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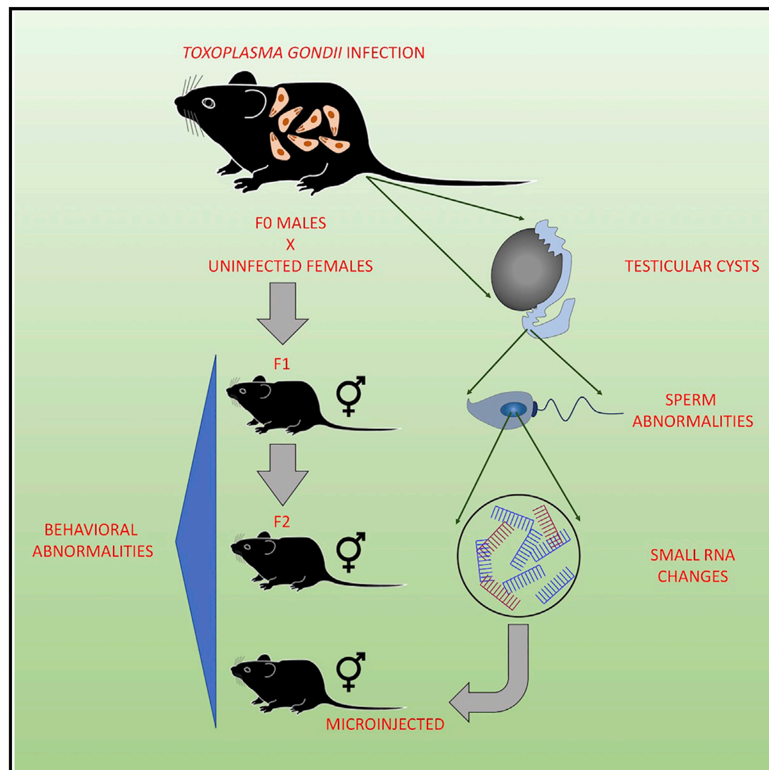
Pathogenic Infection in Male Mice Changes Sperm Small RNA Profiles and Transgenerationally Alters Offspring Behavior

Graphical Abstract



Highlights

- F1 and F2 generation of *T. gondii*-infected males display behavioral abnormalities
- Offspring behavioral changes display sexual dimorphism
- *T. gondii* infection leads to changes in sperm small RNA levels
- Zygotic microinjection of isolated sperm small RNA recapitulates behavioral changes

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In Brief

Tyebji et al. provide evidence that paternal pathogenic infection has transgenerational impacts on offspring phenotypes. They discover that *Toxoplasma gondii* infection induces epigenetic changes, involving small RNAs, in mouse sperm and demonstrate a mechanism involving these non-coding RNAs. This mechanism mediates transgenerational inheritance modulating offspring phenotype, including behavior.

Tyebji et al., 2020, Cell Reports *31*, 107573
April 28, 2020 © 2020 The Author(s).
https://doi.org/10.1016/j.celrep.2020.107573

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Pathogenic Infection in Male Mice Changes Sperm Small RNA Profiles and Transgenerationally Alters Offspring Behavior

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SUMMARY

Germline epigenetic factors influence transgenerational inheritance of behavioral traits upon changes in experience and environment. Immune activation due to infection can also modulate brain function, but whether this experience can be passed down to offspring remains unknown. Here, we show that infection of the male lineage with the common human parasite *Toxoplasma* results in transgenerational behavioral changes in offspring in a sex-dependent manner. Small RNA sequencing of sperm reveals significant transcriptional differences of infected animals compared to controls. Zygote microinjection of total small RNA from sperm of infected mice partially recapitulates the behavioral phenotype of naturally born offspring, suggesting an epigenetic mechanism of behavioral inheritance in the first generation. Our results demonstrate that sperm epigenetic factors can contribute to intergenerational inheritance of behavioral changes after pathogenic infection, which could have major public health implications.

INTRODUCTION

Perturbations in the environment—such as stress, malnutrition, and infection—can negatively influence an animal's homeostasis, affecting the growth and development of cells by a process known as reprogramming (Bale et al., 2010). The timing of such environmental insults, as well as their duration, can determine whether the epigenetic effects are limited or occur more broadly. In the latter case, they can be incorporated into the germ cells, thus having repercussions across generations (Bale, 2014). Until recently, it was believed that sperm contribute very little to early zygotic development, apart from their haploid genome (Sutovsky and Schatten, 1999) and some epigenetic marks "retained" by the oocytes (Rodgers et al., 2015). But now, it has become increasingly clear that the sperm transmit a substantial amount of information from the fathers that heavily impinges on the developmental trajectory of the fetus and affects the phenotype of the mature offspring over multiple generations (Perez and Lehner, 2019; Yeshurun and Hannan, 2019). Recent evidence from a study of non-genetic inheritance of parental environmental influences on offspring behavior and cognition has revealed a striking role of epigenetic mechanisms such as DNA methylation (Feinberg et al., 2015), histone modifications (Soubry, 2015), and small (Gapp et al., 2014; Reilly et al., 2016; Rodgers et al., 2015, 2013; Short et al., 2017) and long (Gapp et al., 2018) non-coding RNAs in the sperm that can modulate traits of psychiatric relevance via the male lineage.

Several reports indicate that pathogens are able to hijack host epigenetic regulators and target processes such as chromatin regulation, transcription, enzyme activity, and protein phosphorylation that promote pathogenesis and persistence within hosts (Cheeseman and Weitzman, 2015; Heard and Martienssen, 2014; Silmon de Monerri and Kim, 2014). *Toxoplasma gondii*, one of the most successful human parasites, infects approximately one-third of the world's human population (Montoya and Liesenfeld, 2004). These single-celled obligate intracellular parasites have the ability to invade the CNS and establish a latent chronic infection by differentiating into encysted bradyzoite forms, where they can persist throughout the host's lifetime (Wohlfert et al., 2017). In mice as well as in humans, such parasitic activity has been demonstrated to lead to a plethora of host behavior modifications (Tyebji et al., 2019), although an explanation of mechanisms by which *Toxoplasma* infection leads to such changes remains elusive. Nevertheless, current literature posits that a combination of parasitic activity altering neural function and the effects of peripheral host immune activation on the CNS results in the appearance of neuropsychiatric disturbances in the host (Tyebji et al., 2019).

One outcome of such a host-parasite interaction is the reshaping of host epigenetic profile. Transgenerational epigenetic inheritance, and the effects of pathogens on this process, has been relatively well studied in plants (Hauser et al., 2011; Heard and Martienssen, 2014). However, such events in mammalian systems are only beginning to be unraveled. Interestingly, recent

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studies have indicated that *Toxoplasma* infection is able to alter host microRNA (miRNA) profiles in macrophages (Li et al., 2019), in spleen (He et al., 2016), and in the brain (Cannella et al., 2014; Hu et al., 2018), a potential mechanism by which the parasite alters the host immune repertoire and ensures its persistence (Menard et al., 2019). Also, *Toxoplasma* infection was shown to alter methylation patterns on promoters of genes responsible for spermatogenesis (Dvorakova-Hortova et al., 2014). Furthermore, *Toxoplasma*-infected male rats displayed increased incidence of hypomethylation at the arginine vasopressin promoter in the medial amygdala compared to uninfected rats, leading to increased activation of neurons in this brain region. Such an effect was hypothesized to generate the classical loss of fear of cat odor in the infected rats because systemic hypermethylation rescued the phenotype (Hari Dass and Vyas, 2014). Thus, *Toxoplasma* can introduce epigenetic changes into the host organism, although its consequence on the extended phenotype remains to be explored. Sperm epigenetics, specifically changes in non-coding RNA, has been proposed to be part of a mechanism by which traits can be passed on transgenerationally (Yeshurun and Hannan, 2019); however, the effects of *Toxoplasma* infection have not been previously explored.

To date, all known literature only describes an intergenerational association of *Toxoplasma* infection and changes in offspring mental health via the maternal mode of infection. Studies (Canetta and Brown, 2012; Knuesel et al., 2014) suggest that effects of maternal *Toxoplasma* infection on offspring behavior could likely be the result of exposure to an inflammatory environment during fetal development, rather than the effect of parasite immunoglobulin G (IgG) or the presence of the parasite itself. Interestingly, many infectious agents such as bacteria, fungi, viruses, and parasites are able to interfere with male reproductive function, and infections of the male genito-urinary tract can also lead to infertility (Pellati et al., 2008). Rodent studies have confirmed that males infected with *Toxoplasma* display a reduction in sexual vigor, sperm motility, sperm count, and viability as well as an increase in the number of sperm abnormalities (Abdoli et al., 2012; Dalimi and Abdoli, 2013; Dvorakova-Hortova et al., 2014; Lim et al., 2013; Terpsidis et al., 2009). However, to be able to affect the progeny, *Toxoplasma* needs to influence the host sperm epigenome and thus have phenotypic repercussions. Such an effect on the F1 generation through direct exposure of the F0 germline is known as an intergenerational effect. Furthermore, if changes are observed in the F2 generation, then the effect becomes transgenerational.

Here, we describe that a chronic *Toxoplasma* infection alters sperm small RNA profiles, and these changes contribute to behavioral changes in subsequent generations. We found that chronically infected sires fathered offspring that displayed sexually dimorphic changes in the anxiety-like phenotype, learning, and memory in the first as well as the second generation. When we analyzed the sperm RNA content of infected males, we found a plethora of changes in the small RNA profiles 4 weeks after *Toxoplasma* infection. Furthermore, we show that microinjected small RNA isolated from sperm of infected mice into zygotes recapitulates the effects of paternal infection on behavior. This is the first study to demonstrate the ability of a pathogenic infection to alter male germ cell epigenetics and have a transgenerational influence on offspring behavioral phenotypes.

RESULTS

Changes in Reproduction Parameters in *Toxoplasma*-Infected Male Mice

We wished to assess the transgenerational effect of *Toxoplasma* through the male lineage. We therefore extended our chronic *Toxoplasma* mouse model and first examined the effect of chronic infection on the testes. *Toxoplasma* is known to reside in the testes of warm-blooded mammals (Martínez-García et al., 1996) and alter sperm parameters (Abdoli et al., 2012; Dvorakova-Hortova et al., 2014; Lim et al., 2013; Terpsidis et al., 2009). Thus, we evaluated reproductive parameters in infected male mice 4 weeks post-infection (wpi), a stage in which mice have resolved the acute infection but harbor chronic bradyzoites. Using periodic acid-Schiff (PAS) staining on testicular sections, we discovered *Toxoplasma* cysts in two-thirds of mice that were evaluated (Figure 1B), albeit at levels so low that they were not able to be accurately counted. We found that the total sperm count in the semen obtained from infected mice was significantly lower than that from uninfected mice ($t_{(5)} = 3.084$, $p = 0.0274$; Figure 1C), although the sperm motility remained unchanged ($t_{(5)} = 0.6066$, $p = 0.5706$; Figure 1D). When we measured the total testes weight, we found no significant difference between the two groups ($t_{(5)} = 2.016$, $p = 0.0999$; Figure 1E), although the testes from infected mice tended to be lighter. Interestingly, when we evaluated the morphological abnormalities in sperm, we found that the sperm from infected mice displayed a significant increase in the number of abnormalities compared to that from uninfected mice ($t_{(5)} = 7.834$, $p = 0.0005$; Figure 1F). Therefore, we show that *Toxoplasma* can reside in the mouse testes and form cysts. Also, infection causes changes in sperm count and abnormalities, raising the possibility of germline modifications due to *Toxoplasma* infection.

Toxoplasma Infection of Males Causes Behavioral Changes in the F1 Offspring

Toxoplasma infection *in utero* is associated with impaired fetal brain development and a high risk of developing neuropsychiatric disorders later in life (Brown et al., 2005; Elsheikha et al., 2016; Mortensen et al., 2007a, 2007b). Nevertheless, no studies to date evaluate the effects of paternal *Toxoplasma*, nor any other infection on resulting offspring, despite ample evidence to support the hypothesis that paternal environmental insults might have ramifications on their progeny (Yeshurun and Hannan, 2019). To study the inheritance of the effects of *Toxoplasma* infection via the male lineage, male C57BL/6 mice were injected with PBS or 50,000 tachyzoites of Pru (type II) strain of *Toxoplasma*, and mating was carried out as illustrated in Figure 1A. During the mating procedure, we observed that infected male mice showed significantly reduced sexual vigor, as evaluated by a reduction in mounting behavior and a reduction in the frequency of vaginal plug observed in females when compared to that by uninfected males. To evaluate if infection was passed on to females during mating, as described in some other animals (Flegr et al., 2014), we assessed the infection status in females that mated with infected males post-weaning the offspring and found that they were seronegative for *Toxoplasma* IgG (results not shown). Then, to assess the paternal

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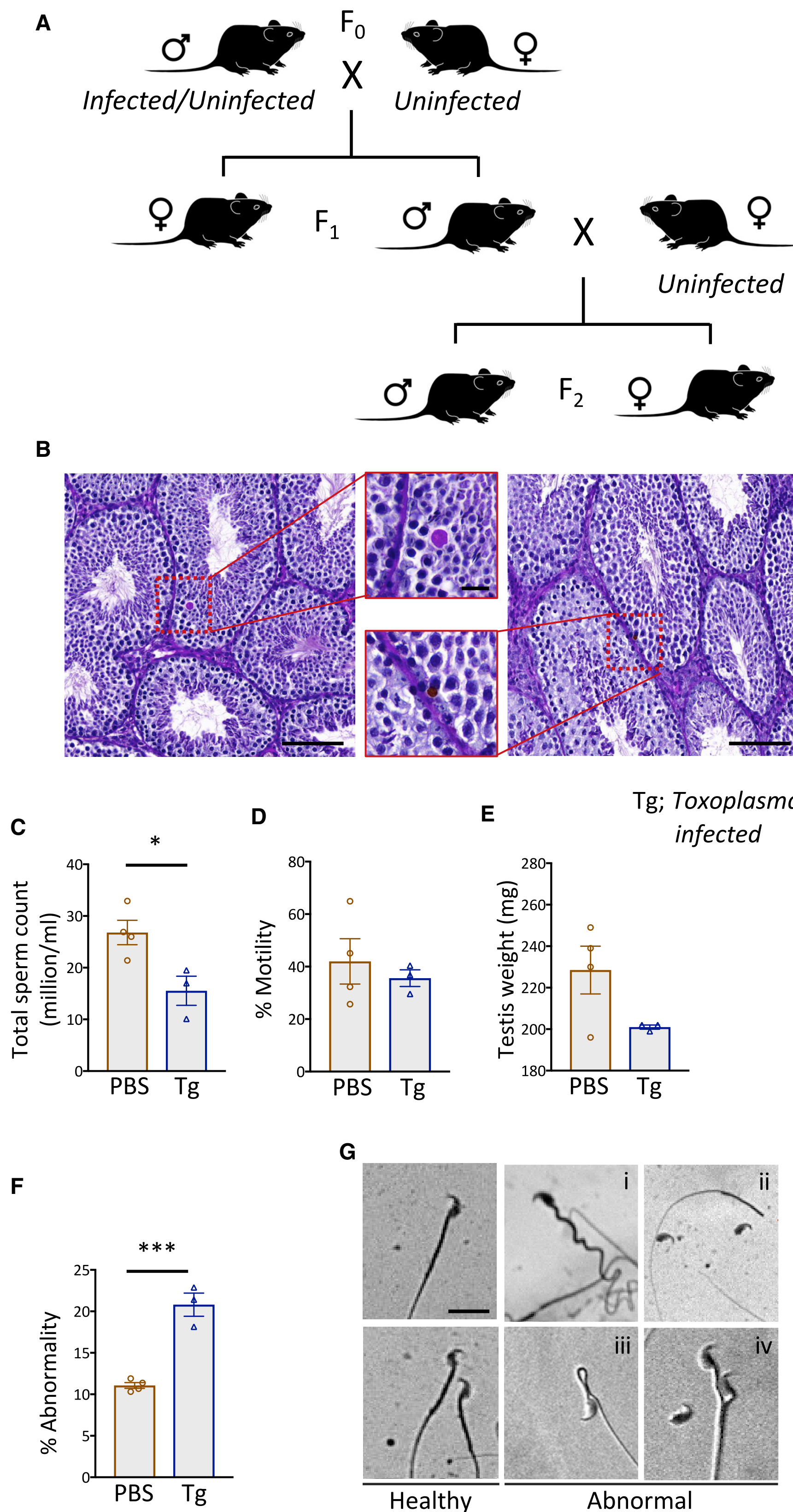

Figure 1. Analysis of *Toxoplasma*-Infected F0 Males

(A–F) A schematic representation of breeding of 6–8-week-old mock (PBS injected) or *Toxoplasma*-infected C57BL/6J males (n = 3 each) with age-matched uninfected females to give rise to F1 and F2 generations via the paternal lineage (A). A separate batch of infected (n = 3) or CNT male (n = 4) mice was generated to study F0 sperm parameters and 4 weeks later used to analyze the following: the presence of *Toxoplasma* cysts in the testes (infected mice only) by Periodic Acid-Schiff staining (cysts were found in testes of 2/3 mice, representative images shown) (B); total sperm count (C); sperm motility (D); testis weight (E); and sperm abnormality (F). Abnormal sperm were classified as described earlier (Seed et al., 1996).

(G) Representative images of healthy and abnormal sperm with (i) abnormal flagellum, (ii) lost head, (iii) bent neck, and (iv) double head are shown.

Scale bars, 100 μm (original) and 20 μm (inset) (B) and 20 μm (G). In all graphs, error bars represent mean ± SEM (n = 3). Data were analyzed by Student's t test. $^{*}p < 0.05$, $^{***}p < 0.001$.

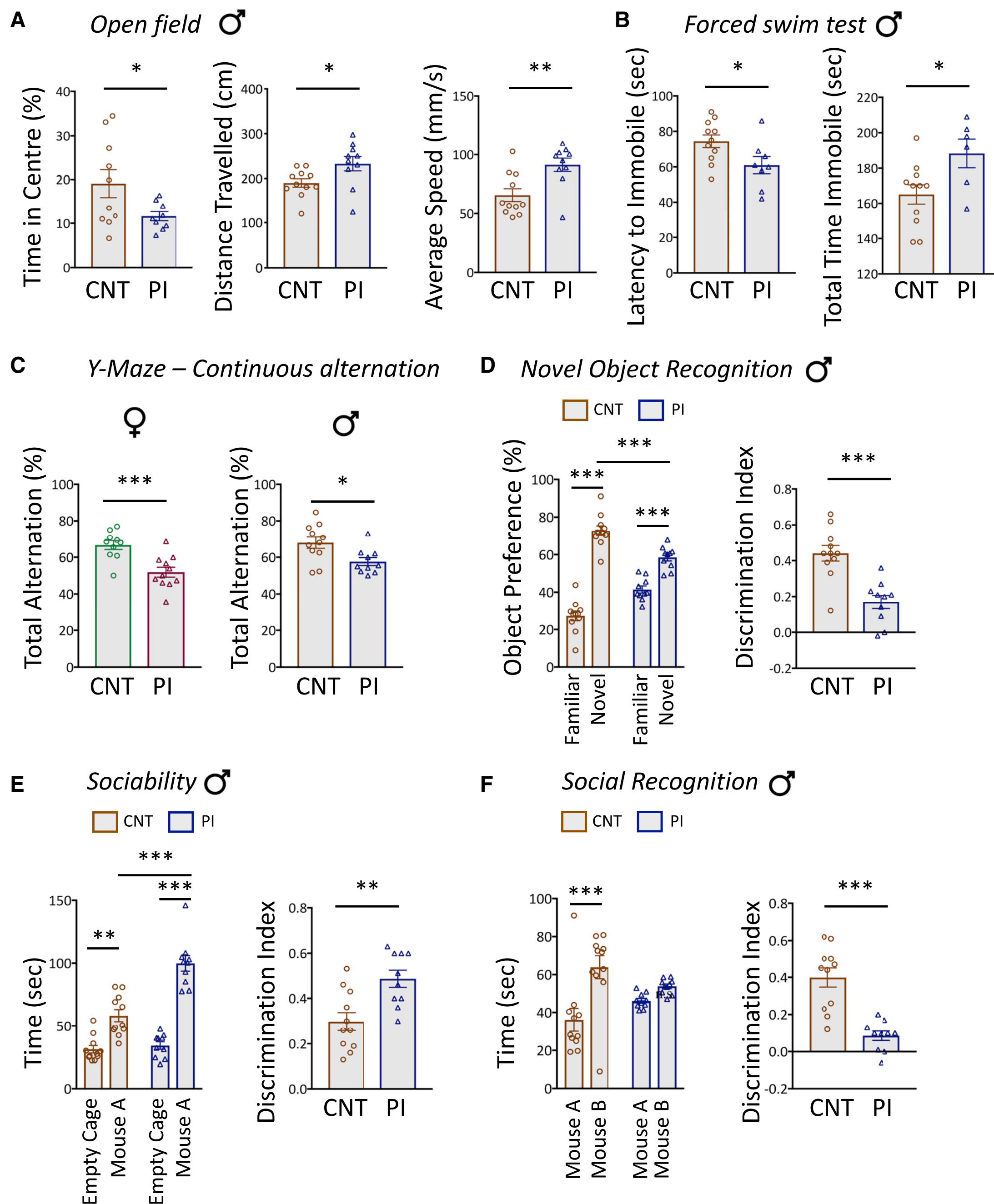

Figure 2. Behavioral Changes in the F1 Progeny of *Toxoplasma*-Infected Male Mice

The 8-week-old offspring of *Toxoplasma*-infected (paternal infection; PI) or mock-infected (CNT) C57BL/6J mice were assessed for behavioral changes.

(A) Anxiety-like phenotype and locomotor activity of male mice was assessed using the open-field test by measuring percentage of time spent in the open area and the distance traveled and average velocity in the open field, respectively.

(B) Depression-like behavior of male mice was measured as the latency to immobility and total time immobile in the forced-swim test.

(C) Spatial working memory in male and female mice was calculated as percentage of alternations on the Y-maze continuous alternation test.

(D) In the object recognition test, time spent by male mice exploring both objects were measured 24 h after training and represented as a percentage of object preference. Discrimination index for the novel object was calculated as the mean difference in time spent exploring both objects divided by total exploration time.

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inheritance of effects of *Toxoplasma* infection on behavior, we conducted a series of behavioral assays at 8 weeks of age (Figure S1A) on offspring (F1) of infected males (paternal infection; PI) and compared them to offspring born to uninfected control mice (CNT). At the end of behavioral testing, F1 offspring were tested and found to be seronegative for *Toxoplasma* IgG (results not shown).

In the open field test, male PI mice spent significantly less time in the center of the arena ($t_{(17)}$ = 2.144, p = 0.0468; Figure 2A), indicating an increase in the anxiety-like phenotype in these mice. However, male PI mice spent an equal amount of time in the light area (Figure S2B) and in the open arms (Figure S2C) during the light-dark box test and elevated-zero maze test, respectively. Also, they covered more total distance ($t_{(19)}$ = 2.375, p = 0.0282) and displayed increased average speed ($t_{(19)}$ = 3.277, p = 0.004) in the open field (Figure 2A), indicating an increase in their general locomotor activity when compared to CNT mice. Furthermore, male PI mice displayed an increased depression-like phenotype as they showed a reduced latency to immobility ($t_{(17)}$ = 2.295, p = 0.0374) and an increased total time immobile ($t_{(15)}$ = 2.449, p = 0.0271) in the forced-swim test (Figure 2B), but they were not different from the CNT mice on the sucrose-preference test (Figure S2D). Interestingly, when we performed the above assays with female PI mice, we found that they showed no changes when compared to CNT female mice (Figure S2).

Next, we measured cognitive changes in PI offspring and found that male as well as female mice displayed intact spatial reference memory (Figure S2F) but impaired spatial working memory in the Y-maze (Figure 2C), as they displayed a significant preference for the novel arm but reduced total alternations (males: $t_{(19)}$ = 2.68, p = 0.0148; females: $t_{(19)}$ = 4.028, p = 0.0007) when compared to CNT mice. Interestingly, in male PI offspring, we observed a significant effect of exposure to novel object in the object recognition test ($F_{(1,38)}$ = 182.5, p < 0.0001), and post hoc analysis revealed that they displayed a significant reduction in the preference for the novel object (p = 0.006). Further, they displayed a significantly reduced discrimination index for the novel object ($t_{(19)}$ = 4.702, p = 0.0002; Figure 2D) when compared to CNT mice. In the sociability phase of the three-chambered social interaction test (Figure 2E), we observed a significant effect of exposure to novel mouse ($F_{(1,38)}$ = 109.4, p < 0.0001), and strikingly, the male PI mice showed increased sociability as they spent more time exploring the cage with a novel mouse (p < 0.0001) compared to CNT mice and displayed an increased discrimination index ($t_{(19)}$ = 3.427, p = 0.0028). However, in the social recall phase (Figure 2F), despite a significant effect of first exposure to a novel mouse ($F_{(1,38)}$ = 15.06, p = 0.0004), male PI mice failed to discriminate between a novel and familiar mouse (p = 0.8247) and also displayed significantly less discrimination index for the novel mouse ($t_{(19)}$ = 5.298, p < 0.0001). When we performed these tests on female PI mice, we found no impairment in their object recognition memory, sociability, or social recognition memory (Figures S2G–S2I). Our results indicate that male F1 offspring of *Toxoplasma*-infected male mice display more significant behavioral changes than female offspring.

Toxoplasma Infection of Males Causes Behavioral Changes in the F2 Offspring

The transmission of the effects of prenatal insult on second-generation offspring's mental health and well-being is known to occur in mice as well as in humans (Bale, 2015; Dunn et al., 2011); however, recent evidence suggests that environmental manipulation in fathers such as chronic stress (Bohacek et al., 2015; Franklin et al., 2010; Short et al., 2016), social instability (Saavedra-Rodríguez and Feig, 2013), diet (Carone et al., 2010), and drug treatment (Crews et al., 2012) can recapitulate its negative effects not only on the resulting F1, but also in the F2 offspring. In some cases, such effects can skip a generation and manifest only in the F2 offspring (Saavedra-Rodríguez and Feig, 2013). Therefore, we evaluated whether paternal (F0) *Toxoplasma* infection affected the behavioral phenotype of the second-generation (F2) offspring. At the end of behavioral testing, F2 offspring were tested and found to be seronegative for *Toxoplasma* IgG (data not shown).

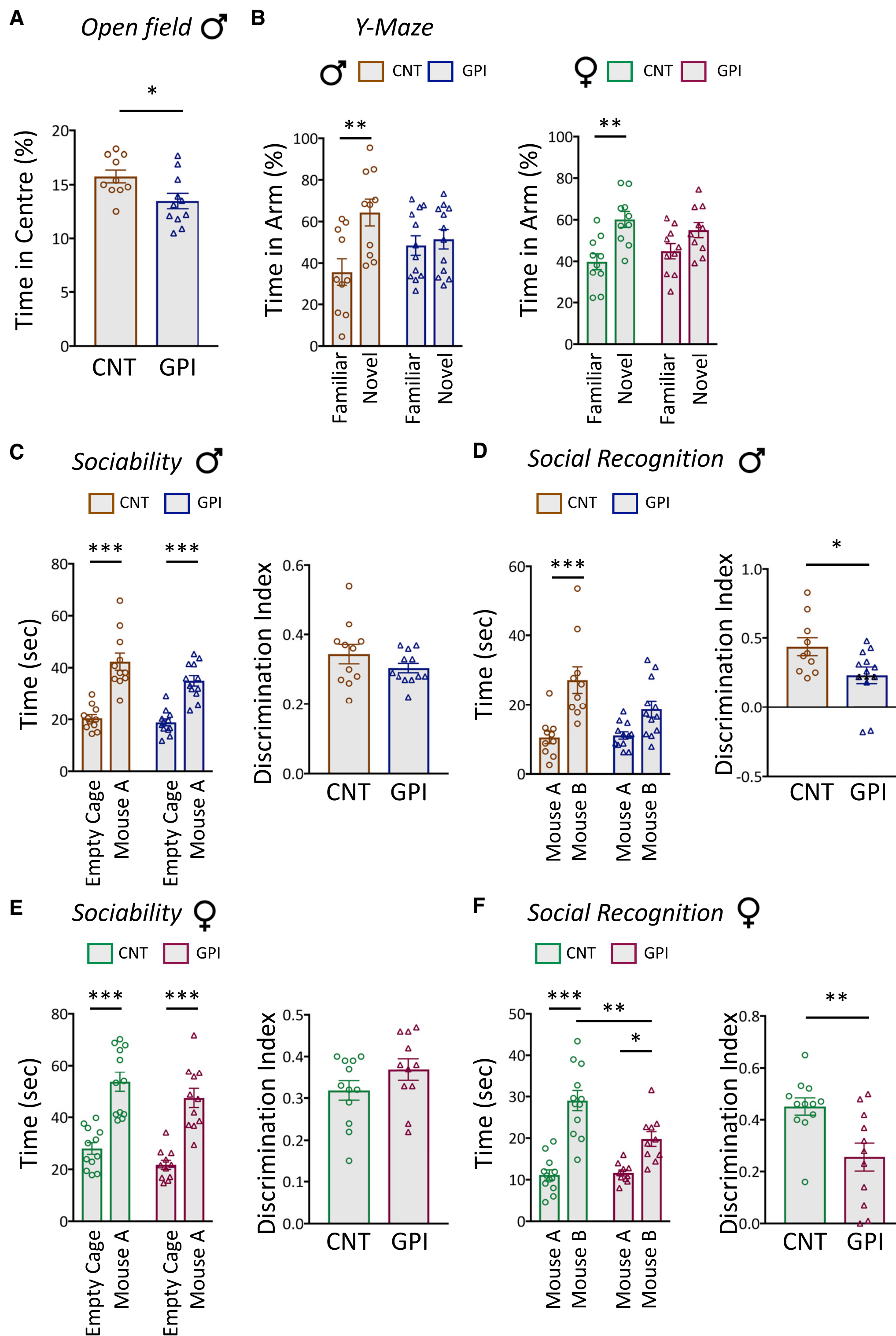
At 8 weeks of age, male F2 mice born to male PI mice (grandpaternal infection; GPI) displayed an increased anxiety-like phenotype in the open-field test, as they spent significantly less time in the center compared to CNT mice ($t_{(19)}$ = 2.455, p = 0.0239; Figure 3A); however, their locomotor ability in this test was unchanged (Figure S3A). Also, their performance in the light-dark box test, elevated-zero maze, sucrose-preference test, and forced-swim test was comparable to CNT mice (Figures S3B, S3C, and S3E). Furthermore, we did not detect any deficits in the female GPI mice in any of these assays (Figures S3A–S3C and S3E).

Interestingly, in the Y-maze test, although we found a significant effect of novel arm preference in male ($F_{(1,40)}$ = 8.213, p = 0.0066) as well as female ($F_{(1,36)}$ = 16.7, p = 0.0002) mice, post hoc analysis showed that GPI mice explored the novel arm equally to the familiar arm (male: p = 0.991, female: p = 0.3202; Figure 3B), indicating an impaired spatial reference memory. Their spatial working memory in the Y-maze continuous alternation test, however, was intact (Figure S3D). In the three-chambered social interaction test, male as well as female GPI mice displayed intact sociability (Figures 3C and 3E). However, in the social recall phase of the test, although male ($F_{(1,40)}$ = 25.81, p < 0.0001) and female ($F_{(1,40)}$ = 56.73, p < 0.0001) mice displayed a significant effect of exposure to novel mouse, post hoc analysis revealed that male GPI mice displayed no preference (p = 0.129) for the novel mouse, while the female mice

(E and F) Sociability (E) and social recognition memory (F) (1 h later) in male mice were measured as total time spent exploring a cage with a novel conspecific versus an empty cage or familiar mouse, respectively. Discrimination indices for sociability and social recognition were calculated as the mean difference in time spent exploring both cages divided by total exploration time.

In all graphs, error bars represent mean ± SEM (n = 8–11). Data were analyzed by Student's t test (A–C; discrimination indices in D–F) and two-way ANOVA with Sidak's multiple comparison as a post hoc test (D–F). *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S2.



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displayed a significantly reduced preference ($p = 0.0031$) for the novel mouse when compared to the CNT mice (Figures 3D and 3F). Also, their discrimination indices in the social recognition test were significantly lower than the CNT mice (male: $t_{(20)} = 2.319$, $p = 0.0311$; female: $t_{(21)} = 3.102$, $p = 0.0054$) indicating that F2 offspring of *Toxoplasma*-infected mice show impaired social recognition memory. Nevertheless, we observed no impairment in their object recognition memory (Figure S3G) when compared to CNT mice. Thus, our results show that infection with *Toxoplasma* influences offspring behavior transgenerationally via the male lineage.

Toxoplasma Infection Alters Small RNA Profiles in the F0 Sperm

Toxoplasma infection is known to induce a global change in the expression profiles of small non-coding RNAs in various host tissues (Menard et al., 2019), which is thought to assist the parasite in evading the host immune system. Thus, we hypothesized that *Toxoplasma* infection could also induce changes in the small RNA cargo of the sperm. As this is now a well-described mechanism of transgenerational inheritance of paternal phenotypes, it could explain the observed behavioral changes in the F1 and F2 progeny of *Toxoplasma*-infected males. Using high-throughput Illumina sequencing, we revealed differences between the sperm small RNA profiles of *Toxoplasma*-infected and CNT animals (Figure 4A). We found 25.88% of small RNAs were differentially expressed after correcting for false discovery rate (FDR), of which 9.21% and 16.67% were up- and downregulated, respectively (Figure S4B). Analysis of the read length of total transcripts (>14 and <38 nt) showed that CNT samples had significantly more read counts of 25-nt and 28-nt fragments and significantly fewer of 17-nt and 27-nt fragments ($F_{(24, 200)} = 414.4$, $p < 0.0001$; Figure S4A). Further quantitative analysis revealed enriched small RNA load in our samples, and biotypes composition analysis showed a majority tRNA population followed by miRNA and piwi-interacting RNA (piRNA) populations, while the rest contained various other small RNA transcripts (Figures S4C and S4D), a distribution profile consistent with what was shown earlier in mature spermatozoa (Hutcheon et al., 2017). Interestingly, when we compared the total counts of the three major subtypes between CNT and *Toxoplasma*-infected samples, we observed that *Toxoplasma* samples had a significantly increased miRNA count ($t_{(8)} = 4.848$, $p = 0.0013$) and decreased piRNA count ($t_{(8)} = 4.303$, $p = 0.0026$; Figure S4E), reminiscent of immature caudal spermatozoa development (Hutcheon et al., 2017; Sharma et al., 2018). When we looked at miRNA changes between *Toxoplasma*-infected and CNT samples, we found that expression of several miRNAs was changed (Figure 4B). Of the total miRNAs mapped (585), 174 were differentially expressed in the *Toxoplasma*-infected group (FDR < 0.05), of which 35 were downregulated and 75 were upregulated with a $\log_2$FC < −2 and > 2, respectively (Figure 4C).

We found that small RNAs targeted distinct biological pathways. Using gene enrichment analysis, we identified pathways regulated by small RNAs that are differentially expressed (DE; $p < 0.05$) between infected and CNT samples and obtained 20 annotated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were over-represented in our dataset (Figure 5A). These contain DE genes that were either up- or downregulated in the *Toxoplasma*-infected group, out of which 10 pathways contained downregulated genes that reached significance in a one-sided hypergeometric test. Gene Ontology (GO) term analysis was performed to identify pathways represented by DE genes in the *Toxoplasma*-infected group relative to the CNT groups. We found 36 pathways that were enriched ($p < 0.05$), with an over-representation of pathways involved in biological processes involved in reproduction, male gamete development and maturation, and cellular organization (Figure 5B). Next, we analyzed each pathway in the *Mus musculus* Reactome database to identify pathways with a significant positive or negative average fold change using DE genes from our dataset (Figure 5C). We found that the pathways of the immune system and the gene expression had the highest positive fold change in the *Toxoplasma*-infected group. On the other side, reproduction and metabolism pathways showed the highest negative fold change in the *Toxoplasma*-infected group. Interestingly, pathways of the *Mus musculus* neuronal system also displayed small but significant downregulation, indicating possible dysregulation of neuronal development in offspring conceived from *Toxoplasma*-infected mouse sperm. Overall, small RNA sequencing revealed large differences in the epigenetic cargo carried by the sperm from the infected mice, a potential mechanism by which effects of *Toxoplasma* infection could influence the progeny.

Microinjection of Small RNA from Infected Sperm Recapitulates Behavioral Effects of Paternal *Toxoplasma* Infection

The above results and those of others (Gapp et al., 2018, 2014; Rodgers et al., 2015, 2013) indicate a strong possibility that the altered sperm small RNA cargo in *Toxoplasma*-infected mice could modify offspring behavior. To establish a causative link between the two, we purified total small RNAs from the PBS-injected (CNT) and *Toxoplasma*-infected samples used for RNA

Figure 3. Behavioral Changes in the F2 Progeny of *Toxoplasma*-Infected Male Mice

The 8-week-old grand-offspring of *Toxoplasma*-infected (grand-paternal infection; GPI) or CNT male C57BL/6J mice were assessed for behavioral changes.

(A) Anxiety-like phenotype of male mice was assessed using the open-field test by measuring percentage of time spent in the open area.

(B) Spatial reference memory in male and female mice was measured in the Y-maze as the percentage of time spent in familiar and novel arms 1 h after training.

(C–F) Sociability in male (C) and female (E) mice was measured as total time spent exploring a cage with a novel conspecific versus an empty cage. Social recognition memory in male (D) and female (F) mice was measured 1 h later as times spent exploring familiar versus novel mouse. Discrimination indices for sociability and social recognition were calculated as the mean difference in time spent exploring both cages divided by total exploration time.

In all graphs, error bars represent mean ± SEM (n = 10–12). Data were analyzed by Student's t test (A; discrimination indices in C–F) and two-way ANOVA with Sidak's multiple comparison as a post hoc test (B–F). *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S3.

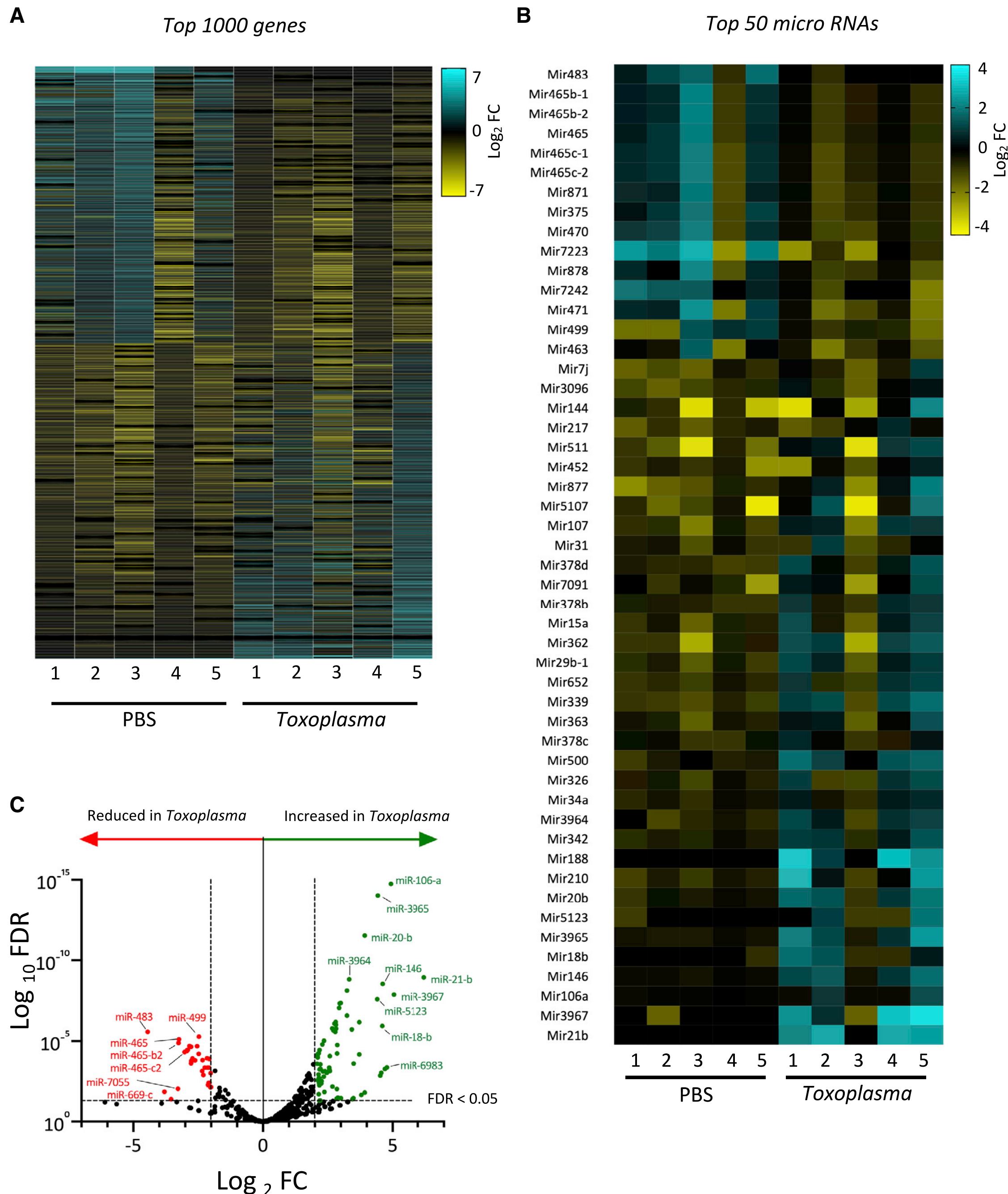

Figure 4. Effects of *Toxoplasma* Infection on Sperm Small RNA Profile

(A and B) Heatmap of top 1000 genes exhibiting the most significant changes (FDR < 0.01) between sperm of *Toxoplasma*-infected and CNT C57BL/6J males 4 wpi (A) and heatmap of 50 miRNAs that showed the highest fold change between *Toxoplasma*-infected and CNT males (B). Columns show the five biological replicates in each group, with each replicate consisting of sperm pooled from two individual mice. Cells within the matrix depict the relative abundance ($\log_2$ fold change) of each transcript after being zero-centered for each row according to the median abundance across all 10 samples. Blue and yellow color indicates quantities above and below the median accumulation of each transcript.

(C) Volcano plot depicting all miRNAs detected in our experiment and displayed according to the fold change (x axis) and FDR (y axis). miRNAs that passed the threshold (dotted lines) of change are colored red (reduced in Tg) or green (increased in Tg; threshold = $\log_2$ fold change ± 2 and FDR rate of < 0.05).

See also Figure S4.

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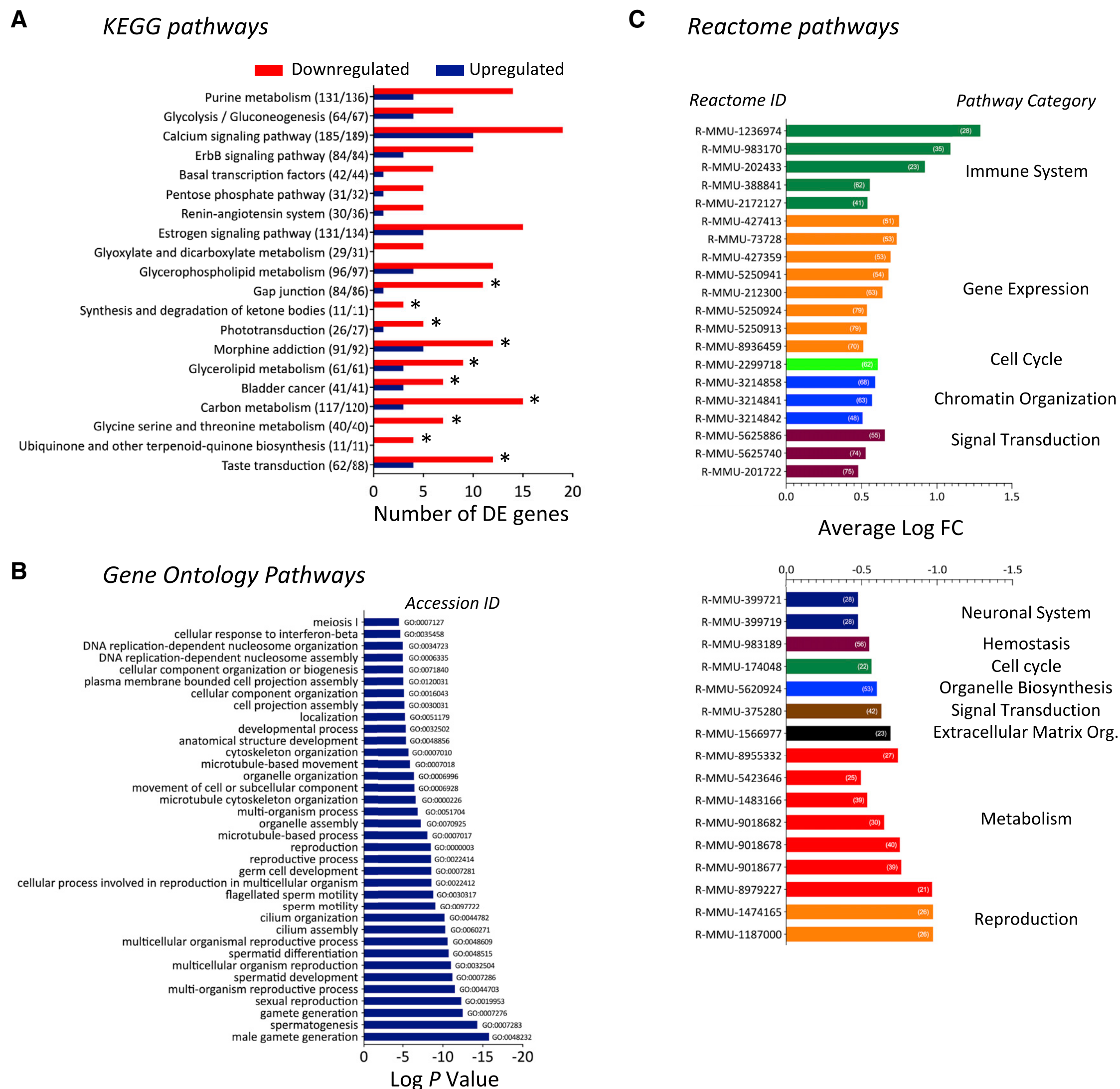

Figure 5. Functional Annotation and Pathway Analysis of Gene Targets

(A) Enrichment analysis identified annotated KEGG pathways (y axis) that were over-represented in our dataset and are shown here as number of differentially expressed (DE) genes (x axis) in the Tg group compared to the Cnt group associated with the pathway. Numbers in parentheses indicate the number of genes detected out of total annotated genes. *p < 0.05 using a one-sided hypergeometric test with adjustment bias for over-representation.

(B) GO analysis reveals enrichment of DE genes in the Tg group relating to different biological functions. Bars represent log p values of each GO term.

(C) Analysis of the *Mus musculus* reactome database identified pathways that were significantly up- or downregulated in the Tg group. Numbers in parentheses indicate genes from the pathway found in our dataset.

See also Table S1.

sequencing and microinjected a physiologically relevant amount (Gapp et al., 2018; Rodgers et al., 2015) (1 fg) into the pronucleus of naive C57BL/6J mice oocytes. The resulting male and female offspring were subjected to behavior tests as done above (Figure S1C).

At 8 weeks of age, male mice with microinjections of small RNA from *Toxoplasma*-infected sires displayed impairments in object recognition memory. We noticed a significant effect of novel object exposure in CNT and test male mice ($F_{(1,36)}$ = 704.1, p < 0.0001), and post hoc analysis revealed that test mice had a significantly lower novel object preference (p = 0.016) when compared to CNT mice (Figure 6A). Also, their discrimination index for the novel object was significantly less ($t_{(18)}$ = 4.087, p = 0.0007) than CNT mice. In the sociability test (Figure 6B), although we found a significant effect of exposure to mouse cage ($F_{(1,36)}$ = 59.19, p < 0.0001), their preference index for the cage with the mouse was significantly less ($t_{(18)}$ = 2.341, p = 0.0309) when compared to CNT mice. Moreover, in the social recall phase (Figure 6C), *Toxoplasma*-infected small-RNA-injected mice showed no preference for the novel mouse (effect of novel mouse: $F_{(1,36)}$ = 36.17, p < 0.0001; post hoc familiar versus novel: p = 0.0978) and displayed a significantly reduced

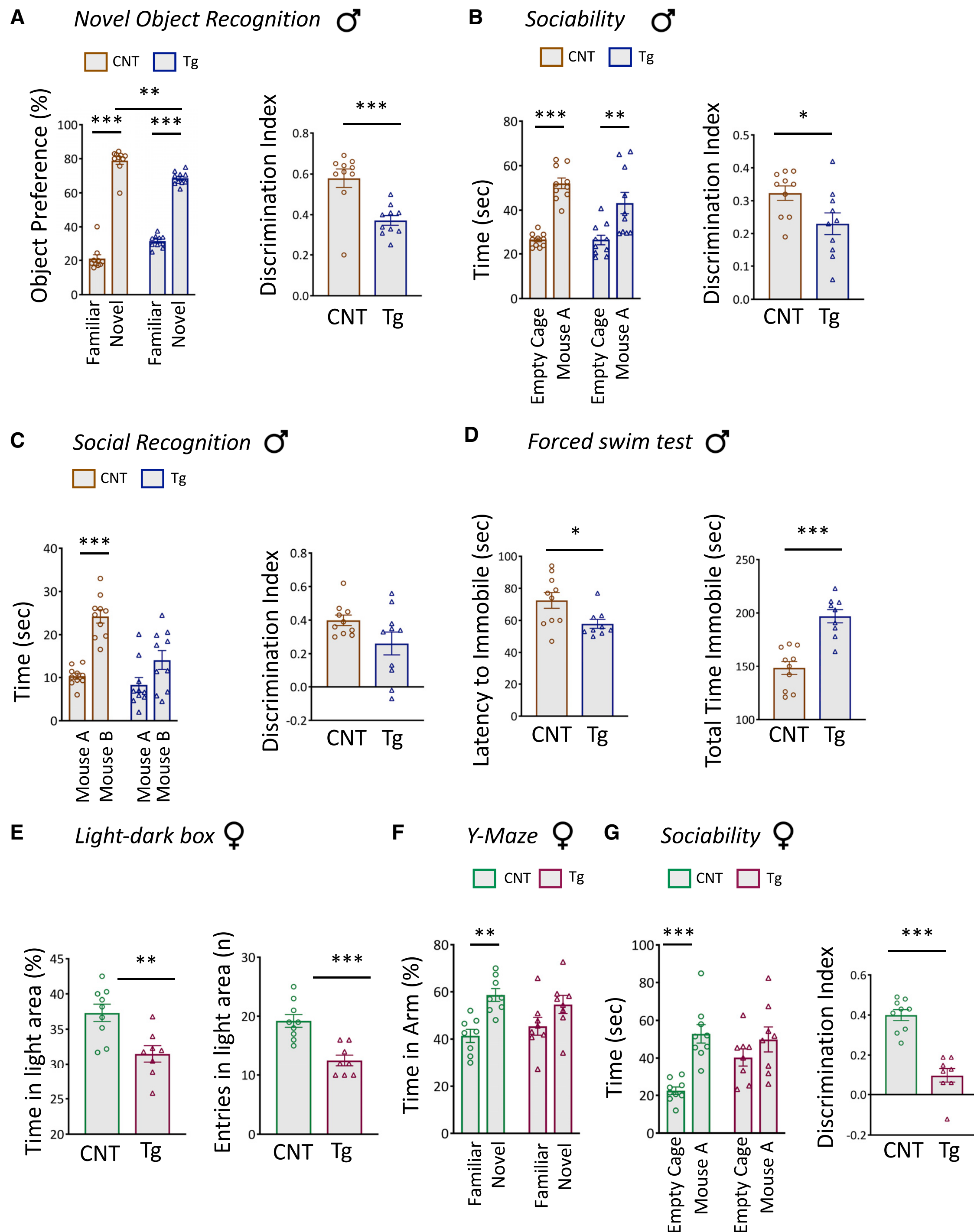

Figure 6. Behavioral Changes in Mice Born with Small RNA of *Toxoplasma*-Infected Mice

Mice born after the microinjection of sperm small RNA of CNT or infected (Tg) mice were assessed for behavioral changes at adulthood.

(A) In male mice, object recognition memory was assessed by calculating the time spent by mice exploring a novel and a familiar object 24 h after training and represented as percentage of object preference. Discrimination index for the novel object was calculated as the mean difference in time spent exploring both objects divided by total exploration time.

(legend continued on next page)

discrimination index ($t_{(18)} = 1.847$, $p = 0.0813$) compared to the CNT littermates. In the forced-swim test (Figure 6D), male mice with small RNA of *Toxoplasma*-infected sires showed decreased latency to immobility ($t_{(17)} = 2.495$, $p = 0.0232$) and increased total immobility ($t_{(17)} = 5.531$, $p < 0.0001$), indicating an increase in depression-like phenotype in these mice. It is interesting to note that these impairments were also recorded in naturally born F1 male progeny of *Toxoplasma*-infected sires. However, we could not find any changes in the anxiety-like phenotype, anhedonia, working memory, or spatial memory in male Tg mice (Figures S5A–S5F).

In female mice with small RNA of *Toxoplasma*-infected sires, we found an increased anxiety-like phenotype, as they spent less time in the light area ($t_{(15)} = 3.409$, $p = 0.0039$) and made fewer entries into the light area ($t_{(15)} = 4.671$, $p = 0.0003$) in the light-dark box test (Figure 6E). We also found that they had impaired spatial reference memory in the Y-maze (Figure 6F), as they spent an equal amount of time in the familiar and novel arms (novel arm effect: $F_{(1,28)} = 16.02$, $p = 0.0004$; post hoc familiar versus novel: $p = 0.3048$). Furthermore, female mice displayed reduced sociability (Figure 6G), as they had no preference for the cage containing the mouse when compared to an empty cage (effect of mouse cage: $F_{(1,30)} = 17.95$, $p = 0.0002$; post hoc empty versus mouse: $p = 0.6967$) and significantly reduced recognition index ($t_{(15)} = 6.843$, $p < 0.0001$) compared to CNT mice, although they had no impairments in social recognition memory 1 h later (Figure S5I). We did not find any impairment in other tests performed on female test mice (Figures S5A–S5H). Interestingly, impairments in Y-maze spatial reference memory and sociability were found in the female mice of the second generation of *Toxoplasma*-infected sires.

Thus, we show that sperm small RNA from *Toxoplasma*-infected mice is able to reshape the zygotic development in a way that recapitulates the behavioral impairments in male as well as female offspring. Interestingly, some of these impairments are similar to those observed in naturally born offspring of infected males. Our results provide definitive evidence that remodeling of the sperm epigenetic environment after *Toxoplasma* infection plays a role in altering offspring behavior.

DISCUSSION

Environmental experiences during an animal's lifetime can induce germline modifications, which can have repercussions on offspring mental health. Infection with pathogens represents another type of environmental experience, which, until now, has not been tested for its ability to be inherited through the paternal lineage. Therefore, we decided to investigate the impact of chronic *Toxoplasma* infection on offspring, as this parasite is one of the most ubiquitous human pathogens, with 30%–80% of the population chronically infected with this parasite. Here, we show that F1 and F2 progeny of infected male mice display changes in behavior in a sex-dependent manner despite being seronegative for the infection, indicating a transgenerational effect of first exposure to the parasite. Furthermore, we demonstrate changes in sperm small RNA after *Toxoplasma* infection. We also provide evidence that sperm small RNA is involved in the transmission of changes in F1 offspring behavior observed after *Toxoplasma* infection.

We show that the presence of *Toxoplasma* modifies sperm small RNA profiles such that it alters the neurodevelopmental trajectory in the resulting F1 offspring. Current knowledge posits several possibilities as to how *Toxoplasma* might be influencing this process. Our results indicate that *Toxoplasma* can infect the cells of the testes and the epididymis, regions where sperm production and maturation occur. Therefore, the possibility exists that defective sperm maturation might lead to changes in the small RNA load it carries. This is supported by our observation that sperm health parameters are changed after *Toxoplasma* infection. An abnormally high quantity of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TFN-α) and interleukin 1 beta (IL-1β), in the reproductive tract lead to reduced sperm count, sperm motility, and sperm morphology (Azenabor et al., 2015). *Toxoplasma* infection has also been described to cause an upregulation of these pro-inflammatory cytokines (Scanga et al., 2002) and therefore could contribute to the observed decline in sperm quality. Spermatozoa development begins in the testis and continues as the sperm cells transit the epididymis. Evidence suggests that this post-testicular window of sperm maturation, which occurs in the epididymis, is the key point at which the host environment can alter sperm programming (Morgan et al., 2019). Our data suggest that infection somehow restricts sperm maturation in the epididymis because the miRNA and piRNA composition profile of infected sperm (Figure S4) most closely matches that of immature sperm from the caput epididymis (Sharma et al., 2018). A likely mechanistic candidate to induce these changes is the extracellular vesicles (EVs) originating from the epithelial cells of the epididymis (Conine et al., 2018; Nixon et al., 2015; Reilly et al., 2016; Sharma et al., 2018). Most eukaryotic cells produce EVs, which are small-membrane bound particles, often carrying small RNA cargo (Sharma et al., 2018) that can be delivered to the maturing sperm in the caput epididymis by endocytosis. Interestingly, recent studies have shown that *Toxoplasma* produce their own

(B) Sociability was measured as total time spent exploring a cage with a novel conspecific versus an empty cage.
(C) Social recognition memory was measured 1 h later as times spent exploring familiar versus novel mouse.
(D) Depression-like behavior was measured as the latency to immobility and total time immobile in the forced-swim test.
(E) In female mice, the anxiety-like phenotype was assessed using the light-dark box test by measuring percentage of time spent and entries made into in the light area.
(F) Spatial reference memory was measured in the Y-maze as the time spent in familiar or novel arms 1 h after training.
(G) Sociability was measured as total time spent exploring a cage with a novel conspecific versus an empty cage. Discrimination indices for sociability and social recognition were calculated as the mean difference in time spent exploring both cages divided by total exploration time.
In all graphs, error bars represent mean ± SEM (n = 8–10). Data were analyzed by Student's t test (D and E; discrimination indices in A–C and G) and two-way ANOVA with Sidak's multiple comparison as a post hoc test (A–C, F, and G). *p < 0.05, **p < 0.01, ***p < 0.001.
See also Figure S5.

EVs that can be recognized by the host cells, some containing miRNA, and are one of the ways the parasite delivers its cargo into the host cells (Li et al., 2018; Silva et al., 2018; Wowk et al., 2017). Although *Toxoplasma* EVs were shown to be immunomodulatory, their effects on host sperm are unknown. Thus, further studies are required to explore whether parasite- and/or host-derived EVs influence the sperm epigenome upon *Toxoplasma* infection.

Recently, several epigenetic mechanisms have been proposed by which neurodevelopmental reprogramming can occur that also underpin the effects on offspring mental health (Bale, 2015). Such mechanisms include DNA methylation, histone modifications, and actions of small non-coding RNAs, which when delivered to the next generation can either confer positive physiological and metabolic adaptations or lead to metabolic diseases and mental health disorders (Soubry, 2015). Effects of *Toxoplasma* infection on host somatic cell non-coding RNA profiles have recently been discussed in detail elsewhere (Menard et al., 2019). *Toxoplasma* is known to modify the host epigenome by secreting effector proteins that can lead to chromatin remodeling and deactivation of transcription factors and can regulate gene expression (Cheeseman and Weitzman, 2015; Silmon de Monerri and Kim, 2014). However, in our model, this is unlikely, given the low level of testicular infection that we observed. Therefore, the most likely scenario is that general inflammation in regions of sperm generation and maturation could change small RNA expression in ways described above, implying that such a scenario is possible in any infectious disease. Indeed, studies have shown that miRNAs and the immune system can impinge on each other, fine-tuning the response to inflammatory events (Tahamtan et al., 2018). In our sequencing data, we found a significant change in several miRNAs that are described to exert anti-inflammatory functions. For example, we found an upregulation of mir-10a, mir-21, mir-181, mir-132, mir-146a, and mir-145 in the sperm of *Toxoplasma*-infected mice, all of which play crucial anti-inflammatory roles either by downregulating the expression of pro-inflammatory cytokines or negatively regulating the TLR4-NF-κB signaling (Tahamtan et al., 2018). This suggests that host responses to inflammation are being incorporated into the sperm epigenome, likely via EVs, and thus delivering an altered immune regulatory signature to the offspring and modifying neurodevelopment. Interestingly, mir-132 is a regulator of neural development (Wanet et al., 2012) and is known to be increased after *Toxoplasma* infection (Xiao et al., 2014). Furthermore, mir-124 positively regulates glutamate signaling as well as social behavior in mice (Gascon et al., 2014) and was downregulated in our sperm data. Thus, our data indicate that *Toxoplasma* infection can alter levels of brain function modulating miRNAs in the sperm of infected mice. Nevertheless, how these changes function to alter zygotic development remains unknown. Future studies will need to evaluate heritable changes in F1 offspring that might explain the F2 phenotype that we observe in this study.

When we tested F1 and F2 offspring behavior, we observed that males and females only had a partial overlap in their degree of cognitive impairments. This suggests sexual dimorphism in paternal inheritance, a commonly observed phenomenon in rodent behavior that plays an important role in disease susceptibility, presentation, and outcome. Our results are in line with various studies describing paternal experiences shaping offspring behavior for up to three generations, many of which report sex-dependent outcomes (Bale, 2015), and also concur with studies describing sex-dependent effects of *Toxoplasma* infection (Li et al., 2015). Transgenerational epigenetic effects depend on the sex of the parent as well as the offspring. The epigenetic machinery can influence or be influenced by gonadal hormones. Furthermore, epigenetic modifications can include DNA methylation of genes, found on the X or Y chromosomes, thus mediating sex-specific outcomes (Bale, 2015). Females can impart sex-specific effects on fetal development during gestation via the placenta (Mueller and Bale, 2008). Although in our study the females were uninfected, altered social interaction with an infected male sire can affect the female hormonal stress response (Bohacek and Mansuy, 2017), affecting the developing fetus in a sex-dependent manner. Gonadal hormones can interact with epigenetic mechanisms, broadly influencing neurodevelopment and producing a sexually dimorphic brain (McCarthy et al., 2009). About 250 of the most abundantly expressed miRNAs in the brain differ in their expression in males and females (Morgan and Bale, 2011), and these sex differences are dependent on the conversion of gonadal testosterone to oestradiol in males (McCarthy, 1994). Therefore, hormonal changes due to epigenomic effects might alter miRNAs in the early neonatal brain. As the female brain is not exposed to gonadal hormones during early development, this indicates a point of disruption in males. Furthermore, because the Y chromosome contains no microRNA genes while the X chromosome is enriched for them (Morgan and Bale, 2012), if genes escape X inactivation due to inflammation-mediated events, females would have twice the expression level of males, thereby altering their behavioral responses. Thus, our results complement and substantially extend the existing literature describing paternal environmental insults as an antecedent to sex-specific changes in offspring behavior.

In a laboratory environment, because the rodent sire has minimal postpartum influence on offspring maturation, transmission of paternal traits can faithfully be traced back to the genetic and epigenetic material in his sperm, which is accessible to the analysis. In this study, we confirm this hypothesis using sperm small RNA microinjections. However, the behavioral changes observed in the offspring generated by small RNA microinjections were only partially identical to what we observed in the naturally born offspring of *Toxoplasma*-infected males. This could be due to the procedural differences between the two scenarios, which might impact offspring development, as well as the fact that we microinjected only the small RNA fraction, and there are other epigenetic factors at play that determine the final outcome. During breeding experiments, female rodents can change their maternal investment based on factors such as unusual social interaction with their mates, which might release stress hormones or cytokines, ultimately altering the developing embryo and/or maternal behaviors post-delivery (Drickamer et al., 2000). Such confounds can be removed by sperm RNA microinjections; however, this procedure involves ovarian stimulation for superovulation, *in vitro* maturation of oocytes, and embryo culture, all of which can alter the epigenetic profiles of

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germ cells and/or embryos (Denomme and Mann, 2012). Another potential confounder is the effects during transport of mice from the microinjection facility to where the behavioral tests were performed, which might alter the behavioral baseline differently in male and females, removing the dimorphism. Microinjecting small RNA does not recapitulate the effects of DNA methylation, histone post-translational modification, and long non-coding RNAs on the offspring development, which might explain phenotypic differences between the two groups. Gapp et al. (2018) recently showed that microinjecting small and long RNA from sperm of mice subjected to postnatal trauma replicated different hallmarks of naturally born offspring. Also, abnormal sperm DNA methylation (Milekic et al., 2015) and changes in sperm histones (Siklenka et al., 2015) were associated with changes in offspring health transgenerationally. Therefore, we corroborate and extend our current understanding that epigenetic signals cooperate to propagate intergenerational effects. However, in this study, we have not analyzed the sperm from the F1 males that were used to generate the F2 offspring. This is a limitation of the current study that restricts our interpretation of sperm epigenetics to intergenerational inheritance only.

This study demonstrates that pathogenic infections can cause epigenetic changes in the male germline as well as modulate the offspring phenotype. This opens a domain of host-parasite interactions, indicating that paternal infection can modulate offspring phenotypes for at least two generations. Considering that *Toxoplasma* is such a common human infection, this has major implications for public health. Our findings should stimulate further research to understand the link among *Toxoplasma*, other infections, and mental health, as well as the mechanisms by with epigenetic changes are encoded and transmitted to future generations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethics Statement
  - Mice infection
  - Mating of mice
- METHOD DETAILS
  - Sperm analysis
  - Histology
  - Behavioral tests
  - Open field
  - Light-dark box test
  - Elevated-zero maze
  - Sucrose preference test
  - Y-maze for spatial and working memory
  - 3-chambered sociability and social recognition memory test
  - Novel object recognition test
  - Forced swim test (FST)
  - Illumina sequencing
  - Sequencing data analysis
  - Functional analysis
  - Small RNA injection into oocytes
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107573.

ACKNOWLEDGMENTS

We would like to acknowledge the expertise of Carolina Alvarado, Shannon Oliver, Julie Scott, Andrea Briffa, and Fiona Waters from the WEHI Bioservices; Ellen Tsui and Cary Tsui from WEHI Histology department; and Lachlan Whitehead and Verena Wimmer from WEHI's Centre of Dynamic Imaging. We would also like to thank Marie Everest and Lesley Gray from the Australian Genomics Research Facility (AGRF, Melbourne, Australia) and Ashley Portbury from the Florey Institute of Neuroscience and Mental Health. A.J.H. is an NHMRC Principal Research Fellow and is supported by the DHB Foundation, Equity Trustees. We also gratefully acknowledge The David Winston Turner Endowment for funding this work. We are also grateful for institutional support from the Victorian State Government Operational Infrastructure Support and the Australian Government NHMRC IRIISS.

AUTHOR CONTRIBUTIONS

S.T. was responsible for conception and experimental design, data acquisition, interpretation and analysis of data, and drafting and revising the article; A.J.H. and C.J.T. were responsible for conception and experimental design, interpretation and analysis of data, and drafting and revising the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 16, 2019
Revised: March 1, 2020
Accepted: April 2, 2020
Published: April 28, 2020

REFERENCES

Abdoli, A., Dalimi, A., and Movahedin, M. (2012). Impaired reproductive function of male rats infected with Toxoplasma gondii. Andrologia *44*, 679–687.

Azenabor, A., Ekun, A.O., and Akinloye, O. (2015). Impact of Inflammation on Male Reproductive Tract. J. Reprod. Infertil. *16*, 123–129.

Bale, T.L. (2014). Lifetime stress experience: transgenerational epigenetics and germ cell programming. Dialogues Clin. Neurosci. *16*, 297–305.

Bale, T.L. (2015). Epigenetic and transgenerational reprogramming of brain development. Nat. Rev. Neurosci. *16*, 332–344.

Bale, T.L., Baram, T.Z., Brown, A.S., Goldstein, J.M., Insel, T.R., McCarthy, M.M., Nemeroff, C.B., Reyes, T.M., Simerly, R.B., Susser, E.S., and Nestler, E.J. (2010). Early life programming and neurodevelopmental disorders. Biol. Psychiatry *68*, 314–319.

Bohacek, J., and Mansuy, I.M. (2017). A guide to designing germline-dependent epigenetic inheritance experiments in mammals. Nat. Methods *14*, 243–249.

Bohacek, J., Farinelli, M., Mirante, O., Steiner, G., Gapp, K., Coiret, G., Ebeling, M., Durán-Pacheco, G., Iniguez, A.L., Manuella, F., et al. (2015).

Pathological brain plasticity and cognition in the offspring of males subjected to postnatal traumatic stress. Mol. Psychiatry *20*, 621–631.

Brown, A.S., Schaefer, C.A., Quesenberry, C.P., Jr., Liu, L., Babulas, V.P., and Susser, E.S. (2005). Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. Am. J. Psychiatry *162*, 767–773.

Canetta, S.E., and Brown, A.S. (2012). Prenatal infection, maternal immune activation, and risk for schizophrenia. Transl. Neurosci. *3*, 320–327.

Cannella, D., Brenier-Pinchart, M.-P., Braun, L., van Rooyen, J.M., Bougdour, A., Bastien, O., Behnke, M.S., Curt, R.-L., Curt, A., Saeij, J.P.J., et al. (2014). miR-146a and miR-155 delineate a MicroRNA fingerprint associated with Toxoplasma persistence in the host brain. Cell Rep. *6*, 928–937.

Carone, B.R., Fauquier, L., Habib, N., Shea, J.M., Hart, C.E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P.D., et al. (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell *143*, 1084–1096.

Cheeseman, K., and Weitzman, J.B. (2015). Host-parasite interactions: an intimate epigenetic relationship. Cell. Microbiol. *17*, 1121–1132.

Conine, C.C., Sun, F., Song, L., Rivera-Pérez, J.A., and Rando, O.J. (2018). Small RNAs Gained during Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. Dev. Cell *46*, 470–480.e3.

Crews, D., Gillette, R., Scarpino, S.V., Manikkam, M., Savenkova, M.I., and Skinner, M.K. (2012). Epigenetic transgenerational inheritance of altered stress responses. Proc. Natl. Acad. Sci. USA *109*, 9143–9148.

Dalimi, A., and Abdoli, A. (2013). Toxoplasma gondii and male reproduction impairment: A new aspect of toxoplasmosis research. Jundishapur J. Microbiol. *6*, 1–5.

Denomme, M.M., and Mann, M.R.W. (2012). Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies. Reproduction *144*, 393–409.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Donald, R.G., and Roos, D.S. (1998). Gene knock-outs and allelic replacements in Toxoplasma gondii: HXGPRT as a selectable marker for hit-and-run mutagenesis. Mol. Biochem. Parasitol. *91*, 295–305.

Drickamer, L.C., Gowaty, P.A., and Holmes, C.M. (2000). Free female mate choice in house mice affects reproductive success and offspring viability and performance. Anim. Behav. *59*, 371–378.

Dunn, G.A., Morgan, C.P., and Bale, T.L. (2011). Sex-specificity in transgenerational epigenetic programming. Horm. Behav. *59*, 290–295.

Dvorakova-Hortova, K., Sidlova, A., Ded, L., Hladovcova, D., Vieweg, M., Weidner, W., Steger, K., Stopka, P., and Paradowska-Dogan, A. (2014). Toxoplasma gondii decreases the reproductive fitness in mice. PLoS ONE *9*, e96770.

Elsheikha, H.M., Büsselberg, D., and Zhu, X.Q. (2016). The known and missing links between Toxoplasma gondii and schizophrenia. Metab. Brain Dis. *31*, 749–759.

Falcon, S., and Gentleman, R. (2007). Using GOstats to test gene lists for GO term association. Bioinformatics *23*, 257–258.

Feinberg, J.I., Bakulski, K.M., Jaffe, A.E., Tryggvadottir, R., Brown, S.C., Goldman, L.R., Croen, L.A., Hertz-Picciotto, I., Newschaffer, C.J., Fallin, M.D., and Feinberg, A.P. (2015). Paternal sperm DNA methylation associated with early signs of autism risk in an autism-enriched cohort. Int. J. Epidemiol. *44*, 1199–1210.

Flegr, J., Klapilová, K., and Kaňková, S. (2014). Toxoplasmosis can be a sexually transmitted infection with serious clinical consequences. Not all routes of infection are created equal. Med. Hypotheses *83*, 286–289.

Franklin, T.B., Russig, H., Weiss, I.C., Gräff, J., Linder, N., Michalon, A., Vizi, S., and Mansuy, I.M. (2010). Epigenetic transmission of the impact of early stress across generations. Biol. Psychiatry *68*, 408–415.

Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., and Mansuy, I.M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. Nat. Neurosci. *17*, 667–669.

Gapp, K., van Steenwyk, G., Germain, P.L., Matsushima, W., Rudolph, K.L.M., Manuella, F., Roszkowski, M., Vernaz, G., Ghosh, T., Pelczar, P., et al. (2018). Alterations in sperm long RNA contribute to the epigenetic inheritance of the effects of postnatal trauma. Mol. Psychiatry.

Gascon, E., Lynch, K., Ruan, H., Almeida, S., Verheyden, J.M., Seeley, W.W., Dickson, D.W., Petrucelli, L., Sun, D., Jiao, J., et al. (2014). Alterations in microRNA-124 and AMPA receptors contribute to social behavioral deficits in frontotemporal dementia. Nat. Med. *20*, 1444–1451.

Gebert, D., Hewel, C., and Rosenkranz, D. (2017). unitas: the universal tool for annotation of small RNAs. BMC Genomics *18*, 644.

Hari Dass, S.A., and Vyas, A. (2014). Toxoplasma gondii infection reduces predator aversion in rats through epigenetic modulation in the host medial amygdala. Mol. Ecol. *23*, 6114–6122.

Hauser, M.-T., Aufsatz, W., Jonak, C., and Luschnig, C. (2011). Transgenerational epigenetic inheritance in plants. Biochim. Biophys. Acta *1809*, 459–468.

He, J.-J., Ma, J., Wang, J.-L., Xu, M.-J., and Zhu, X.-Q. (2016). Analysis of miRNA expression profiling in mouse spleen affected by acute Toxoplasma gondii infection. Infect. Genet. Evol. *37*, 137–142.

Heard, E., and Martienssen, R.A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. Cell *157*, 95–109.

Hu, R.-S., He, J.-J., Elsheikha, H.M., Zhang, F.-K., Zou, Y., Zhao, G.-H., Cong, W., and Zhu, X.-Q. (2018). Differential Brain MicroRNA Expression Profiles After Acute and Chronic Infection of Mice With *Toxoplasma gondii* Oocysts. Front. Microbiol. *9*, 2316.

Hutcheon, K., McLaughlin, E.A., Stanger, S.J., Bernstein, I.R., Dun, M.D., Eamens, A.L., and Nixon, B. (2017). Analysis of the small non-protein-coding RNA profile of mouse spermatozoa reveals specific enrichment of piRNAs within mature spermatozoa. RNA Biol. *14*, 1776–1790.

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., and Woodgett, J.R. (2011). Assessment of social interaction behaviors. J. Vis. Exp. *48*, 2473.

Knuesel, I., Chicha, L., Britschgi, M., Schobel, S.A., Bodmer, M., Hellings, J.A., Toovey, S., and Prinssen, E.P. (2014). Maternal immune activation and abnormal brain development across CNS disorders. Nat. Rev. Neurol. *10*, 643–660.

Kulkarni, S.K., Singh, K., and Bishnoi, M. (2007). Elevated zero maze: a paradigm to evaluate antianxiety effects of drugs. Methods Find. Exp. Clin. Pharmacol. *29*, 343–348.

Li, Y.E., Kannan, G., Pletnikov, M.V., Yolken, R.H., and Xiao, J. (2015). Chronic infection of Toxoplasma gondii downregulates miR-132 expression in multiple brain regions in a sex-dependent manner. Parasitology *142*, 623–632.

Li, Y., Liu, Y., Xiu, F., Wang, J., Cong, H., He, S., Shi, Y., Wang, X., Li, X., and Zhou, H. (2018). Characterization of exosomes derived from *Toxoplasma gondii* and their functions in modulating immune responses. Int. J. Nanomedicine *13*, 467–477.

Li, S., Yang, J., Wang, L., Du, F., Zhao, J., and Fang, R. (2019). Expression profile of microRNAs in porcine alveolar macrophages after Toxoplasma gondii infection. Parasit. Vectors *12*, 65.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*, 923–930.

Lim, A., Kumar, V., Hari Dass, S.A., and Vyas, A. (2013). Toxoplasma gondii infection enhances testicular steroidogenesis in rats. Mol. Ecol. *22*, 102–110.

Martínez-García, F., Regadera, J., Mayer, R., Sanchez, S., and Nistal, M. (1996). Protozoan infections in the male genital tract. J. Urol. *156*, 340–349.

McCarthy, M.M. (1994). Molecular aspects of sexual differentiation of the rodent brain. Psychoneuroendocrinology *19*, 415–427.

McCarthy, M.M., Auger, A.P., Bale, T.L., De Vries, G.J., Dunn, G.A., Forger, N.G., Murray, E.K., Nugent, B.M., Schwarz, J.M., and Wilson, M.E. (2009). The epigenetics of sex differences in the brain. J. Neurosci. *29*, 12815–12823.

Menard, K.L., Haskins, B.E., and Denkers, E.Y. (2019). Impact of *Toxoplasma gondii* Infection on Host Non-coding RNA Responses. Front. Cell. Infect. Microbiol. *9*, 132.

Milekic, M.H., Xin, Y., O'Donnell, A., Kumar, K.K., Bradley-Moore, M., Malaspina, D., Moore, H., Brunner, D., Ge, Y., Edwards, J., et al. (2015). Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. Mol. Psychiatry *20*, 995–1001.

Montoya, J.G., and Liesenfeld, O. (2004). Toxoplasmosis. Lancet *363*, 1965–1976.

Morgan, C.P., and Bale, T.L. (2011). Early prenatal stress epigenetically programs dysmasculinization in second-generation offspring via the paternal lineage. J. Neurosci. *31*, 11748–11755.

Morgan, C.P., and Bale, T.L. (2012). Sex differences in microRNA regulation of gene expression: no smoke, just miRs. Biol. Sex Differ. *3*, 22.

Morgan, C.P., Chan, J.C., and Bale, T.L. (2019). Driving the Next Generation: Paternal Lifetime Experiences Transmitted via Extracellular Vesicles and Their Small RNA Cargo. Biol. Psychiatry *85*, 164–171.

Mortensen, P.B., Nørgaard-Pedersen, B., Waltoft, B.L., Sørensen, T.L., Hougaard, D., Torrey, E.F., and Yolken, R.H. (2007a). Toxoplasma gondii as a risk factor for early-onset schizophrenia: analysis of filter paper blood samples obtained at birth. Biol. Psychiatry *61*, 688–693.

Mortensen, P.B., Nørgaard-Pedersen, B., Waltoft, B.L., Sørensen, T.L., Hougaard, D., and Yolken, R.H. (2007b). Early infections of Toxoplasma gondii and the later development of schizophrenia. Schizophr. Bull. *33*, 741–744.

Mueller, B.R., and Bale, T.L. (2008). Sex-specific programming of offspring emotionality after stress early in pregnancy. J. Neurosci. *28*, 9055–9065.

Nixon, B., Stanger, S.J., Mihalas, B.P., Reilly, J.N., Anderson, A.L., Tyagi, S., Holt, J.E., and McLaughlin, E.A. (2015). The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. Biol. Reprod. *93*, 91.

Pellati, D., Mylonakis, I., Bertoloni, G., Fiore, C., Andrisani, A., Ambrosini, G., and Armanini, D. (2008). Genital tract infections and infertility. Eur. J. Obstet. Gynecol. Reprod. Biol. *140*, 3–11.

Perez, M.F., and Lehner, B. (2019). Intergenerational and transgenerational epigenetic inheritance in animals. Nat. Cell Biol. *21*, 143–151.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.-C., Mendell, J.T., and Salzberg, S.L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat. Biotechnol. *33*, 290–295.

Reilly, J.N., McLaughlin, E.A., Stanger, S.J., Anderson, A.L., Hutcheon, K., Church, K., Mihalas, B.P., Tyagi, S., Holt, J.E., Eamens, A.L., and Nixon, B. (2016). Characterisation of mouse epididymosomes reveals a complex profile of microRNAs and a potential mechanism for modification of the sperm epigenome. Sci. Rep. *6*, 31794.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. *43*, e47.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140.

Rodgers, A.B., Morgan, C.P., Bronson, S.L., Revello, S., and Bale, T.L. (2013). Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. J. Neurosci. *33*, 9003–9012.

Rodgers, A.B., Morgan, C.P., Leu, N.A., and Bale, T.L. (2015). Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. Proc. Natl. Acad. Sci. USA *112*, 13699–13704.

Saavedra-Rodríguez, L., and Feig, L.A. (2013). Chronic social instability induces anxiety and defective social interactions across generations. Biol. Psychiatry *73*, 44–53.

Scanga, C.A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E.Y., Medzhitov, R., and Sher, A. (2002). Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. J. Immunol. *168*, 5997–6001.

Seed, J., Chapin, R.E., Clegg, E.D., Dostal, L.A., Foote, R.H., Hurtt, M.E., Klinefelter, G.R., Makris, S.L., Perreault, S.D., Schrader, S., et al.; ILSI Risk Science Institute Expert Working Group on Sperm Evaluation (1996). Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reprod. Toxicol. *10*, 237–244.

Sharma, U., Sun, F., Conine, C.C., Reichholf, B., Kukreja, S., Herzog, V.A., Ameres, S.L., and Rando, O.J. (2018). Small RNAs Are Trafficked from the Epididymis to Developing Mammalian Sperm. Dev. Cell *46*, 481–494.e6.

Short, A.K., Fennell, K.A., Perreau, V.M., Fox, A., O'Bryan, M.K., Kim, J.H., Bredy, T.W., Pang, T.Y., and Hannan, A.J. (2016). Elevated paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and modifies anxiety and depressive phenotypes in the offspring. Transl. Psychiatry *6*, e837.

Short, A.K., Yeshurun, S., Powell, R., Perreau, V.M., Fox, A., Kim, J.H., Pang, T.Y., and Hannan, A.J. (2017). Exercise alters mouse sperm small noncoding RNAs and induces a transgenerational modification of male offspring conditioned fear and anxiety. Transl. Psychiatry *7*, e1114.

Siklenka, K., Erkek, S., Godmann, M., Lambrot, R., McGraw, S., Lafleur, C., Cohen, T., Xia, J., Suderman, M., Hallett, M., et al. (2015). Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. Science *350*, aab2006.

Silmon de Monerri, N.C., and Kim, K. (2014). Pathogens hijack the epigenome: a new twist on host-pathogen interactions. Am. J. Pathol. *184*, 897–911.

Silva, V.O., Maia, M.M., Torrecilhas, A.C., Taniwaki, N.N., Namiyama, G.M., Oliveira, K.C., Ribeiro, K.S., Toledo, M.D.S., Xander, P., and Pereira-Chioccola, V.L. (2018). Extracellular vesicles isolated from Toxoplasma gondii induce host immune response. Parasite Immunol. *40*, e12571.

Soubry, A. (2015). Epigenetic inheritance and evolution: A paternal perspective on dietary influences. Prog. Biophys. Mol. Biol. *118*, 79–85.

Sutovsky, P., and Schatten, G. (1999). Paternal Contributions to the Mammalian Zygote: Fertilization after Sperm-Egg Fusion. Int. Rev. Cytol. *195*, 1–65.

Tahamtan, A., Teymoori-Rad, M., Nakstad, B., and Salimi, V. (2018). Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. Front. Immunol. *9*, 1377.

Terpsidis, K.I., Papazahariadou, M.G., Taitzoglou, I.A., Papaioannou, N.G., Georgiadis, M.P., and Theodoridis, I.T. (2009). Toxoplasma gondii: reproductive parameters in experimentally infected male rats. Exp. Parasitol. *121*, 238–241.

Tyebji, S., Saavedra, A., Canas, P.M., Pliassova, A., Delgado-García, J.M., Alberch, J., Cunha, R.A., Gruart, A., and Pérez-Navarro, E. (2015). Hyperactivation of D1 and A2A receptors contributes to cognitive dysfunction in Huntington's disease. Neurobiol. Dis. *74*, 41–57.

Tyebji, S., Seizova, S., Hannan, A.J., and Tonkin, C.J. (2019). Toxoplasmosis: A pathway to neuropsychiatric disorders. Neurosci. Biobehav. Rev. *96*, 72–92.

Wanet, A., Tacheny, A., Arnould, T., and Renard, P. (2012). miR-212/132 expression and functions: within and beyond the neuronal compartment. Nucleic Acids Res. *40*, 4742–4753.

Wohlfert, E.A., Blader, I.J., and Wilson, E.H. (2017). Brains and Brawn: Toxoplasma Infections of the Central Nervous System and Skeletal Muscle. Trends Parasitol. *33*, 519–531.

Wowk, P.F., Zardo, M.L., Miot, H.T., Goldenberg, S., Carvalho, P.C., and Mörking, P.A. (2017). Proteomic profiling of extracellular vesicles secreted from Toxoplasma gondii. Proteomics *17*, 1600477.

Xiao, J., Li, Y., Prandovszky, E., Karuppagounder, S.S., Talbot, C.C., Jr., Dawson, V.L., Dawson, T.M., and Yolken, R.H. (2014). MicroRNA-132 dysregulation in Toxoplasma gondii infection has implications for dopamine signaling pathway. Neuroscience *268*, 128–138.

Yeshurun, S., and Hannan, A.J. (2019). Transgenerational epigenetic influences of paternal environmental exposures on brain function and predisposition to psychiatric disorders. Mol. Psychiatry *24*, 536–548.

Yu, G., and He, Q.Y. (2016). ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. Mol. Biosyst. *12*, 477–479.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Sulfadiazine Sodium	Sigma	Cat#: S6387
QIAzol lysis reagent	QIAGEN	Cat#: 79306
Tris-EDTA microinjection buffer	Sigma	Cat#: T9285
Sucrose	Sigma	Cat#: S8501
Critical Commercial Assays		
Cell-VU prestained morphology slides	Millennium Sciences	Cat#: DRM-900
Cell-VU sperm counting chamber	Millennium Sciences	Cat#: DRM-600
miRNeasy Mini Kit	QIAGEN	Cat#: 217004
RNeasy Min Elute Cleanup Kit	QIAGEN	Cat#: 74204
NEBNext Multiplex Small RNA Library Prep Set	New England Biolabs	Cat#: E7300
Deposited Data		
Raw fastq RNA-seq files	This paper	SGE137224
Experimental Models: Cell Lines		
Human foreskin fibroblasts (HFFs)	ATCC	SCRC-1041
Experimental Models: Organisms/Strains		
Mouse: C57BL/J6	The Clive and Vera Ramaciotti Laboratory	N/A
T. gondii: PruΔ*hxgprt*	(Donald and Roos, 1998)	N/A
Software and Algorithms		
CaseCentre slide management application v2.8	3DHISTECH	https://www.3dhistech.com/CaseCenter
TopScan mouse tracking software	Cleversys	http://cleversysinc.com/CleverSysInc/csi_products/topscan-suite/
GraphPad Prism version 7	Graphpad	https://www.graphpad.com/scientific-software/prism/
ImageJ (Fiji)	NIH	https://imagej.net/Fiji
STAR aligner (v2.5.3a)	(Dobin et al., 2013)	https://github.com/alexdobin/STAR/blob/master/doc/STARmanual.pdf
Featurecounts (v1.5.3)	(Liao et al., 2014)	http://subread.sourceforge.net/
Stringtie tool v1.3.3	(Pertea et al., 2015)	http://ccb.jhu.edu/software/stringtie/
edgeR (version 3.22.3)	(Robinson et al., 2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Unitas (v1.7.0)	(Gebert et al., 2017)	https://www.smallrnagroup.uni-mainz.de/software/unitas_documentation_1.7.0.pdf
Trim Galore (v0.5.0)	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
ReactomePA	(Yu and He, 2016)	https://bioconductor.org/packages/release/bioc/html/ReactomePA.html
LIMMA toolkit	(Ritchie et al., 2015)	http://www.bioconductor.org/packages/release/bioc/html/limma.html
GOstats package	(Falcon and Gentleman, 2007)	http://bioconductor.org/packages/release/bioc/html/GOstats.html
ZEN Imaging Software	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen-imaging.html

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Pannoramic SCAN II slide scanner	3DHISTECH	https://www.3dhistech.com/pannoramic_scan
Mouse behavior testing apparatus	San Diego Instruments	https://sandiegoinstruments.com/
Wire cup	Officeworks Australia	Cat#: ES47547BK

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Christopher J Tonkin (tonkin@wehi.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The accession number for the sequencing data reported in the paper is GEO: SGE137224 at NCBI's Gene Expression Omnibus.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics Statement

All animal experimentation in this study comply with the ARRIVE guidelines and were carried out in accordance with the U.K Animals (Scientific Procedures) Act, 1986 and of European Directive (2010/63/ EU) for the care and use of laboratory animals. These were also in accordance with the regulatory standards of and were approved by the Walter and Eliza Hall Institute Animal Ethics Committees under approval numbers 2014.021 and 2018.048.

Mice infection

All experiments were performed using inbred C57BL/6J mice purchased from The Clive and Vera Ramaciotti Laboratory (Melbourne, Australia) and habituated to the facility for 1 week before beginning of any procedure. F0 male mice were intraperitoneally injected with tachyzoites of *Toxoplasma* Prugniuand strain (Pru) that were maintained by passage in human foreskin fibroblast cell cultures. Parasites, harvested using a 27-guage needle, were pelleted and resuspended in phosphate-buffered saline (PBS) and then counted. Six- to eight-week-old male mice weighing between 22 g-24 g each were randomly assigned to either control (n = 3) or infected (n = 3) groups and received 100ul PBS (vehicle control) or 50 000 tachyzoites (infected; Tg) resuspended in 100ul PBS. They were monitored and weighted daily for the next 21 days. Mice were group housed with a 12-hr light-dark cycle, with access to food *ad libitum*. To establish a chronic infection, mice were provided with sulfadiazine sodium (Sigma) in drinking water (100ug/ml) from day 5 to 10 post-infection. Sulfadiazine treatment controls tachyzoite proliferations in the acute stages of infection and helps avoid animal death.

Mating of mice

Mating was performed as illustrated in Figure 1A. 25-30 days post infection (dpi), a time when *Toxoplasma* infection has been reported to induce alterations in rodent sperm parameters (Abdoli et al., 2012; Dvorakova-Hortova et al., 2014; Terpsidis et al., 2009), F0 male Ctr and Tg mice were mated with age-matched uninfected female mice, and females were checked for vaginal plug each morning. Upon observation of vaginal plug, the females were separated and single housed until they littered. The male mouse was introduced to a different female to continue breeding to generate sufficient offspring numbers. Female and male F1 offspring were weaned at the age of 2 weeks into separate boxes of 4-6 mouse each until behavioral testing began at 8 weeks of age. To generate F2 progeny, male F1 offspring of F0 Tg or Ctr mice were mated as described above with a separate cohort of age-matched uninfected females after completing all the behavioral assays. Males (n = 3-4), whose performance on the behavioral tasks was close to the mean of the cohort, were selected for mating to remove any bias. F2 offspring were weaned and housed as described above and were tested for behavioral changes at 8 weeks of age.

METHOD DETAILS

Sperm analysis

To analyze the effect of *Toxoplasma* infection on the sperm parameters at a time when the F0 males were used for mating, a separate cohort of male C57BL/6 mice (n = 3-4) were injected with either PBS or tachyzoites of *Toxoplasma* (Pru) and left for infection to

develop as described above. 25 dpi, mature spermatozoa from control and infected mice was collected using the swim-up method. Briefly, left and right cauda epididymis were dissected, placed into 20ul sterile PBS, chopped using a sterile scalpel and left for 5 min at room temperature for the sperm to be released.

To analyze sperm morphology, 2ul of spermatozoa sample was placed onto Cell-VU pre-stained morphology slides (DRM-900; Millennium Sciences, NY, USA) and covered using a coverslip. Bright-field images of stained spermatozoa were obtained at 20x magnification using Zeiss Axiovert 200M wide field microscope equipped with an AxioCam MRn CCD detector. For each sample, 3 fields of view were obtained and the total (approximately 300) spermatozoa were counted and classified as normal and abnormal. Abnormalities in sperm were classified as: head lost, double head, pin head, loss of hook shape, bent neck, two-tail and abnormal flagellum (Seed et al., 1996) and presented as percentage of total spermatozoa.

Sperm count and motility was assessed using the Cell-VU sperm counting chamber (DRM-600; Millennium Sciences, NY, USA) as per the manufacturer's instructions. Briefly, 2ul of spermatozoa sample was placed on the counting area and the grid containing coverslip placed on top. Sperm were immediately counted and classified as motile and non-motile sperm. Total sperm count (motile + non-motile) was presented as million spermatozoa per milliliter of undiluted sample. Motility was calculated as percentage of total sperm.

Histology

The testes from control and infected mice were removed and weighed (paired). Testes from infected mice were then PFA fixed, paraffin embedded, sliced (5um) using a microtome and mounted on microscope slides before undergoing Periodic Acid-Schiff (PAS) staining according to standard protocol. Slides were then scanned using the Pannoramic SCAN II slide scanner and images processed using the CaseCentre slide management application v2.8 (3DHISTECH, Budapest, Hungary). Images were visually scanned to find *Toxoplasma* cysts.

Behavioral tests

Behavioral testing was performed in the light phase of the light/dark cycle and each test was performed on a different day. The light intensity in the testing room was 100 lux and the room temperature was maintained at 22°C. Mice were acclimatised to the testing room 1 hr prior to beginning the tests. All behavioral testing apparatus was cleaned with 70% ethanol and F10 disinfectant between each session and at the end of the last session each day. For analysis of behavioral videos, the TopScan mouse tracking software (CleverSys, Reston, VA, USA) was used by an experimenter blinded to the classification of the mice. Mice that failed to perform a test were excluded from the analysis.

Open field

The open-field arena was made out of a white Perspex box (40 × 40 cm) and was used to analyze anxiety and locomotor activity. Mice were placed near the wall of the arena and allowed to freely explore for 10 min while being recorded from a top mounted camera. Tracking software determined the speed, location and total distance covered by the mice. Centre region of the open-field was defined as 60% of the total area. Light intensity in the center of the arena was 50 lux.

Light-dark box test

The apparatus for the light-dark box test consisted of the open-field box described earlier and contained an insert made out of black Perspex with an opening at the bottom to allow free movement of mice in either directions. The insert divided the field into two zones of equal area. At the start of the test, mice were placed inside the dark insert and their movement recorded for 5 min. Video tracking software computed the number of entries made into and the total time spent in the light zone which had a light intensity of 700 lux.

Elevated-zero maze

The maze (San Diego Instruments, CA, USA) is a modification of the classical elevated-plus maze, and contains an elevated (60 cm above the floor) annular platform (10 cm wide). Its two opposite enclosed quadrants and two open quadrants allows uninterrupted exploration of the maze, thus removing ambiguity in the interpretation of time spent in the central zone of the traditional plus maze design (Kulkarni et al., 2007). Mice were placed at the entrance of any one of the closed areas and its activity recorded over the next 5 min. Video tracking software quantified the time spent in the closed and open quadrants. Entry was defined as all four limbs being inside the quadrant.

Sucrose preference test

The sucrose preference test was used to measure anhedonia-like behavior in mice. For this, mice were singly housed and habituated to drinking from two water containing bottles for 24 hr. Later, one of the bottles was switched to 1% sucrose and the weight of both the bottles was recorded. Next day, the bottles were weighed again to determine total sucrose or water consumption and the mice were group housed again. Sucrose preference was calculated as: [sucrose intake/(water intake + sucrose intake)] x 100.

Y-maze for spatial and working memory

The apparatus (San Diego Instruments, CA, USA) was constructed from beige-colored Perspex and contained three arms (38 cm long, 12 cm high and 7 cm wide) at 120 degrees to each other. Two test arms (A and B) contained sliding doors at their stem to block mouse entry, while the third arm, designated as home (H) arm, contained a 10 cm start area.

To test the spatial reference memory task, during training, mice were placed in the start area of the arm H and allowed to explore that arm and any one (A or B) of the test arms (familiar arm) for 10 min, while the other arm remained blocked off (novel arm). 1 hr later, to test the spatial memory, both arms A and B were made available and mice were allowed to explore the maze for 5 min while being video recorded. Tracking software determined time spent in each arm. Allocation of novel and familiar arms (either A or B) was randomized between each trial. Arm preference was calculated as: (time spent in arms A or B) x 100/Time spent in arms A+B.

To test spatial working memory, mice were placed in the start area of the arm H and allowed unrestricted exploration of all three arms for 5 min. Sequence of entry into each arm was noted. Entry was defined as all four limbs being inside the arm. One alternation was defined as entry into a different arm of the maze in each of the 3 consecutive arm entries made. Percentage alternation was calculated as: number alternations x 100/ total possible alternations.

The working memory task was performed at least one week prior to the spatial memory task to avoid interference from the previous exposure to the apparatus.

3-chambered sociability and social recognition memory test

Crawley's sociability and preference for social novelty test, better known as the 3-chambered test, is commonly used to detect changes in social cognition in mouse models of CNS disorders. The apparatus (San Diego Instruments, CA, USA) was made of clear Perspex and consisted of two end chambers (21 × 27 cm) and a central chamber (21 × 12 cm), separated by guillotine doors. The test was performed as previously described (Kaidanovich-Beilin et al., 2011). Briefly, the test mice were habituated to the apparatus for 10 min and then held in the center chamber. Then, an empty wire cup and a wire cup containing an age and sex-matched control mouse (stranger A) were placed in the two end chambers. To evaluate sociability, the sliding doors were opened and the test mouse was allowed to explore for 5 min while being video recorded. Control and test mice were then returned to their home cage. To evaluate social recognition memory 1 hr later, another age and sex-matched control mouse (stranger B) was placed in a wire cup into the chamber that previously contained the empty cup, and the stranger A placed as it was earlier. Test mouse was again allowed 5 min of exploration while being video recorded. In these sessions, time spent by the test mouse sniffing and exploring the empty cage (t1) and cup with stranger A (t2) or B (t2) was manually quantified. Discrimination/recognition index was calculated as: t2-t1/(t2+t1) or t3-t2/(t2+t3). Chambers used to place the empty cup or cup with stranger mice were randomized across trials. Before the tests began, stranger mice were acclimatised to being in the cup for 10 min once a day for 3 days.

Novel object recognition test

The object recognition test was performed as described earlier (Tyebji et al., 2015). Briefly, after habituation to the square arena for 10 min, mice were trained to explore and remember two similar objects (A and A', placed diagonally opposite to each other and equidistant from the center of the arena) for 10 min while being video recorded. Mice were then returned to their home cage. 24 hr later, to test their recognition memory, mice were reintroduced for 5 min to the arena containing one of the previously explored familiar objects (A or A') and a novel unexplored object (B). In both training and testing sessions, time spent by the mice sniffing and exploring each object was manually scored. Object preference was calculated as the time exploring each object x 100/ time exploring both objects. Discrimination index was calculated as: [(time exploring novel object – time exploring familiar object)/ time exploring both objects].

Forced swim test (FST)

Depression-like behavior was evaluated using the FST as described previously. Mice were individually placed into a 2 ltr glass beaker filled with water (26°C, 15 cm water depth) for 5 min and video recorded. Latency to first immobility and total immobility time was manually scored. Immobility was defined as remaining motionless, except for certain necessary movements to remain afloat. Mice were then removed from the water, towel dried, placed under a heat lamp for 5 mins before returning them to the home cage. FST was the last behavioral assay performed.

Illumina sequencing

Mature spermatozoa were collected using the swim-up method as described above from a separate cohort of Cnt or Tg mice 4 weeks post-infection. Each biological replicate consisted of sperm samples pooled from two individual animals. Fresh sperm samples were homogenized using the QIAzol lysis reagent and immediately processed for total RNA extraction using the miRNeasy Mini Kit (-QIAGEN, # 217004). Sample analysis was performed using the Agilent 2100 Bioanalyzer RNA 600 Nano and the Bioanalyser Small RNA kit (Agilent Technologies, USA) to check for quality and quantity of RNA. Sequencing was done at the Australian Genomics Research Facility (AGRF, Melbourne, Australia) using the Illumina HiSeq 2500 workflow. An amount of 1000 ng of total RNA was used for library prep using NEB's NEBNext Multiplex Small RNA Library Prep Set (New England BioLabs Inc.) according to the manufacturers' instructions. Following gel-purification, the size of the library was assessed via electrophoresis (Agilent TapeStation TapeScreen DNA 1000 Assay) and quantified by qPCR (KAPA Library Quantification Kits for Illumina). Quality control was performed following cDNA conversion using Agilent 2100 Bioanalyzer. The libraries were normalized to 2nM and pooled for sequencing on

the HiSeq 2500 with 50 bp single reads. After demultiplexing and adaptor removal, an average of 15,597,171 single reads were obtained in each sperm library.

Sequencing data analysis

Read quality was assessed using FastQC and per base read quality was determined to be above Q30 for > 95% bases across all 10 samples. Reads were then screened for the presence of any Illumina adaptor/overrepresented sequences and cross-species contamination. Cleaned sequence reads were aligned against the *Mus musculus* genome (build version mm10) using the STAR aligner (Dobin et al., 2013) (v2.5.3a) to map reads to the genomic sequences. Reads were mapped to each known gene and summarized using the Featurecounts (Liao et al., 2014) (v1.5.3) utility of the subread package. Multi-mapping was turned on and features mapped to the GRCm38 Gencode GTF containing small RNA annotations. The transcripts were assembled with the Stringtie tool (Pertea et al., 2015) (v1.3.3) utilizing the reads alignment with M6 and reference annotation based assembly option (RABT) which generates assembly for known and potentially novel transcripts. The Gencode annotation containing both coding and non-coding annotation for mouse genome version GRcm38 (Ensemble release 81) were used as a guide. edgeR (Robinson et al., 2010) (v3.22.3) was used to perform normalization and differential expression (DE) analysis using R-3.5.0. Genes with CPM < 0.5 and counts in < 3 replicates were removed. Filtered counts were normalized with the TMM normalization and differential expression undertaken with the negative binomial GLM. Small RNA biotypes were classified using the hierarchical read mapping procedure implemented in Unitas (Gebert et al., 2017). Reads were trimmed to a minimum length of 14 bp with Trim_Galore! V0.5.0 () and 5 bp clipped from read 1. Illumina adapters were scanned and removed. Reads with length > 38 bp were removed. Unitas (v1.7.0) was executed using the most recent *Mus musculus* database (Supplementary Table. 1) and the '-riborase' flag. In-house scripts at AGRF were used to summarize the results; including quality control after read QC, the percentage of reads mapping to each biotype and to quantify the diversity within each sample. RNA biotypes estimates were calculated by summing the reads of transcripts belonging to the same RNA biotype and then displayed as a percentage of the total number of assigned reads in that samples.

Functional analysis

Pathway analysis was assessed using ReactomePA (Yu and He, 2016). Each pathway in the Reactome *Mus musculus* database was assessed. All genes in the pathway with a false discovery rate (FDR) measure from the DE analysis are considered (that is, no P value filter). A one-sample t test is used to assess the mean logFC of the pathway genes relative to the sample mean. By default, the mean is 0. Multiple testing was assessed with Bonferroni corrected P values. We extracted significant pathways ($p < 0.05$) and displayed them ranked by average logFC. Gene Enrichment was assessed using the kegga() function that is part of the LIMMA toolkit in R (Ritchie et al., 2015). Significant DE genes ($p < 0.05$) were extracted and separated to lists of upregulated or downregulated genes based on a logFC > 0 or logFC < 0. The gene sets were individually assessed for enrichment using kegga() which employs a one-sided hypergeometric test with adjustment bias for over-representation. Gene ontology annotations were added using the GOstats() package in R (Falcon and Gentleman, 2007). Significant GO terms were assessed using the built-in hypergeometic test function hyperGTest().

Small RNA injection into oocytes

The total RNA samples used for RNA sequencing were pooled and small RNA fraction was enriched using the RNeasy Min Elute Cleanup Kit (QIAGEN, Cat. # 74204). Samples were analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to check for quality and quantity of small RNA. Female C57BL/6J were superovulated by subcutaneous injection of 5 IU of PMSG (Folligon) on day 1 and then 5 IU of hCG (Chorulon) on day 3, and then mated with male C57BL/6J mice to obtain fertilized oocytes. One day later, 0.5 day old embryos were collected and 1-2 pl of 1 ng/ul (approx. 1 fg) of total small RNA suspended in Tris-EDTA microinjection buffer (10mM Tris and 0.1mM EDTA, pH 7.4) was injected into the pronucleus of fertilized eggs using standard DNA microinjecting method. Surviving zygotes were tested and 12-15 zygotes were implanted into each surrogate mother 5 days later. Male and female offspring thus generated were weaned at 2 weeks and housed 4-6 animals per cage, as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis for all behavioral and molecular data was performed using the GraphPad Prism version 7 (GraphPad Software, La Jolla, USA). Statistical analysis was performed using the Student's t test (two-tailed) or the two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison post hoc test and indicated in the figure legends. A 95% confidence interval was used and values of $p < 0.05$ were considered as statistically significant. Data are presented as mean ± SEM. Although the sample sizes were not predetermined using any statistical procedures, our sample sizes are similar to those used in the field. Details such as biological replicates for each experiment can be found in the figure legend.

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Title:
Pathogenic Infection in Male Mice Changes Sperm Small RNA Profiles and Transgenerationally Alters Offspring Behavior

Date:
2020-04-28

Citation:
Tyebji, S., Hannan, A. J. & Tonkin, C. J. (2020). Pathogenic Infection in Male Mice Changes Sperm Small RNA Profiles and Transgenerationally Alters Offspring Behavior. CELL REPORTS, 31 (4), https://doi.org/10.1016/j.celrep.2020.107573.

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