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ORIGINAL ARTICLE

Gestational immune activation and *Tsc2* haploinsufficiency cooperate to disrupt fetal survival and may perturb social behavior in adult mice

D Ehninger[1,2], Y Sano[2], PJ de Vries[3,4], K Dies[5], D Franz[6], DH Geschwind[7], M Kaur[2], Y-S Lee[2], W Li[2], JK Lowe[7], JA Nakagawa[8], M Sahin[5], K Smith[8], V Whittemore[8] and AJ Silva[2]

[1]DZNE, German Center for Neurodegenerative Diseases, Bonn, Germany; [2]Departments of Neurobiology, Psychiatry and Biobehavioral Sciences, Psychology and the Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, USA; [3]Neurodevelopmental Service, Cambridgeshire and Peterborough NHS Foundation Trust, Peterborough, UK; [4]Developmental Psychiatry Section, University of Cambridge, Cambridge, UK; [5]The F.M. Kirby Neurobiology Center, Department of Neurology, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA; [6]Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; [7]Neurogenetics Program, University of California, Los Angeles, Los Angeles, CA, USA and [8]Tuberous Sclerosis Alliance, Silver Spring, MD, USA

Approximately 40–50% of individuals affected by tuberous sclerosis (TSC) develop autism spectrum disorders (ASDs). One possible explanation for this partial penetrance is an interaction between TSC gene mutations and other risk factors such as gestational immune activation. In this study, we report the interactive effects of these two ASD risk factors in a mouse model of TSC. Combined, but not single, exposure had adverse effects on intrauterine survival. Additionally, provisional results suggest that these factors synergize to disrupt social approach behavior in adult mice. Moreover, studies in human populations are consistent with an interaction between high seasonal flu activity in late gestation and TSC mutations in ASD. Taken together, our studies raise the possibility of a gene × environment interaction between heterozygous TSC gene mutations and gestational immune activation in the pathogenesis of TSC-related ASD.

Introduction

An emerging theme in the biology of autism spectrum disorder (ASD) is the involvement of signaling mechanisms that regulate protein synthesis, such as those involving mammalian target of rapamycin (mTOR).[1,2] For example, heterozygous mutations in the *TSC1* or *TSC2* genes, the key regulators of mTOR signaling, cause tuberous sclerosis (TSC) and elevate the risk for autism 100-fold compared with the general population.[3–5] In addition, upstream regulators (PTEN, MET, NF1)[6–9] and a downstream effector (eIF4E)[10] of the TSC–mTOR pathway have been implicated in ASD. Moreover, alterations in mTOR-dependent translational control are also associated with fragile X syndrome,[11,12] a single-gene disorder

caused by mutations in the *FMR1* gene and associated with high rates of ASD. Collectively, these findings suggest an overlapping genetic signature in a subset of ASD-related disorders.

Prenatal viral infections also constitute a risk factor for neuropsychiatric disorders, including schizophrenia and ASD.[13–22] Prenatal rubella virus infections increase the risk of autism in the offspring > 200-fold,[14–16] and available evidence indicates other viral infections confer risk as well.[19–21] Together with the presence of inflammatory brain changes,[13,23,24] these findings suggest a role of infections and/or immunological processes in at least a subset of ASD.

Gestational viral infections trigger a maternal immune response, which can perturb fetal brain development, at least in part because of the effects of cytokines in the developing nervous system.[13,25–27] In animals, gestational viral infections can be mimicked by systemic administration of polyinosinic:polycytidylic acid (poly I:C), a synthetic double-stranded RNA, which elicits an innate immune response via activation of Toll-like receptor 3.[13,27,28]

An interaction between TSC mutations and gestational immune activation could explain the partial penetrance of ASD phenotypes in TSC. TSC–mTOR

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signaling is downstream of poly I:C/Toll-like receptor 3, poly I:C-induced cytokines,[29,30] and TSC–mTOR signaling modulates immune responses.[30–33] To test for possible interactive effects, we combined *Tsc2* haploinsufficiency and the poly I:C model of gestational immune activation in mice and assessed the impact on behavior in adult animals.

Materials and methods

Mice

To test for interactive effects between a $Tsc2^{+/-}$ mutation and immune activation during pregnancy, we first mated male $Tsc2^{+/-}$ mice[34] with female wild-type (WT) mice (see below). $Tsc2^{+/-}$ male breeders were on a C57BL/6Ncrl genetic background. We used C57BL/6J females for breeding because previous immune activation studies were conducted in the context of which $Tsc2^{+/-}$ males were crossed with a mutant line on a C57BL/6J genetic background. Female breeders with a vaginal plug subsequent to overnight mating (designated E0) were single housed and were left undisturbed, except for weekly cage change. The establishment of pregnancy was determined by checking for abdominal distension at E12. Pregnant females were injected (intraperitoneally; injected volume: $10\,\mu l\,g^{-1}$ body weight) with either $20\,mg\,kg^{-1}$ poly I:C (Sigma, St Louis, MO, USA, potassium salt; poly I:C is supplied at 10% of the total weight of the salt; dosage was based on the weight of poly I:C itself) or vehicle only (0.9% sterile saline) at E12.5.

Litter composition with respect to numbers and genotypes was assessed in a subset of surviving litters (that is, live births). In these litters, animals were closely monitored from birth to weaning. Tail biopsies for genotyping were taken at weaning (P21) or whenever individual pups were lost postnatally (no group differences in terms of postnatal loss of pups were observed). The litter composition data provided refer to the observations carried out at birth.

To assess potential interactive effects between a heterozygous *Tsc2* mutation and advanced paternal age, we generated $Tsc2^{+/-}$ and WT offspring derived from old (18–22 months at conception) or young (3–6 months at conception) fathers, by mating male $Tsc2^{+/-}$ mice (C57BL/6Ncrl) with female WT animals (C57BL/6Ncrl; 3 to 4 months at conception). Litters of each paternal age group were derived from at least four different fathers.

All experiments were conducted blind to genotype and treatment. The Chancellor's Animal Research Committee at the University of California, Los Angeles, approved the research protocols used here.

Behavior

Behavioral experiments were conducted in 3- to 6-month-old male mice. Social approach behavior was studied in a three-compartment apparatus and the procedure was similar to previously reported protocols.[35,36] Initially, test animals were allowed to explore the three-compartment apparatus freely for a 20-min period (habituation). The next day, animals were briefly (5 min) re-habituated to the apparatus. For the social behavior phase of the task, an unfamiliar conspecific (same sex, age and genetic background as the test subject) was placed into one of the side compartments and restrained by a small wire object ('social cage'). The compartment on the other side contained an empty wire object ('empty cage'). The test subject was then released into the center compartment and allowed to explore the three-compartment apparatus freely for 10 min. Behavior was videotaped and scored offline by an experienced observer; we measured the time(s) that the test subject spent exploring (sniffing, rearing) the social cage and the empty cage. In addition, we used commercially available software (TopScan, CleverSys, Reston, VA, USA) for automated analyses of social exploration. To this end, we defined an annular zone around the social and empty cage and used the software to track nose entries of the subject mouse into this annular zone. These automated analyses yielded estimates of total sniff time, number of sniff episodes and average duration of sniff episodes.

Activity was assessed in an open field assay as previously described.[36] In brief, mice were placed in a square open field made of acrylic and activity was recorded by an automated system (Med Associates, St Albans, VT, USA) for 10 min. Total distance moved (cm) was the primary measure of interest for the assessment of activity levels.

For the olfactory sensitivity test, an unfamiliar food item high in carbohydrate content (one froot loop from Kellogg's cereal) was placed into the home cages of test subjects for 2 days before testing. Testing was performed in novel standard cages containing a 3 cm deep layer of bedding. Test subjects were initially habituated to the novel cages for 5 min. After habituation, we buried one froot loop in the bedding of the test cages. For retrieval of the froot loop, test subjects were placed into the test cages for 15 min. Behavior was videotaped for offline analysis. We measured the latency to retrieve the froot loop.

Human TSC and ASD data

Birth dates and clinical information of TSC individuals was contributed by four sources: the TSC Natural History Database (TS Alliance) and the patient records of Drs Petrus de Vries, David Franz and Mustafa Sahin. All individuals included in the analysis were born in the United States or the United Kingdom. The following clinical information was available: intellectual disability (yes/no), ASD (yes/no) and lifetime history of infantile spasms (yes/no). We analyzed data of TSC individuals affected by ASD (TSC–ASD individuals; $n = 230$) and TSC individuals who were not affected by ASD, intellectual disability or infantile spasms ($n = 265$). Details regarding year of birth, gender composition, clinical features and mutational status of these clinical samples are summarized in Supplementary Table 1.

Date of conception was estimated for each individual by subtracting 267 days (average duration of a pregnancy) from the birth date. Seasonal influenza infections typically peak between January and March (average mid-February). To estimate gestational age during peak influenza activity for each individual, we calculated gestational age at 14 February following conception. Subsequently, we assigned each individual to one of the four groups (all covering 13-week periods): individuals for whom peak seasonal flu activity coincided with the first trimester (weeks 1–13), second trimester (weeks 14–26) or third trimester (weeks 27–39) of pregnancy or for whom peak seasonal flu activity was outside of gestational periods. Using χ^2 analyses, we determined, for each population (TSC–ASD, TSC-no ASD or other neurodevelopmental phenotypes), if the observed frequency of cases in the four different groups (peak seasonal flu activity: coinciding either with the first trimester, second trimester, third trimester or outside of gestation) differed significantly from the expected frequencies (that is, from an even distribution of cases across groups).

For ASD individuals unaffected by TSC, obtained through the Autism Genetic Resource Exchange (www.agre.org), birth month information (rather than precise birth dates) was available. Individuals born between August and October were considered to be in first trimester of gestation during peak seasonal flu activity; births in May, June or July were considered to correspond to second trimester of gestation during peak seasonal flu activity; births between February and April were considered to be in third trimester of gestation during peak seasonal flu activity. In November, December and January births, gestation was assumed to not overlap with peak seasonal flu activity. The χ^2 analysis was performed as described above.

Statistics
All statistical tests (analysis of variance (ANOVA), *post hoc* analyses, *t*-tests, χ^2 analyses) were chosen *a priori* and are described in more detail in the main text and figure legends.

Results

To test for an interaction between the $Tsc2^{+/-}$ mutation in mice and gestational poly I:C (a model of gestational immune activation), we performed timed matings of $Tsc2^{+/-}$ males in the C57BL/6Ncrl genetic background and C57BL/6J WT females, and injected pregnant females at E12.5 with either poly I:C $(20\,\mathrm{mg\,kg^{-1}})^{27}$ or saline control.

Although established pregnancies were successfully carried to term in the vast majority of saline-injected breeders (96.43% survived; 3.57% lost; $n = 28$ pregnancies), abdominal collapse or frank abortion of dead fetuses occurred in a considerable portion of poly I:C-injected mice (68.89% survived; 31.11% lost; $n = 45$ pregnancies; Figure 1).
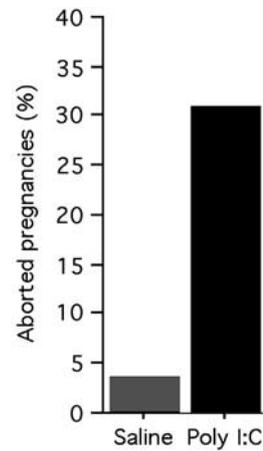
Type of pregnancy loss	Number of cases	Time interval between Poly I:C injection and discharge of dead fetuses
Resorption	9 (64.29%)	N/A
Frank abortion	5 (35.71%)	2 days (2 cases)
		4 days (2 cases)
		6 days (1 case)

Figure 1 Percentage of pregnancies lost in polyinosinic:-polycytidylic acid (poly I:C)-injected and saline-treated animals. The vast majority of saline-injected females carried pregnancy to term; in contrast, a considerable portion of pregnancies was aborted as a consequence of poly I:C injection. The table shows the proportion of poly I:C-related lost pregnancies associated with either fetal resorption (abdominal collapse without abortion of dead fetuses) or frank abortion (premature discharge of dead fetuses).

We compared the number of $Tsc2^{+/-}$ and WT pups in litters carried to term. Analyses revealed that fewer $Tsc2^{+/-}$ pups per litter were born to poly I:C-injected mice than to saline controls, whereas the number of WT pups per litter did not differ between the poly I:C and saline control group (two-way ANOVA with treatment and genotype as between-subject factors: effect of treatment $F(1, 80) = 4.968$; $P = 0.0286$; poly I:C: $n = 21$ litters; saline: $n = 21$ litters; Bonferroni/Dunn *post hoc* analysis: $Tsc2^{+/-}$/poly I:C vs $Tsc2^{+/-}$/saline, $P = 0.0038$; WT/poly I:C vs WT/saline, $P = 0.8358$; Figure 2a). These findings suggest that $Tsc2^{+/-}$ fetuses are more vulnerable to poly I:C-induced intrauterine death than their WT counterparts. Indeed, when we isolated fetuses at 6.5 h after poly I:C injection, we found signs of fetal resorption. Our observations indicated that at 6.5 h after poly I:C, 3 out of 21 $Tsc2^{+/-}$ fetuses (14.29%) appeared to be affected by resorption (pale, deformed, mushy consistency) compared with 1 out of 18 WT control fetuses (5.56%) tested.

Next, we wanted to determine if $Tsc2$ haploinsufficiency and poly I:C-related gestational immune activation interacted to affect mouse behavioral models of autistic phenotypes. As abnormalities in social behaviors represent a core feature of ASD, we assayed social approach behavior in mice, using a three-compartment apparatus.[35] In brief, the three-compartment apparatus comprised one side compartment containing an empty wire object ('empty cage')
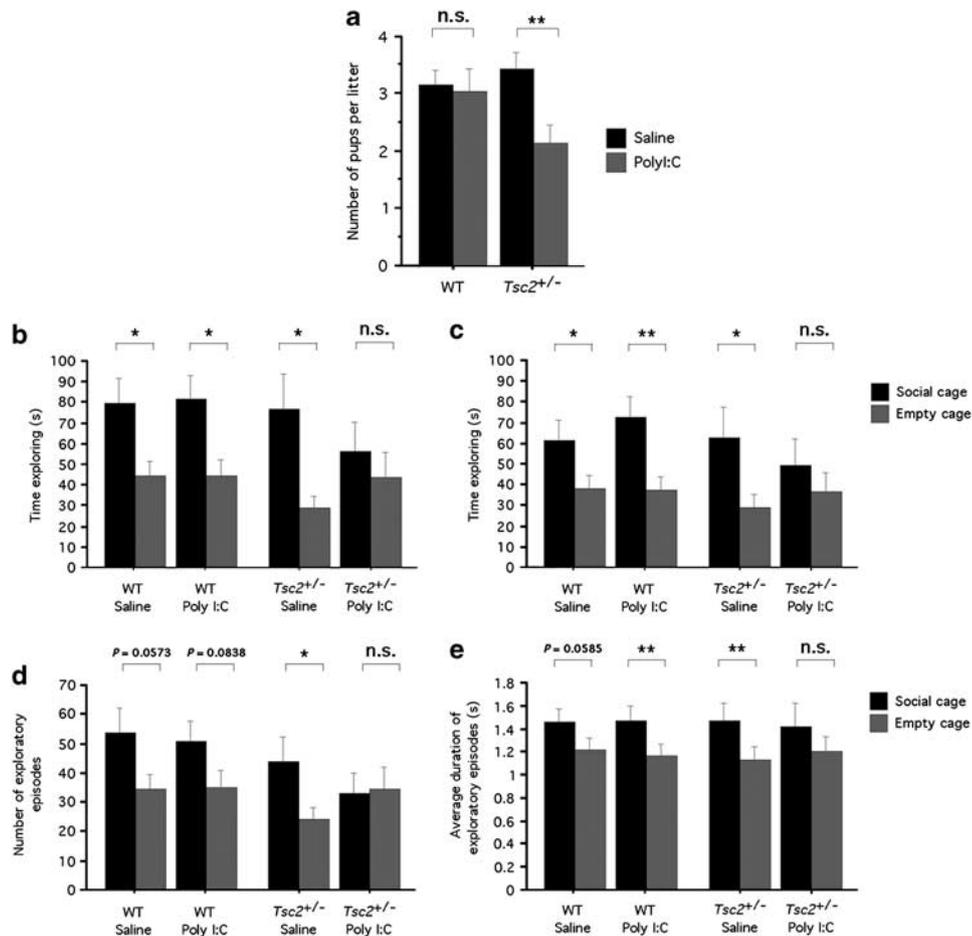
Figure 2 Interactive effects of *Tsc2* haploinsufficiency and gestational polyinosinic:polycytidylic acid (poly I:C) on intrauterine survival and adult social approach behavior. (**a**) Graph shows the number of pups born in surviving litters, plotted by genotype (*Tsc2*$^{+/-}$ and WT) and treatment (poly I:C and saline). Fewer *Tsc2*$^{+/-}$ pups were born to poly I:C-injected females compared with saline-injected controls. (**b**) Graph shows the time(s) spent actively exploring the social cage and the empty cage (sniffing, rearing), as determined by an experienced observer. WT/saline, *Tsc2*$^{+/-}$/saline and WT/poly I:C mice spent significantly more time exploring the social cage than the empty cage, demonstrating normal social approach behavior. *Tsc2*$^{+/-}$/poly I:C mice, in contrast, did not spent more time exploring the social cage than the empty cage. (**c–e**) These graphs show the time spent exploring (**c**), the number of exploratory episodes (**d**) and the average duration of exploratory episodes (**e**) during the social approach behavioral task as quantified with an automated system. Whereas WT/saline, *Tsc2*$^{+/-}$/saline and WT/poly I:C mice tended to show higher values for the social cage than the empty cage, this was not the case for *Tsc2*$^{+/-}$/poly I:C mice. **$P < 0.01$, *$P < 0.05$, NS $P > 0.05$. Data represent means ± s.e.m.

and one side compartment containing a small wire object constraining a mouse ('social cage'). To quantify social approach behavior, we let test mice freely explore the three-compartment apparatus and measured the time these mice spent exploring the social cage versus the empty cage.

WT and *Tsc2*$^{+/-}$ mice born to saline-injected mothers and WT mice born to poly I:C-injected females spent significantly more time exploring the social cage than the empty cage (WT/saline: planned *t*-test, $P = 0.0140$, $n = 16$ mice; *Tsc2*$^{+/-}$/saline: planned *t*-test: $P = 0.0116$, $n = 13$ mice; WT/poly I:C: planned *t*-test, $P = 0.0101$, $n = 16$ mice; Figure 2b), demonstrating robust social approach behavior. In contrast, mice exposed to both factors, the *Tsc2*$^{+/-}$ mutation and gestational poly I:C (*Tsc2*$^{+/-}$/poly I:C

mice), did not spend significantly more time exploring the social cage than the empty cage (planned *t*-test: $P = 0.5062$, $n = 13$ mice; Figure 2b), demonstrating a lack of normal social approach behavior. To explore this further, we performed automated analyses of social exploration in *Tsc2*$^{+/-}$/poly I:C mice and the other experimental groups (for details, see Materials and methods). These analyses also showed that all groups except the *Tsc2*$^{+/-}$/poly I:C group spent significantly more time exploring the social cage than the empty cage (WT/saline: planned *t*-test, $P = 0.0232$, $n = 16$ mice; *Tsc2*$^{+/-}$/saline: planned *t*-test: $P = 0.0405$, $n = 13$ mice; WT/poly I:C: planned *t*-test, $P = 0.0025$, $n = 16$ mice; *Tsc2*$^{+/-}$/poly I:C: planned *t*-test: $P = 0.2070$, $n = 13$ mice; Figure 2c). Next, we determined the number of exploratory episodes and the

average duration of an exploratory episode for each animal. With respect to these measures, WT/saline, $Tsc2^{+/-}$/saline and WT/poly I:C animals tended to show higher values for the social cage than the empty cage, although not all comparisons yielded statistical significance (number of exploratory episodes: WT/saline, planned *t*-test, $P = 0.0573$, $n = 16$ mice; $Tsc2^{+/-}$/saline, planned *t*-test: $P = 0.0204$, $n = 13$ mice; WT/poly I:C, planned *t*-test, $P = 0.0838$, $n = 16$ mice; average duration of exploratory episodes: WT/saline, planned *t*-test, $P = 0.0585$, $n = 16$ mice; $Tsc2^{+/-}$/saline, planned *t*-test: $P = 0.0089$, $n = 13$ mice; WT/poly I:C, planned *t*-test, $P = 0.0029$, $n = 16$ mice; Figures 2d and e). $Tsc2^{+/-}$/poly I:C mice, in contrast, did not show more exploratory episodes and longer exploration of the social cage than the empty cage (number of exploratory episodes: planned *t*-test, $P = 0.8351$, average duration of exploratory episodes: planned *t*-test, $P = 0.2135$; $n = 13$ mice; Figures 2d and e).

Additional behavioral testing with the open field revealed no group difference with respect to general exploratory behavior (ambulatory distance; two-way ANOVA with genotype and treatments as between-subject factors: genotype effect, $F(1, 45) = 0.154$, $P = 0.6964$; treatment effect: $F(1, 45) = 1.822$, $P = 0.1839$; genotype × treatment interaction: $F(1, 45) = 0.846$, $P = 0.3625$; WT/saline, $n = 13$ mice; WT/poly I:C, $n = 18$ mice; $Tsc2^{+/-}$/saline, $n = 11$ mice; $Tsc2^{+/-}$/poly I:C, $n = 7$ mice; Figure 3a). The open field assay also revealed that ambulatory distance in the center zone did not differ between groups (ambulatory distance in center zone of the open field; two-way ANOVA with genotype and treatments as between-subject factors: genotype effect, $F(1, 45) = 0.448$, $P = 0.5067$; treatment effect: $F(1, 45) = 0.949$, $P = 0.3351$; genotype × treatment interaction: $F(1, 45) = 0.129$, $P = 0.7213$; WT/saline, $n = 13$ mice; WT/poly I:C, $n = 18$ mice; $Tsc2^{+/-}$/saline, $n = 11$ mice; $Tsc2^{+/-}$/poly I:C, $n = 7$ mice; Figure 3b), suggesting normal levels of anxiety-related behaviors in $Tsc2^{+/-}$/poly I:C mice. To evaluate general olfactory function, animals were tested with an olfactory sensitivity test that uses a food (froot loop) retrieval assay. There was no significant difference with respect to latencies to retrieve the food item across the experimental groups (two-way ANOVA with genotype and treatment as between-subject factors, latency to retrieve food item: genotype effect, $F(1, 39) = 0.187$, $P = 0.6681$; treatment effect, $F(1, 39) = 1.401$, $P = 0.2437$; genotype × treatment interaction, $F(1, 39) = 0.011$, $P = 0.9182$; Figure 3c), suggesting normal general olfaction in $Tsc2^{+/-}$/poly I:C mice. Taken together, these findings suggest that abnormalities in social approach behavior described above for the $Tsc2^{+/-}$/poly I:C mice were not simply because of altered activity levels or perturbations of general olfactory function.

Next, we determined if *Tsc2* haploinsufficiency also interacts with other ASD risk factors. Advanced paternal age confers ASD risk,[37] but has no obvious links to TSC–mTOR signaling. To combine *Tsc2*
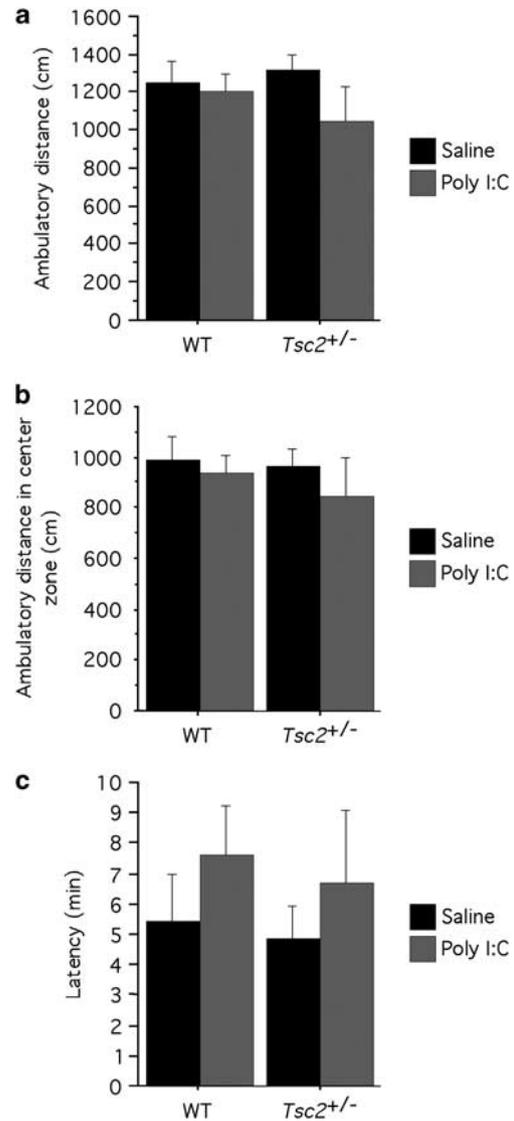

Figure 3 (**a**) Behavioral testing, using the open field, revealed no significant effect of *Tsc2* haploinsufficiency or gestational polyinosinic:polycytidylic acid (poly I:C) on ambulatory behavior, demonstrating normal locomotion in $Tsc2^{+/-}$/poly I:C mice. (**b**) Ambulatory distance in the center zone also did not differ between groups, suggesting normal levels of anxiety-related behaviors in $Tsc2^{+/-}$/poly I:C mice. (**c**) The graph shows the latency (min) to retrieve a buried food item in an olfactory sensitivity test. There was no significant difference with respect to latencies across the experimental groups, suggesting that olfactory dysfunction did not account for the behavioral impairments in $Tsc2^{+/-}$/poly I:C mice. Data represent means ± s.e.m.

haploinsufficiency with advanced paternal age in mice, we bred old $Tsc2^{+/-}$ males with WT females (for details, see Materials and methods); we generated controls by crossing young $Tsc2^{+/-}$ males with WT females and assessed social behavior in the adult offspring. Adult $Tsc2^{+/-}$ and WT mice conceived by either young or old fathers showed normal social approach behavior (WT/young father: planned *t*-test, time exploring social cage vs time exploring empty
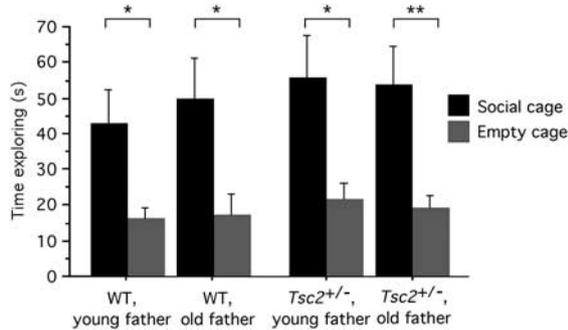
Figure 4 Advanced paternal age confers autism suscept-ibility and also is a risk factor for other neurodevelopmental disorders. To combine *Tsc2* haploinsufficiency with advanced paternal age in mice, we bred old *Tsc2*$^{+/-}$ males with WT females and generated controls by crossing young *Tsc2*$^{+/-}$ males with WT females and assessed social behavior in the adult offspring. *Tsc2*$^{+/-}$ and WT offspring of both young and old fathers showed normal social approach behavior. Plotted is the time(s) test subjects spent exploring either a cage containing a conspecific (social cage) or an empty cage. *$P < 0.05$, **$P > 0.01$. Data represent means ± s.e.m.

cage, $P = 0.0129$, $n = 14$ mice; WT/old father: planned *t*-test, time exploring social cage vs time exploring empty cage, $P = 0.0229$, $n = 9$ mice; *Tsc2*$^{+/-}$/young father, planned *t*-test, time exploring social cage vs time exploring empty cage, $P = 0.0177$, $n = 9$ mice; *Tsc2*$^{+/-}$/old father, planned *t*-test, time exploring social cage vs time exploring empty cage: $P = 0.0042$, $n = 16$ mice; Figure 4). These data show that our studies did not detect an interaction between the *Tsc2*$^{+/-}$ mutation and advanced paternal age as we had uncovered in the poly I:C studies. These findings attest to the selectivity of the interactive effects between *Tsc2* haploinsufficiency and gestational poly I:C.

The animal model data described above suggest that gestational viral infections might contribute to autistic phenotypes in human TSC populations. Seasonal influenza infections typically peak between the beginning of January and late March each year (www.cdc.gov/flu/weekly/fluactivity.htm) and they are a common source of gestational immune activation. To test if seasonal influenza infections interact with TSC gene mutations in the pathogenesis of TSC-related ASD, we obtained birth date and clinical information from human TSC populations (clinical samples from the United States and the United Kingdom; for details, see Materials and methods) and estimated gestational age during peak seasonal flu activity for TSC–ASD individuals ($n = 230$). As a reference group, we used TSC individuals without major neurodevelopmental phenotypes (that is, without intellectual disability, ASD or a history of infantile spasms; $n = 265$; for details, see Materials and methods).

Our analyses revealed an uneven distribution of peak seasonal flu activity across gestational periods

in TSC–ASD individuals (peak seasonal flu activity: coinciding with the first trimester, $n = 47$ individuals; second trimester, $n = 57$ individuals; third trimester, $n = 75$ individuals; outside gestational periods, $n = 51$ individuals; $\chi^2 = 7.983$, $P = 0.0463$; Table 1), suggesting an excess of TSC–ASD individuals, for whom late stages of pregnancy coincided with peak seasonal flu activity. In contrast, TSC controls without ASD or other neurodevelopmental phenotypes showed no obvious association between gestational periods and seasonal flu activity (peak seasonal flu activity: coinciding with the first trimester, $n = 67$ individuals; second trimester, $n = 74$ individuals; third trimester, $n = 58$ individuals; outside gestational periods, $n = 66$ individuals; $\chi^2 = 1.943$, $P = 0.5843$; Table 1).

We also obtained birth date information of ASD individuals unaffected by TSC from the Autism Genetic Resource Exchange database (www.agre.org; $n = 6734$ individuals). There was no obvious association between gestational periods and peak seasonal flu activity in ASD individuals unaffected by TSC (peak seasonal flu activity: coinciding with the first trimester, $n = 1741$ individuals; second trimester, $n = 1687$ individuals; third trimester, $n = 1606$ individuals; outside gestational periods, $n = 1700$ individuals; $\chi^2 = 5.701$, $P = 0.1271$; Table 1). These findings suggest that the association of late gestation and peak seasonal flu activity was specific for TSC-related ASD.

Discussion

In this study we presented evidence indicating that *Tsc2* haploinsufficiency and the poly I:C model of gestational immune activation cooperate to disrupt intrauterine survival and adult social approach behavior in mice. In addition, studies in human populations were consistent with an interaction between high seasonal flu activity in late gestation and TSC mutations in ASD.

Dysfunctional social behaviors represent a core feature of ASD. Social approach behavior in the three-compartment apparatus was used as a model to study social interactions in mice.[35,38] Our provisional results suggest abnormal social approach behavior in *Tsc2*$^{+/-}$/poly I:C mice, which could not be explained by altered levels of general exploratory behavior (activity in the open field was normal) or by general olfactory dysfunction (which was normal in an olfactory sensitivity test). Alterations in higher-order circuits could form the basis of dysfunctional social approach behavior in *Tsc2*$^{+/-}$/poly I:C mice. Nevertheless, it is also conceivable that altered social behavior in the *Tsc2*$^{+/-}$/poly I:C mice is related to perturbations in the accessory olfactory system, which has an important role in detecting socially relevant odors.[39]

For the poly I:C experiments we used a single injection of $20 \, \text{mg} \, \text{kg}^{-1}$ at E12.5 (for details, see Materials and methods). Pilot experiments had

Table 1 The number of cases (*n*) in which peak seasonal flu activity coincided with the first, second or third trimester of gestation or was outside gestational periods

First trimester (n)	Second trimester (n)	Third trimester (n)	Outside gestation (n)	χ^2	P-value
TSC and ASD					
47	57	75	51	7.983	0.0463*
TSC, no ASD or other neurodevelopmental phenotypes					
67	74	58	66	1.943	0.5843
ASD, no TSC					
1741	1687	1606	1700	5.701	0.1271

Abbreviations: ASD, autism spectrum disorder; TSC, tuberous sclerosis.
Data are shown for TSC individuals affected by ASD (TSC and ASD), TSC individuals unaffected by ASD or other neurodevelopmental phenotypes (TSC, no ASD or other neurodevelopmental phenotypes) and ASD cases unrelated to tuberous sclerosis (ASD, no TSC), along with results from the corresponding χ^2 analyses.
*$P < 0.05$.

established that this poly I:C dose does not adversely affect social approach behavior in C57BL/6Ncrl × C57BL/6J F1 WT animals (data not shown; see also Figures 2b–e). This poly I:C dose also did not have an effect on behavior in the open field assay, either in WT mice or in $Tsc2^{+/-}$ mutants (Figures 3a and b). A previous report[27] indicated effects of this dose in pure C57BL/6J WT animals. One possibility is that differences in the genetic backgrounds used in the two studies modulated behavioral phenotypes, although we have not formally addressed this possibility.

As our behavioral analyses were naturally performed in only those $Tsc2^{+/-}$ mice that survived poly I:C treatment, it is in principle possible that this analysis inevitably focused on a specialized subset of unique individuals (for example, selected for certain genetic traits by the poly I:C treatment). The possibility that the surviving mice are genetically distinct from those that died during gestation is unlikely, as in our experiments we used isogenic mice derived from a cross between C57BL/6NCrl and C57BL/6J mice (homogeneous with respect to genetic background). Given the lack of an assortment of background genes in the tested offspring, it appears unlikely that the synergistic effect between the $Tsc2^{+/-}$ mutation and poly I:C is because of an interaction between the $Tsc2^{+/-}$ mutation and modifier genes segregating in the genetic background of the mice studied. We conclude that an interaction between the $Tsc2^{+/-}$ mutation and gestational immune activation represents the most parsimonious explanation for the synergistic effect on adult social behavior in $Tsc2^{+/-}$/poly I:C mice.

There are several ways in which the $Tsc2^{+/-}$ mutation could interact with gestational immune activation to result in neurological damage. Gestational immune activation is thought to perturb fetal brain development, at least in part because of the effects of cytokines in the developing nervous

system.[13,25–27] Importantly, TSC/mTOR signaling is downstream of multiple factors implicated in gestational immune activation, including various cytokines and growth factors.[29,30] Accordingly, it is possible that disinhibited TSC/mTOR signaling downstream of mediators of gestational immune activation effects (that is, cytokines, growth factors) amplifies their impact on the $Tsc2^{+/-}$ fetal brain.

Additionally, TSC/mTOR signaling also has an important role in the modulation of immune responses. The mTOR pathway promotes the activation of the innate immune system in response to Toll-like receptor engagement or viral infections.[30,32] At least in part via its effect on the innate immune system, TSC/mTOR signaling is also involved in the regulation of the adaptive immune response, including T-cell proliferation and cytokine production. For instance, mTOR inhibition dampened the T-cell response to virus vaccination[32] and favored the stimulation of tolerogenic regulatory T cells relative to that of effector T cells.[33] Taken together, the poly I:C-related immune activation may be more pronounced in $Tsc2^{+/-}$ mutants, which could explain why the ensuing damage is larger in $Tsc2^{+/-}$ mice than in WT controls.

Interestingly, deletion of *Pten*, an upstream regulator of the TSC/mTOR pathway, led to severe autoimmune disease in mice because of deficient removal of autoreactive lymphocytes.[40] Consistent with these results in *Pten* haploinsufficient mutants, increased phosphatidylinositol 3-kinase (PI3K) activity also caused lymphoproliferative autoimmune disorder in mice.[41] In another mouse model of autoimmune lymphoproliferative syndrome, also associated with disinhibited PI3K/AKT/mTOR signaling, pharmacological mTOR inhibition ameliorated lymphoproliferation and modulated levels of serum IgG nuclear auto-antibodies.[42] Collectively, these findings suggest that disinhibition of PI3K/AKT signaling is sufficient to generate an autoimmune

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phenotype, which depends, at least in part, on disinhibited mTOR signaling. $Tsc2^{+/-}$ mice do not show obvious signs of autoimmune disease but, conceivably, the poly I:C-related immune challenge may uncover a latent predisposition for autoimmunity in $Tsc2^{+/-}$ mutants,[43–46] a possibility that has to be addressed in future studies. Additionally, it will be of interest to determine if autoimmune phenomena are present in human TSC individuals affected by ASD.

It is noteworthy that preliminary observations suggest that immune responses may be significantly altered in human TSC subjects.[47] Additionally, findings consistent with inflammatory changes in tubers have been reported with respect to brain tissue from TSC individuals.[48] These observations, together with the data discussed above, should prompt further research into the relationship of immunological and neurological phenotypes in TSC.

To determine if gestational immune activation may interact with TSC mutations in generating ASD-related phenotypes in human populations, we analyzed the temporal relationship of peak seasonal flu activity and gestational periods in TSC individuals affected by ASD. As a comparison, we carried out similar studies in TSC individuals unaffected by ASD or other neurodevelopmental disorders. An excess of TSC–ASD individuals had late-trimester pregnancy coincide with peak seasonal flu activity, whereas this was not the case with TSC individuals without either ASD or other neurodevelopmental disorders. In contrast to the data in TSC populations, peak seasonal flu activity was equally distributed across gestational and extra-gestational periods in a general ASD population, suggesting that the association of late gestation with peak seasonal flu activity was specific for TSC–ASD individuals.

Several studies in general ASD populations have examined the relationship of ASD and seasonality of birth and have yielded mixed results.[49–55] Our findings suggest that certain ASD subpopulations (that is, ASD associated with TSC) may be prone to an interaction with seasonal infections, whereas this may not be the case for ASD of other etiologies. In the future, it may be possible to stratify ASD populations according to genetic risk factors and more specifically test which ASD sub-populations show interaction with seasonal infections. Genetic factors might interact with gestational viral infections in the pathogenesis of ASD by influencing the susceptibility for infection, through the qualitative and/or quantitative modulation of the host immune response or by influencing the extent to which the immune response interacts with other downstream processes (for example, cross-talk with neurodevelopmental processes).

Direct evidence for a role of gestational viral infections in ASD comes from studies that show an elevated risk in subjects that were gestationally exposed to specific viral infections,[14–16,19,20] such as rubella, or in subjects that were hospitalized because of viral infections.[21] Further studies on the relationship of TSC-related ASD and gestational viral infections in populations with proven maternal viral infection would be useful, but are difficult to perform because of the relatively low birth incidence of TSC.

Taken together, the data described above show that a heterozygous *Tsc2* mutation and the poly I:C model of gestational viral infections display interactive effects on intrauterine survival and may also have a synergistic impact on social behavior in adult mice. Studies in human TSC populations indicated an association between high seasonal flu activity in late gestation and ASD. Collectively, our data raise the possibility that TSC gene mutations interact with gestational viral infections in the pathogenesis of TSC-related ASD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)