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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The specific aims of this concept award are 1) to conduct a battery of behavioral tests on a cerebellar mouse model of Tuberous sclerosis complex to determine whether Tsc2 mediated Purkinje cell loss induces ASD-like deficits; and 2) to determine whether rapamycin, an mTORC1 inhibitor, rescues the Purkinje cell degeneration and behavioral deficits in mutant mice. Here we report substantial progress on Aim 1. We have performed the battery of behavioral tests on the cerebellar mouse model of TSC. Here we demonstrate that mild social deficits occur in a Tsc2 haploinsufficient environment and are exacerbated by loss of Purkinje cells. Purkinke cell loss leads to repetitive behavior. Autistic-like behaviors are more severe in male Tsc2f/-;Cre mice than female mice. We have also submitted out findings to the Neurobiology of Disease for review.

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## Introduction:

Tuberous sclerosis complex (TSC) is a dominant neurogenetic disorder affecting about 1 in 6,000 people [1]. TSC is caused by heterozygous inactivation of either the *TSC1* or *TSC2* gene, encoding the proteins hamartin or tuberin, respectively [2, 3]. Hamartin and tuberin form a complex that inhibits the mammalian target of rapamycin complex 1 (mTORC1), a kinase that controls translation and cell growth [4]. Many lesions in TSC patients demonstrate loss of both alleles of either *TSC1* or *TSC2*, suggesting that the two-hit mechanism is important for pathogenesis [5-8]. However, there is also evidence that haploinsufficiency also plays a role in disease progression [9, 10]. The brain pathology is the most debilitating aspect of TSC and is often associated with Autism Spectrum Disorders (ASD) [11-14]. Traditionally, the neurological basis of ASD has been thought to lie mainly in the cerebral cortex [15-18]. Recent evidence suggests that the involvement of the cerebellum may also be an important determinant in ASD [19-22]. In TSC, the severity of the autistic phenotype is associated with number and severity of cerebellar lesions [23]. The cerebellum communicates with the cerebral cortex via the inhibitory GABAergic axons of the Purkinje cell that project to the deep cerebellar nuclei [24]. The deep cerebellar nuclei then send projections to the thalamus and cerebral cortex [25, 26]. Purkinje cell loss is one of the most common anatomical abnormalities seen in autopsy studies of autistic patients [21, 27, 28]. Given the comorbidity of TSC and ASD, we hypothesized that abnormalities in the cerebellum of TSC patients might be an important determinant of autism. In support of this hypothesis, Purkinje cell loss has been reported in some patients with TSC [29, 30]. In an effort to study the relationship between the cerebellum, TSC, and ASD, we created and characterized a novel mouse model with Purkinje cell loss due to Purkinje cell specific *Tsc2* deletion [30]. 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. Therefore, we proposed to: **1) to conduct a battery of behavioral tests to determine whether Tsc2 mediated Purkinje cell loss induces ASD-like deficits; and 2) to determine whether rapamycin, an mTORC1 inhibitor, rescues the Purkinje cell degeneration and behavioral deficits in mutant mice.**

**Body:**

## Task 1 - Specific Aim 1. To conduct behavioral tests to determine whether Tsc2 mediated Purkinje cell loss induces ASD-like deficits (timeframe, months 1-12).

Aim 1 has been completed and we have recently submitted a manuscript to the journal Neurobiology of Disease (appendix). Behavior testing was performed on Tsc2f/-;Cre mice to determine if they have autistic-like behavior. All mice were determined to have intact reflexes, olfaction, and vision. Motor abnormalities were examined as a loss of Purkinje cells can lead to ataxia [30-33]. At two months of age, there was no difference in performance on an accelerating RotaRod, but gait analysis did detect a slightly wider gait (p=0.079) in Tsc2f/-;Cre mice compared to Tsc2f/+ mice.

Since social behaviors are one well reported deficit in ASD [34], we used the three chambered apparatus [35-38] to detect differences in sociability and social novelty. Males and females were analyzed separately to detect sex specific differences similar to that seen in human ASD. Male Tsc2f/+ mice spent significantly more time (p=0.0001) in the chamber with the stranger mouse than the chamber with the inanimate object. Male Tsc2f/- mice spent slightly more time in the chamber with the stranger mouse than in the chamber with the inanimate object, but this was not statistically significant. Male Tsc2f/-;Cre mice spent approximately equal time in both chambers (Fig 1A). Tsc2f/+ female mice showed a slight preference for the chamber with the stranger mouse than the chamber with the inanimate object, but this was not statistically significant. However, both Tsc2f/- and Tsc2f/-;Cre females did not show a preference for either chamber (Fig 1B). 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 more time with the novel mouse than the familiar mouse (p=0.078). 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 1C). Tsc2f/+ female mice spent slightly more time with the novel mice than the familiar mice (p=0.076) (Fig 1D). Interestingly, Tsc2f/- female mice showed a significant preference for the novel mouse (p=0.015). However, female Tsc2f/-;Cre mice also showed a slight preference (NS) for the novel mouse compared with the familiar mouse. These data are in agreement with the social preference testing, supporting a social behavioral deficit in Tsc2f/- mice that progresses in Tsc2f/-;Cre mice.

Since repetitive behaviors are another well reported deficit in ASD [34], we used the marble burying assay [39] to assay repetitive behaviors. 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 2). Similarly, female Tsc2f/-;Cre mice also buried more marbles than either Tsc2f/+ (p=0.070) or Tsc2f/- (p=0.028) mice (Fig 2). 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.

In summary, we have demonstrated a novel concept: Loss of Tsc2 in Purkinje cells leads to autistic-related behavior in mice. This finding is an important foundation for future work to understand the neurobiologic mechanisms, and shed light on the many paths that lead to the autistic phenotype.

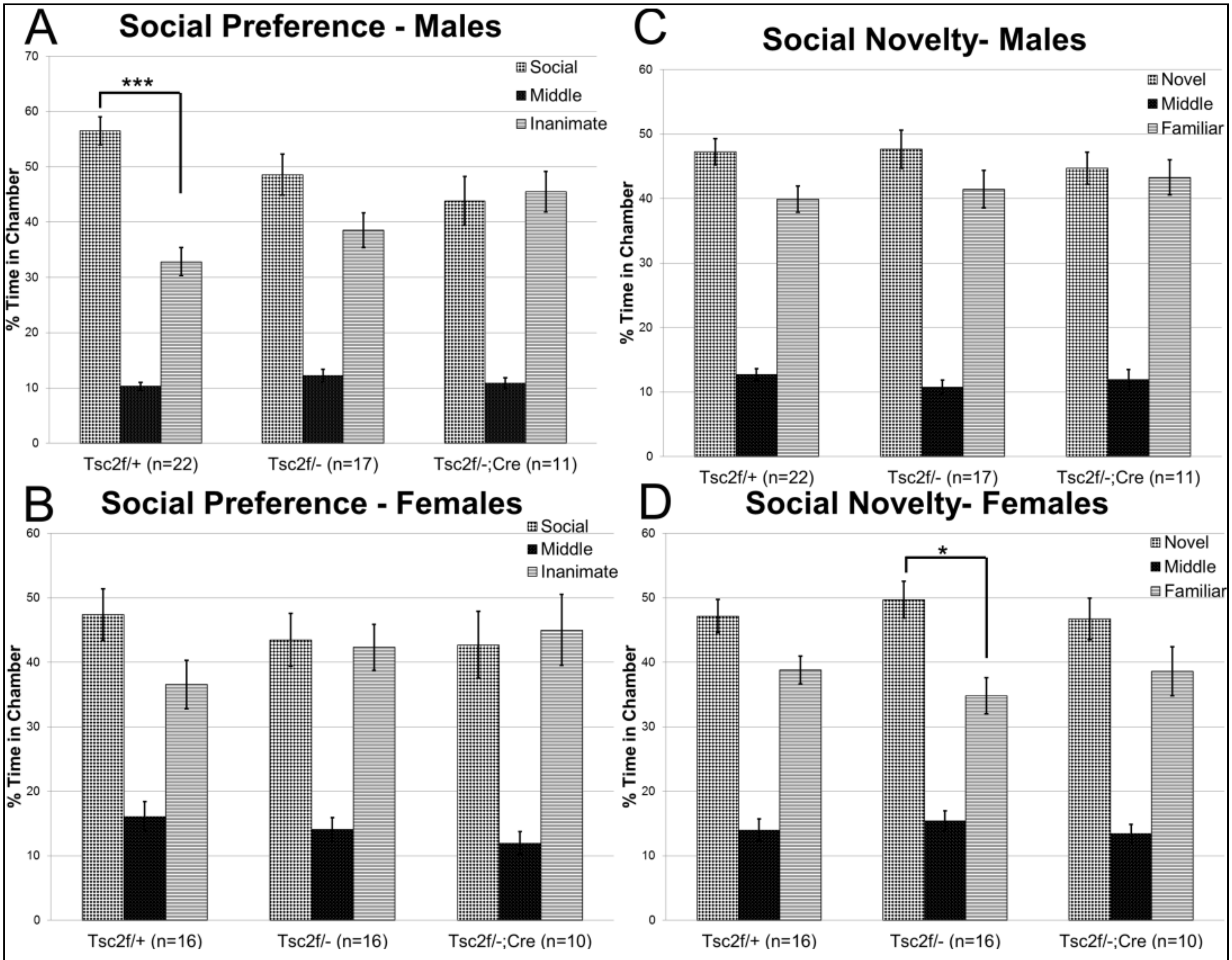

Figure 1: Social behavior deficits. (A) Male Tsc2f/+ mice spent more time (p=0.0001) in the social chamber than in the inanimate object chamber. Tsc2f/- mice showed a slight preference (NS) for the social chamber. However, Tsc2f/-;Cre mice spent equal time in both chambers. (B) Female Tsc2f/+ mice showed a slight preference (NS) for the stranger mouse compared with the inanimate object. However, both Tsc2f/- and Tsc2f/-;Cre mice spent equal time in both chambers. (C) Male Tsc2f/+ mice spent slightly (p=0.078) more time 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=0.076) and Tsc2f/- (p=0.0008) mice spent more time with the novel mice than the familiar mice. Tsc2f/-;Cre mice also spent slightly more time (NS) with the novel mouse than with the familiar mouse.

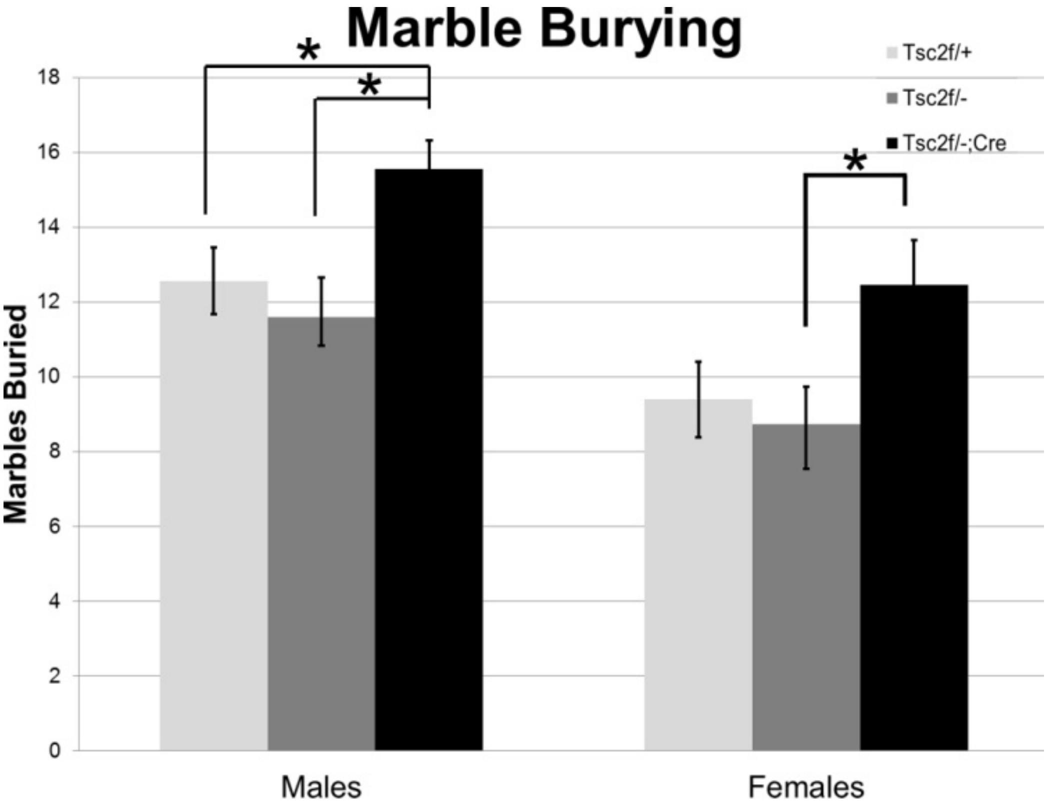

Figure 2: Repetitive behaviors. Male Tsc2f/-;Cre (n=11) mice buried significantly more marbles than either Tsc2f/+ (n=23) (p=0.043) or Tsc2f/- (n=20) (p=0.016) mice. Female Tsc2f/-;Cre (n=11) mice buried more marbles than either Tsc2f/+ (n=20) (p=0.070) or Tsc2f/- (n=19) (p=0.028) mice.

**Task 2 - Specific Aim 2: To determine whether rapamycin, an mTORC1 inhibitor currently in trials to treat TSC, can rescue the Purkinje cell degeneration, and behavioral deficits in mutant mice (timeframe, months 12-24).**

The PI for this project, Dr. Gambello, has accepted a new faculty position at Emory University in the Department of Human Genetics. Since Tasks 1 and 2 constitute a doctoral thesis for Michelle Reith, task 2 will be performed in part at UT Houston, and in part at Emory University. Jim McKenna, research coordinator, will also move with Dr. Gambello. We are asking that the next year's funding be sent to Emory University. We then will contract out to UT to pay for the costs of the animals that will be treated at UT. This will far less expensive than rederiving animals at Emory University.

As activation of mTORC1 is an important mechanism of pathogenesis [40, 41], and mTORC1 activity is elevated in Purkinje cells following deletion of *Tsc2* [30], we hypothesize that the mTORC1 inhibitor, rapamycin, will ameliorate behavioral deficits in Tsc2f/-;Cre mice. Since the male mice exhibited the most severe behavioral deficits, we decided to treat male Tsc2f/+ and male Tsc2f/-;Cre mice with rapamycin to determine if we could rescue behavioral deficits. The mice are currently undergoing treatment and behavioral assessment, but the testing is not yet completed.

## Key Research Accomplishments:

- Mild social deficits occur in a *Tsc2* haploinsufficient environment and are exacerbated following *Tsc2*-mediated Purkinje cell loss
- Purkinje cell loss leads to increased repetitive behaviors
- Autistic-like behaviors are more severe in male Tsc2f/-;Cre mice than female Tsc2f/-;Cre mice
- Manuscript submission based on findings from Task 1.

## Reportable Outcomes:

- This work was presented by RMR at the National Graduate Student Research Conference hosted at the National Institutes of Health in Bethesda, MD. The poster was titled: "Loss of Tuberin in the Purkinje Cell: A Possible Link Between Tuberous Sclerosis Complex and Autism." Manuscript submission based on findings from Task 1.

## Conclusions:

TSC is a heterogeneous disorder that affects the brain in almost all affected individuals. The effects of this disease on the cerebellum have not been well studied. Our data suggests that the cerebellar Purkinje cell may be particularly susceptible to dysfunction and death in TSC leading to ASD-like behavior. In particular *Tsc2*-mediated Purkinje cell loss leads to social behavior deficits and increased repetitive behaviors. These studies reveal new insight into the cerebellar pathology of TSC and ASD. The rapamycin rescue experiments will offer promise for future clinical trials in humans. These model mice will also be a useful reagent for the testing of other pharmacologic agents for the treatment of TSC-associated autism and quite possibly idiopathic autism.

## References:

1. Osborne, J.P., A. Fryer, and D. Webb, *Epidemiology of tuberous sclerosis.* Ann N Y Acad Sci, 1991. 615: p. 125-7.
2. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. 277(5327): p. 805-8.
3. *Identification and characterization of the tuberous sclerosis gene on chromosome 16.* Cell, 1993. 75(7): p. 1305-15.
4. Inoki, K., et al., *TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.* Nat Cell Biol, 2002. 4(9): p. 648-57.
5. Henske, E.P., et al., *Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogenesis of tuberous sclerosis tumors.* Am J Pathol, 1997. 151(6): p. 1639-47.
6. Au, K.S., et al., *Complete inactivation of the TSC2 gene leads to formation of hamartomas.* Am J Hum Genet, 1999. 65(6): p. 1790-5.
7. Green, A.J., P.H. Johnson, and J.R. Yates, *The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor.* Hum Mol Genet, 1994. 3(10): p. 1833-4.
8. Green, A.J., M. Smith, and J.R. Yates, *Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients.* Nat Genet, 1994. 6(2): p. 193-6.
9. Henske, E.P., et al., *Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions.* Am J Hum Genet, 1996. 59(2): p. 400-6.
10. Niida, Y., et al., *Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions.* Am J Hum Genet, 2001. 69(3): p. 493-503.
11. Gillberg, I.C., C. Gillberg, and G. Ahlsen, *Autistic behaviour and attention deficits in tuberous sclerosis: a population-based study.* Dev Med Child Neurol, 1994. 36(1): p. 50-6.
12. Hunt, A. and J. Dennis, *Psychiatric disorder among children with tuberous sclerosis.* Dev Med Child Neurol, 1987. 29(2): p. 190-8.
13. Hunt, A. and C. Shepherd, *A prevalence study of autism in tuberous sclerosis.* J Autism Dev Disord, 1993. 23(2): p. 323-39.
14. Smalley, S.L., et al., *Autism and tuberous sclerosis.* J Autism Dev Disord, 1992. 22(3): p. 339-55.
15. Aylward, E.H., et al., *MRI volumes of amygdala and hippocampus in non-mentally retarded autistic adolescents and adults.* Neurology, 1999. 53(9): p. 2145-50.
16. Courchesne, E. and K. Pierce, *Brain overgrowth in autism during a critical time in development: implications for frontal pyramidal neuron and interneuron development and connectivity.* Int J Dev Neurosci, 2005. 23(2-3): p. 153-70.
17. Abell, F., et al., *The neuroanatomy of autism: a voxel-based whole brain analysis of structural scans.* Neuroreport, 1999. 10(8): p. 1647-51.
18. Dawson, G., et al., *Neuropsychological correlates of early symptoms of autism.* Child Dev, 1998. 69(5): p. 1276-85.
19. Vargas, D.L., et al., *Neuroglial activation and neuroinflammation in the brain of patients with autism.* Ann Neurol, 2005. 57(1): p. 67-81.
20. Yip, J., J.J. Soghomonian, and G.J. Blatt, *Decreased GAD65 mRNA levels in select subpopulations of neurons in the cerebellar dentate nuclei in autism: an in situ hybridization study.* Autism Res, 2009. 2(1): p. 50-9.
21. Palmen, S.J., et al., *Neuropathological findings in autism.* Brain, 2004. 127(Pt 12): p. 2572-83.
22. Fatemi, S.H., et al., *Consensus Paper: Pathological Role of the Cerebellum in Autism.* Cerebellum, 2012.
23. Weber, A.M., et al., *Autism and the cerebellum: evidence from tuberous sclerosis.* J Autism Dev Disord, 2000. 30(6): p. 511-7.
24. Saab, C.Y. and W.D. Willis, *The cerebellum: organization, functions and its role in nociception.* Brain Res Brain Res Rev, 2003. 42(1): p. 85-95.

25. Yamamoto, T., et al., *The medial dorsal nucleus is one of the thalamic relays of the cerebellocerebral responses to the frontal association cortex in the monkey: horseradish peroxidase and fluorescent dye double staining study.* Brain Res, 1992. 579(2): p. 315-20.
26. Gonzalo-Ruiz, A. and G.R. Leichnetz, *Connections of the caudal cerebellar interpositus complex in a new world monkey (Cebus apella).* Brain Res Bull, 1990. 25(6): p. 919-27.
27. Fatemi, S.H., et al., *Purkinje cell size is reduced in cerebellum of patients with autism.* Cell Mol Neurobiol, 2002. 22(2): p. 171-5.
28. Bailey, A., et al., *A clinicopathological study of autism.* Brain, 1998. 121 ( Pt 5): p. 889-905.
29. Boer, K., et al., *Clinicopathological and immunohistochemical findings in an autopsy case of tuberous sclerosis complex.* Neuropathology, 2008. 28(6): p. 577-90.
30. Reith, R.M., et al., *Loss of the tuberous sclerosis complex protein tuberin causes Purkinje cell degeneration.* Neurobiol Dis, 2011. 43(1): p. 113-22.
31. Sarna, J.R. and R. Hawkes, *Patterned Purkinje cell loss in the ataxic sticky mouse.* Eur J Neurosci, 2011. 34(1): p. 79-86.
32. Caddy, K.W. and T.J. Biscoe, *Structural and quantitative studies on the normal C3H and Lurcher mutant mouse.* Philos Trans R Soc Lond B Biol Sci, 1979. 287(1020): p. 167-201.
33. Landis, S.C. and R.J. Mullen, *The development and degeneration of Purkinje cells in pcd mutant mice.* J Comp Neurol, 1978. 177(1): p. 125-43.
34. Association, A.P., *Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR Fourth Edition*. 4 ed. 2000: Amer Psychiatric Pub. 943.
35. Kwon, C.H., et al., *Pten regulates neuronal arborization and social interaction in mice.* Neuron, 2006. 50(3): p. 377-88.
36. Moy, S.S., et al., *Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice.* Genes Brain Behav, 2004. 3(5): p. 287-302.
37. Peca, J., et al., *Shank3 mutant mice display autistic-like behaviours and striatal dysfunction.* Nature, 2011. 472(7344): p. 437-42.
38. Crawley, J.N., *Mouse behavioral assays relevant to the symptoms of autism.* Brain Pathol, 2007. 17(4): p. 448-59.
39. Thomas, A., et al., *Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety.* Psychopharmacology (Berl), 2009. 204(2): p. 361-73.
40. Chan, J.A., et al., *Pathogenesis of tuberous sclerosis subependymal giant cell astrocytomas: biallelic inactivation of TSC1 or TSC2 leads to mTOR activation.* J Neuropathol Exp Neurol, 2004. 63(12): p. 1236-42.
41. Crino, P.B., K.L. Nathanson, and E.P. Henske, *The tuberous sclerosis complex.* N Engl J Med, 2006. 355(13): p. 1345-56.

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Keywords: Purkinje cell; Tuberous sclerosis; mouse; Tsc2; autism; social; repetitive behavior

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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. 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. Tsc2f/-;Cre mice also demonstrated increased repetitive behavior as assessed with marble burying activity. Altogether, these results demonstrate that loss of Tsc2 in Purkinje cells in a haploinsufficient background lead to behavioral deficits that are characteristic of human autism. Therefore, Purkinje cells loss and/or dysfunction may be an important link between TSC and ASD.

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Done behavioral neuroscience
examining cerebellum models

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Works with mTORC1 pathway and has extensive experience with mouse models

Opposed Reviewers:

To the Editors of the Neurobiology of Disease:

We are submitting our manuscript, “Loss of *Tsc2* in Purkinje cells is associated with autistic-like behavior in a mouse model of Tuberous Sclerosis Complex” for your consideration. We believe that our data strengthens the association between the cerebellum and autistic behavior in a novel mouse model of tuberous sclerosis complex. This model system is a powerful foundation for future studies examining the role of the cerebellum, and in particular, the Purkinje cell, in behavior. As such, we believe that our findings are very much in accordance with the goals of your journal.

Sincerely

Michael J. Gambello, MD, PhD

***Highlights (for review)**

- Mice have social behavioral deficits following *Tsc2*-mediated Purkinje cell loss
- Mild social deficits occur in a *Tsc2* haploinsufficient environment
- Purkinje cell loss leads to increased repetitive behaviors
- Autistic-like behaviors are more severe in male mice than female mice

# Loss of *Tsc2* in Purkinje cells is associated with autistic-like behavior in a mouse model of Tuberous Sclerosis Complex

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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. 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. Tsc2f/-;Cre mice also demonstrated increased repetitive behavior as assessed with marble burying activity. Altogether, these results demonstrate that loss of *Tsc2* in Purkinje cells in a haploinsufficient background lead to behavioral deficits that are characteristic of human autism. Therefore, Purkinje cells loss and/or dysfunction may be an important link between TSC and ASD.

## Keywords:

Purkinje cell, Tuberous sclerosis, mouse, Tsc2, autism, social, repetitive behavior

## Abbreviations:

Tuberous sclerosis complex (TSC), autism spectrum disorder (ASD), mammalian target of rapamycin complex 1 (mTORC1)

## Introduction:

Tuberous sclerosis complex (TSC) is a dominant neurogenetic disorder affecting about 1 in 6,000 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 proteins hamartin or tuberin, respectively (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; Green et al., 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). 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 and Silva; Lee et al.; 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 provide 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. 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). Behavioral studies of Lurcher mice revealed decreased anxiety-related behaviors, increased activity levels, and increased repetitive behaviors (Hilber et al., 2004; Martin et al.). As repetitive behaviors are a hallmark of ASD (Association, 2000), the Lurcher mice provides 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 be an important determinant of autism. In support of this hypothesis, Purkinje cell loss has been reported in some patients with TSC (Boer et al., 2008; Reith et al.). In an effort to study the relationship between the cerebellum, TSC, and ASD, we created and characterized a novel mouse model with Purkinje cell loss due to Purkinje cell specific *Tsc2* deletion (Reith et al.). 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. 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 TSC 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 129 and C57BL/6J background. Generation of the *Tsc2*$^{+/flox}$ and *Tsc2*$^{+/KO}$ mice have 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; Reith et al.).

### 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 video tapped for 20 minutes at 8:00, 12:00, and 4:00 for a total of one hour of video.

#### Reflexes

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

#### Marble Burying

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 minutes. 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 x 16 inch) plexiglass chamber with photobeams. Mice were placed in the chamber for 30 minutes. Total horizontal activity (distance traveled) as well as average speed were measured. To access for anxiety related behaviors, the percent of time in the center of the chamber was recorded.

#### Buried Food

To asses 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 x 40 x 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 x 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 ten minutes. 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 minutes 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) paw 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 (180s max) on an accelerating (4-40 rpm over 200s) ENV-576M RotaRod (Med Associates, Georgia, VT). Two trials were conducted on one day with approximately two hours between trials. The average of the two trials was used in the analysis.

### Light/Dark Box

The light/dark box was a 60 x 40 x 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 x 8 cm opening in the center. The light side was 40x 40 cm and lined with white contact paper. The dark side was 20 x 40 and was lined with black contact paper and covered. Mice were placed in the light side and allowed to freely explore for 10 minutes. ANY-maze software (Stoelting Wood Dale, IL) tracked the mice.

### Morris Water Maze

The Morris water maze was performed essentially as previously described (Dash et al., 2009). Mice were given four trials a day for five days with a hidden platform. Each trial began from each of four random starting positions. Mice were given a maximum of 60 second to find the platform. If a mouse failed to find the platform after 60 seconds, it was lead there by the experimenter. Mice were allowed to remain on the platform for 10 seconds before being placed in a 37$^{o}$C warming cage between trials. The intertrial interval was four minutes. 24 hours following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 seconds. Latency to first platform location and total number of platform crossings were recorded.

### Reversal Water Maze

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 hours following the end of the hidden platform testing, the platform was removed and a probe trial was given for 60 seconds. 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 5μm. Sections were rehydrated and subjected to antigen retrieval in a microwave with 10mM sodium citrate buffer, pH 6. Sections were blocked with 10% goat serum and 0.5% Triton X-100 in 1x PBS for 20 min. Slides were incubated in primary antibody solution overnight at 4$^{o}$C. Sections were then washed in 1x PBS and incubated with secondary antibody for one hour at room temperature. Sections were then washed in 1x 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-Adrich, 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), 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 $IgG_1$), Alexa Fluor 555 (anti-rabbit IgG) (anti-mouse $IgG_1$) (anti-mouse $IgG_{2a}$).

### Statistical analysis

Statistical analyses were conducted using analysis of variance (ANOVA) followed by Tukey post-hoc comparisons to compare the results of the control, heterozygous, and knockout geneotypes. When appropriate, sex was also used as an additional variable. For social preference and social novelty, a paired 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:

### General Health Assessment

Behavioral testing was conducted on Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice. At one month of age, the mice were given a general physical examination. All mice were healthy and had normal reflexes (eye blink, ear twitch, whisker twitch, grasping, and forepaw reach). At six weeks of age, 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 1A). There was no difference in latency to find food suggesting intact olfactory function in all genotypes.

Since Pcp2-Cre expression also occurs in retinal bipolar cells (Barski et al., 2000), we performed a vision dependent Morris Water maze at three months of age to determine if the animals could navigate to a visible platform. Mice were placed in a Morris Water maze arena with a visible platform. The latency to locate the platform was measured across three trials (Fig 1B). All mice found the platform in a similar time suggesting normal vision. Furthermore, retinal cell type-specific staining at 5 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 1).

### Motor Function

Purkinje cell specific homozygous deletion of *Tsc2* causes Purkinje cell loss as previously described (Reith et al.) (Fig 2G-I, J). Purkinje cell loss is not uniform across all folia. At one month of age, there is approximately 15% cell loss in folium II and 22% cell loss in folium IX. By three months of age, Purkinje cell loss progresses to about 86% in folium II and 43% in folium IX. All behavior testing was performed between these two time points. Interestingly, *Tsc2* haploinsufficiency does not affect Purkinje cell viability, as Tsc2f/- mice do not have any Purkinje cell loss at three months of age (Fig 2A-D). To examine if either complete *Tsc2* loss or haploinsufficiency in Purkinje cells affects motor function, mice were tested on the RotaRod at two months of age (Fig 3A). There was no significant difference in latency to fall among any of the groups. To further explore the motor system, gait analysis was performed by placing ink on the fore and hindpaw of the mice. Tsc2f/-;Cre mice had a slightly wider gait compared to controls (p=0.079) (Fig 3B) suggesting a mild ataxia. 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 3C).

### Social Behavior Testing

Impaired social behavior is one well reported deficit in 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 many mouse models of autism (Kwon et al., 2006; Moy et al., 2004; Peca et al., 2011). We used the three chambered apparatus 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. Male Tsc2f/+ mice spent significantly more time (p=0.0001) in the chamber with the stranger mouse than the chamber with the inanimate object (Fig 4A). Male Tsc2f/- mice spent slightly more time in the chamber with the stranger mouse than in the chamber with the inanimate object, but this was not statistically significant. Male Tsc2f/-;Cre mice spent approximately equal time in both chambers (Fig 4A). Tsc2f/+ female mice showed a slight preference for the chamber with the stranger mouse than the chamber with the inanimate object, but this was not statistically significant. 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 more time with the novel mouse than the familiar mouse (p=0.078) (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/+ female mice spent slightly more time with the novel mice than the familiar mice (p=0.076) (Fig 4D). Interestingly, Tsc2f/- female mice showed a significant preference for the novel mouse (p=0.015). 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.

### Repetitive Behavior and Anxiety

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 5A). Similarly, female Tsc2f/-;Cre mice also buried more marbles than either Tsc2f/+ (p=0.070) or Tsc2f/- (p=0.028) mice (Fig 5A). 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.

To determine anxiety levels, mice were placed in an open field arena and the percentage of time spent in the middle was determined. Anxious animals spend more time along the perimeter of the chamber rather than the middle. Male Tsc2f/-;Cre mice spent slightly (NS) more time in the middle of the chamber than Tsc2f/+ and Tsc2f/- (Fig 5B). Conversely, female Tsc2f/-;Cre mice spent slightly less time (NS) in the middle than Tsc2f/+ and Tsc2f/- mice possibly suggesting increased anxiety levels (Fig 5A). These data suggest sex specific differences in anxiety-related behaviors.

### Spatial Learning and Memory

To asses 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 Figure 2A). One aspect of restricted behaviors often seen in patients with autism 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 Figure 2B-C).

## Discussion:

Between 17%-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.; Ehninger et al.; Goorden et al., 2007; Waltereit et al.; Young et al.). Here we have developed and behaviorally characterized a new mouse model of TSC-associated ASD. More importantly, our data supports the hypothesis that loss of heterozygosity of *Tsc2* in cerebellar Purkinje cells and/or frank Purkinje cell loss contributes to ASD-like behavior.

We detected mild social deficits in heterozygous Tsc2f/- mice. These results add to the murine behavioral deficits detected in haploinsufficient *Tsc1* or *Tsc2* backgrounds (Ehninger et al.; Goorden et al., 2007; Waltereit et al.; Young et al.). Goorden et al detected social behavioral deficits in a $Tsc1^{+/-}$ model. Additionally, animals haploinsufficient for *Tsc2* showed social behavioral deficits when combined with seizures (Waltereit et al.) or gestational immune activation (Ehninger et al.). 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 plausibly 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.

Since there is mounting evidence that cerebellar abnormalities play a role in ASD, particularly Purkinje cell loss (Bailey et al., 1998a; Fatemi et al., 2012; Fatemi et al., 2002; 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 (Reith et al.). Tsc2f/-;Cre mice loose 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.). 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; Middleton and Strick, 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. Assessment of sociability at different time points will answer that question.

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.; 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 both repetitive behaviors and social deficits suggesting that gender does 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 *Tsc2*$^{+/-}$ mice having learning deficits (Ehninger et al., 2008). One possible explanation behind these conflicting results might be due to strain differences of the mice. Ehninger et al. 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 summary, we demonstrate that deletion of *Tsc2* in Purkinje cells, leads to social behavior deficits and increased repetitive behaviors. This provides a novel mouse model of TSC-associated autism that would 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 therefore ameliorating behavioral deficits.

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## Figure Legends:

Figure 1: General health assessment. (A) Latency to buried food as a measure of olfaction. There was no significant difference in latency to the food across any of the trials. (B) Vision dependent water maze was conducted to determine the ability of the mice to see. There was no significant difference in latency to the visible platform across any of the trials.

Figure 2: Loss of *Tsc2* causes Purkinje cell loss. (A-C) Calbindin staining at 3 months of age shows loss of Purkinje cell is 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.

Figure 3: Motor Function. (A) RotaRod performance at 2 months of age shows no difference in latency to fall among Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre. (B) Gait width in Tsc2f/-;Cre mice was slightly increased (p=0.079) compared to Tsc2f/+. (C) Average speed in an open-field did not differ between Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice.

Figure 4: Social behavior deficits. (A) Male Tsc2f/+ mice spent more time (p=0.0001) in the social chamber than in the inanimate object chamber. Tsc2f/- mice showed a slight preference (NS) for the social chamber. However, Tsc2f/-;Cre mice spent equal time in both chambers. (B) Female Tsc2f/+ mice showed a slight preference (NS) for the stranger mouse

compared with the inanimate object. However, both Tsc2f/- and Tsc2f/-;Cre mice spent equal time in both chambers. (C) Male Tsc2f/+ mice spent slightly (p=0.078) more time 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=0.076) and Tsc2f/- (p=0.0008) mice spent more time with the novel mice than the familiar mice. Tsc2f/-;Cre mice also spent slightly more time (NS) with the novel mouse than with the familiar mouse.

Figure 5: Repetitive behaviors and anxiety. (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. (B) In an open-field arena, male Tsc2f/-;Cre (n=7) mice spent slightly (NS) more time in the middle than either Tsc2f/+ (n=17) or Tsc2f/- (n=17) mice. Female Tsc2f/-;Cre (n=11) mice, however, spent less time in the middle (NS) than either Tsc2f/+ (n=18) or Tsc2f/- (n=16) mice.

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.

Supplemental Figure 1: Layer specific staining of Tsc2f/f;Cre retina at 5 months of age. (A-B) H&E staining of Tsc2f/+ (A) and Tsc2f/f;Cre retina (B). (C-D) Pax6 staining for amacrine, ganglion, and horizontal cells in the Tscf/+ (C) and Tsc2f/f;Cre (D). (E-F) GS staining for muller glia cells in the Tsc2f/+ (E) and Tsc2f/f;Cre (F). (G-H) PKCa for rod bipolar cells in the Tsc2f/+ (G) and Tsc2f/f;Cre (H). (I-J) R4D2 staining for rod photoreceptor cells in the Tsc2f/+ (I) and Tsc2f/f;Cre (J). (K-L) Cone arrestin staining for cone cells in the Tsc2f/+ (K) and Tsc2f/f;Cre (L).

Supplemental Figure 2: Morris Water Maze assessment of learning and reversal learning. (A) Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice did not show any differences in spatial learning. (B) There were no differences in reversal learning once the platform was moved. (C) A probe trial was performed after 24 hours after the reverse water maze and did not show any difference among Tsc2f/+, Tsc2f/-, and Tsc2f/-;Cre mice.

## References

1993. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell. 75, 1305-15.

Abell, F., et al., 1999. The neuroanatomy of autism: a voxel-based whole brain analysis of structural scans. Neuroreport. 10, 1647-51.

Allan, A. M., et al., 2008. The loss of methyl-CpG binding protein 1 leads to autism-like behavioral deficits. Hum Mol Genet. 17, 2047-57.

Allen, G., Courchesne, E., 2003. Differential effects of developmental cerebellar abnormality on cognitive and motor functions in the cerebellum: an fMRI study of autism. Am J Psychiatry. 160, 262-73.

Asano, E., et al., 2001. Autism in tuberous sclerosis complex is related to both cortical and subcortical dysfunction. Neurology. 57, 1269-77.

Association, A. P., 2000. Diagnostic and Statistical Manual of Mental Disorders DSM-IV-TR Fourth Edition. Amer Psychiatric Pub.

Astrinidis, A., et al., 2002. Tuberin, the tuberous sclerosis complex 2 tumor suppressor gene product, regulates Rho activation, cell adhesion and migration. Oncogene. 21, 8470-6.

Au, K. S., et al., 1999. Complete inactivation of the TSC2 gene leads to formation of hamartomas. Am J Hum Genet. 65, 1790-5.

Au, K. S., et al., 2007. Genotype/phenotype correlation in 325 individuals referred for a diagnosis of tuberous sclerosis complex in the United States. Genet Med. 9, 88-100.

Aylward, E. H., et al., 1999. MRI volumes of amygdala and hippocampus in non-mentally retarded autistic adolescents and adults. Neurology. 53, 2145-50.

Bailey, A., et al., 1998a. A clinicopathological study of autism. Brain. 121 ( Pt 5), 889-905.

Bailey, D. B., Jr., et al., 1998b. Autistic behavior in young boys with fragile X syndrome. J Autism Dev Disord. 28, 499-508.

Barski, J. J., et al., 2000. Cre recombinase expression in cerebellar Purkinje cells. Genesis. 28, 93-8.

Bertrand, J., et al., 2001. Prevalence of autism in a United States population: the Brick Township, New Jersey, investigation. Pediatrics. 108, 1155-61.

Bhaskar, P. T., Hay, N., 2007. The two TORCs and Akt. Dev Cell. 12, 487-502.

Boer, K., et al., 2008. Clinicopathological and immunohistochemical findings in an autopsy case of tuberous sclerosis complex. Neuropathology. 28, 577-90.

Butler, M. G., et al., 2005. Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations. J Med Genet. 42, 318-21.

Caddy, K. W., Biscoe, T. J., 1979. Structural and quantitative studies on the normal C3H and Lurcher mutant mouse. Philos Trans R Soc Lond B Biol Sci. 287, 167-201.

Cappon, D., 1953. Clinical manifestations of autism and schizophrenia in childhood. Can Med Assoc J. 69, 44-9.

Chan, J. A., et al., 2004. Pathogenesis of tuberous sclerosis subependymal giant cell astrocytomas: biallelic inactivation of TSC1 or TSC2 leads to mTOR activation. J Neuropathol Exp Neurol. 63, 1236-42.

Chevere-Torres, I., et al., Impaired social interactions and motor learning skills in tuberous sclerosis complex model mice expressing a dominant/negative form of tuberin. Neurobiol Dis. 45, 156-64.

Coldren, J. T., Halloran, C., 2003. Spatial reversal as a measure of executive functioning in children with autism. J Genet Psychol. 164, 29-41.

Corbett, B. A., et al., 2009. Examining executive functioning in children with autism spectrum disorder, attention deficit hyperactivity disorder and typical development. Psychiatry Res. 166, 210-22.

Courchesne, E., 1997. Brainstem, cerebellar and limbic neuroanatomical abnormalities in autism. Curr Opin Neurobiol. 7, 269-78.

Courchesne, E., Pierce, K., 2005. Brain overgrowth in autism during a critical time in development: implications for frontal pyramidal neuron and interneuron development and connectivity. Int J Dev Neurosci. 23, 153-70.

Courchesne, E., et al., 1994. The brain in infantile autism: posterior fossa structures are abnormal. Neurology. 44, 214-23.

Crawley, J. N., 2007. Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol. 17, 448-59.

Crino, P. B., et al., 2006. The tuberous sclerosis complex. N Engl J Med. 355, 1345-56.

Curatolo, P., et al., Autism spectrum disorders in tuberous sclerosis: pathogenetic pathways and implications for treatment. J Child Neurol. 25, 873-80.

Dash, P. K., et al., 2009. Sulforaphane improves cognitive function administered following traumatic brain injury. Neurosci Lett. 460, 103-7.

Dawson, G., et al., 1998. Neuropsychological correlates of early symptoms of autism. Child Dev. 69, 1276-85.

de Vries, P. J., et al., 2007. The psychopathologies of children and adolescents with tuberous sclerosis complex (TSC): a postal survey of UK families. Eur Child Adolesc Psychiatry. 16, 16-24.

DiMario, F. J., Jr., 2004. Brain abnormalities in tuberous sclerosis complex. J Child Neurol. 19, 650-7.

Ehninger, D., et al., 2008. Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. Nat Med. 14, 843-8.

Ehninger, D., et al., Gestational immune activation and Tsc2 haploinsufficiency cooperate to disrupt fetal survival and may perturb social behavior in adult mice. Mol Psychiatry. 17, 62-70.

Ehninger, D., Silva, A. J., Rapamycin for treating Tuberous sclerosis and Autism spectrum disorders. Trends Mol Med. 17, 78-87.

Eluvathingal, T. J., et al., 2006. Cerebellar lesions in tuberous sclerosis complex: neurobehavioral and neuroimaging correlates. J Child Neurol. 21, 846-51.

Exner, C., et al., 2004. Cerebellar lesions in the PICA but not SCA territory impair cognition. Neurology. 63, 2132-5.

Fatemi, S. H., et al., 2012. Consensus Paper: Pathological Role of the Cerebellum in Autism. Cerebellum.

Fatemi, S. H., et al., 2002. Purkinje cell size is reduced in cerebellum of patients with autism. Cell Mol Neurobiol. 22, 171-5.

Folstein, S., Rutter, M., 1977. Infantile autism: a genetic study of 21 twin pairs. J Child Psychol Psychiatry. 18, 297-321.

Gillberg, I. C., et al., 1994. Autistic behaviour and attention deficits in tuberous sclerosis: a population-based study. Dev Med Child Neurol. 36, 50-6.

Gonzalo-Ruiz, A., Leichnetz, G. R., 1990. Connections of the caudal cerebellar interpositus complex in a new world monkey (Cebus apella). Brain Res Bull. 25, 919-27.

Goorden, S. M., et al., 2007. Cognitive deficits in Tsc1+/- mice in the absence of cerebral lesions and seizures. Ann Neurol. 62, 648-55.

Gordon, N., 2007. The cerebellum and cognition. Eur J Paediatr Neurol. 11, 232-4.

Green, A. J., et al., 1994a. The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet. 3, 1833-4.

Green, A. J., et al., 1994b. Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet. 6, 193-6.

Guess, D., Carr, E., 1991. Emergence and maintenance of stereotypy and self-injury. Am J Ment Retard. 96, 299-319; discussion 321-44.

Hashimoto, T., et al., 1995. Development of the brainstem and cerebellum in autistic patients. J Autism Dev Disord. 25**,** 1-18.

Henske, E. P., et al., 1996. Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. Am J Hum Genet. 59**,** 400-6.

Henske, E. P., et al., 1997. Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogenesis of tuberous sclerosis tumors. Am J Pathol. 151**,** 1639-47.

Hilber, P., et al., 2004. Stress and anxious-related behaviors in Lurcher mutant mice. Brain Res. 1003**,** 108-12.

Hoke, K. L., et al., 2007. Integration of sensory and motor processing underlying social behaviour in tungara frogs. Proc Biol Sci. 274**,** 641-9.

Huang, J., Manning, B. D., 2008. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 412**,** 179-90.

Hunt, A., Dennis, J., 1987. Psychiatric disorder among children with tuberous sclerosis. Dev Med Child Neurol. 29**,** 190-8.

Hunt, A., Shepherd, C., 1993. A prevalence study of autism in tuberous sclerosis. J Autism Dev Disord. 23**,** 323-39.

Inoki, K., et al., 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 17**,** 1829-34.

Inoki, K., et al., 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 4**,** 648-57.

Kaufmann, W. E., et al., 2003. Specificity of cerebellar vermian abnormalities in autism: a quantitative magnetic resonance imaging study. J Child Neurol. 18**,** 463-70.

Kelly, R. M., Strick, P. L., 2003. Cerebellar loops with motor cortex and prefrontal cortex of a nonhuman primate. J Neurosci. 23**,** 8432-44.

Kennedy, D. P., et al., 2006. Failing to deactivate: resting functional abnormalities in autism. Proc Natl Acad Sci U S A. 103**,** 8275-80.

Krienen, F. M., Buckner, R. L., 2009. Segregated fronto-cerebellar circuits revealed by intrinsic functional connectivity. Cereb Cortex. 19**,** 2485-97.

Kwon, C. H., et al., 2006. Pten regulates neuronal arborization and social interaction in mice. Neuron. 50**,** 377-88.

Lee, T. L., et al., Integrative gene network analysis provides novel regulatory relationships, genetic contributions and susceptible targets in autism spectrum disorders. Gene. 496**,** 88-96.

Martin, L. A., et al., Repetitive behavior and increased activity in mice with Purkinje cell loss: a model for understanding the role of cerebellar pathology in autism. Eur J Neurosci. 31**,** 544-55.

Marui, T., et al., 2004. Association between the neurofibromatosis-1 (NF1) locus and autism in the Japanese population. Am J Med Genet B Neuropsychiatr Genet. 131B**,** 43-7.

Meikle, L., et al., 2007. A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. J Neurosci. 27**,** 5546-58.

Middleton, F. A., Strick, P. L., 2000. Basal ganglia and cerebellar loops: motor and cognitive circuits. Brain Res Brain Res Rev. 31**,** 236-50.

Middleton, F. A., Strick, P. L., 2001. Cerebellar projections to the prefrontal cortex of the primate. J Neurosci. 21**,** 700-12.

Moy, S. S., et al., 2004. Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav. 3**,** 287-302.

Muhle, R., et al., 2004. The genetics of autism. Pediatrics. 113**,** e472-86.

Nie, D., et al., 2010. Tsc2-Rheb signaling regulates EphA-mediated axon guidance. Nat Neurosci. 13, 163-72.

Niida, Y., et al., 2001. Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. Am J Hum Genet. 69, 493-503.

Numis, A. L., et al., 2011. Identification of risk factors for autism spectrum disorders in tuberous sclerosis complex. Neurology. 76, 981-7.

Osborne, J. P., et al., 1991. Epidemiology of tuberous sclerosis. Ann N Y Acad Sci. 615, 125-7.

Palmen, S. J., et al., 2004. Neuropathological findings in autism. Brain. 127, 2572-83.

Paulus, K. S., et al., 2004. Pure post-stroke cerebellar cognitive affective syndrome: a case report. Neurol Sci. 25, 220-4.

Peca, J., et al., 2011. Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. Nature. 472, 437-42.

Prevention, C. f. D. C. a., Prevalence of Autism Spectrum Disorders --- Autism and Developmental Disabilities Monitoring Network, United States, 2006. Vol. 58. Centers for Disease Control and Prevention, Atlanta, 2006, pp. 1-20.

Reith, R. M., et al., Loss of the tuberous sclerosis complex protein tuberin causes Purkinje cell degeneration. Neurobiology of Disease. In Press, Corrected Proof.

Rogers, T. D., et al., Connecting the dots of the cerebro-cerebellar role in cognitive function: Neuronal pathways for cerebellar modulation of dopamine release in the prefrontal cortex. Synapse. 65, 1204-12.

Rutter, M., 1978. Diagnosis and definition of childhood autism. J Autism Child Schizophr. 8, 139-61.

Saab, C. Y., Willis, W. D., 2003. The cerebellum: organization, functions and its role in nociception. Brain Res Brain Res Rev. 42, 85-95.

Sarbassov, D. D., et al., 2005. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 17, 596-603.

Schmahmann, J. D., Sherman, J. C., 1998. The cerebellar cognitive affective syndrome. Brain. 121 ( Pt 4), 561-79.

Smalley, S. L., et al., 1992. Autism and tuberous sclerosis. J Autism Dev Disord. 22, 339-55.

Tavano, A., et al., 2007. Disorders of cognitive and affective development in cerebellar malformations. Brain. 130, 2646-60.

Tavazoie, S. F., et al., 2005. Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. Nat Neurosci. 8, 1727-34.

Thomas, A., et al., 2009. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology (Berl). 204, 361-73.

van Slegtenhorst, M., et al., 1997. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science. 277, 805-8.

Vargas, D. L., et al., 2005. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol. 57, 67-81.

Waltereit, R., et al., Epilepsy and Tsc2 haploinsufficiency lead to autistic-like social deficit behaviors in rats. Behav Genet. 41, 364-72.

Way, S. W., et al., 2009. Loss of Tsc2 in radial glia models the brain pathology of tuberous sclerosis complex in the mouse. Hum Mol Genet. 18, 1252-65.

Weber, A. M., et al., 2000. Autism and the cerebellum: evidence from tuberous sclerosis. J Autism Dev Disord. 30, 511-7.

Wing, L., 1981. Language, social, and cognitive impairments in autism and severe mental retardation. J Autism Dev Disord. 11, 31-44.

Wiznitzer, M., 2004. Autism and tuberous sclerosis. J Child Neurol. 19, 675-9.

Wolpert, D. M., et al., 2003. A unifying computational framework for motor control and social interaction. Philos Trans R Soc Lond B Biol Sci. 358**,** 593-602.

Wong, V., 2006. Study of the relationship between tuberous sclerosis complex and autistic disorder. J Child Neurol. 21**,** 199-204.

Yamamoto, T., et al., 1992. The medial dorsal nucleus is one of the thalamic relays of the cerebellocerebral responses to the frontal association cortex in the monkey: horseradish peroxidase and fluorescent dye double staining study. Brain Res. 579**,** 315-20.

Yip, J., et al., 2009. Decreased GAD65 mRNA levels in select subpopulations of neurons in the cerebellar dentate nuclei in autism: an in situ hybridization study. Autism Res. 2**,** 50-9.

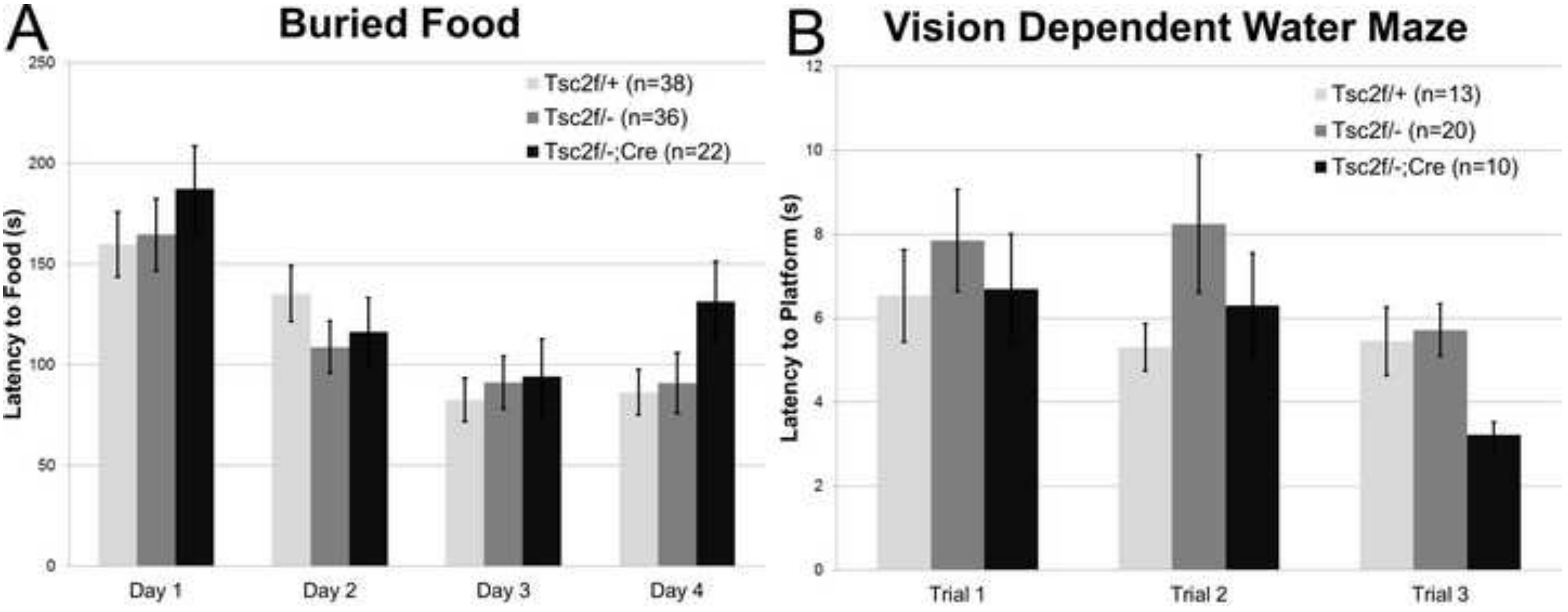
Young, D. M., et al., Altered ultrasonic vocalizations in a tuberous sclerosis mouse model of autism. Proc Natl Acad Sci U S A. 107**,** 11074-9.

Zhang, Y., et al., 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol. 5**,** 578-81.

Zhou, J., et al., 2011. Tsc1 mutant neural stem/progenitor cells exhibit migration deficits and give rise to subependymal lesions in the lateral ventricle. Genes Dev. 25**,** 1595-600.

Zuo, J., et al., 1997. Neurodegeneration in Lurcher mice caused by mutation in delta2 glutamate receptor gene. Nature. 388**,** 769-73.

Figure1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Buried Food
Latency to Food (s)
250
200
150
100
50
0
Tsc2f/+ (n=38)
Tsc2f/- (n=36)
Tsc2f/-;Cre (n=22)
Day 1
Day 2
Day 3
Day 4
B
Vision Dependent Water Maze
Latency to Platform (s)
12
10
8
6
4
2
0
Tsc2f/+ (n=13)
Tsc2f/- (n=20)
Tsc2f/-;Cre (n=10)
Trial 1
Trial 2
Trial 3

**Figure2**

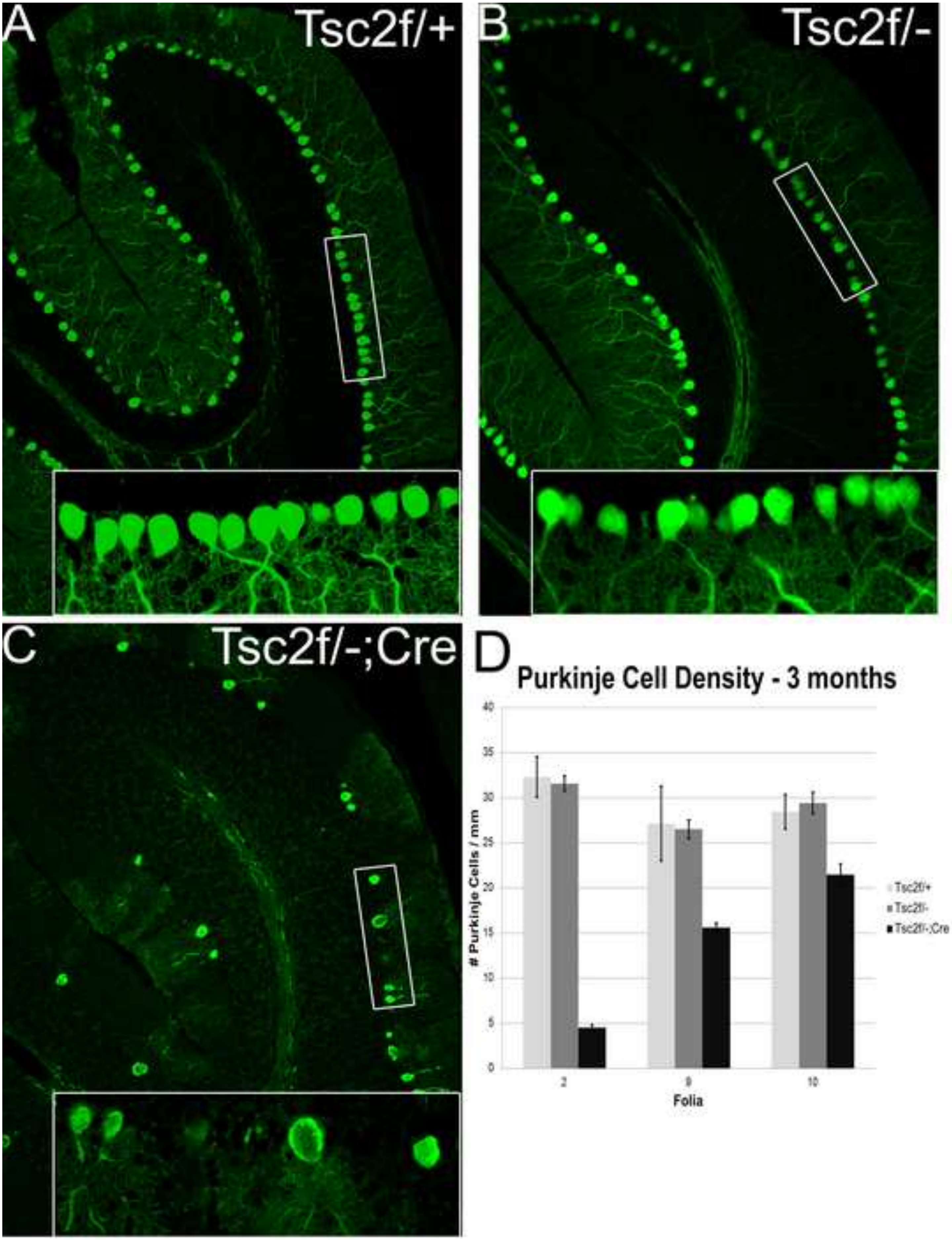
A
Tsc2f/+
B
Tsc2f/-
C
Tsc2f/-;Cre
D
Purkinje Cell Density - 3 months
# Purkinje Cells / mm
0
5
10
15
20
25
30
35
40
2
9
10
Folia
▒ Tsc2f/+
■ Tsc2f/-
■ Tsc2f/-;Cre

**Figure3**

Click here to download high resolution image

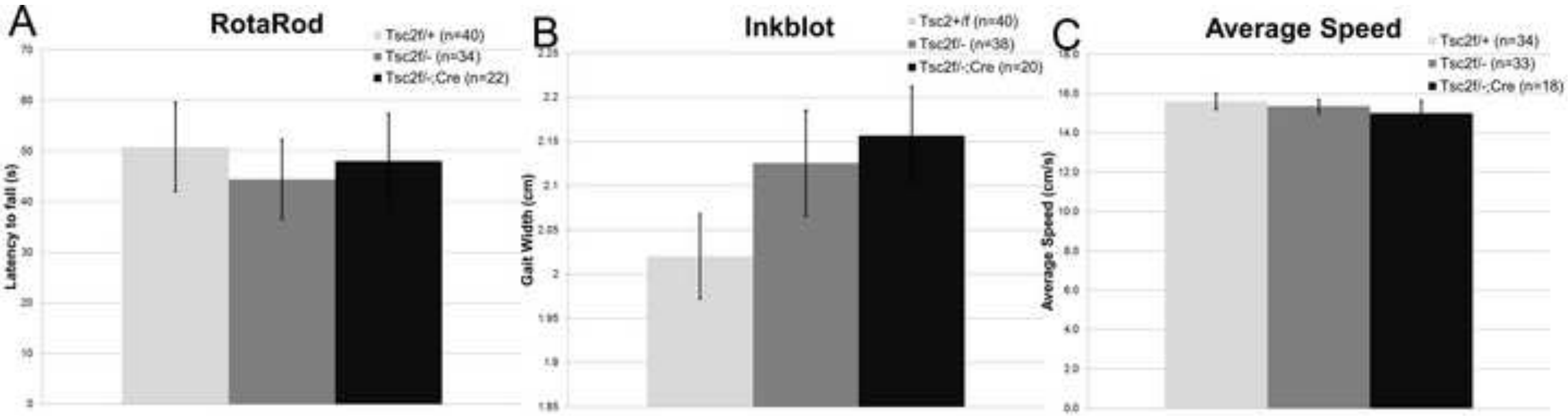

Figure4

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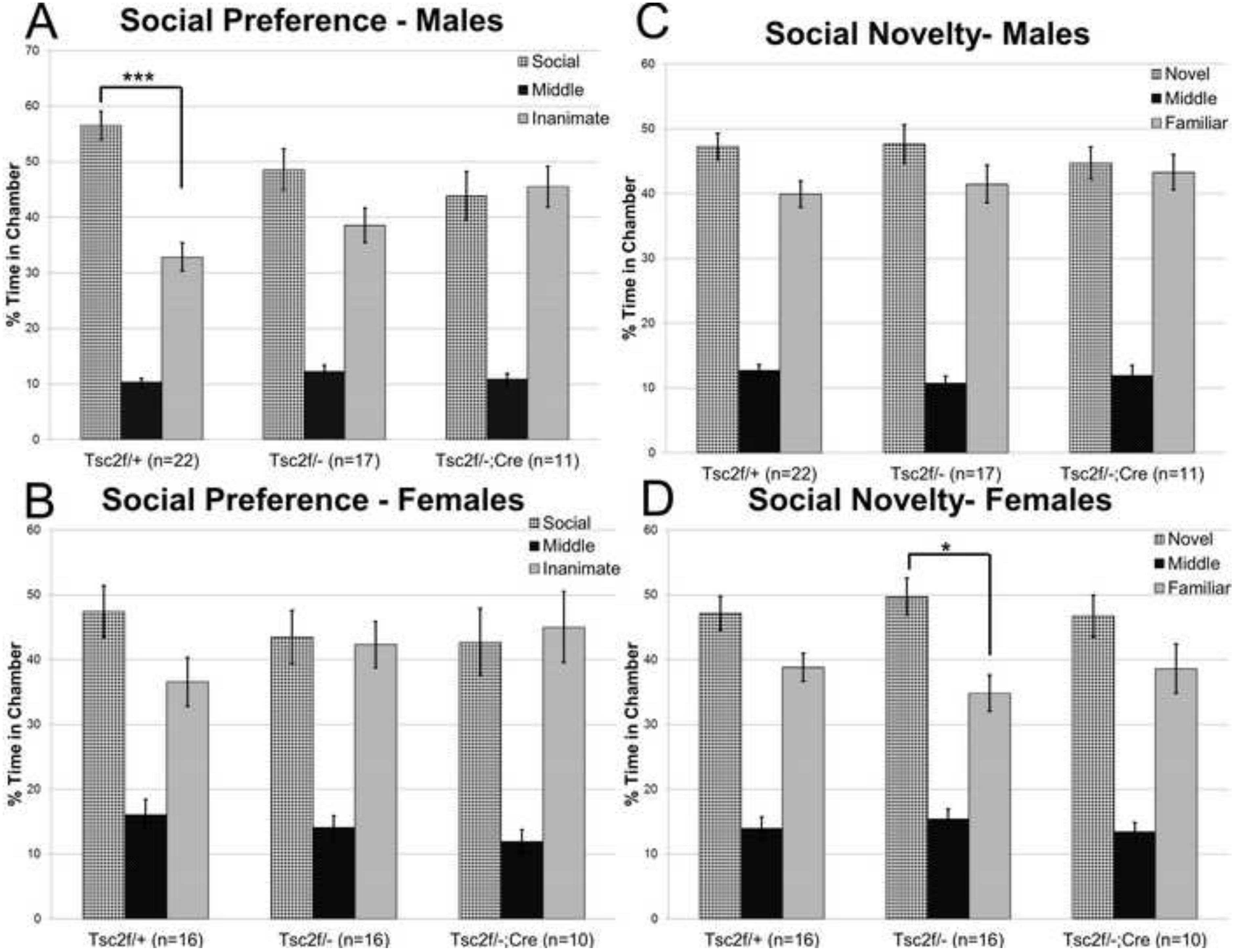
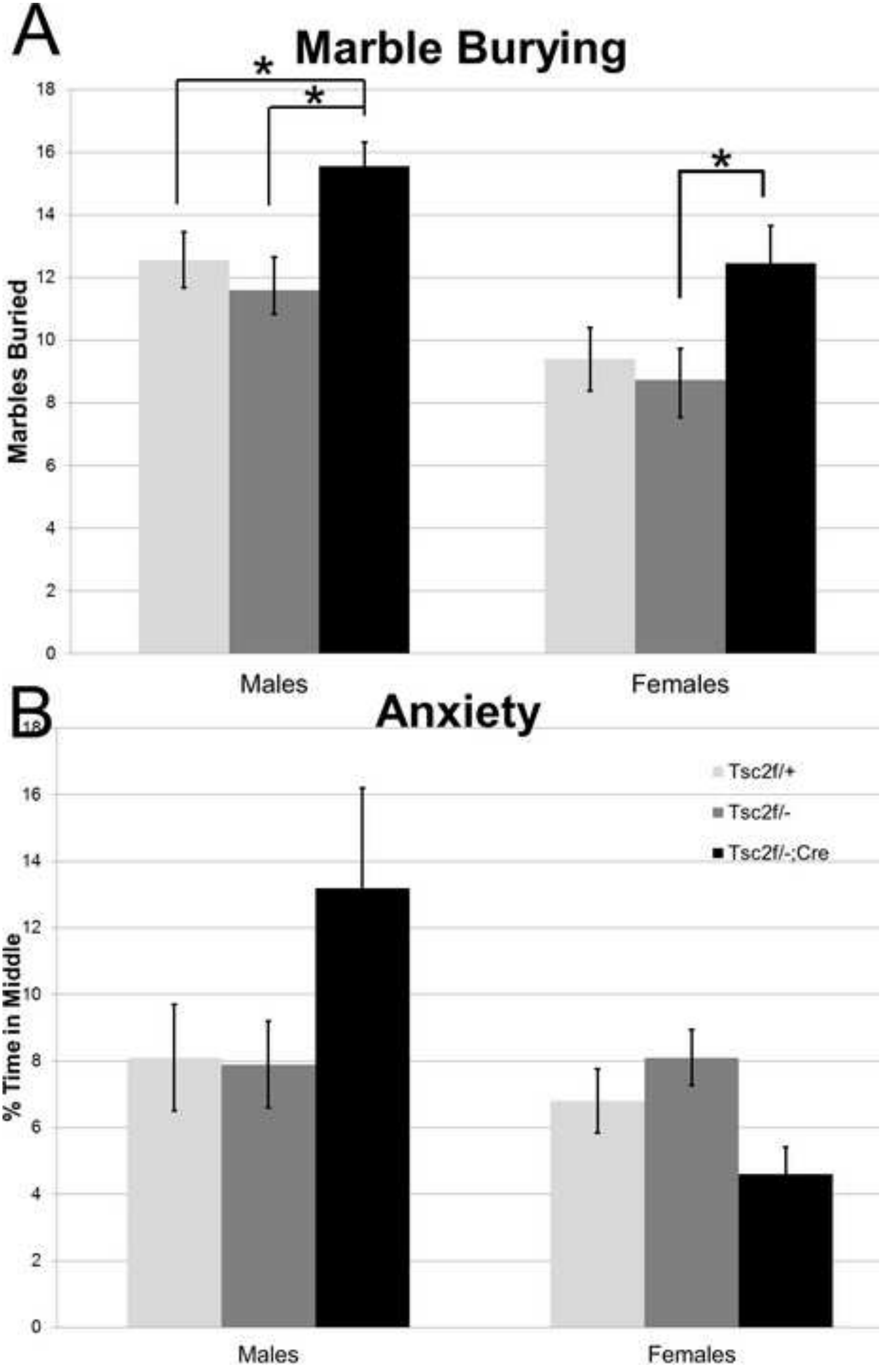


Figure5


A
Marble Burying
Marbles Buried
18
16
14
12
10
8
6
4
2
0
*
*
*
Males
Females
B
Anxiety
% Time in Middle
18
16
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6
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Tsc2f/+
Tsc2f/-
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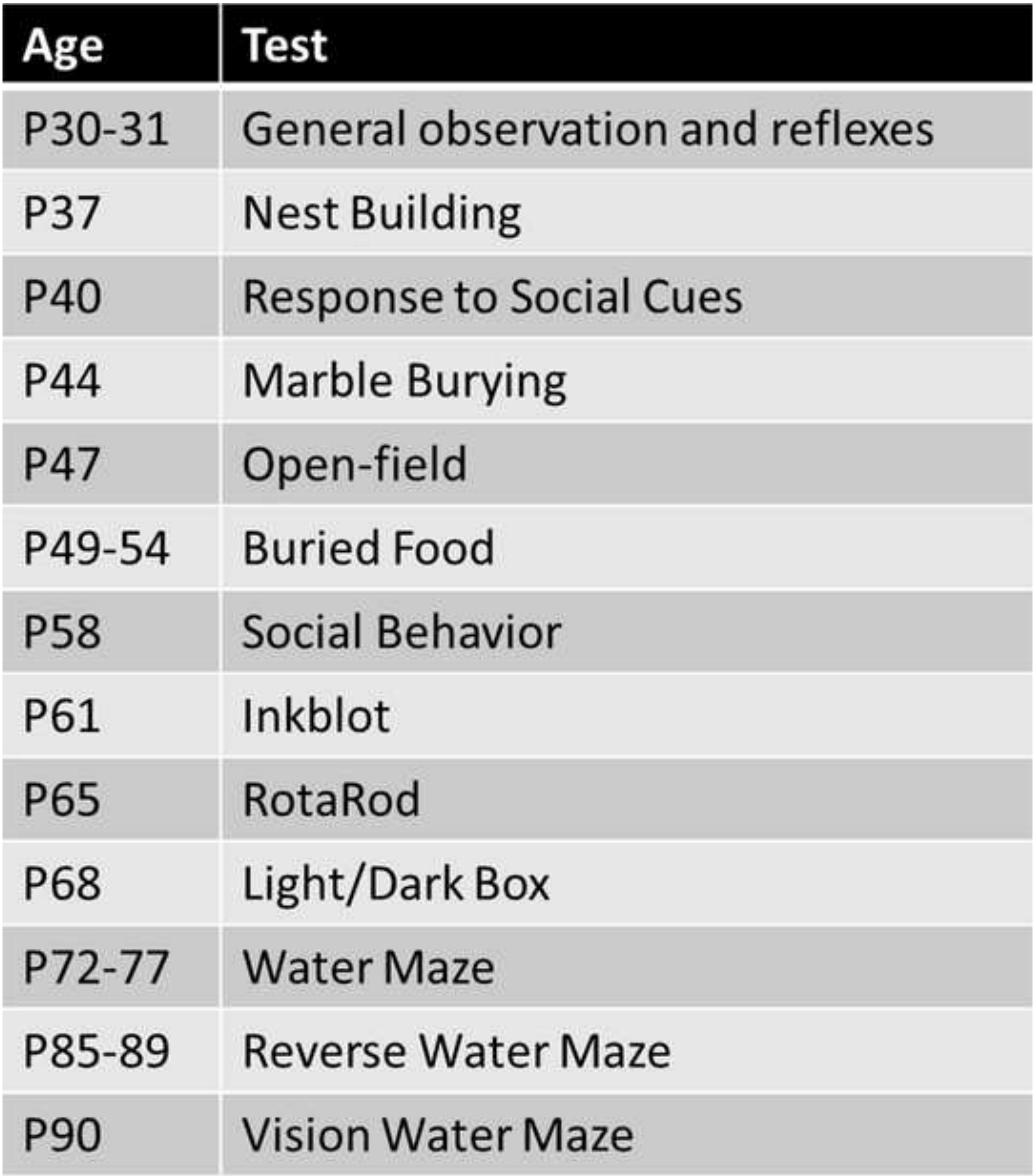
Males
Females



| 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 |

Supplementary Material 1

Supplementary Material 2