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Impaired Social Interactions and Motor Learning Skills in Tuberous Sclerosis Complex Model Mice Expressing a Dominant/Negative Form of Tuberin

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Abstract

Tuberous sclerosis complex (TSC) is a genetic disorder characterized by the development of hamartomas in multiple organs. Neurological manifestation includes cortical dysplasia, epilepsy, and cognitive deficits such as mental impairment and autism. We measured the impact of TSC2-GAP mutations on cognitive processes and behavior in, Δ RG transgenic mice that express a dominant/negative TSC2 that binds to TSC1, but has mutations affecting its GAP domain and its rabaptin-5 binding motif, resulting in inactivation of the TSC1/2 complex. We performed a behavioral characterization of the Δ RG transgenic mice and found that they display mild, but significant impairments in social behavior and rotarod motor learning. These findings suggest that the Δ RG transgenic mice recapitulate some behavioral abnormalities observed in human TSC patients.

Keywords

tuberous sclerosis complex; autism; TSC2; GAP domain; mTORC1; social interaction; motor skills; reversal learning; spatial learning

Introduction

Tuberous sclerosis complex (TSC), first described as Bourneville's Disease in the 1880s, is a genetic disorder that is manifested early in childhood. TSC is characterized by the development of hamartomas (benign tumors) and tubers in multiple organs, including the skin, retina, heart, kidney, lung, and brain (Curatolo et al., 2008). It is postulated that the presence of these tubers in the brain contributes to the neurological abnormalities in the disease, which includes cortical dysplasia, subependymal giant cell astrocytomas (SEGA), seizures, mental impairment, attention deficit hyperactivity disorder (ADHD), and autism (de Vries et al., 2009; Orlova and Crino, 2010). At the molecular level, deletion or genetic mutations of the tumor suppressor genes hamartin (*tsc1*) and tuberlin (*tsc2*) have been identified as the cause of TSC in humans (Cheadle et al., 2000a; Cheadle et al., 2000b). It has been reported that *tsc2* gene mutations are more frequent and result in a more severe phenotype (i.e. seizures and learning disability) in TSC patients, with the exception of

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reported cases of patients with TSC but no mutation identified, as well as one *tsc2* mutation that causes a more mild phenotype (Camposano et al., 2009; Dabora et al., 2001; Jansen et al., 2006; Kwiatkowski et al., 2003). In addition, the *tsc2* gene is more prone to large deletions, rearrangements, and missense mutations than the *tsc1* gene. Of particular interest is the finding of missense mutations clustered within the *tsc2* exons 34–38 which encode for a region with homology to the GAP domain of rap1GAP or GAP3 (Maheshwar et al., 1997).

TSC2 is a GTPase-activating protein (GAP) that regulates the small G protein Rheb (Tee et al., 2003). It forms a heterodimer with TSC1 in an interaction that confers stability to both proteins (Chong-Kopera et al., 2006; Henske, 2003; Krymskaya and Shipley, 2003; Nellist et al., 1999). The TSC1/TSC2 heterodimer functions as a negative regulator of the protein kinase mammalian target of rapamycin (mTOR) (Fingar and Blenis, 2004; Jozwiak, 2006; Krymskaya, 2003), a key regulator of protein synthesis that is known to be critical for synaptic plasticity and memory (Hoeffer and Klann, 2010; Richter and Klann, 2009). Activation of the phosphatidylinositol 3-kinase (PI3K/Akt) and extracellular signal-regulated kinase (ERK) pathways results in the phosphorylation of TSC2 and inhibition of TSC2-GAP activity, thereby increasing the levels of Rheb-GTP. This type of signaling triggers the phosphorylation of the mTOR complex 1 (mTORC1) substrates p70 S6 kinase (S6K1) and eukaryote initiation factor 4E-binding protein (4E-BP), which are key translation initiation regulators (Jozwiak et al., 2005; Orlova and Crino, 2010; Yang et al., 2006). Consequently, loss or malfunction of either TSC1 or TSC2 results in hyperactivation of S6K1 and ribosomal protein S6 phosphorylation and as a result, defective regulation of cell size and proliferation (Krymskaya, 2003; Uhlmann et al., 2004). Moreover, studies in hippocampal pyramidal neurons have shown that the TSC pathway regulates soma size, the density and size of dendritic spines, and the properties of excitatory synapses (Tavazoie et al., 2005). In humans, analyses of TSC-associated lesions have shown aberrant hyperactivation of the mTORC1 signaling pathway as indicated by increased levels of phosphorylated S6K1, S6, and 4E-BP (Orlova and Crino, 2010).

Epilepsy is the most common neurological abnormality in TSC, occurring in 60 to 90% of individuals (Holmes and Stafstrom, 2007). Attention deficits also have been observed in TSC patients, with 50% of the individuals presenting ADHD (de Vries et al., 2009; Prather and de Vries, 2004). On the other hand, learning disability affects around 40% of individuals with TSC (Joinson et al., 2003). Studies have suggested that when TSC individuals suffer from learning disability, it tends to be severe and profound (Harrison and Bolton, 1997). In addition, sporadic cases of TSC with mutations in *tsc2* gene, are frequently associated with intellectual disabilities (Jones et al., 1997). Recent studies have shown that TSC1 and TSC2 heterozygous knockout mice have spatial learning deficits (Ehninger et al., 2008; Goorden et al., 2007).

The first description of autistic behavior in tuberous sclerosis patients was made in 1932 (Critchley and Earle, 1932). Subsequently, it has been estimated that TSC patients have high rates of autism, ranging from 20 to 60%, whereas 3–4% of autistic children may have TSC (Bolton et al., 2002; Curatolo et al., 2004; Smalley, 1998). Different candidate genes have been tested for their involvement in autism, and *tsc1/2* genes are of particular interest (Kwon et al., 2006; Wassink et al., 2004). In addition, an analysis of a family with both TSC and a high incidence of anxiety disorder suggested that alterations in the *tsc2* gene might predispose individuals to autism (Smalley et al., 1994). Interestingly, the *tsc2* gene is localized in the region of chromosome 16p13.3 which has been linked to bipolar affective disorder, epilepsy, and autism (Consortium, 1993; Daniels et al., 2001). However, the molecular basis of autism in TSC is still largely unknown, as is whether mutations that disrupt TSC1/2 function are associated with altered behaviors that would be consistent with mental impairment and autistic-like phenotypes. An array of social interaction paradigms are

now used in mice with targeted mutations to test the genetic and molecular basis underlying aspects of ASD (Moy et al., 2007; Silverman et al., 2010b). Behavioral analyses of TSC2 heterozygous knockout mice revealed normal social preference, whereas analyses of TSC1 heterozygous knockout mice have shown decreased social interaction (Ehninger et al., 2008; Goorden et al., 2007).

We studied mice expressing a dominant/negative TSC2 transgene (termed Δ RG transgenic mice) that binds to TSC1, but has mutations affecting its GAP domain and its rabaptin-5 binding motif (Govindarajan et al., 2005; Pasumarthi et al., 2000). The dominant negative TSC2 protein then displaces the endogenous protein and disrupts its GAP function and rabaptin-5 binding resulting in altered mTORC1 signaling (Govindarajan et al., 2005) and vesicle trafficking (Pasumarthi et al., 2000). In contrast to previous TSC mouse models, (Ess et al., 2004; Ghosh et al., 2006; Hernandez et al., 2007; Kobayashi et al., 1999; Onda et al., 2002; Piedimonte et al., 2006; Tavazoie et al., 2005; Uhlmann et al., 2004; Uhlmann et al., 2002; Wang et al., 2007; Wilson et al., 2006; Wilson et al., 2005; Zeng et al., 2011), Δ RG transgenic mice express the dominant negative TSC2 in all tissues, including the brain, making them an excellent model system to study the impact of TSC2 mutations on synaptic plasticity, learning and memory, and social behavior. Recent studies showed that the Δ RG mice have increased anxiety levels and impaired hippocampus-dependent memory (Ehninger and Silva, 2010). Herein we present a more complete behavioral characterization of Δ RG mice, showing that disruption of TSC2-GAP function results in behavioral abnormalities, including mild impairments in social behavior, motor learning skills, and spatial learning, consistent with TSC and autism in humans.

Materials and methods

Animals

Δ RG transgenic mice—Generation of Δ RG mice has been described previously (Govindarajan et al., 2005). Original breeders were provided by Dr. Jack Arbiser of Emory University School of Medicine. To generate experimental mice, Δ RG mice were mated with C57Bl/6 wild-type mice. Mouse genotyping was performed by PCR using transgene- and wild-type- specific primer sets. Mice were housed in groups of 2–3 animals per cage and kept on a 12 h light/dark cycle. Behavioral testing was performed on male Δ RG transgenic mice and their male wild-type littermates (2–6 months of age). For all experiments, mice were acclimated to the testing room one hr prior to behavioral training. The experimenter was blind to the genotype while performing the behavioral tasks. All behavioral tasks were performed starting with the least aversive task first (social behaviors) and ending with the most aversive (contextual fear conditioning). Food and water were available at all times. All procedures were approved by the New York University Animal Care and Use Committee and followed the NIH Guidelines for the use of animals in research.

Social interaction task—Sociability tendencies of Δ RG transgenic mice and their wild-type littermate mice were measured in a three-chambered social box (Crawley, 2004; Moy et al., 2007). Initially, mice received two habituation sessions (30 min each) in the social arena. First, they freely explored the empty social arena and then a second exploration was allowed in the presence of two wire cages, one in each of the chamber sides. A 10 min social preference test followed in which the mice were allowed to explore the three-chambered arena containing in one side, a caged mouse (social target) and a caged object (non-social target) in the other side. The placement of both social and non-social targets was counterbalanced between animals. The measured parameters of social interaction were time spent in each chamber and time spent sniffing each target, calculated by Ethovision XT video tracking software (Noldus). The following day the test mice were placed in the central

chamber and were subjected to a 10 min social novelty test. During this test a novel caged mouse replaced the previous non-social target. Doors between the chambers were opened and the test mice were given the choice to interact with a familiar mouse versus a novel mouse. Similar parameters were measured as in the social preference test. Direct reciprocal interaction was measured as previously described (Blundell et al., 2010). Briefly, mice were placed in a novel mouse cage and left to habituate for one hr under dim lights conditions for two consecutive days. On the third day, mice were placed back in the cage and allowed to directly interact with the young adult target mouse for three min. Time spent interacting with the target mouse was scored by an observer blind to genotype. A Student's *t*-test was used for statistical analysis with $p < 0.05$ as significance criteria.

Marble burying task—Mice were subjected to the marble burying task as previously described (Hoeffer et al., 2008; Thomas et al., 2009) to test for repetitive behavior. Briefly, mice were placed individually in clean cages containing fresh bedding (5 cm deep) and 20 black marbles arranged in five evenly spaced rows of four marbles each. Testing consisted of a 30 min period under white noise conditions. The number of marbles buried at the end of this period was recorded. A Student's *t*-test was used for statistical analysis with $p < 0.05$ as significance criteria.

Self-grooming behavior—Mice were individually placed in clean empty cages without bedding for a period of 20 min under conditions of white noise. During the first 10 min mice were allowed to habituate to the empty cage. Cumulative time spent in spontaneous repetitive grooming behavior was scored during the last 10 min (McFarlane et al., 2008). A Student's *t*-test was used for statistical analysis with $p < 0.05$ as significance criteria.

Rotating rod task—Motor coordination and balance were measured using an accelerating Rota-Rod (Ugo Basile, Collegetown, PA). Testing was performed for two sequential days with four trials per day spaced 30 min apart. The test protocol involved an accelerating protocol from 4 to 40 rpm over five min period that ended whenever a test mouse fell or when the protocol was completed. Two episodes of holding onto the rod rotating 360° also were scored as a fall. All mice used were similar in weight in order to eliminate the effect of weight on balance performance. The time to fall (latency) was recorded, and a mean for the four trials was calculated for each day. The data were pooled according to genotype, and a mean value was determined for each group. Two-way ANOVA was used for statistical analysis with $p < 0.05$ as significance criteria.

Novel object recognition—Mice were habituated in a round testing arena for 10 min on day one. On day two mice were habituated again to the arena for 10 min, in the presence of equally spaced objects. Mice then were presented with two familiar objects (least preferred objects) on days three and four for 10 min. On day five, one of the objects was replaced with a novel object and the mice were allowed to explore the environment for 10 min. Time spent exploring each object during the first five min was recorded. Exploration was defined as contact with the object (tail only excluded) or facing the object (distance < 2 cm). The amount of time spent exploring each object was divided by the amount of time exploring both novel and familiar objects using the Ethovision XT video tracking software (Noldus). The resulting value was multiplied by 100 to generate a percent of interacting time. A two-way ANOVA was used for statistical analysis with $p < 0.05$ as significance criteria.

Morris water maze task—Spatial learning and memory was tested by using the Morris water maze. Mice were trained in the hidden platform version of the Morris water maze, which consisted of four trials (60 s/trial) each day for six consecutive days. The swim-start position was varied from trial to trial. A probe trial was administered one hr after the end of

training on day six. On day seven, mice received reversal training during the next four consecutive days to test for perseverative behavior, four trials each day (60s/trial), in which the hidden platform was moved to the opposite quadrant. On the next two days mice were trained on visible platform task which consisted of four trials each day (60s/trial) with the escape platform and swim-start position moved randomly between each trial. Memory was assessed as the time (s) required for the mouse to find the platform for each consecutive trial or for each consecutive training day (escape latency). During probe trials and reversal learning training, the number of times the mouse crosses the space where the platform originally was located was monitored, as well as the time spent in each of the four quadrants. The trajectories of the mice were recorded with the Ethovision XT video tracking software (Noldus). A two-way ANOVA was used for statistical analysis with $p < 0.05$ as significance criteria.

Y-water maze task—Perseverative behavior was measured using the y-water maze task (Hoeffler et al., 2008). On day one mice received three habituation trials (60s/trial) to the maze (ITI = 15 min). The next day mice were trained to locate a submerged escape platform in one arm or another of a y-shaped maze with six blocks with five trials for each block. During day three, mice were tested for memory of the platform location and performance in achieving an escape success criterion of 90%. The escape arm was reversed and only mice that achieved the criterion were tested to determine the latency to find the new platform location. Mice were assigned randomly to either left or right arms at the beginning of training. Repeated measures ANOVA tests were used for statistical analysis with $p < 0.05$ as significance criteria.

Pre-pulse Inhibition—Sensorimotor gating was measured by testing the startle response of the mice and the prepulse inhibition (PPI) of the startle response as previously described (Banko et al., 2007). Mice were placed in a Plexiglass cylinder connected to a startle detector (Med Associates Inc, St. Albans, VT). They were left undisturbed for five min to habituate to the background 70 dB noise. Afterwards, mice were presented with six blocks of a series of different acoustic prepulses (74, 78, 82, 86 and 90dB) followed by the acoustic startle stimulus (120dB), in addition to a no stimulus trial that consisted of background 70 dB noise and a startle stimulus only trial (120 dB). The presentation of each acoustic prepulse, the no stimulus trial and the startle stimulus only trial, were in a pseudorandom order within each block. The maximum startle amplitude was recorded and PPI was calculated as follows: $\%PPI = 100 - [(startle\ on\ prepulse + stimulus) / startle\ alone \times 100]$. The acoustic response amplitude data was analyzed using One-way ANOVA. Prepulse inhibition data was analyzed using a two-way ANOVA with repeated measures with $p < 0.05$ as significance criteria.

Contextual fear conditioning—Associative memory was tested by using a contextual fear conditioning paradigm in which the mice were trained to associate a foot shock with the training context chamber. The training consisted of a three min exploration followed by a two s foot shock (0.7mA). A second foot shock was delivered one min later and mice remained in the chamber for another 30 seconds. Contextual tests (seven min total duration) were performed in the same chamber at 24 h and seven days after training. Memory was assessed as the percentage of time mice spent freezing when re-exposed to the training context. A student's t-test will be used for statistical analysis with $p < 0.05$ as significance criteria, as previously described.

Results

Assessment of social tendencies in the three-chambered social box

Studies have suggested high rates of autism in TSC patients (Bolton, 2004; Curatolo et al., 2004). However, at present there is little data relating autistic-like behavior in TSC due to mutations in *tsc2* gene. To investigate the association between TSC2-GAP mutations and autism we examined social behaviors of Δ RG mice in a three-chambered social arena (Crawley, 2004; Moy et al., 2007). In the social preference task, we observed that Δ RG mice, similar to their wild-type littermates, have a normal preference toward interacting with a social target versus a non-social target (Fig. 1A). However, Δ RG mice exhibited social impairment when challenged 24 hours later in a social novelty test (Fig. 1B). Specifically, we found that wild-type mice spent significantly more time interacting with a novel social target than with a familiar one (Fig. 1B). In contrast, Δ RG mice spent similar amount of time interacting with both novel and familiar social targets (Fig. 1B). Interestingly, both wild-type and Δ RG mice showed habituation to the familiar social target, exhibiting a significant decrease in interaction with this target 24 hours after their initial interaction on day one (Fig. 1C). In addition, in a novel object recognition task the Δ RG mice spent significantly more time exploring a novel object in the presence of a previous familiar object, similar to their wild-type littermates (Fig. 1D), suggesting lack of neophobia. Moreover, results from a reciprocal social interaction task showed that Δ RG mice spent less time interacting with a conspecific mouse compared to their wild-type littermates (Fig. 1E). These findings suggest a possible association between TSC2-GAP disruptive mutations and impaired social tendencies in TSC patients with autistic-like phenotypes.

Evaluation of motor coordination and motor learning in Δ RG mice

A recent clinical study found the presence of cerebellar lesions in 33% of the cases from children and young adult TSC patients (Ertan et al., 2010). Moreover, both TSC2 mRNA and protein are highly expressed in the cerebellum of humans and rodents (Geist and Gutmann, 1995; Geist et al., 1996; Kerfoot et al., 1996). Therefore, the rotarod test was used to assess motor coordination and motor learning of the Δ RG mice. Both wild-type and Δ RG mice were able to acquire and learn the task using an accelerating protocol (4–40rpm), which is a coordination-demanding task (Fig. 2A). However, the Δ RG mice had a mild, but significantly reduced latency to fall in consecutive trials on day two compared to their wild-type littermates (Fig. 2B). These results indicate that Δ RG mice have a modest impairment in motor learning skills.

Analysis of repetitive and perseverative behavior in Δ RG mice

Another major symptom for the diagnosis of autism is the presence of stereotyped repetitive and ritualistic behaviors, and resistance to change in habit (perseverative behavior) (Moy et al., 2007; Pelphrey et al., 2004; Wing, 1996). Therefore, we examined the Δ RG mice for enhanced repetitive and/or perseverance behaviors. First, we used the marble-burying task to measure repetitive-like behavior in Δ RG mice. The Δ RG mice and their wild-type littermates buried the same number of marbles during the 30 min test (Fig. 3A). Stereotyped repetitive behavior in mouse models of autism also can be studied by measuring self-grooming behavior (Gandal et al., 2010; McFarlane et al., 2008; Silverman et al., 2010a). Δ RG mice spent a similar amount of time performing self-grooming compared to their wild-type littermates (Fig. 3B). Taken together, these findings suggest that Δ RG mice do not exhibit enhanced repetitive behaviors.

We proceeded to determine whether the Δ RG mice exhibit increased perseveration in reversal learning tasks. In the water-based Y-maze, we found that Δ RG mice learned the initial task in a manner similar to their wild-type littermates (Fig. 4A). During a memory test

performed 24 hours later, both groups of mice chose the correct arm where the hidden platform was located the day before (Fig. 4A). Furthermore, when the platform was moved to the other arm, we found that Δ RG mice and their wild-type littermates were able to reverse their previous learning with equal efficacy (Fig. 4A).

We then examined the Δ RG mice on the hidden platform version of the Morris water maze, a hippocampus-dependent spatial learning task (Morris, 1984). During the acquisition phase, both Δ RG mice and their wild-type littermates learned to find the platform, as shown by a similar decrease in escape latency across six consecutive days (Fig. 4B). However, Δ RG mice started to show a slight increase in escape latency towards the end of the training. In addition, a probe trial test showed that Δ RG mice and their wild-type littermates both displayed a preference for the target quadrant in which the platform was located during the training (Fig. 4C) and crossed the previous platform location an equal number of times (Fig. 4D). However, analysis of the latency to find the location of the platform during the first probe trial indicated that the Δ RG mice had a significantly higher latency compared to their wild-type littermates having reached a plateau in their performance starting from day five of training (Fig. 4E). To test the Δ RG mice for reversal learning deficits and perseverative behavior, the hidden platform was moved to a new location. The Δ RG mice displayed reversal learning similar to their wild-type littermates as evidenced by a decrease in the latency to find the platform in the new location across training (Fig. 4B). In a visible platform test, the Δ RG mice were not different from wild-type mice in latencies to find the platform, indicating that they have normal visual acuity, swimming ability, and motivation to escape from the water (Fig. 4B). These results indicate that the Δ RG mice have normal spatial learning and reversal learning in the Morris water maze. Moreover, taken together with the results from the Y-maze experiments, these findings indicate that the Δ RG mice exhibit normal perseverative behavior.

Evaluation of sensorimotor gating and fear responses in Δ RG mice

Clinical studies have found that patients with autistic disorders have abnormal sensorimotor gating as measured by decreased prepulse inhibition (PPI) compared to normal controls (McAlonan et al., 2002; Perry et al., 2007). Therefore, we determined whether PPI was decreased in Δ RG mice. We found that the startle response was similar in both Δ RG and wild-type mice for background noise (70dB) (Fig. 5A). In the absence of prepulses, Δ RG mice had normal startle reactivity to the presentation of the acoustic startle stimulus (120dB) alone (Fig. 5B). All acoustic prepulses (74, 78, 82, 86 and 90dB) followed by the acoustic startle stimulus (120dB) were able to inhibit the startle response in both Δ RG and wild-type mice with similar efficacy (Fig. 5C). These results suggest that sensorimotor gating is not affected in Δ RG mice.

Interestingly, studies using a fear conditioning startle paradigm found that both autistic and normal individuals have a potentiated startle response following fear conditioning (Bernier et al., 2005). Therefore, using a contextual fear conditioning paradigm, we determined whether the Δ RG mice had impairments in associative fear learning and memory. Δ RG mice and wild-type mice responded similarly during the acquisition phase of this task (Fig. 6A) suggesting normal associative fear learning. When exposed to the same training context 24 hours later, both Δ RG mice and wild-type littermates showed similar freezing responses (Fig. 6B). Furthermore, memory tests performed seven days after training showed that longer-term fear memory in both groups was stable and indistinguishable (Fig. 6C). These findings indicate that disruption of TSC2-GAP domain does not affect the formation of contextual fear memories in Δ RG mice.

Discussion

Because mutations in TSC patients have been reported to be clustered in the region of the *tsc2* gene encoding the GAP domain of TSC2 (Maheshwar et al., 1997), Δ RG transgenic mouse model of TSC provide an opportunity to assess the neurological consequences of mutations in TSC2-GAP domain and their correlation with the neuropsychiatric phenotypes observed in human TSC patients and humans with autism.

Studies in children and adolescents suggest that autism may be seen as inappropriate or indiscriminate approaches to strangers instead of lack of social interaction (Loveland et al., 2001). In mice, this behavior is measured as equal or less exploration of a novel social target over a familiar one (Crawley, 2004; Moy et al., 2007). Initial social behavior studies using the three-chambered social task (Silverman et al., 2010b) failed to produce sociability and social novelty preference in the wild-type mice in our studies (Supplementary Fig. 1A and 1B). Based on this observation and the fact that Δ RG mice exhibit anxiety-like phenotypes (Ehninger and Silva, 2010) that could confound the interpretation of the social behavior experiments (Carter et al., 2011; Silverman et al., 2010b), we decided to modify the three-chambered social task to include longer habituation sessions (30 min). This modified version of the task was able to produce normal social behaviors in wild-type mice (Fig. 1A and Fig. 1B). In addition, we found that although Δ RG mice exhibit normal social preference, they exhibit inappropriate social behavior when exposed to novel social experiences by equally exploring both familiar and novel social targets (Fig. 1A and Fig. 1B). This inappropriate social behavior was not a consequence of either a failure in social learning or a failure in recognizing novelty. Δ RG mice showed normal social recognition learning of the familiar social target 24 h after their initial encounter (Fig. 1C). In addition, Δ RG mice had deficits in a direct reciprocal social task, a more natural social behavior (Fig. 1E), although it should be noted that the three-minute test session we used is short compared to 10-minute sessions used in most social interaction studies (Roullet and Crawley, 2011; Silverman et al., 2010b). Interestingly, the average time of total direct reciprocal social interactions of wild-type mice using session test lengths of 10 minutes or more (Matsuo et al., 2009; McFarlane et al., 2008; Radyushkin et al., 2009) is 85 seconds, similar to the behavior of the wild-type mice (90 seconds, 50% interacting time) in our studies. In addition, Δ RG mice exhibited less interaction with a freely moving target mouse, perhaps due to increased levels of social anxiety caused by direct contact with the target mouse. In support of this argument, increased levels of anxiety behaviors in Δ RG mice were reported previously (Ehninger and Silva, 2010). Moreover, the most common psychiatric disorder in children with autism is social anxiety (Simonoff et al., 2008).

Although TSC often has been categorized as a cortical disorder, other brain regions, such as the cerebellum are also affected in TSC patients (Ertan et al., 2010; Ridler et al., 2007). Consistent with this idea, clinical studies of autistic children have suggested a correlation between lower performance in motor coordination skills and a high score in autistic diagnostic criteria (Halayem et al., 2010). It has been shown that Δ RG mice suffer from cerebellar developmental problems, including enhanced proliferation and failure in migration of cerebellar granule cells (Bhatia et al., 2009; Govindarajan et al., 2005), consistent with the pathology of TSC in humans (Crino and Henske, 1999). We observed that Δ RG mice have mild, but significant motor learning impairments as measured with an accelerating rotarod paradigm (Fig. 2). In contrast to our findings with Δ RG mice, TSC2 heterozygous knockout mice have been reported to have normal motor skills (Ehninger et al., 2008). In addition, no cerebellar abnormality has been reported in TSC2 heterozygous knockout mice, suggesting that disruption of the GAP domain of TSC2 might specifically impact the cerebellum. Moreover, recent study using conditional deletion of TSC2 in the

cerebellum, showed increased purkinje cell apoptosis, as well as motor skills deficits (Reith et al., 2011).

The motor learning impairments observed in Δ RG mice are likely related to cerebellar deficits, and it is possible that the social deficits observed in Δ RG mice also are related to cerebellar dysfunction. Clinical evidence suggests an involvement of the cerebellum in the impaired attentional and orienting skills, stereotypical behaviors, impaired social interactions and impaired communication in children with TSC and autism (Asano et al., 2001; Courchesne, 2004; Courchesne et al., 2004). Similarly, a *gabrb3*^{-/-} mouse model of autism, which also displays cerebellar abnormalities, exhibits social interaction and attentional function deficits (DeLorey et al., 2008).

It was shown previously that Δ RG mice have normal spatial memory in a Morris water maze task, but a minor impairment throughout the acquisition of the task and during a probe trial test (Ehninger and Silva, 2010). Similarly, in our studies we observed that the Δ RG mice exhibit normal spatial learning, but a mild impairment was observed on the last day of acquisition of the Morris water maze task (Fig. 4B). In contrast to previous studies (Ehninger and Silva, 2010), we found that Δ RG mice have a significant preference for the target quadrant, indicating normal spatial memory. The protocols used in these studies are slightly different in that spaced training was used in the former study, and a combination of massed and spaced training was used in the latter study. It has been shown that rodents trained with spaced trials perform better and have better long-lasting memories of the platform location than those trained with massed trials (Commins et al., 2003; Spreng et al., 2002). Moreover, we also examined the latency to first find the platform zone during the probe trial and found that Δ RG mice reached a plateau in their performance as reflected by a significantly higher latency to find the platform zone (Fig. 4E). However, this plateau in performance did not affect the spatial reference memory of the Δ RG mice (Fig. 4C and 4D).

The Δ RG mice were shown to have normal fear responses to the training context but were not able to discriminate between distinct contexts using a context fear discrimination paradigm (Ehninger and Silva, 2010). Herein, we report that Δ RG mice have normal contextual fear memory that persists up to seven days after the initial training (Fig. 6B and 6C). Because it has been suggested that amygdala dysfunction is an important component of autism (Baron-Cohen et al., 2000; Corbett et al., 2009; Monk et al., 2010; Schultz et al., 2000), it would be interesting to investigate whether cued fear conditioning and partial reinforcement fear conditioning learning are altered in Δ RG mice resulting in indiscriminate fear responses as well.

Other mouse models of TSC have been used to investigate the behavioral consequences of decreasing TSC1 and TSC2 protein levels. TSC2 heterozygous knockout mice were shown to have normal social preference tendencies (Ehninger et al., 2008), similar to what we observed with Δ RG mice (Fig. 1A). This suggests that the functionality of TSC2-GAP domain is not involved in mediating social preference in mice. However, the previous studies did not determine whether the TSC2 heterozygous knockout mice have alterations in social novelty or reciprocal social interactions. On the other hand, of TSC1 heterozygous knockout mice were shown to have reduced social approach in a reciprocal direct social interaction task (Goorden et al., 2007), similar to our findings with Δ RG mice (Fig. 1E). Because both TSC1 and TSC2 proteins form a complex that confers stability to both proteins (Chong-Kopera et al., 2006; Henske, 2003; Krymskaya and Shipley, 2003; Nellist et al., 1999), decreasing TSC1 protein levels might affect the stability of the TSC1/TSC2 heterodimer. A decrease in TSC1/TSC2 heterodimer stability might compromise the GAP activity of TSC2, which would be similar to disruptive mutations in TSC2-GAP domain as

in the Δ RG mice. Taken together, these findings suggest that normal function of the GAP domain of TSC2 is required for normal reciprocal social interactions.

Both TSC1 and TSC2 heterozygous knockout mice have impaired spatial memory in the Morris water maze task, with the impairments being more severe in the TSC1 heterozygous knockout mice (Ehninger et al., 2008; Goorden et al., 2007). Taken together with the alterations we observed in the Δ RG mice (Fig. 4B–4E), these differences in spatial learning and memory in TSC model mice suggest a differential role or function for the two proteins (Orlova and Crino, 2010) during hippocampus-dependent spatial memory, and that the GAP activity of TSC2 is not engaged in these processes. In addition, both Δ RG mice and TSC2 heterozygous knockout mice have generalized contextual fear responses (Ehninger et al., 2008; Ehninger and Silva, 2010), possibly due to decreased GAP activity in TSC2 in both mouse lines. However, TSC1 heterozygous knockout mice have significantly lower contextual fear memory (Goorden et al., 2007). These findings suggest a differential role for TSC1 and TSC2 in associative fear learning and memory.

Finally, although seizures have been detected in other TSC mouse models (Erbayat-Altay et al., 2007; Meikle et al., 2007; Uhlmann et al., 2002; Zeng et al., 2011) we did not observe any spontaneous seizures in Δ RG mice while either performing behavioral experiments or while the mice were in their home cages.

Conclusions

Our studies herein indicate that Δ RG mice exhibit some behavioral phenotypes associated with core symptoms of autism, including social interaction deficits and mild impairments in motor learning skills. Mutations in the GAP domain of TSC2 affect specific aspects of social behavior in Δ RG mice, particularly those engaging novel experiences and reciprocal interactions. The inappropriate social approach observed in Δ RG mice represent a failure for the mice to adapt socially. Moreover, the decrease in reciprocal social interactions displayed by the Δ RG mice might be an indication of social anxiety in these mice. Finally, abnormal cerebellar development observed previously in Δ RG mice is correlated with deficits in motor learning and memory. Thus, the Δ RG transgenic mouse is a mouse model of TSC that could be exploited to further investigate the role of the GAP domain of TSC2 in social anxiety, as well as its potential role in the cerebellum for social interactions and attentional functions that are relevant to autism associated with TSC.

Research highlights

- Δ RG mice exhibit deficits in preference for social novelty and social reciprocal interaction.
- Δ RG mice exhibit a mild, but significant impairment in rotarod motor learning.
- Δ RG mice do not exhibit either repetitive or perseverative behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ADHD	attention deficit hyperactivity disorder
ASD	autism spectrum disorders

GAP	GTPase-activating protein
TSC	tuberous sclerosis complex
TSC2-DN	TSC2-dominant/negative

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Figure 1. Social behavior of wild-type and ΔRG transgenic mice

(A) social preference: interaction with social target was significantly higher for both wild-type (WT) and ΔRG mice than their interaction with a non-social target (WT, n=10 ***p<0.0001; ΔRG , n=10 ***p<0.0001, Student's *t*-test). (B) social novelty: WT mice spent significantly more time interacting with the novel social target than with the familiar social target, whereas ΔRG mice spend equal amount of time interacting with both social targets (WT, n=9 **p<0.01; ΔRG , n=10 p>0.05, Student's *t*-test). (C) social learning: both WT and ΔRG mice showed decreased interaction with the familiar social target 24 hour compared with their initial interaction on the previous day (WT, n=9 *p<0.05; ΔRG , n=9 *p<0.05, Student's *t*-test). (D) novel object recognition: both WT and ΔRG mice spent significantly

more time interacting with the novel object than with the familiar object (WT, n=6 *** $p<0.001$; Δ RG, n=6 ** $p<0.01$, Student's *t*-test). (E) reciprocal social interaction: compared to WT mice, Δ RG mice displayed significantly less interaction time with the freely moving target mouse (WT, n=10; Δ RG, n=10. * $p<0.05$, Student's *t*-test).

Figure 2. Impaired motor skill learning and coordination in Δ RG transgenic mice

Motor skill learning and coordination were evaluated with the rotarod task. (A) wild-type (WT) mice exhibited normal motor learning and coordination, whereas Δ RG mice exhibited deficits in motor coordination as shown by significant decreased latency to fall off the rod (WT, $n=12$; Δ RG, $n=12$. $*p<0.05$, Two-way ANOVA). (B) Compared to WT mice, Δ RG mice displayed poor performance during day two of training, as shown by a significant decrease in the average of latency to fall off the rod (WT, $n=12$; Δ RG mice, $n=12$. $**p<0.01$, Student's t -test).

Figure 3. Δ RG mice show normal repetitive behaviors

(A) marble burying: both wild-type (WT) and Δ RG mice buried similar number of marbles during a 30 min period (WT, $n=10$; Δ RG, $n=10$. $p>0.05$, Student's t -test). (B) self-grooming: both WT and Δ RG mice spent similar amounts of time in self-grooming behavior (WT, $n=12$; Δ RG, $n=17$. $p>0.05$, Student's t -test).

Figure 4. ΔRG mice exhibit normal perseverative behaviors

(A) y-water maze learning and memory, and reversal learning: both WT and ΔRG mice learned to choose the correct arm with the same efficacy. When tested (T) 24 hours later, both groups showed normal memory for the correct arm, followed by normal reversal learning of the original task (WT, n=13; ΔRG, n=17. $p > 0.05$, two-way ANOVA). (B) Morris water maze: both WT and ΔRG mice learned to find the hidden platform with similar escape latency times during the initial six days of training. During reversal learning, both WT and ΔRG mice learned to find the new hidden platform location with similar escape latency times during the four days of reversal training. Both groups were able to find the escape platform with similar escape latency times during the two days of the visible version of the

water maze. (WT, n=12; Δ RG, n=12. $p>0.05$, Two-way ANOVA). (C) probe trial quadrant occupancy: both WT and Δ RG mice spent significantly more time searching in the target quadrant compared to the other quadrants during the probe trial test (WT, n=12; Δ RG, n=12. $p>0.05$, [Quadrants, *** $p<0.0001$] two-way ANOVA). (D) probe trial platform crossings; both WT and Δ RG mice had a similar number of platform crossings in the target quadrant during the probe trial test (WT, n=12; Δ RG, n=12. $p>0.05$, two-way ANOVA). (E) probe trial latency: Δ RG mice spent significantly more time trying to find the platform zone than WT mice during the probe trial test (WT, n=12; Δ RG, n=12. * $p<0.05$, Student's *t*-test).

Figure 5. Normal sensorimotor gating in Δ RG mice

(A) Both wild-type (WT) and Δ RG mice displayed similar startle responses for the 70db background stimulus (WT, n=12; Δ RG, n=12. $p>0.05$, Student's t -test). (B) Both WT and Δ RG mice displayed similar startle responses for the 120db startle stimulus (WT, n=12; Δ RG, n=12. $p>0.05$, Student's t -test) (C) Δ RG mice showed similar levels of pre-pulse inhibition as WT mice (WT, n=10; Δ RG, n=13. $p>0.05$, Two-way ANOVA).

Figure 6. Δ RG mice have normal long-lasting contextual fear memory

(A) wild-type (WT) and Δ RG mice responded similarly during the acquisition of a contextual fear paradigm where two footshocks were presented one minute apart after a three minute period of habituation (WT, $n=17$; Δ RG, $n=15$. $p>0.05$, [training *** $p<0.0001$] Two-way ANOVA). (B) both WT and Δ RG mice had similar levels of freezing to the context 24 hours after training (WT, $n=17$; Δ RG, $n=17$. $p>0.05$, Student's t -test) (C) both WT and Δ RG mice had similar levels of freezing to the context 7 days after training (WT, $n=17$; Δ RG, $n=17$. $p>0.05$, Student's t -test).