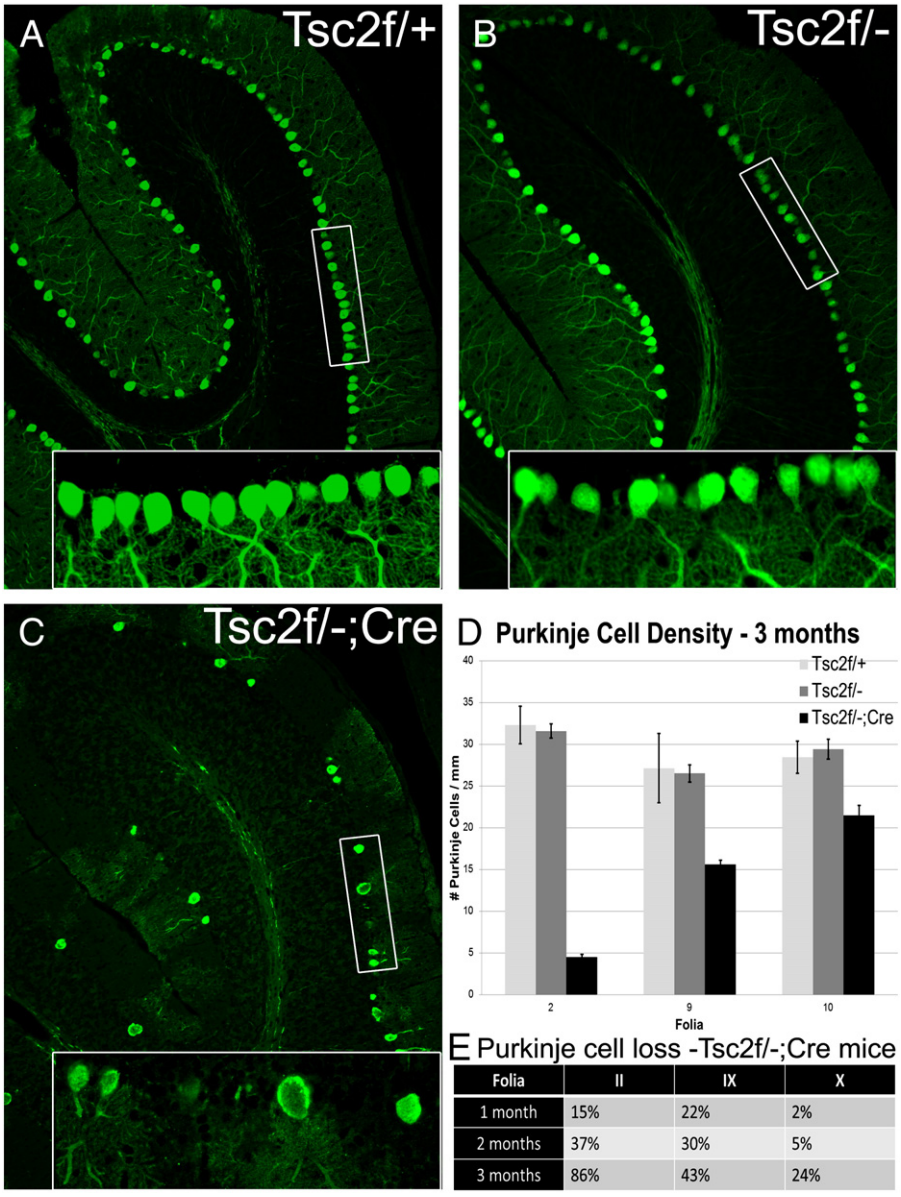
Purkinje cell loss is a progressive phenotype in the Tsc2f/−;Cre mice within the testing interval. The progressive decrease in Purkinje cell density in selected folia is shown (Fig. 1E). Interestingly, *Tsc2* haploinsufficiency did not affect Purkinje cell viability, as Tsc2f/− mice did not have any Purkinje cell loss at three months of age (Fig. 1B). Gross motor coordination was examined using an open-field arena. There was no difference in average speed over a 30 minute interval in any of the groups (Fig. 2A). At two months of age, gait analysis (using inkblot testing) indicated that Tsc2f/−;Cre mice had a slightly wider gait compared to controls ($p=0.079$) (Fig. 2B). However, there was no significant difference in latency to fall among any of the groups on an accelerating RotaRod at two months of age (Fig. 2C). Some of the mice, though, were re-tested on the RotaRod at five months of age, after more Purkinje cell loss had occurred. At five months of age, Tsc2f/−;Cre mice had a significant ($p=0.029$) shorter latency to fall indicating more severe cerebellar dysfunction (Fig. 2D).

Repetitive behavior

Repetitive behavior is a well described feature of ASD (Association, 2000). The number of marbles that a mouse will bury in a specific time period is an established assay for repetitive behavior (Thomas et al., 2009). We determined the number of marbles buried in a 30 minute period by male and female mice of all genotypes. Male Tsc2f/−;Cre mice buried significantly more marbles than either Tsc2f/+ ($p=0.042$) or Tsc2f/− ($p=0.016$) mice (Fig. 3A). Similarly, female Tsc2f/−;Cre mice also buried more marbles than either Tsc2f/+ ($p=0.070$) or Tsc2f/− ($p=0.028$) mice (Fig. 3A). There was no difference between Tsc2f/+ and Tsc2f/− of either sex. These data show an increase in repetitive behavior associated with the loss of Tsc2 in Purkinje cells.

Social behavior testing

Impaired social behavior is a prominent feature of ASD (Association, 2000). A number of behavioral paradigms have been developed to asses social interactions in mice (Crawley, 2007). One widely used assay is the three chambered apparatus which has detected social deficits in multiple mouse models of autism (Moy et al., 2004; Kwon et al., 2006; Peca et al., 2011). The three chambered apparatus was used to determine sociability and social novelty preference of the mice at two months of age. We tested males and females separately to detect sex specific differences similar to that seen in human ASD. The stranger mouse was female. Male Tsc2f/+ mice spent significantly more time ($p<0.001$) in the chamber with the stranger mouse than the chamber with the inanimate object (Fig. 4A). Male Tsc2f/− mice also spent significantly more



Folia	II	IX	X
1 month	15%	22%	2%
2 months	37%	30%	5%
3 months	86%	43%	24%

Fig. 1. Loss of *Tsc2* causes Purkinje cell loss. (A–C) Calbindin staining at 3 months of age shows loss of Purkinje cells in the Tsc2f/−;Cre (C) compared to the Tsc2f/+ (A) and Tsc2f/− (B). (D) Quantitation of Purkinje cell density across Folia 2, 9, and 10 shows Purkinje cell loss in Tsc2f/−;Cre mice but not in Ts2f/− with respect to Tsc2f/+ mice. (E) Quantitation of percent Purkinje cell loss in Tsc2f/−;Cre mice with respect to Tsc2f/+ mice across Folia 2,9, and 10 throughout the difference time points of testing.

time ($p = 0.047$) in the chamber with the stranger mouse than the chamber with the inanimate object. Male Tsc2f/−;Cre mice, however, did not show a preference for the stranger mouse (Fig. 4A), Tsc2f/+ female mice spent more time ($p = 0.057$) in the chamber with the stranger mouse than the chamber with the inanimate object. However, both Tsc2f/− and Tsc2f/−;Cre females did not show a preference for either chamber (Fig. 4B). These data suggest abnormalities in sociability in the Tsc2f/− mice that increase upon deletion of the second copy of *Tsc2* in Purkinje cells.

When social novelty was assessed, male Tsc2f/+ mice spent significantly ($p = 0.014$) more time with the novel mouse than the familiar mouse (Fig. 4C). Male Tsc2f/− mice showed a slight preference for the novel mouse than the familiar mouse, but this was not statistically significant. However, male Tsc2f/−;Cre mice spent about equal time with the novel mouse as with the familiar mouse (Fig. 4C). Tsc2f/+ and Tsc2f/− female mice spent significantly ($p = 0.018$, $p = 0.0008$, respectively) more time with the novel mouse than with the familiar mouse. Female Tsc2f/−;Cre mice did not show significant preference for the novel mouse compared with the familiar mouse. The social novelty data are in agreement with the social preference testing, supporting a social behavioral deficit in Tsc2f/− mice that is exaggerated in Tsc2f/−;Cre mice.

To ensure that social deficits were not due to lack of olfaction, olfaction was assessed using a buried food test. The latency to find a buried food pellet was measured once a day for four days (Fig. 4E). There was no difference in latency to find food suggesting intact olfaction in all genotypes.

These results showed that loss of Purkinje cells (or loss of *Tsc2* in Purkinje cells) in a haploinsufficient environment led to social behavior deficits. A second hit event in a haploinsufficient environment mimics the LOH hypothesis for TSC patients. However, to isolate the effects of the Purkinje cells in the role of social behavior, male Tsc2f/f;Cre (only Purkinje cells affected by loss of *Tsc2*) mice were examined along with their littermate controls. In this case, male Tsc2f/+ mice showed a slight preference (NS) for time spent in the social chamber over time spent in the inanimate object chamber (Fig. 5A). However, male Tsc2f/f;Cre mice

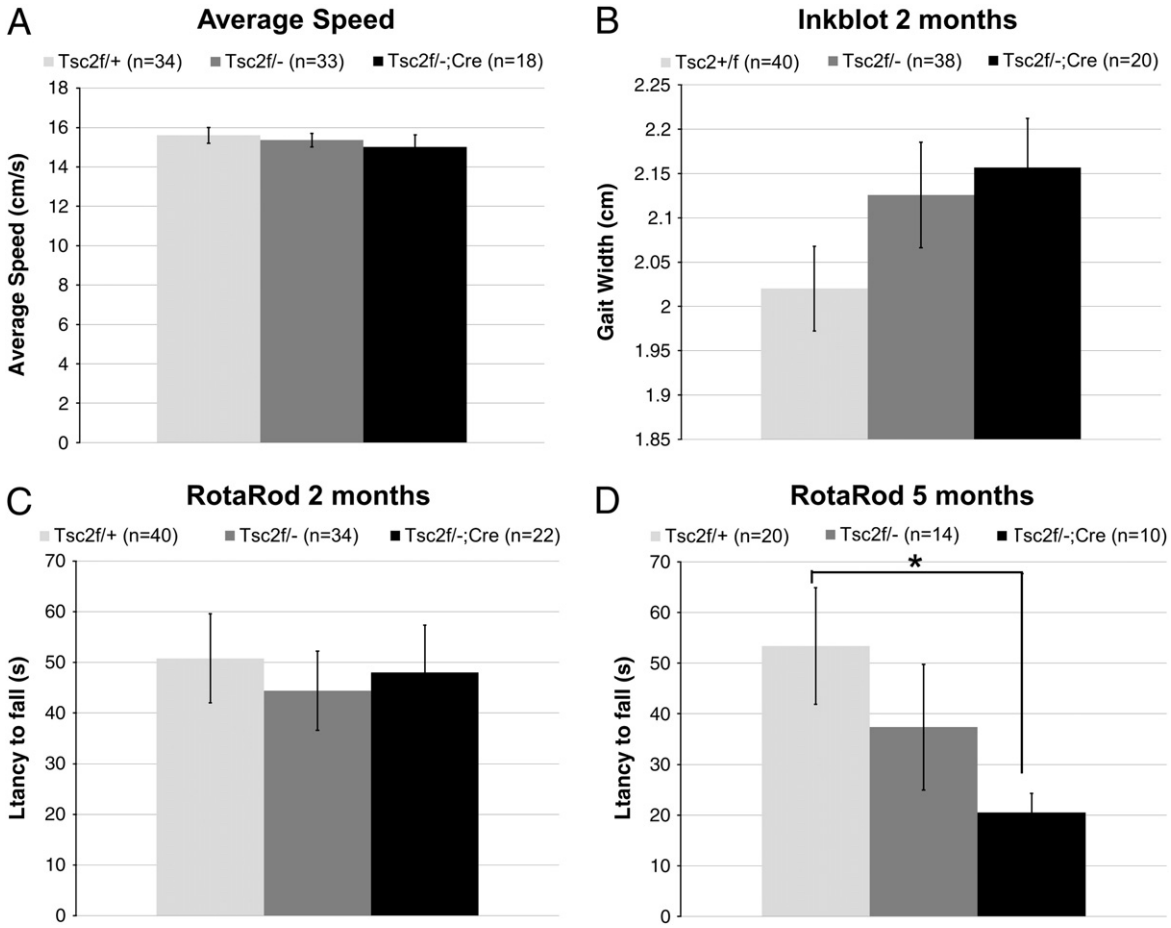


Fig. 2. Motor function. (A) Average speed in an open-field did not differ between Tsc2f/+, Tsc2f/−, and Tsc2f/−;Cre mice. (B) Gait width in Tsc2f/−;Cre mice was slightly increased ($p=0.079$) compared to Tsc2f/+. (C) RotaRod performance at 2 months of age shows no difference in latency to fall among Tsc2f/+, Tsc2f/−, and Tsc2f/−;Cre. (D) RotaRod performance at 5 months of age shows a significantly ($p=0.029$) shorter latency to fall in Tsc2f/−;Cre mice compared to Tsc2f/+ mice.

spent equal time in both chambers (Fig. 5A). When assessing social novelty, male Tsc2f/+ mice spent significantly ($p=0.0029$) more time in the chamber with the novel mouse than the familiar mouse (Fig. 5B).

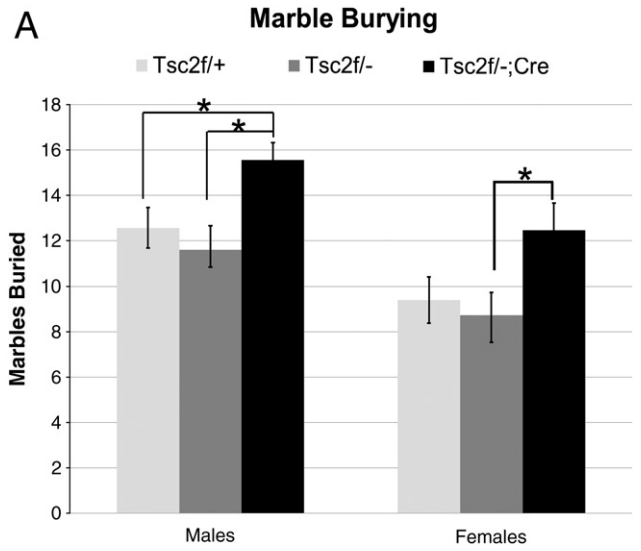


Fig. 3. Repetitive behaviors. (A) Male Tsc2f/−;Cre ($n=11$) mice buried significantly more marbles than either Tsc2f/+ ($n=23$) ($p=0.043$) or Tsc2f/− ($n=20$) ($p=0.016$) mice. Female Tsc2f/−;Cre ($n=11$) mice buried more marbles than either Tsc2f/+ ($n=20$) ($p=0.070$) or Tsc2f/− ($n=19$) ($p=0.028$) mice.

However, Tsc2f/f;Cre mice spent equal time in both chambers (Fig. 5B). These results suggest that isolated Purkinje cell pathology due to *Tsc2* loss can cause social deficits.

Inhibition of mTORC1 prevents social behavior deficits

Previous characterization of Tsc2f/−;Cre mice showed increased phosphorylated S6 (pS6) expression in Purkinje cells, indicating increased activation of mTORC1 (Reith et al., 2011). Furthermore, treatment with the mTORC1 inhibitor, rapamycin, was able to prevent Purkinje cell death (Reith et al., 2011). Therefore, we sought to investigate if rapamycin treatment could affect the social behavior deficits in male mice. Both rapamycin and vehicle treated mice of all genotypes had intact reflexes and no differences were noted on olfaction (data not shown).

Importantly, vehicle treated Tsc2f/+ mice retained preference for the chamber with the social mouse rather than the chamber with the inanimate object ($p=0.0076$) (Fig. 6A). Vehicle treated Tsc2f/−;Cre mice, however, did not show a preference for the social chamber (Fig. 6A), validating the social behavioral deficit noted in untreated Tsc2f/−;Cre mice. Notably, rapamycin treated Tsc2f/−;Cre mice did show preference for the social chamber over the chamber with the inanimate object ($p=0.013$) (Fig. 6A), indicating that rapamycin treatment prevented the social behavioral deficit. However, rapamycin treated Tsc2f/+ mice did not show significant preference for the social chamber (Fig. 6A).

When we tested for social novelty, vehicle treated Tsc2f/+ mice still showed preference for time spent in the chamber with the

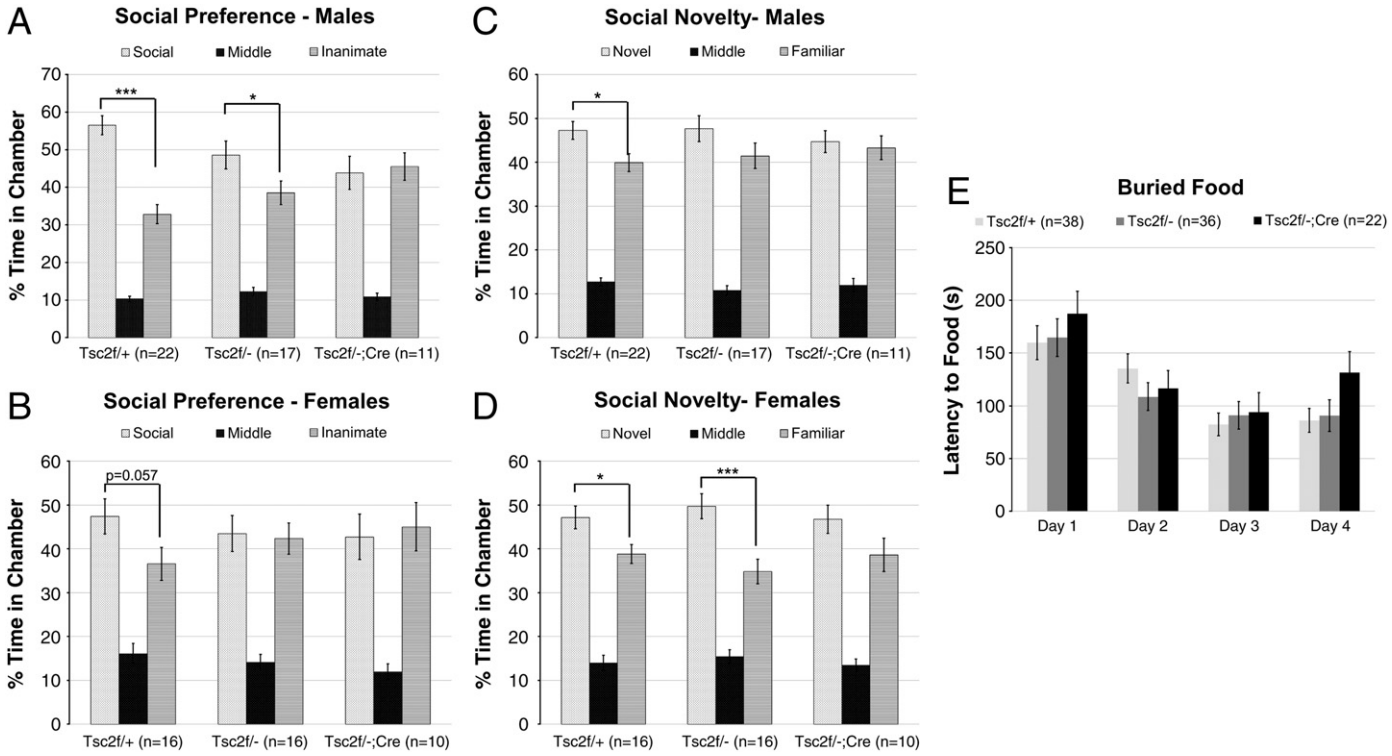


Fig. 4. Social behavior deficits in Tsc2f/−;Cre mice. (A) Male Tsc2f/+ mice spent more time ($p<0.001$) in the social chamber than in the inanimate object chamber. Tsc2f/− spent more time ($p=0.047$) in the social chamber. However, Tsc2f/−;Cre mice spent equal time in both chambers. (B) Female Tsc2f/+ mice spent more time ($p=0.057$) with the stranger mouse than with the inanimate object. Conversely, both Tsc2f/− and Tsc2f/−;Cre mice spent equal time in both chambers. (C) Male Tsc2f/+ mice spent more time ($p=0.014$) with a novel mouse than with a familiar mouse. Tsc2f/− mice showed a slight preference (NS) for time spent with the novel mouse. However, Tsc2f/−;Cre mice spent equal time in both chambers. (D) Female Tsc2f/+ ($p=0.018$) and Tsc2f/− ($p=0.0008$) mice spent more time with the novel mouse than the familiar mouse. Tsc2f/−;Cre mice also spent slightly more time (NS) with the novel mouse than with the familiar mouse. (E) Latency to buried food as a measure of olfaction. There was no significant difference in latency to the food across any of the trials.

novel mouse than with the familiar mouse ($p=0.031$) (Fig. 6B). Vehicle treated Tsc2f/−;Cre mice, however, did not show preference for social novelty (Fig. 6B), confirming the results obtained in untreated mice. Again, rapamycin treated Tsc2f/−;Cre mice demonstrated a preference for social novelty, spending significantly more time in the chamber with the novel mouse than in the chamber with the familiar mouse ($p=0.022$) (Fig. 6B). Rapamycin treated Tsc2f/+ mice also displayed preference for social novelty, spending more time with the novel mouse than the familiar mouse ($p=0.048$) (Fig. 6B).

Anxiety behaviors

Anxiety levels are often increased in patients with ASD (Brereton et al., 2006; Hofvander et al., 2009; Skokauskas and Gallagher, 2012). To determine anxiety levels, mice were place in an open-field arena and the percentage of time spent in the middle was determined. Anxious animals spent more time along the perimeter of the chamber rather than the middle. Female Tsc2f/−;Cre mice spent slightly less time (NS) in the middle than Tsc2f/+ and Tsc2f/− mice, possibly suggesting

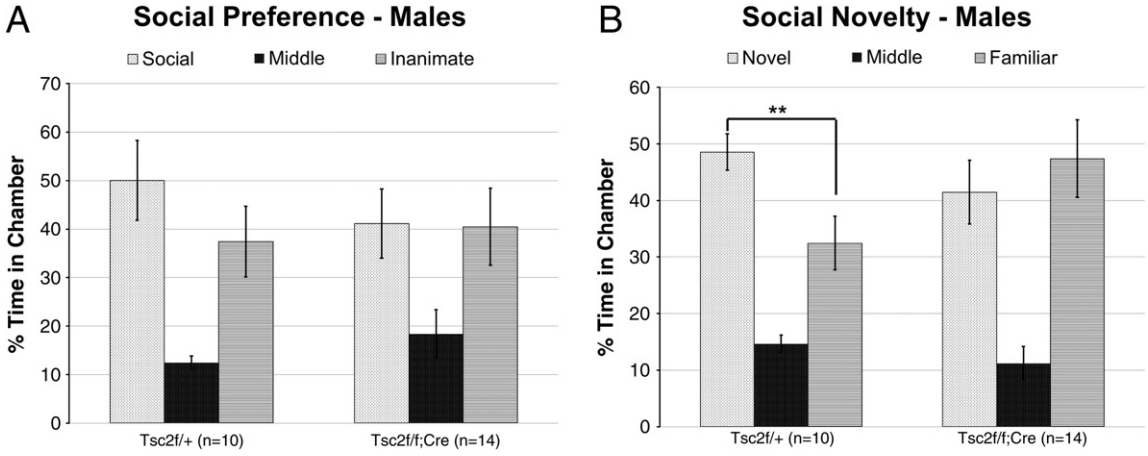


Fig. 5. Social behavior deficits in Tsc2f/f;Cre mice. (A) Male Tsc2f/+ mice spent slightly more time (NS) in the social chamber than in the inanimate object chamber. However, Tsc2f/f;Cre mice spent equal time in both chambers. (B) Male Tsc2f/+ mice spent more time ($p=0.0029$) more time with a novel mouse than with a familiar mouse. However, Tsc2f/f;Cre mice spent equal time in both chambers.

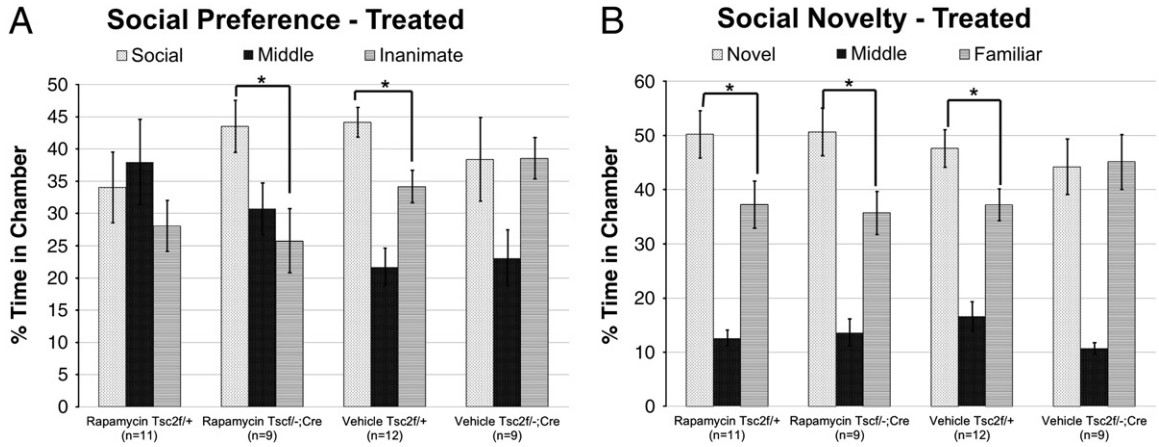


Fig. 6. Rapamycin prevents social behavior deficits. (A) Vehicle treated Tsc2f/+ mice spent more time ($p=0.0076$) in the social chamber than in the inanimate object chamber. However, vehicle treated Tsc2f/−;Cre mice spent equal time in both chambers. With rapamycin treatment, Tsc2f/−;Cre mice spent more time ($p=0.013$) in the social chamber than in the inanimate object chamber. However, rapamycin treated Tsc2f/+ mice did not show a preference for the social chamber. (B) Vehicle treated Tsc2f/+ mice spent more time ($p=0.031$) with a novel mouse than with a familiar mouse. However, vehicle treated Tsc2f/−;Cre mice spent equal time in both chambers. With rapamycin treatment, Tsc2f/−;Cre mice spent more time ($p=0.022$) with a novel mouse than with a familiar mouse. Rapamycin treated Tsc2f/+ mice also spent more time ($p=0.048$) with a novel mouse than with a familiar mouse.

increased anxiety levels (Fig. 7A). Conversely, male Tsc2f/−;Cre mice spent slightly (NS) more time in the middle of the chamber than Tsc2f/+ and Tsc2f/− (Fig. 7B), possibly suggesting decreased anxiety levels. These data suggest sex-specific differences in anxiety-related behaviors.

Anxiety levels were also assessed in treated mice (all males). Vehicle treated Tsc2f/−;Cre mice spent more time in the middle of an open-field arena than vehicle treated Tsc2f/+ mice ($p=0.059$) and rapamycin treated Tsc2f/−;Cre mice ($p=0.0078$) (Fig. 7C). This reaffirms that Tsc2f/−;Cre males have decreased levels of anxiety which was also noted in untreated mice. However, when compared to untreated mice, all treatment groups (both vehicle and rapamycin) spent less time in the middle, suggesting that added handling and/or injections exacerbated anxiety levels (Figs. 7B–C).

Spatial learning and memory

There is a high association of intellectual disability and ASD, and the severity of ASD tends to parallel the severity of intellectual disability (Matson and Shoemaker, 2009). Therefore, to assess for deficits in spatial learning and memory, mice were trained on a Morris water maze. Tsc2f/−;Cre mice did not show any deficits in spatial learning over a five day training interval (Supplemental Fig. 3A). One aspect of restricted behaviors often seen in patients with ASD is resistance to change (Coldren and Halloran, 2003; Corbett et al., 2009). To test this, we measured reversal learning on the Morris water maze by changing the location of the hidden platform after the acquisition phase. Tsc2f/−;Cre mice did not show any deficits in reversal learning (Supplemental Figs. 3B–C). We performed a vision dependent Morris water maze at three months of age to determine if the animals could navigate to a visible platform. The latency to locate the visible platform was measured across three trials (Supplemental Fig. 3D). All mice found the platform in a similar time demonstrating comparable visual acuity for the specific test.

Discussion

Between 17% and 60% of patients with TSC have ASD (de Vries et al., 2007; Smalley et al., 1992; Wong, 2006), a much higher prevalence than in the general population. This high comorbidity underscores the importance of understanding how mutations in either *TSC1* or *TSC2* lead to the ASD phenotype. Genetically altered mice have been important tools in dissecting out the link between these two disorders (Chevere-Torres et al., 2012; Ehninger et al., 2012; Goorden et al., 2007; Tsai et al., 2012; Waltereit et al., 2011; Young et al., 2010). Here we have developed and behaviorally characterized a new mouse model of TSC-associated ASD. These mice have increased repetitive behaviors and social behavior deficits. More

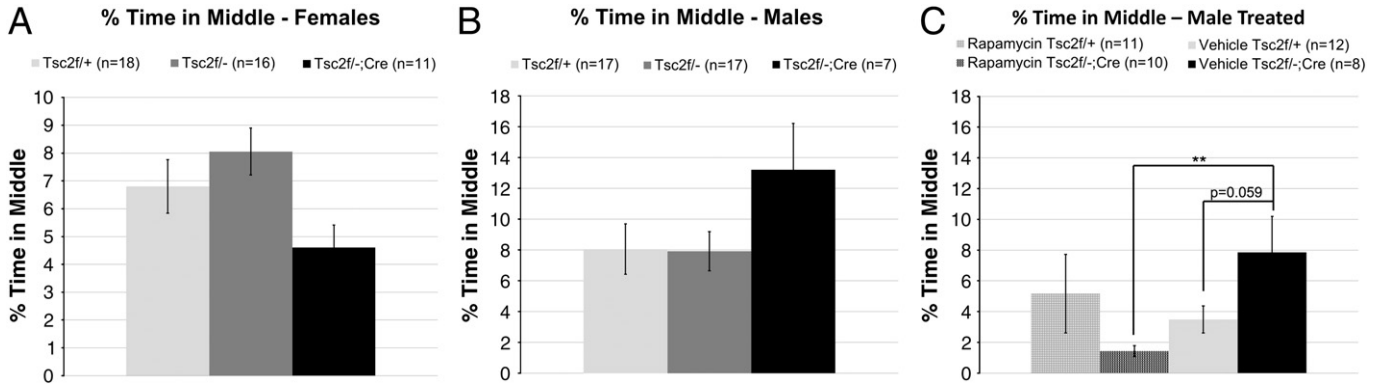


Fig. 7. Anxiety behaviors. (A) In an open-field arena, female Tsc2f/−;Cre mice spent less time in the middle (NS) than either Tsc2f/+ or Tsc2f/− mice. (B) However, male Tsc2f/−;Cre mice spent more time (NS) in the middle than either Tsc2f/+ or Tsc2f/− mice. (C) Vehicle treated male Tsc2f/−;Cre mice spent more time in the middle of an open-field chamber than vehicle treated Tsc2f/+ ($p=0.059$) or rapamycin treated Tsc2f/−;Cre ($p=0.0078$) mice. However, both genotypes of both treatment groups spent less time in the middle of an open-field arena than untreated male mice (B).

importantly, our data supports the hypothesis that loss of heterozygosity (LOH) of *Tsc2* in cerebellar Purkinje cells and/or frank Purkinje cell loss contributes to ASD-like behavior. Furthermore, these behaviors are prevented by rapamycin treatment.

Since there is mounting evidence that cerebellar abnormalities play a role in ASD, particularly Purkinje cell loss (Bailey et al., 1998a; Fatemi et al., 2002, 2012; Palmen et al., 2004; Vargas et al., 2005; Yip et al., 2009), we behaviorally characterized a previously generated mouse strain with Purkinje cell specific *Tsc2* loss. Tsc2f/−;Cre mice lose the remaining copy of *Tsc2* in all Purkinje cells by one month of age, when Purkinje cells begin to progressively die. Here we demonstrate that Tsc2f/−;Cre mice have more severe social deficits than the haploinsufficient group (Tsc2f/−). These data reveal a role for *Tsc2* in Purkinje cells that is important for normal murine sociability. The mechanism of this observation is unclear and warrants further study. The cerebellum is thought to be involved in higher order processes similar to its role in motor coordination. It has been postulated that in order to decode someone else's actions (like in social behavior), sub-threshold activation of your own actions is required (Hoke et al., 2007; Wolpert et al., 2003). This behavior is believed to be modulated by the connections of the cerebellum to the prefrontal cortex (Kelly and Strick, 2003; Krienen and Buckner, 2009; Rogers et al., 2011). Purkinje cells are the sole inhibitory output of the cerebellum, synapsing on the deep cerebellar nuclei (Saab and Willis, 2003). The deep cerebellar nuclei then relay projections through the thalamus to various cortical regions including the prefrontal cortex, a region important in autism pathology (Gonzalo-Ruiz and Leichnetz, 1990; Middleton and Strick, 2000, 2001; Yamamoto et al., 1992). This circuit is altered or abolished with loss of *Tsc2* in Purkinje cells or frank Purkinje cell loss. It is unclear if complete loss of Purkinje cells per se and/or dysfunctional *Tsc2*-null remaining Purkinje cells are important for this autistic-related phenotype. The assessment of sociability at different time-points may reveal whether dysfunctional Purkinje cells and/or Purkinje cell loss per se cause the behavioral deficits.

Mild social deficits were detected in heterozygous Tsc2f/− mice suggesting that haploinsufficiency of *Tsc2* is sufficient to cause a behavioral phenotype. These results add to the murine behavioral deficits detected in haploinsufficient *Tsc1* or *Tsc2* backgrounds (Ehninger et al., 2012; Goorden et al., 2007; Tsai et al., 2012; Waltereit al., 2011; Young et al., 2010). Goorden et al. (2007) detected social behavioral deficits in a $Tsc1^{+/-}$ model even in the absence of seizures and cerebral lesions. Tsai et al. (2012) also recently detected social behavior deficits in $Tsc1^{+/-}$ mice (Tsai et al., 2012). It is known, however, that patients with mutations in *TSC2* have a more severe neurological phenotype including an increased risk for autism (Au et al., 2007; Dabora et al., 2001). Animals haploinsufficient for *Tsc2* showed social behavioral deficits when combined with seizures (Waltereit et al., 2011) or gestational immune activation (Ehninger et al., 2012). The association of haploinsufficiency of *Tsc1/2* and behavioral deficits is compelling, though the precise cellular mechanisms remain obscure. Haploinsufficiency of *Tsc1* alters dendritic spine structure in vitro by increasing dendritic length and decreasing spine density (Tavazoie et al., 2005), possibly leading to altered cellular input. Also, haploinsufficiency of *Tsc2* leads to growth cone collapse and subsequent abnormalities in axonal pathfinding (Nie et al., 2010) leading to altered cellular output. These effects could possibly induce social behavioral deficits. Furthermore, complete loss of *Tsc1/2* leads to cell migration abnormalities and reduced myelination (Astrinidis et al., 2002; Meikle et al., 2007; Way et al., 2009; Zhou et al., 2011). Haploinsufficiency might cause subtle abnormalities in the position or signaling of neurons, possibly affecting connectivity and leading to abnormal behavior.

Contradictory to the data presented here, Ehninger et al. (2012) reported that $Tsc2^{+/-}$ mice do not have social deficits in the three chambered apparatus (Ehninger al., 2012; Ehninger et al., 2008). Their model, however, was on a pure C57BL/6 background. Interestingly, Goorden et al. and Tsai et al. (2012) did detect social deficits in a C57BL6 $Tsc1^{+/-}$ model (Goorden et al., 2007; Tsai et al., 2012). Perhaps, however, there are modifier genes in the different strains that contribute to the behavioral effect. This could partially explain why ASD is not 100% penetrant in patients with TSC, suggesting additional influences in the development of behavioral deficits.

Treatment with the mTORC1 inhibitor, rapamycin, ameliorates social behavior deficits in Tsc2f/−;Cre mice. These results indicate that rapamycin can have a positive effect on social behavior deficits associated with TSC. However, caution needs to be taken as rapamycin induced a deficit in social preference in Tsc2f/+ mice. Given that mTOR is an essential gene for proper cellular function (mTOR knockout mice are embryonic lethal; Gangloff et al., 2004; Murakami et al., 2004), it is reasonable to expect adverse consequences of mTORC1 inhibition, if starting at control levels.

Treatment also seemed to cause increased anxiety. Not only was this detected in the open-field arena, but it was also observed in the general handling of the mice. This effect occurred in both rapamycin and vehicle treated animals. Although vehicle treated male Tsc2f/−;Cre mice were still less anxious than the other treatment groups, they were notably more anxious than their untreated counterparts. Because the effect was not isolated to rapamycin therapy, it is likely to be a generalized effect. Mice were given two behavior tests and three injections per week. Therefore, when they were handled, they did not know which of these two events was about to take place. Therefore, one hypothesis is that they associated handling with the pain caused from the injection, leading to the increased anxiety.

Rosa26 Cre expression studies suggest that the behavioral phenotype observed was most likely Purkinje cell specific event as we did not find β-galactosidase-positive cells in the thalamus and cortex, suggesting that Cre recombinase was not active. Though we cannot exclude the possibility that ectopic Cre expression in the cortex may be contributing to ASD-like behaviors in the mice, Cre expression is largely focused in the Purkinje cells.

The data suggest that both *Tsc2* haploinsufficiency and Purkinje cell loss contribute to the autistic-like behavioral deficits. To test whether Purkinje cell loss in a wildtype background could also lead to social deficits, Tsc2f/f;Cre mice were subjected to behavioral testing. Tsc2f/f;Cre mice only lose *Tsc2* in Purkinje cells, and all remaining cells are Tsc2f/f which is equivalent to $Tsc2^{+/+}$ (Hernandez et al., 2007). Tsc2f/f;Cre mice also demonstrated social deficits, suggesting that Purkinje cell loss/dysfunction is sufficient to induce social deficits. However, the Tsc2f/+ littermates also did not show significant social preference (though they did retain preference for social novelty). There is a slight strain difference between Tsc2f/−;Cre mice and Tsc2f/f;Cre mice. Tsc2f/−;Cre mice and respective controls are approximately 74% C57BL/6J and 26% 129X1/SvJ. Tsc2f/f;Cre mice and respective controls are approximately 62% C57BL/6J and 38% 129X1/SvJ. While C57BL/6J mice are noted to have normal social behavior, 129 mice show abnormalities in social behavior tasks (Moy et al., 2007). Therefore, Tsc2f/+ mice, with increased percentage of 129X1/SvJ stain, do not show significant social preference.

Repetitive behavior is another hallmark of ASD (Association, 2000). We detected increased marble burying activity in Ts2f/−;Cre mice, indicating increased repetitive behaviors. Interestingly, haploinsufficiency of *Tsc2* was not sufficient to cause an increase in repetitive behaviors, suggesting that either complete loss of *Tsc2* in Purkinje cells and/or Purkinje cell loss is required for this phenotype. The role of the cerebellum in repetitive behaviors may lie in its role to coordinate motor functions. Since dysfunction and/or loss of GABAergic Purkinje cells leads to decreased inhibitory efferents to the deep cerebellar nuclei and consequently other parts of the brain, this could lead to behavioral disinhibition. It has been hypothesized that autistic patients are constantly in a state of overstimulation (Kennedy et al., 2006). Therefore, performing repetitive behaviors may have a calming effect on this overstimulated state (Guess and Carr, 1991).

Although autism occurs in a 4:1 male female ratio in the general population (Bertrand et al., 2001), TSC-associated autism occurs in a

1:1 male female ratio (Curatolo et al., 2012; Numis et al., 2011; Smalley et al., 1992; Wiznitzer, 2004). However, a genotype–phenotype study found that male TSC patients had more severe neurological findings (Au et al., 2007). In our mouse model correlate, we find autistic-like behaviors in both male and female mice. However, the male mice show the greater increase in social novelty deficits suggesting that gender may influence the severity of these characteristics. How sex affects the neurologic phenotypes remains unclear.

Finally, we show that there are no learning deficits in Tsc2f/−;Cre mice. This finding was a bit surprising given the reports of learning deficits in *Tsc2*$^{+/-}$ mice (Ehninger et al., 2008). Ehninger et al. measured time spent in quadrant as an indicator of learning deficits, while we examined latency to the platform. Another possible explanation behind these conflicting results might be due to strain differences of the mice. Ehninger et al. (2012) conducted their studies on a C57BL/6NCrl background. Our studies, however, are on a mixed C57Bl6/129 background. Therefore, there are likely modifier genes contributing to this effect. In support of our findings, Eker rats, a heterozygous Tsc2$^{+/-}$ based model, also show no evidence for learning deficits (Waltereit et al., 2011).

In summary, we demonstrate that deletion of *Tsc2* in Purkinje cells, leads to social behavior deficits and increased repetitive behaviors. These studies establish a novel mouse model of TSC-associated autism that will allow for the exploration of cerebellocortical projections and their ability to modulate autistic-like behaviors. This mouse model also paves the way for potential therapeutic targets aimed at preventing Purkinje cell degeneration and ameliorating behavioral deficits. These pre-clinical studies have exciting implications for the future treatment of TSC-associated ASD.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2012.10.014.

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