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Prenatal rapamycin results in early and late behavioral abnormalities in wildtype C57Bl/6 mice

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Abstract

Mammalian target of rapamycin (mTOR) signaling has been shown to be deregulated in a number of genetic, neurodevelopmental disorders including Tuberous Sclerosis Complex, Neurofibromatosis, Fragile X, and Rett syndromes. As a result, mTOR inhibitors, such as rapamycin and its analogs, offer potential therapeutic avenues for these disorders. Some of these disorders – such as Tuberous Sclerosis Complex – can be diagnosed prenatally. Thus, prenatal administration of these inhibitors could potentially prevent the development of the devastating symptoms associated with these disorders. To assess the possible detrimental effects of prenatal rapamycin treatment, we evaluated both early and late behavioral effects of a single rapamycin treatment at embryonic day 16.5 in wildtype C57Bl/6 mice. This treatment adversely impacted early developmental milestones as well as motor function in adult animals. Rapamycin also resulted in anxiety-like behaviors during both early development and adulthood but did not affect adult social behaviors. Together, these results indicate that a single, prenatal rapamycin treatment not only adversely affects early postnatal development but also results in long lasting negative effects, persisting into adulthood. These findings are of importance in considering prenatal administration of rapamycin and related drugs in the treatment of patients with neurogenetic, neurodevelopmental disorders.

Keywords

mTOR; tuberous sclerosis; embryonic; mouse

Introduction

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays critical roles in cell growth, proliferation, and protein synthesis. mTOR is the critical kinase component of two protein complexes, mTOR complex 1 (mTORC1) and mTORC2, distinguishable by their distinct protein components and kinase targets. These complexes play critical roles in the regulation of protein synthesis, mitochondrial and lipid metabolism, cytoskeletal organization, cell growth and proliferation, and autophagy in response to changes in metabolic cues such as cellular stress and growth factors (Laplante and Sabatini 2012).

Dysregulated mTOR signaling is thought to contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases

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(Bove et al. 2011). In addition to these age-related neurodegenerative disorders, abnormal mTOR signaling has been implicated in a number of genetic, neurologic diseases of childhood (Ehninger and Silva 2011; Kelleher and Bear 2008). Mutations of phosphatase and tensin homolog deleted in chromosome 10 (PTEN), which acts to negatively regulate PI3K/AKT upstream of mTOR, is associated with autism, while variants of the MET receptor tyrosine kinase, which signals upstream of AKT, are also associated with a greater risk of autism. Furthermore, *neurofibromin 1 (NF1)*, the gene mutated in neurofibromatosis, diminishes mTOR signaling. In addition, *Fragile X mental retardation protein (FMRP)*, the gene mutated in Fragile X, also acts to modulate mTOR signaling (Kelleher and Bear 2008). Both of these latter diseases are associated with high rates of neurodevelopmental disorders, such as intellectual disability, learning disability, and autism.

Tuberous Sclerosis Complex (TSC) is another disorder in which deregulated mTOR signaling plays a central role in disease pathogenesis. TSC is associated with a high frequency of several neuropsychiatric disorders including epilepsy, intellectual disability, and autism (De Vries 2010; de Vries et al. 2007; Prather and de Vries 2004; Tsai and Sahin 2011). TSC results from mutation in either *TSC1* or *TSC2*, and the protein products of these genes form a complex, which acts to negatively regulate mTORC1. Hence, loss of either *TSC1* or *TSC2* leads to activated mTORC1 and downstream events. Multiple TSC mouse models have been generated, including constitutive knockouts as well as neuronal or glial specific conditional mutants. Homozygous loss of either *Tsc1* or *Tsc2* is embryonic lethal, while *Tsc2*^{+/-} mice display axonal pathfinding deficits (Nie et al. 2010). In addition, glial, neuronal, and neural progenitor specific mouse models display cellular hypertrophy, white matter abnormalities, and gliosis, as well as failure to thrive, seizures, and early mortality (Meikle et al. 2007; Uhlmann et al. 2002; Way et al. 2009; Zeng et al. 2008).

As mTORC1 signaling is abnormally elevated in these neurogenetic, neurodevelopmental disorders, rapamycin (sirolimus) and its analogs have been considered as potential therapies. Rapamycin is the founding member of a large family of related compounds (collectively termed rapalogues) that act by binding to the 12kDa protein FKBP12. Rapamycin and FKBP12 together act as a specific allosteric modulator of mTORC1, inhibiting most though not all of its functions (Benjamin et al. 2011).

Clinically, rapamycin has been widely used as an immunosuppressive agent to prevent rejection after organ transplantation. In addition to this well-characterized and FDA-approved role, rapamycin is also being studied in a number of diseases including cancer (Benjamin et al. 2011; Wander et al. 2011) and age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (Bove et al. 2011). Rapamycin also offers a potential therapeutic option for neurogenetic disorders such as TSC. In brain specific TSC mutant mouse models, early postnatal rapamycin treatment results in a remarkable rescue of anatomic deficits – including cellular hypertrophy and white matter abnormalities – along with prevention of the early mortality in these animals (Meikle et al. 2008; Tsai et al. 2012; Way et al. 2009; Zeng et al. 2008). Furthermore, rapamycin and its analogue everolimus have resulted in significant clinical regression of subependymal giant cell astrocytomas (SEGA) in TSC patients (Franz et al. 2006; Krueger et al. 2010).

A role for rapamycin in treating the neuropsychiatric abnormalities in TSC has also been tested in various TSC mouse models. Neuronal and glial specific TSC mutants demonstrate significant seizure activity, which is prevented by treatment prior to the onset of seizures (Meikle et al. 2008; Zeng et al. 2008). Furthermore, rapamycin treatment also corrects autistic-like behaviors in cerebellar *Tsc1* mutant mice (Tsai et al. 2012). In addition, both *Tsc1*^{+/-} and *Tsc2*^{+/-} mice demonstrate deficient hippocampal dependent learning and social impairment (Ehninger et al. 2008; Goorden et al. 2007). Rapamycin treatment of these mice

– even with treatment onset in adulthood – results in rescue of their demonstrated hippocampal dependent learning deficits (Ehninger et al. 2008).

The ability to diagnose TSC both pre- and peri-natally (Datta et al. 2008; Tworetzky et al. 2003) raises the possibility of treating TSC patients at these same very early time points, possibly improving outcomes in these individuals for such conditions as autism, intellectual disability, and epilepsy. Recently, we have reported that in a neural progenitor model of TSC, prenatal rapamycin treatment led to an improvement in survival without apparent deleterious impact on pregnancy or delivery (Anderl et al. 2011). However, the severity of the phenotype in this model made it difficult or impossible to assess possible adverse effects of treatment. Since rapamycin has been demonstrated to have adverse behavioral consequences when administered to mice during adulthood (Dash et al. 2006), here we examined the potential negative impacts of prenatal rapamycin treatment.

MATERIALS AND METHODS

Mice

The C57Bl/6J mice used in this study were obtained from Jackson Laboratories. We used males and females for experimental procedures unless otherwise specified. All experimental protocols were approved by Animal Research Committee at Boston Children's Hospital.

Rapamycin Treatment

Rapamycin was dissolved in 0.25% Tween-20, 0.25% polyethylene glycol prior to usage. Pregnant mice were injected with vehicle (0.25% polyethylene glycol, 0.25% tween) or rapamycin (1 mg/kg) intraperitoneally once on embryonic day (E)16.5 (morning of birth designated as post natal day (P) 0.5).

Developmental Milestones

Developmental milestone testing commenced on postnatal day P4 and continued every other day until P14 for all animals. Tests performed were: righting reflex, negative geotaxis, level screen test, cliff aversion, and auditory startle, described in detail below (Heyser 2004; Roubertoux et al. 2006; Scattoni et al. 2008). Pups were weighed prior to each testing period and were reunited with their mother immediately after testing. Testing environment, time period, and investigator were consistent across all trials. Investigators were blinded to treatment status (rapamycin vs. vehicle).

Righting Reflex—Pups were placed on their backs and latency to turn over (place all four paws on the surface) was recorded.

Negative Geotaxis—Pups were positioned with nose pointing towards the bottom of a piece of wire mesh (1/16"; 8"×10") positioned at a 45-degree angle. Latency to rotate 180 degrees and re-position facing towards the top of the screen was recorded.

Level screen test—Pups were placed on a piece of wire mesh positioned horizontally on flat surface and were gently dragged down the length of the screen by the tail. Strength of grip was measured by resistance to pulling. Since investigator scoring was based on subjective judgment of pup resistance, one observer performed this and all subsequent tests. Pup responses were scored on a scale of 0–3 with 0 indicating no response and 3 indicating strong resistance to pulling (i.e. pup attempted to grip wire mesh and cling).

Cliff Aversion—Pups were positioned on wire mesh held horizontally 6 inches above the bench with both front paws hanging approximately 1 cm over the edge of the screen. Pups

were scored on a scale of 0–3 based on how rapidly they retreated from the edge of the screen. No response was recorded as 0, and immediate and rapid withdrawal from the edge of the screen was recorded as 3.

Auditory Startle—Pups were allowed to rest on the heating pad while the experimenter snapped her fingers approximately 1 inch behind pup's head. Startle response was recorded and scored 0–3. No response merited a score of 0, and a significant flinch or twitch as judged by the observer was scored as 3.

Open Field

Open field testing was performed as described (Holmes et al. 2001). Mice were placed in a lit (50 lux) open field (45 × 45cm square apparatus) and allowed to run freely for a 15 minute period. Mice were not previously habituated to the locomotor activity chamber. Movement was recorded by camera and analyzed by Noldus analysis software. Measurements were taken from 7 week old mice.

Accelerating Rotarod

Animals were tested on an accelerating rotarod at 6 weeks of age as described (Buitrago et al. 2004). Animals were placed on the rotarod set for a rotational velocity of 4 rpm increasing to 40 rpm by 5 minutes. Latency to fall was recorded (seconds). Animals were given three trials per day over 5 consecutive days. Testing was performed at consistent periods in circadian cycle for all experiments.

Ultrasonic Vocalizations

On P4, P6, P9, and P11, vehicle and rapamycin treated pup vocalizations were evaluated as previously described (Tsai et al. 2012). Briefly, pups were separated from mothers for a 5 minute period during which they were placed in a soundproof chamber containing ultrasonic detectors. Vocalizations were recorded and analyzed using Ultravox software (Noldus, VA).

Elevated Plus Maze

Testing was performed as described (30). Briefly, mice were placed in the center of the elevated plus maze apparatus for a 5 minute trial. Mouse movements were recorded by camera and analyzed with Noldus software. Mice were determined to be on an open arm when all four limbs were fully on the open arm. The time spent in the open arm and the number of entries were recorded. Mice were tested at 7 weeks of age.

Social Interaction

Animals were tested for social interaction as previously described (Tsai et al. 2012). Animals were tested in a 3 chambered apparatus, designed by J. Crawley (Yang et al. 2011). Animals were initially habituated to the apparatus. After habituation, animals were placed individually into the middle chamber while a novel animal was placed into an inverted wire cup (novel animal) in one side chamber while an empty wire cup (novel object) was placed into the other side chamber. The tested animal was then allowed to explore the apparatus for a 10 minute trial. Time spent interacting with novel animal and object was recorded by the examiner with stopwatch. After the 10 minute trial, the animal was returned to the middle chamber while another unfamiliar age-matched male animal (novel animal) was placed under the previously empty wire cup. The tested animal was then allowed to explore the apparatus for an additional 10 minute trial and time in each chamber and time spent interacting with the novel and familiar animals were recorded as above. Animals were tested at 8 weeks of age.

Statistics

Data are reported as mean $\pm$ SEM, and statistical analysis was carried out using the Student's t-test (2-tailed, unpaired) or two-way ANOVA with Bonferroni's post hoc analysis.

RESULTS

Many neuropsychiatric conditions of childhood, such as autism and intellectual disability, are thought to have onset at pre- or peri-natal time points. Many neurogenetic diseases including Neurofibromatosis, Fragile X Syndrome, Rett Syndrome, and TSC have not only been associated with high rates of these conditions but also with elevated mTOR signaling. mTOR inhibitors, such as rapamycin, offer a potential therapeutic strategy for these disorders. To investigate the potential effects of prenatal rapamycin treatment on development and behavior, we treated pregnant C57Bl/6 dams at E16.5. This time point was chosen for several reasons. First, it is identical to a treatment time we have used previously to rescue neonatal lethality in a *Tsc1* mouse model (Anderl et al. 2011). Second, it is late enough to avoid the potential deleterious effects of treatment during early embryonic brain and organ development, where mTOR signaling plays important roles. Third, this date is several days removed from the expected delivery date in the mice so that the stress of drug administration itself would not complicate the birthing process. Finally, this time point in mouse gestation is roughly equivalent to the second trimester of a human pregnancy, after major organ development has occurred (Clancy et al. 2001) and arguably the earliest time at which rapamycin might be administered to a patient. We injected pregnant mothers at E16.5 with a single dose of either 1 mg/kg rapamycin or vehicle given intraperitoneally (IP). Injections and all subsequent behavioral examinations were performed blinded to treatment status.

Prenatal rapamycin treatment results in abnormal sensorimotor milestones

We first evaluated whether treatment would affect growth in wildtype (WT) mice and found significant differences in weight at P6 (30% reduction) and P11 (18% reduction) with rapamycin treatment (Figure 1A; P6 $p < 0.05$, P11, $p < 0.01$ two-way ANOVA, Bonferroni's post hoc analysis). To evaluate the impact of E16.5 rapamycin treatment on early postnatal development, we then evaluated sensorimotor milestones in vehicle and rapamycin treated mice. We examined the righting reflex and negative geotaxis but found no significant difference between the treatment groups in either of these tests (Figure 1B–C, $p > 0.05$, two-way ANOVA, Bonferroni's post hoc analysis). In contrast, rapamycin-treated mice demonstrated significantly impaired performance at P4 and P11 on the level screen test when compared with vehicle-treated mice (Figure 1D; P4, $p < 0.001$, P11, $p < 0.05$; two-way ANOVA, Bonferroni's post hoc analysis: $f(1,155) = 24.3$). Rapamycin-treated mice also demonstrated a decreased aversive response at P11 and P13 during cliff aversion testing (Figure 1E; P11, $p < 0.01$, P13, $p < 0.05$; two-way ANOVA, Bonferroni's post hoc analysis: $f(1,154) = 12$).

Prenatal rapamycin treatment results in motor abnormalities

In addition to early effects, we also investigated the effects of prenatal treatment on later development. As early impairments were noted in sensorimotor development, we first looked for effects on adult motor function. We evaluated vehicle and rapamycin treated animals in a square open field arena and found that both treated groups demonstrated similar movement in the open field without significant differences noted (Figure 2A; two-way ANOVA, Bonferroni's post hoc analysis: $p > 0.05$, $f(1,390) = 2.5$). We then tested treated mice in the accelerating rotarod. On day (D)1, both treatment groups demonstrated no significant differences in performance on the rotarod (Figure 2B). We then evaluated

whether prenatal treatment would have any effects on motor dependent learning on the rotarod. Rapamycin treated mice displayed significantly decreased latency to fall off the rotarod on D2 ($p < 0.05$) and D5 ($p < 0.01$) (Figure 2B; two-way ANOVA, Bonferroni's post hoc analysis: $f(1,122) = 21.7$).

Prenatal rapamycin treatment results in increased anxiety

Mouse models of disorders that impact mTOR signaling have frequently demonstrated abnormal anxiety-like behaviors (Ehninger and Silva 2011; Kwon et al. 2006; Yuan et al. 2012). As a result, we were interested in evaluating whether prenatal rapamycin treatment might itself have effects on anxiety-related behaviors in C57Bl/6 mice.

We first evaluated for early effects on anxiety-related reflexes in an assay of acoustic startle. Rapamycin treated mice demonstrated significantly increased startle compared to vehicle treated animals at P13 (Figure 3A, $p < 0.001$ at P13; two-way ANOVA, Bonferroni's post hoc analysis: $f(1,60) = 12.3$). We then evaluated pup vocalizations at various time points through early postnatal development. When isolated from their mothers, pups emit vocalizations detected in the ultrasonic register. We recorded these vocalizations in pups on P4, P6, P9, and P11. We noted significantly increased vocalizations in rapamycin treated mice at P6, consistent with increased anxiety-related behavior at this early stage in development (Figure 3B, $p < 0.01$, two-way ANOVA, Bonferroni's post hoc analysis, $f(1,101) = 5.9$).

We further tested whether rapamycin might have long lasting effects on anxiety-related behaviors in treated C57Bl/6 mice. We examined these animals on the elevated plus maze and recorded both time in the open arms and the number of entries into the open arms. Rapamycin treated animals displayed both significantly decreased amount of time spent in the open arms (Figure 4A, $p < 0.05$, unpaired t-test) and significantly decreased number of entries into the open arms (Figure 4B, $p < 0.05$, unpaired t-test).

Prenatal rapamycin treatment does not alter social behavior in C57Bl/6 mice

Patients with TSC also have high rates of comorbid autism spectrum disorders, with reports ranging from 25–60%. Furthermore, many identified ASD-associated genes have been reported to impact mTOR signaling (Ehninger and Silva 2011; Kelleher and Bear 2008). Thus, as rapamycin might be considered for potential therapeutic benefit in children with ASDs, we evaluated whether rapamycin treatment alone might affect social behaviors in C57Bl/6 mice. We tested vehicle and rapamycin treated mice at 8 weeks of age in the three chambered apparatus (Yang et al. 2011). In assays of social approach, vehicle and rapamycin treated animals demonstrated significant social preference for the novel animal (compared to the novel object) when we examined the time spent in their respective chambers (Figure 5A, $p < 0.001$, two-way ANOVA. Bonferroni's post hoc analysis, $f(2,72) = 103.6$). We also examined the amount of time spent in close interaction with the novel animal versus object in this social approach assay and again found that both vehicle and rapamycin treated animals displayed significant preference for the novel animal (Figure 5B, $p < 0.001$, two-way ANOVA. Bonferroni's post hoc analysis, $f(1,48) = 128$). We then investigated vehicle and rapamycin treated animals in an assay of social novelty in the same apparatus. Both treated cohorts demonstrated significant preference for the novel animals as compared to the familiar animal when time spent in each chamber was evaluated (Figure 5C, $p < 0.001$, two-way ANOVA. Bonferroni's post hoc analysis, $f(2,72) = 142.1$). Moreover, both vehicle and rapamycin treated mice also displayed significant preference for the novel animals when time in close interaction was examined (Figure 5D, $p < 0.001$, two-way ANOVA. Bonferroni's post hoc analysis, $f(1,48) = 105.5$).

DISCUSSION

Increased mTOR signaling has been implicated in a number of genetic, neurodevelopmental disorders – including Neurofibromatosis, Rett Syndrome, Fragile X Syndrome, and TSC – which has led to consideration of rapamycin, a specific mTOR inhibitor, as a potential therapeutic option. Some of these disorders, including TSC, can be diagnosed *in utero*, usually due to the presence of cardiac lesions (Datta et al. 2008). Prenatal diagnosis of TSC has raised the possibility that prenatal rapamycin therapy might be used to modify or even prevent the neurologic comorbidities associated with TSC.

In this study, we evaluated both early and late effects of a single, prenatally administered treatment with rapamycin in wildtype C57Bl/6 mice. Previous clinical case studies with rapamycin during pregnancy have not demonstrated any adverse outcomes to mother or child (Chu et al. 2008; Framarino dei Malatesta et al. 2011) while previous studies from our laboratory demonstrated significant survival benefit of prenatal treatment of a severe mouse brain model in which *Tsc1* is deleted in neural progenitor cells (Anderl et al. 2011). In addition, during preparation of this manuscript, a study of a mouse model of *Tsc2*, evaluating the effects of prenatal and postnatal rapamycin treatment noted that daily rapamycin administration beginning at E12.5 resulted in hippocampal dependent learning impairments when compared to treatment started postnatally in this *Tsc2* model (Way et al. 2012). Our study adds significantly to these findings.

We identified early and late sensorimotor phenotypes, in addition to anxiety phenotypes, in a vehicle controlled study of a single dose of rapamycin administered at E16.5. In this study, this single treatment of rapamycin resulted in sensorimotor impairments with changes in sensorimotor function both during early postnatal development and during adult stages. Rapamycin treatment resulted in impairments in level screen and cliff aversion testing during early development in addition to impairments in motor learning in the accelerating rotarod during adulthood.

We also determined that rapamycin treatment at E16.5 resulted in increased anxiety-like behaviors in both early postnatal development and during adult stages. We identified increased startle and increased USVs in mouse pups during early development in addition to decreased number of entries and time spent in the open arm of the elevated plus maze during adulthood. These increased anxiety-like behaviors due to rapamycin fit our recent observations in a different TSC brain model in which decreased anxiety was seen (Yuan et al. 2012). Social behaviors, on the other hand, were not altered with rapamycin treatment. Why social behaviors remain unaffected when motor and anxiety development were adversely affected by rapamycin treatment is unclear. However, this data suggests that at this developmental time point, mTOR signaling is less likely to play critical roles in the development of neural circuits important for social behaviors.

Taken together, these data provide strong evidence that mTOR signaling plays critical roles during late neuronal development such that rapamycin administration results in long lasting behavioral effects. Hence, our data suggest that caution is appropriate in considering use of rapamycin in humans at early developmental time points.

Previous work on numerous mouse models of TSC has demonstrated the significant benefit of early postnatal rapamycin treatment (Ehninger et al. 2008; Meikle et al. 2008; Tsai et al. 2012; Way et al. 2009; Zeng et al. 2008). Treatment of neuronal or glial specific *Tsc* mutants demonstrated significant benefit in pathological, behavioral, and epileptic phenotypes when treatment was initiated at 1 week postnatal age. These later treatments also did not appear to negatively impact mouse behavior, although this possibility could not be excluded in many of these models as untreated mutants had severe phenotypes including

decreased survival. However, even adult treatment of mice heterozygous for *Tsc2* resulted in reversal of learning deficits (Ehninger et al. 2008). These studies, taken together, help to inform the timing of administration of mTOR inhibition as a therapeutic strategy for neurodevelopmental disorders and caution that earlier treatment may not be a superior therapeutic strategy. More detailed studies, however, are required to investigate the effects of rapamycin therapy at additional developmental time points on the neurocognitive development of mice. Thus, this study highlights the challenges that are involved in the development of treatment modalities for the therapy of neurodevelopmental disorders while emphasizing the need for additional pre-clinical trials in the consideration of pre- or perinatal rapamycin therapies.

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Figure 1. Sensorimotor milestones in vehicle and rapamycin treated mice

A. Weight is reduced at P6 and P11 in rapamycin treated animals. **B and C.** Righting and negative geotaxis are not different in rapamycin vs. vehicle treated mice reflex ($n > 13$ each group). Rapamycin treated mice, however, displayed impaired performance compared to vehicle treated mice in **D.** Level screen and **E.** Cliff aversion assays. $n > 7$ each group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Two-way ANOVA, Bonferroni's post hoc analysis.

Figure 2. Prenatal rapamycin treatment affects motor performance in adult mice
A. Rapamycin treatment did not result in changes in open field exploration. $p>0.05$, two-way ANOVA. $n=10$ (vehicle), 18 (rapamycin). **B.** On rotarod testing, however, rapamycin treatment resulted in significantly impaired performance. * $p<0.05$, ** $p<0.01$. Two-way ANOVA, Bonferroni's post hoc analysis. $n=12-13$.

Figure 3. Rapamycin treatment resulted in increased startle and vocalizations

A. Rapamycin treated mice displayed increased startle at P13. **B.** Rapamycin treated mice also demonstrated increased vocalizations at P6 compared to untreated controls. n=13–17.

** p<0.01, *** p<0.001. Two-way ANOVA, Bonferroni's post hoc analysis.

Elevated Plus Maze

Figure 4. Rapamycin treatment resulted in increased anxiety-related behaviors on the elevated plus maze

A. Rapamycin treated mice spent less time in the open arms and **B.** made fewer entries into the open arms. n=12 (vehicle), 19 (rapamycin). * $p < 0.05$. Student's T test.

Figure 5. Rapamycin treatment did not alter social behaviors in C57Bl/6 mice

In an assay of social approach, rapamycin and vehicle treated animals displayed significant preference for the novel animal over novel object both by **A**. Time in the chamber and **B**. Time spent investigating. In an assay of social novelty, rapamycin treated mice acted similarly to vehicle treated animals both by **C**. Time in the chamber and **D**. Time investigating the novel and familiar animals. $n = 11$ (vehicle), $n = 15$ (rapamycin). *** $p < 0.001$, two-way ANOVA, Bonferroni's post hoc analysis.