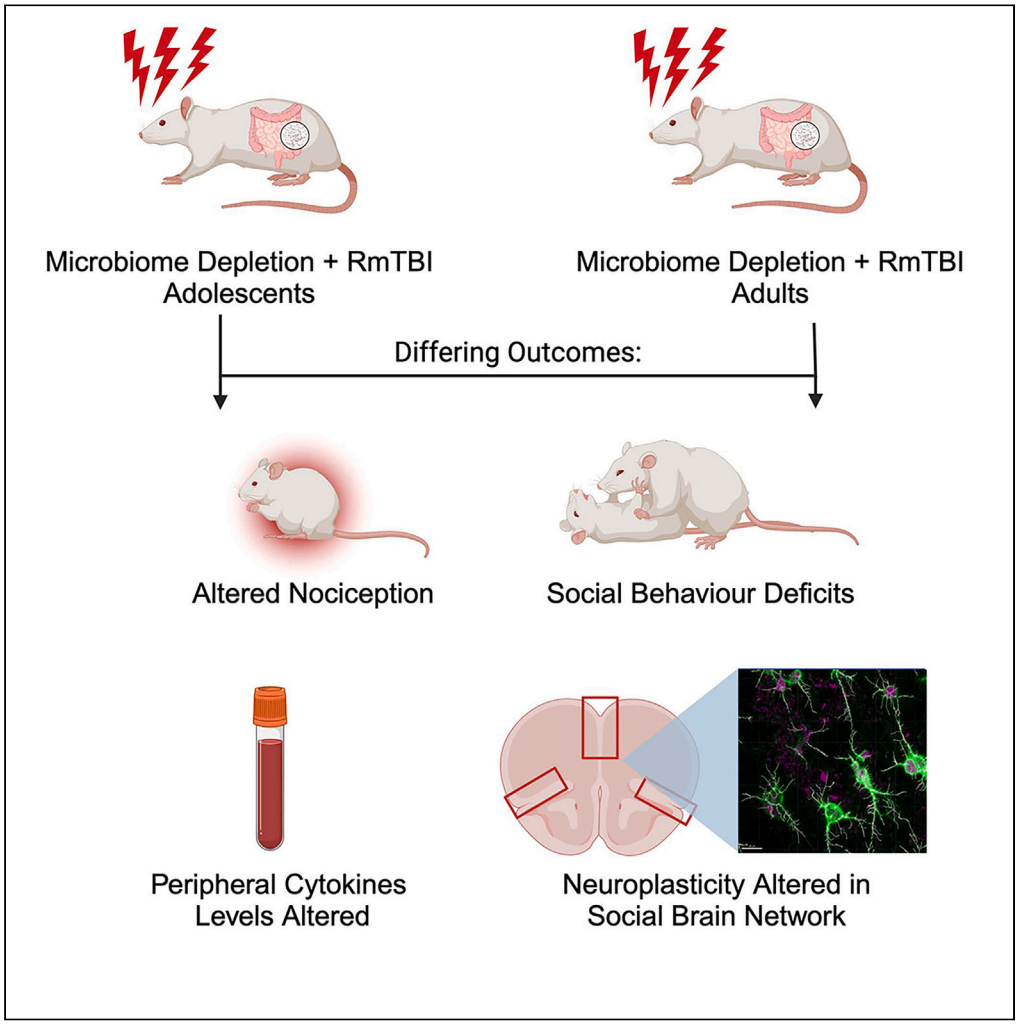
iScience

CellPress
OPEN ACCESS

Article

Microbiome depletion prior to repeat mild TBI differentially alters social deficits and prefrontal cortex plasticity in adolescent and adult rats



Marissa Sgro, Zoe N. Kodila, Crystal Li, ..., Amy C. Reichelt, Glenn R. Yamakawa, Richelle Mychasiuk

richelle.mychasiuk@monash.edu

Highlights

RmTBI modifies perineuronal net structure and function within the prefrontal cortex

Changes in neuroplasticity are linked to age, injury, and microbiome composition

The microbiome mediates RmTBI-induced social deficits in adults and adolescents

The microbiome may be a viable, age-specific, target for RmTBI therapeutics

Sgro et al., iScience *27*, 109395
April 19, 2024 © 2024 The Author(s).
https://doi.org/10.1016/j.isci.2024.109395

iScience

CellPress
OPEN ACCESS

Article

Microbiome depletion prior to repeat mild TBI differentially alters social deficits and prefrontal cortex plasticity in adolescent and adult rats

Marissa Sgro,[1] Zoe N. Kodila,[1] Crystal Li,[1] Irena Carmichael,[2] Samantha Warren,[2] Amy C. Reichelt,[3] Glenn R. Yamakawa,[1] and Richelle Mychasiuk[1,4,*]

SUMMARY

Although aging, repeat mild traumatic brain injury (RmTBI), and microbiome modifications independently change social behavior, there has been no investigation into their cumulative effects on social behavior and neuroplasticity within the prefrontal cortex. Therefore, we examined how microbiome depletion prior to RmTBI affected social behavior and neuroplasticity in adolescent and adult rats. Play, temperament analysis, elevated plus maze, and the hot/cold plate assessed socio-emotional function. Analyses of perineuronal nets (PNNs) and parvalbumin (PV) interneurons was completed. Social-emotional deficits were more pronounced in adults, with microbiome depletion attenuating social behavior deficits associated with RmTBI in both age groups. Microbiome depletion increased branch length and PNN arborization within the PFC but decreased the overall number of PNNs. Adults and males were more vulnerable to RmTBI. Interestingly, microbiome depletion may have attenuated the changes to neuroplasticity and subsequent social deficits, suggesting that the microbiome is a viable, but age-specific, target for RmTBI therapeutics.

INTRODUCTION

Repeat mild traumatic brain injuries (RmTBIs) account for a large proportion of TBI worldwide[1] and generate a significant medical and economic burden on the healthcare system.[2] Both adolescents and adults that experience RmTBIs are at increased risk for cognitive impairments and long-term neurodegeneration.[3] Adolescents are at the highest risk for sustaining RmTBI,[4] with research suggesting that 20% of all adolescents will have sustained a concussion before the age of 10 and experience at least one repeat injury within 2 years of their first concussion.[5] RmTBI is also highly prevalent in adults,[6] with a subset of these individuals going on to develop chronic post-concussion symptomologies.[7] RmTBI has been associated with exacerbated symptomology and worsened pathology when compared to single mTBIs, possibly resulting from cumulative damage to the brain.[8]

Importantly, the adolescent time-period involves maturation across multiple facets, including biological, social, and emotional functioning.[9] Although changes in social and emotional function following TBI are commonly reported in adult populations, as they have adverse consequences for return to work, familial relationships, and the maintenance of intimate relationships,[10] the influence of RmTBI on social behavior deficits is underrepresented in the literature on adolescents.[11] Of importance, adolescence represents a period of significant neurological maturation, particularly pertaining to synaptic pruning[12] and myelination,[13] within the cortical regions that makeup the social brain network (SBN).[14] The SBN encompasses a network of brain regions that underpin social functioning, which include, but are not limited to, the medial prefrontal cortex (mPFC) and orbital frontal cortex (OFC).[14] In addition to maturation-dependent changes across the lifespan, dendritic arborization and pruning of neurons within OFC and mPFC have also been associated with social development and changes to social play dynamics.[15] Therefore, when RmTBI is experienced during adolescence, it potentially disrupts typical developmental trajectories within the SBN, subsequently resulting in a plethora of chronic social deficits.[16] However, in adults, the SBN is thought to be mature; functional connectivity and cortical specialization of regions such as the mPFC and OFC are considered "stable".[17] It is unclear whether this stability in adulthood acts as a protective or detrimental modulator of RmTBI outcomes. However, one would speculate that adolescents and adults will experience different social deficits following RmTBI.

The mPFC and OFC are two key regions of the SBN with distinct but important roles in social functioning,[14] as such injury within these core structures has been associated with altered social behavior.[18] Although many neuronal populations are involved, perineuronal nets (PNNs) and their often colocalized parvalbumin (PV) interneurons, regulate neuroplasticity within the SBN.[19] Increased density of PNNs, and therefore

[1]Department of Neuroscience, Central Clinical School, Monash University, Melbourne, VIC 3004, Australia
[2]Monash Micro Imaging, Central Clinical School, Monash University, Melbourne, VIC 3004, Australia
[3]School of Biomedicine, University of Adelaide, Adelaide, SA 5005, Australia
[4]Lead contact
*Correspondence: richelle.mychasiuk@monash.edu
https://doi.org/10.1016/j.isci.2024.109395

This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

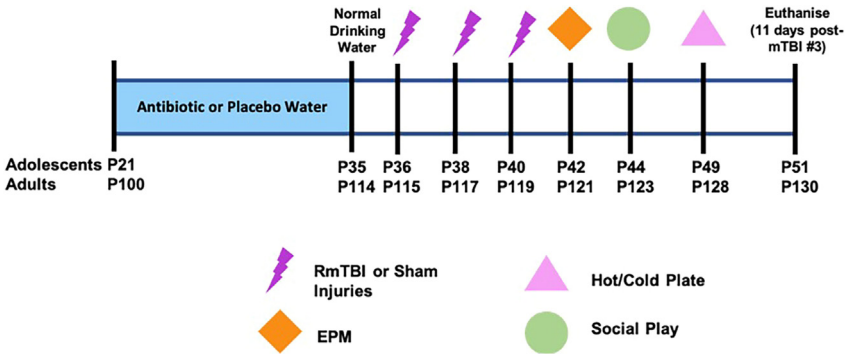


Figure 1. Experimental study design timeline

their coverage of PV cells, has been associated with reduced or the 'closure' of neuroplasticity.[20] PNNs are extracellular matrix lattice structures that are predominantly found surrounding GABAergic PV interneurons, with both the neurons and extracellular structures being vulnerable to environmental changes, as well as mechanical and chemical injury across the lifespan.[20] The PNN lattice structures also regulate synaptogenesis and provide a protective microenvironment for the neurons they encompass.[21] Importantly, changes to the density, connectivity, and quantity of PNNs and PV neurons has been associated with depressive- and anxiety-like behaviors, as well as social deficits in adolescent and adult rodent studies.[22,23]

Adolescence also represents a window of plasticity whereby the microbiome, immune system, and brain undergo parallel maturation.[24] Across the lifespan (in both adolescence and adulthood), one of the primary functions of the gut microbiome is the maintenance of intestinal and systemic homeostasis through the mediation of host pro- and anti-inflammatory pathways.[25] Recent studies demonstrate that a dysbiosis-state in the microbiome can induce social deficits[24] and social behavior disorders, such as autism spectrum disorder (ASD).[26] Given that different rates of neuroplasticity and microbiome development occur throughout adolescence and adulthood, it is possible that dysregulation of the SBN and/or microbiome differentially modify recovery post-RmTBI.

Although studies in rodents have demonstrated changes in social behavior following mTBI and microbiome alterations independently, there have been no studies investigating the cumulative effect of microbiome depletion and RmTBI on social behavior and neuroplasticity within the SBN. Moreover, there is a need to investigate the influence of age on social behavior following RmTBI. Therefore, we sought to examine how microbiome depletion prior to RmTBI influenced social behavior, its comorbidities, and neuroplastic changes within PNN and PV interneurons as well as the PNN lattice network. We specifically focused on quantity and structural co-localization of PNNs and PVs within the mPFC and OFC in adolescent and adult Sprague-Dawley rats (P30 and P100, respectively). To do so, we induced microbiome depletion via systemic administration of a broad-spectrum antibiotic cocktail prior to RmTBI induction in adolescent and adult rats.[27] Socio-emotional functioning was analyzed via play behavior and temperament analysis, as well as comorbid measures including anxiety-like behavior and alterations to pain sensitivity. We hypothesized that adolescent rats exposed to microbiome depletion and RmTBI would demonstrate worsened socio-emotional functioning compared to adult rats, and that these deficits would be associated with reductions in PNNs, PVs, and PNN arborization within the mPFC and OFC.

RESULTS

We validated microbiome depletion prior to injury (see Figure 1 for timeline) and injury induction by demonstrating significant loss of bacterial DNA (Figures 2A and 2B) and significant increases in time-to-right following the injury procedure in RmTBI animals (Table 1 contains all statistics for the ANOVAs; Table S3 contains all statistical output for the post-hoc analyses). However, contrary to our hypotheses, microbiome depletion prior to RmTBI did not result in exacerbation of injury outcomes. We found that all factors (sex, antibiotics, and age), differentially modified anxiety-like and nociceptive behaviors. See Table 1 and Figure 2 for all statistical and graphical data.

Although we filmed both adolescents and adults for play behavior analyses (defined in Table 2), the adults exhibited little or no play behavior, so the results are only reported for adolescents. Within the adolescent cohort we found that males initiated play significantly more often than females, ($F_{(1,79)}$ = 8.949, p = 0.004), and that sham animals also played significantly more than RmTBI animals, ($F_{(1,79)}$ = 13.063, p = 0.001). Interestingly, there was also a significant injury by antibiotic interaction whereby, microbiome depletion protected RmTBI animals from deficits in play initiation, ($F_{(1,79)}$ = 6.714, p = 0.012). See Figure 3A. With respect to rotations (or play continuation) following an initiation from the play partner, we identified similar outcomes, with males and shams rotating significantly more often than females and RmTBIs (($F_{(1,79)}$ = 5.054, p = 0.028) and ($F_{(1,79)}$ = 11.196, p = 0.001)), and microbiome depletion protecting the RmTBI from the deficits ($F_{(1,79)}$ = 4.595, p = 0.035). See Figure 3B.

Given that we were unable to examine social function in adults using play analyses, but were still interested in changes to social behavior, we used temperament-based software to analyze sociability in adolescents and adults. All statistical analyses can be found in Table 1, with graphical representation in Figure 4. We found that compared to adults, adolescents are generally more active, switch between tasks more often, and spent more time in social behaviors such as play and sniffing their partners. Conversely, adults spent significantly more of their time

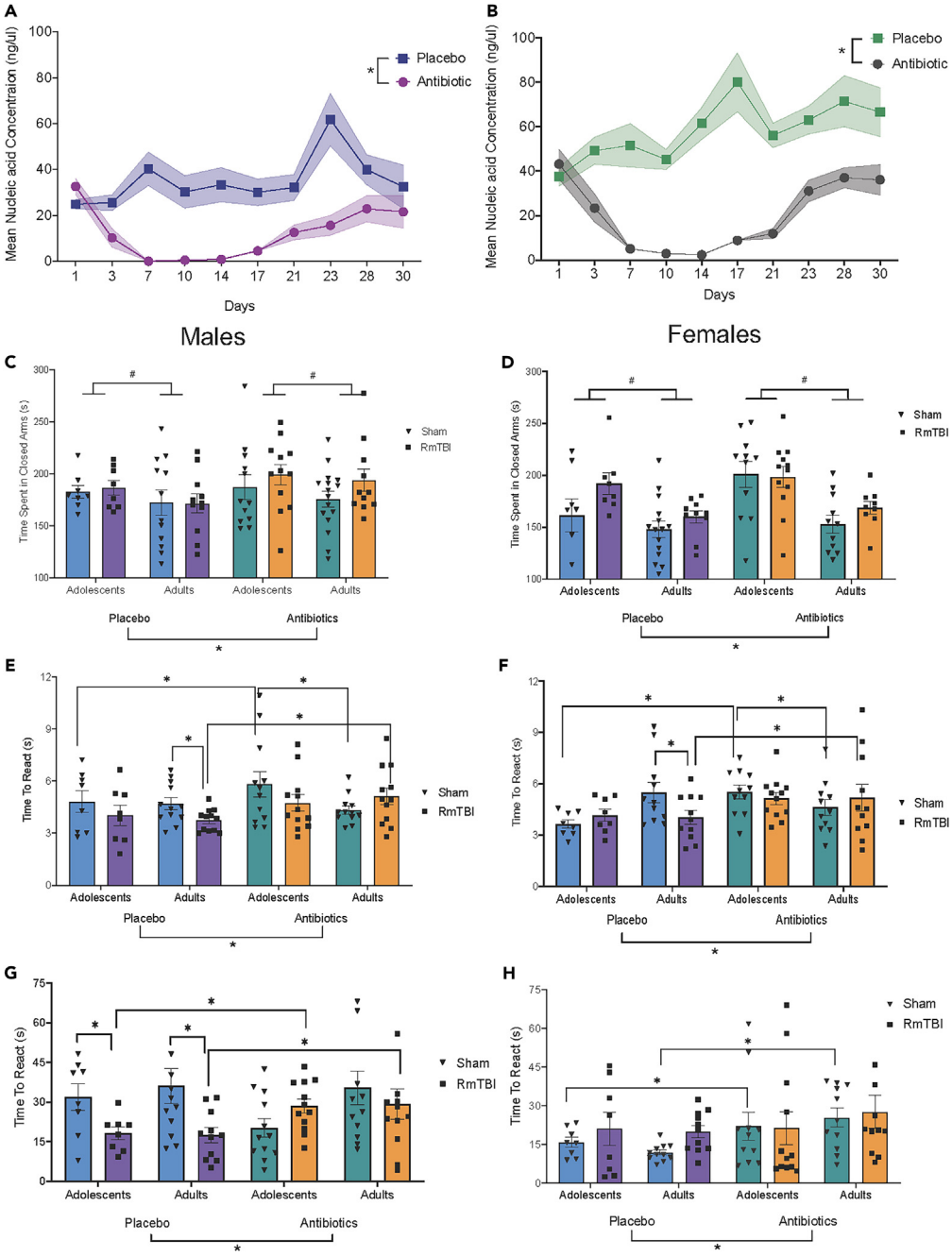


Figure 2. Confirmation of Microbiome Depletion and Injury Induction

(A) Demonstrates depletion of the gut microbiota in adolescents and (B) adults; Antibiotic administration concluded prior to RmTBI induction (C and D); Increases in anxiety-like behavior in adolescents compared to adults and microbiome depleted rats; (E and F) Time to react on the hot plate, with adults exhibiting greater changes to nociceptive thresholds and microbiome depletion attenuating RmTBI alterations; (G and H) Time to react on the cold plate, with microbiome depletion and female sex attenuating RmTBI changes. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

rearing and grooming themselves. RmTBI reduced digging time and increased time spent in no activity. Interestingly, just as microbiome depletion appeared to attenuate the effects of RmTBI on play behavior in adolescents, it also increased the amount of time rats spent engaged in other social behaviors such as sniffing and grooming their cage mates.

To examine systemic inflammation, a Luminex panel was used to examine circulating cytokines within the serum collected at euthanasia. Complete results for the statistical analyses can be found in Table S2, with graphical representation of a subset of the cytokines in Figure 5.

Table 1. Summary of four-way ANOVA statistical analyses for the behavioral and temperament outcomes

Behavioral Measure	Main Effect Sex F(*p*)	Main Effect Antibiotics F(*p*)	Main Effect Injury F(*p*)	Main Effect Age F(*p*)	Significant Interactions
Time-to-Right	0.725 (0.396)	12.440 (<0.001)	167.520 (<0.001)	0.353 (0.553)	None
*EPM – Closed Arms	5.955 (0.016)	7.162 (0.008)	3.692 (0.057)	14.066 (<0.001)	Sex x Age: 5.193 (0.024)
*Hot Plate	0.060 (0.806)	8.678 (0.004)	2.014 (0.158)	0.143 (0.706)	Antibiotics x Injury x Age: 6.200 (0.014)
*Cold Plate	6.791 (0.010)	4.299 (0.040)	0.575 (0.449)	1.388 (0.241)	Sex x Antibiotics x Injury: 4.660 (0.032)
Temperament analyses					
*Allogrooming	1.108 (0.024)	5.176 (0.024)	1.273 (0.261)	0.290 (0.591)	Sex x Antibiotics x Injury: 4.226 (0.042)
*Digging	11.618 (0.001)	0.107 (0.744)	13.109 (<0.001)	31.766 (<0.001)	Injury x Age: 4.784 (0.030) Sex x Antibiotics x Injury: 8.556 (0.004)
Grooming	1.382 (0.242)	1.692 (0.195)	5.056 (0.026)	4.677 (0.032)	None
*No Activity	0.558 (0.456)	1.578 (0.211)	6.427 (0.012)	2.716 (0.101)	None
Playing	10.571 (0.001)	0.046 (0.830)	9.828 (0.002)	77.582 (<0.001)	Sex x Age: 7.594 (0.007) Antibiotics x Injury x Age: 11.677 (0.001)
Rearing	1.911 (0.169)	0.495 (0.483)	3.414 (0.067)	50.66 (<0.001)	Sex x Injury: 4.138 (0.044)
Sitting	0.868 (0.353)	0.025 (0.874)	10.727 (0.001)	11.799 (0.001)	Antibiotics x Injury: 6.833 (0.010)
Sniffing	3.671 (0.057)	3.942 (0.049)	3.229 (0.074)	43.444 (<0.001)	4 Way Interaction: 6.031 (0.015)
*Task Switching	5.622 (0.019)	0.449 (0.504)	2.111 (0.148)	8.553 (0.004)	Sex x Age: 4.886 (0.029) Antibiotics x Age: 6.696 (0.011)

Behaviors denoted by an * are shown in Figures 2 and 3.

However, in summary, all cytokines examined demonstrated a main effect of age, or a significant age interaction, whereby circulating cytokine levels were often much higher in adults. In addition, there was a main effect of sex or significant interaction involving sex, in 12/18 cytokines (67%), with males exhibiting increased responsivity to the manipulations, when compared to females. Conversely, only 2 cytokines (leptin and IP-10) were directly modulated by microbiome depletion, whereas RmTBI exacerbated the adult cytokine response, but did not have the same effect in adolescents.

We also examined the structural changes that occurred within the PNNs of the mPFC and OFC. Graphical representation of these findings can be found in Figures 6 and 7 respectively, while the statistical results for the four-way ANOVAs can be found in Table 3. See Table S4 for statistical results from the post-hoc analyses. To summarize, within the mPFC, RmTBI increased PNN filament/branch length, number of branches, and terminal branch points in a sexually dependent manner. For males the changes in PNN arborization were more pronounced in adults, especially those that were exposed to microbiome treatment. However, in females, the effects were more pronounced in placebo rats, regardless of age. Within the OFC, the dendritic length, number of branches, and terminal branch points were significantly greater in males when compared to females, as well as in adults when compared to adolescents. Similar to the mPFC, the OFC also exhibited sex-dependent changes, whereby in females, the most pronounced changes in PNN arborization were identified in microbiome depleted, RmTBI adults. Although, in males, the largest effects were identified in microbiome depleted sham adults.

Finally, we examined quantitative changes in PNN and PV cell numbers that occurred within the mPFC and OFC. Given technical challenges, we were unable to compare between adolescent and adult cell counts, and as such three-way ANOVAs with sex, antibiotics, and injury were run separately for the adolescent and adult cohorts. Graphical representation of these findings can be found in Figures 8 and 9 respectively, while the statistical results for the three-way ANOVAs can be found in Table 4. See Table S5 for statistical results of all post-hoc analyses. Briefly, microbiome depletion significantly reduced the number of PNNs in both the mPFC and the OFC of adolescents and adults. Interestingly, for placebo treated rats, RmTBI increased PV numbers in adolescents, but reduced PV numbers in adults; with the exception of the female OFC where RmTBI increased PV numbers in a manner similar to that of adolescents. Contrary to expectation, when RmTBI and microbiome depletion were combined, the results were not additive, but rather microbiome depletion attenuated RmTBI-induced deficits.

DISCUSSION

As our understanding of the biological processes underpinning the heterogeneity in recovery from RmTBI continues to advance, a role for the microbiome and associated immune system mediators has emerged. We have previously demonstrated age-dependent temporal changes in microbiota composition and functionality in adolescent and adult rats as a function of microbiome depletion and RmTBI.[28] Based on these previous findings, we hypothesize that the bacteria and metabolites that may be most likely to involved in the modification of plasticity and behavior across the lifespan include bacteria *Clostridium* spp., *Lachnospiraceae,* and *Bacteroides* and the metabolite *glycerophosphocholine* as they exhibit strong abilities to modify inflammatory processes.[28] Although social behavior deficits have been linked

Table 2. Table outlining the behaviors of interest and how they were defined

Behavior	Definition
Allogrooming	Grooming or licking their cage mate
Digging	Burrowing into and causing movement within the wood chip substrate through any limb including forepaws, hind paws and nose.
Eating	Consumption of anything within the cage including fecal matter or the wood chips.
Grooming	Any form of self-grooming including scratching, licking, and kneading.
No Activity	Rat is completely stationary - head, limbs, and tail are all motionless.
Playing	Engaging or attempting to engage in play through pouncing on the nape of cage mate or reciprocating in play through tumbling and rotating in response to a cage mate initiating play.
Rearing	Rising of body until full extension of legs including against wall of cage and with no external support.
Sitting	Only hind paws on the ground but not extended as they are in rearing and with forepaws raised.
Walking	Rat is moving around or surveying cage including temporary or quick halts where movement is still notable.

to both alterations to the microbiota[29] and RmTBI,[10] there is a dearth of literature on the role that the microbiome may play in the development of social behavior deficits following RmTBI. Therefore, we investigated how microbiota depletion prior to injury, age at injury, and sex influenced social behavior, comorbid behaviors, and neuroplasticity in the adolescent and adult rat brain. First, our findings demonstrate that comorbid conditions associated with social deficits (anxiety and changes to nociceptive sensitivity) occurred following microbiome depletion and RmTBI, with the effects being more prominent in adults. Second, microbiome depletion attenuated the social behavior deficits associated with RmTBI in in both adolescent and adults. Third, following RmTBI, adults exhibited the greatest change in circulating pro-inflammatory cytokines, with the effects being most pronounced in males. Lastly, the structure of PNNs within the mPFC and OFC were altered in an age and sex dependent manner, with adults exposed to microbiome depletion and RmTBI, exhibiting the greatest change to arborization.

To understand the role of the microbiome in health and disease, preclinical studies have induced depletion of gut bacteria via antibiotic administration.[28] Following similar protocols, we depleted the microbiome and confirmed that it was absent through injury induction for both adolescents and adults; however, it is important to note that the microbiome was in the re-colonisation phase during behavioral testing. In addition, compared to adults, the adolescent's microbiome concentration was lower, both prior to, and following antibiotic administration.

Multiple preclinical and clinical studies have demonstrated that changes to social behavior are often accompanied by comorbid conditions, including anxiety and depression.[30] Moreover, dysregulation of the microbiota-brain-immune axis has also been implicated in anxiety-related disorders.[31] Within this study, we identified greater anxiety-like behavior in adolescents, when compared to adults. As adolescence encompasses a sensitive period of development for the microbiome,[28] as well as the PFC, amygdala, and hypothalamus,[32] it is often associated with the onset of anxiety disorders,[33] which in turn, greatly impairs social functioning.[34] Conversely, the adult PFC and microbiome are considerably more stable,[35] which may explain our age-specific findings. We also demonstrated that microbiome depletion increased anxiety-like behavior in adolescent males. Gut microbiome dysbiosis and microbial composition modifications have been associated with dysregulation of the HPA axis and appropriate sexual maturation.[28] Therefore, it is possible that the dysbiotic state of the microbiome influenced anxiety-like behavior in a sex-dependent manner, as studies also demonstrate that testosterone levels are positively correlated with anxiety disorder development.[36]

Alterations to nociception are another common comorbidity associated with RmTBI-induced social behavior deficits.[30,37] Importantly for this study, changes within the microbiome have also been suggested to influence nociceptive sensitivity.[38] For example, a reduction in the number of butyrate-producing bacteria, which have been associated with inflammation, modify nociceptive sensitivity and subsequent pain outcomes.[39] Our adolescent groups presented with lower bacterial DNA concentrations, both before and after antibiotic treatment, which

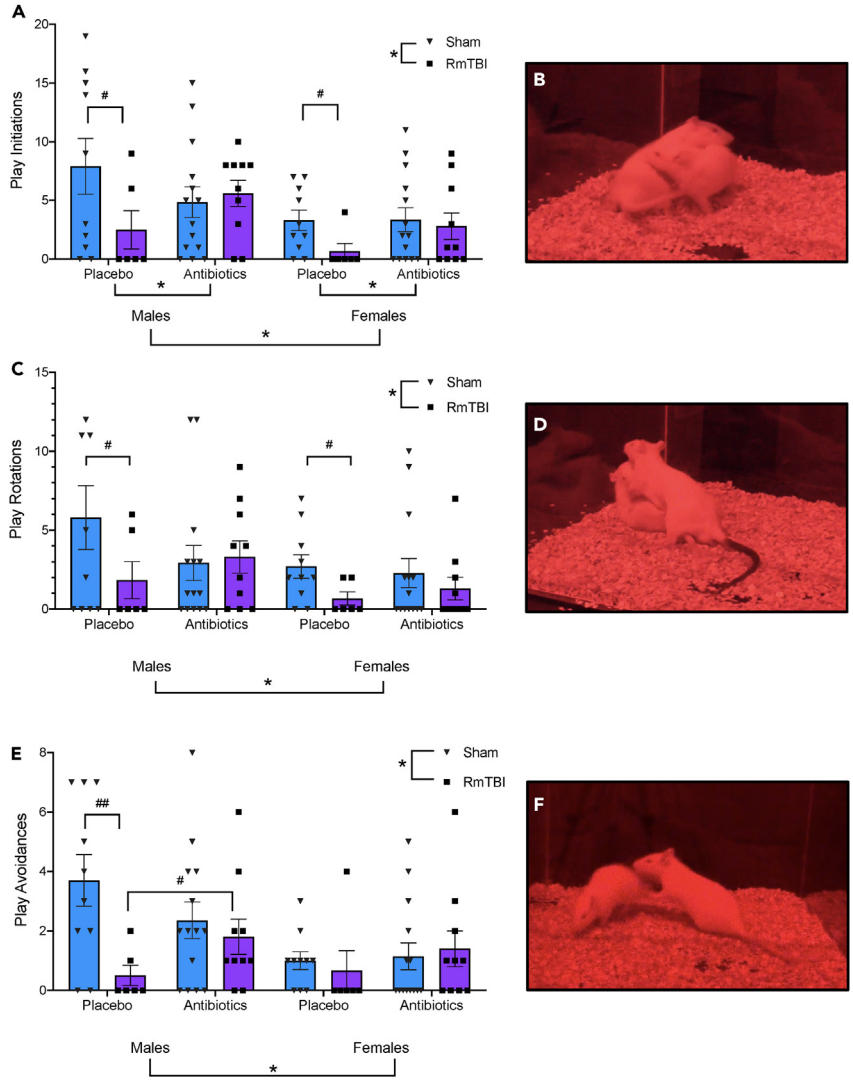


Figure 3. Play behavior in adolescent rats

(A and C) RmTBI reduced play initiation and engagement in both sexes; (E) RmTBI increased play avoidance in males, with microbiome depletion attenuating this deficit when combined with RmTBI. Screen captures of play initiation, engagement and evasion are demonstrated in panels B, D, and F, respectively. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$, ## represent significant difference, $p < 0.01$.

may explain why we found that microbiome depletion prior to RmTBI in adults, altered nociceptive sensitivity to a greater extent than in adolescents. Therefore, microbiome depletion may have provided a protective advantage for adolescents. However, this is contrary to a recent clinical study by Tong et al.[40] in healthy participants, that found adolescents were more likely to exhibit changes to nociceptive thresholds when compared to adults. It is important to note that the healthy brain may respond very differently than an injured brain, especially in the context of microbiome depletion. Nevertheless, the link between RmTBI-induced social deficits and their comorbidities (anxiety and pain) is complex and requires further research to elucidate the underlying mechanisms and determine whether the gut microbiome is a driver of symptomology or complicit in symptom maintenance following RmTBI.

Unsurprisingly, when compared to adults, healthy adolescents spent more of their time engaged in pro-social behaviors, such as play, allogrooming, and sniffing their cage mates. From an evolutionary perspective, adolescence is a period whereby individuals generally leave their home groups to increase their social circles, establish their own identity, and find suitable mates, so these age-differences in sociability were expected.[35] On the contrary, adults spent more time rearing and self-grooming which may be related to their mature and well-established social hierarchies, allowing them to spend more time in self-directed behaviors that do not require extensive social interaction.[41] In addition, we corroborated previous findings that RmTBI reduced play and reward-driven behavior, increased repetitive-type behaviors, while also modifying social function.[42] RmTBI insults may impair the socio-emotional regions of the brain causing disruptions within the SBN and

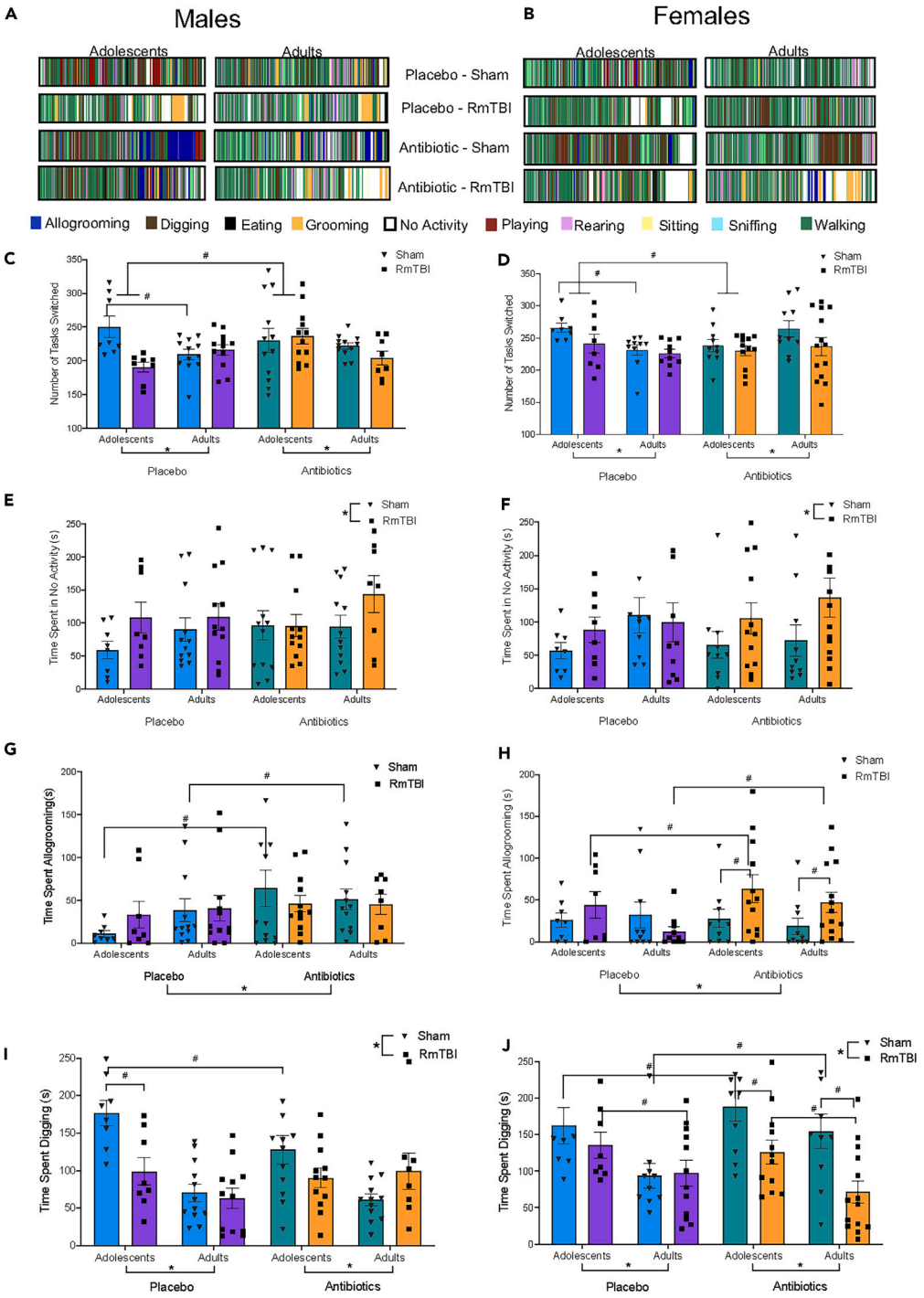


Figure 4. Temperament analysis in adolescent and adult rats

(A and B) Histograms illustrating a representative outcomes, each color is associated with a particular social behavior; (C and D) Number of tasks switched in males and females; (E and F) RmTBI increased the time spent in no activity for both males and females; (G and H) Microbiome depletion increased time spent allogrooming in males and females, whereas microbiome depletion combined with RmTBI increased allogrooming in females; (I and J) Microbiome depletion in combination with RmTBI returned the time spent digging to that of placebo sham levels. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

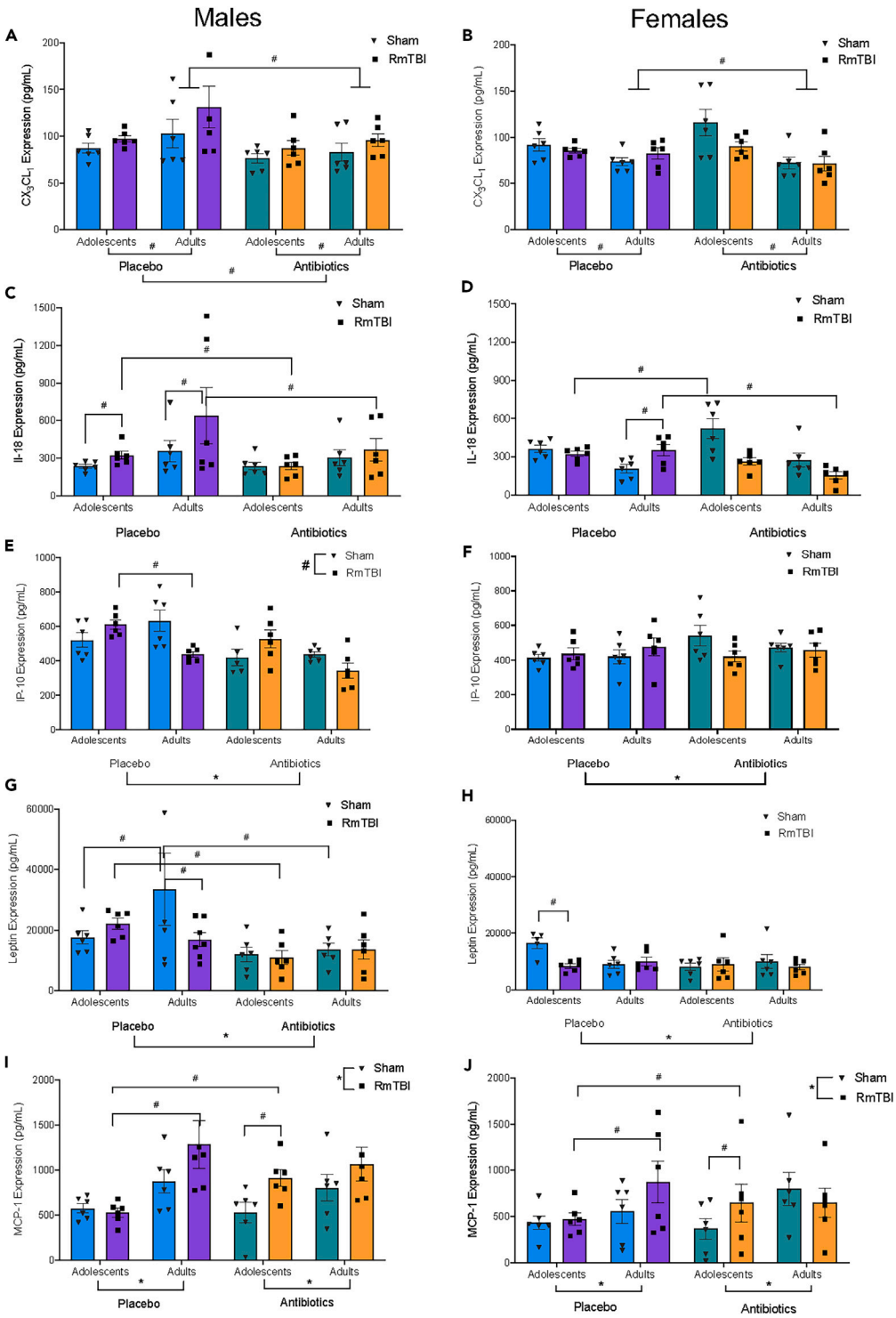


Figure 5. Levels of circulating cytokines from serum collected at euthanasia

(A and B) CX3CL1 expression in males and females; (C and D) IL-18 expression in males and females; (E and F) IP-10 expression in males and females; (G and H) Leptin expression in males and females; (I and J) MCP-1 expression in males and females; Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

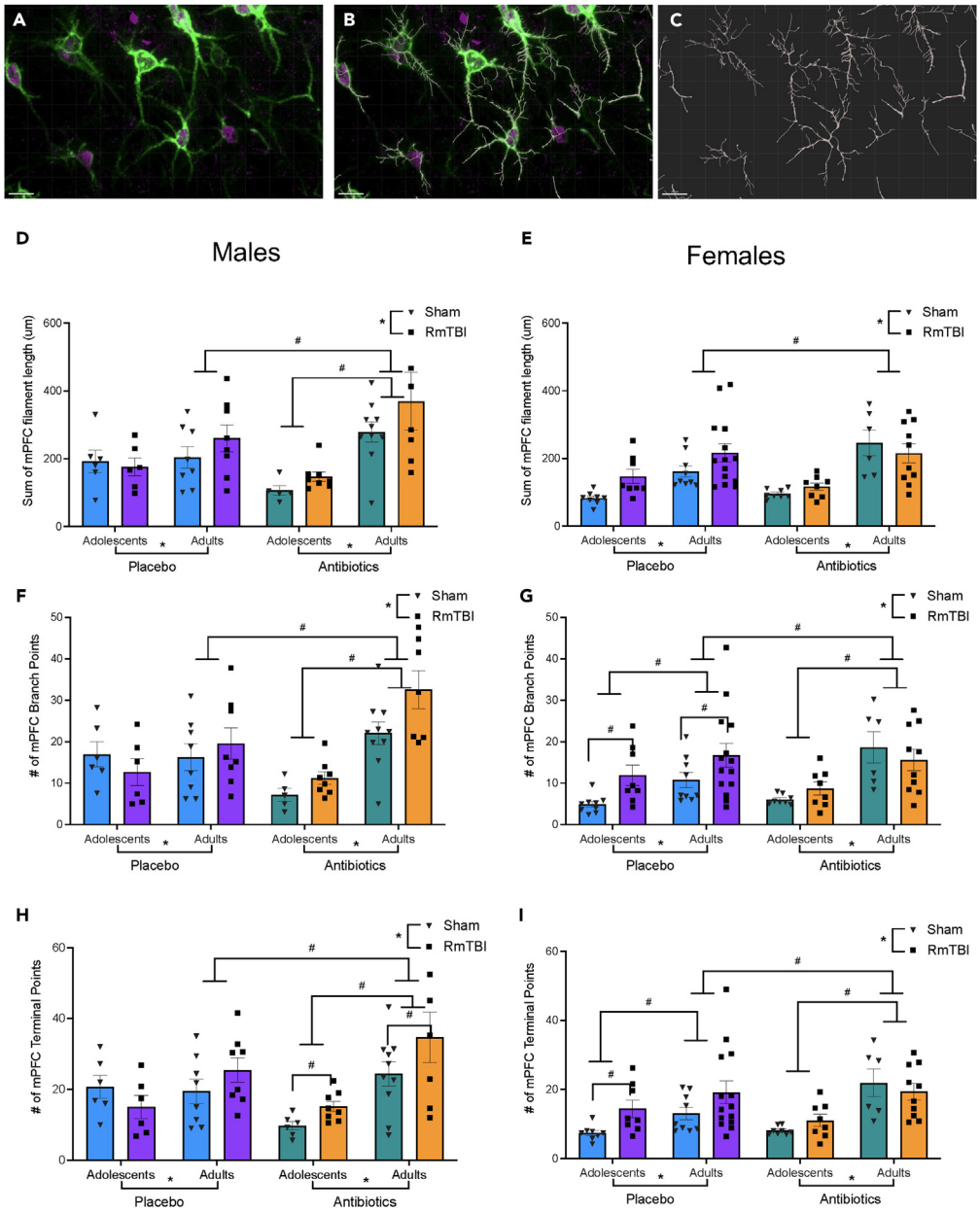


Figure 6. Structure analysis of PNN arborization within the mPFC of adolescent and adult rats

(A) Imaris screen capture of colocalized PNN and PV within mPFC; (B) Imaris screen capture of colocalized PNN with filament overlay; (C) Imaris screen capture of filament overlay alone; (D) Length of PNN branching in males and (E) females; (F) Number of terminal branches in males and (G) females; (H) Number of terminal points in males and (I) females. Microbiome depletion in combination with RmTBI in adults, increased length of branches (D and E), number of branches (F and G) and extent of branching (H and I) in the mPFC. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

reward circuitry.[43] These functional changes could explain RmTBI-induced reductions in the time spent engaged in rewarding behaviors such as digging and play and increases in repetitive-type behaviors as indicated by reduced task switching.

Contrary to our hypothesis, we found that microbiome depletion prior to RmTBI improved social deficits. Previous studies have demonstrated that microbiome depletion altered play and social behaviors, whereby rodents engage in less play and display symptomologies that resemble autism-spectrum disorder phenotypes.[26] This has been attributed to changes in inflammatory cytokines mediated by the gut microbiota, short chain fatty acids (SCFAs), HPA axis functioning, and neuroimmune mediators.[35] Moreover, we previously demonstrated microbiome depletion prior to RmTBI resulted in an increase in the bacterial metabolite *glycerophosphocholine* (precursor of acetylcholine) and an increase in *Clostridium* spp.[28], both of which modulate pro/anti-inflammatory processes and functioning of the HPA axis.[44] However,

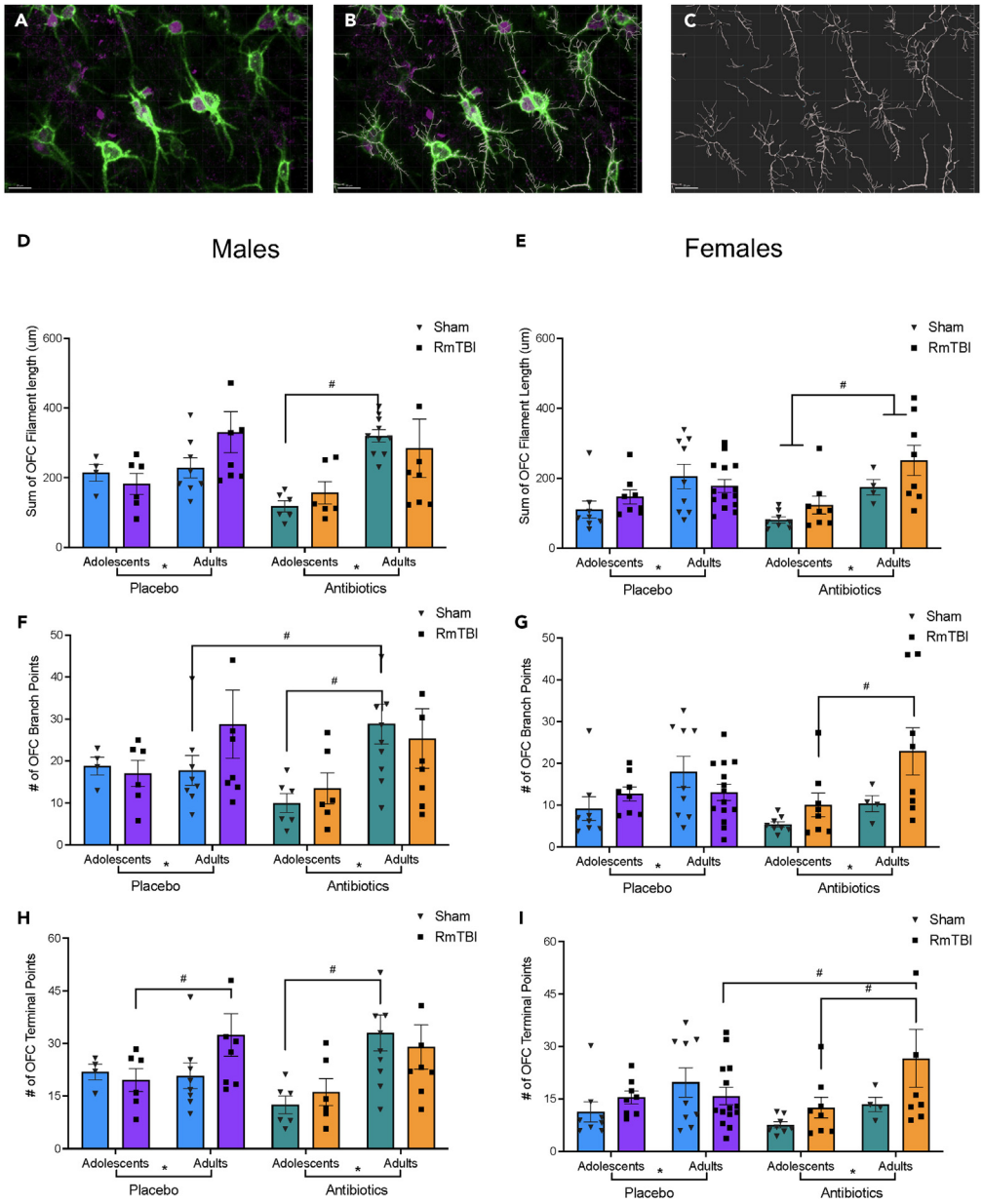


Figure 7. Structure analysis of colocalized PNN arborization within the OFC of adolescent and adult rats

(A) Imaris screen capture of colocalized PNN in the OFC; (B) Imaris screen capture of colocalized PNN with filament overlay; (C) Imaris screen capture of filament overlay alone; (D) Length of PNN branches in males and (E) females; (F) Number of branching points in males and (G) females; (H) Number of terminal points in males and (I) females; Microbiome depletion in combination with RmTBI in adults, increased length of branches (D and E), number of branches (F and G) and extent of branching (H and I) in the OFC. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

limited research has investigated the mechanistic action underpinning how the microbiome mediates these social behaviors following RmTBI. It is possible, that depletion of the microbiome altered the progression of the secondary injury cascade normally induced by RmTBI,[45] thereby limiting peripheral immune components available for transportation into the central nervous system. As a result, whilst there may have been a decrease in SCFAs that are generally beneficial for social behavior and appropriate functioning of the components of the SBN, the loss of the microbiome may have reduced circulating pro-inflammatory cytokines that would have normally been produced by the gut microbiome in response to injury.[46] Given that microbiome depletion and RmTBI differentially modulated aspects of social behavior in male and female, and adolescent and adult rats, we next examined circulating cytokine levels to understand how these manipulations may have affected systemic inflammation.

Table 3. Summary of the four-way ANOVA statistical results for the PNN structural analyses

Brain Region	Anatomical Measure of PNN surrounding PV + neuron	Main Effect Sex F(*p*)	Main Effect Antibiotics F(*p*)	Main Effect Injury F(*p*)	Main Effect Age F(*p*)	Significant Interactions
mPFC	Total Filament Length	12.336 (<0.001)	1.087 (0.299)	4.707 (0.032)	46.971 (<0.001)	Antibiotics x Age: 9.497 (0.003)
	Number of Branches	11.838 (<0.001)	0.036 (0.849)	3.256 (0.074)	35.761 (<0.001)	Antibiotics x Age: 5.786 (0.018) Sex x Antibiotics x Injury: 6.116 (0.015)
	Number of Terminal Points	15.255 (<0.001)	0.563 (0.455)	5.338 (0.023)	34.978 (<0.001)	Sex x Antibiotics x Injury: 4.827 (0.030)
OFC	Total Filament Length	13.462 (<0.001)	0.325 (0.570)	1.740 (0.190)	30.340 (<0.001)	4 Way Interaction: 4.066 (0.046)
	Number of Branches	9.218 (0.003)	0.003 (0.958)	0.761 (0.385)	16.783 (<0.001)	None
	Number of Terminal Points	11.613 (<0.001)	0.108 (0.744)	2.195 (0.141)	16.137 (<0.001)	4 Way Interaction: 4.248 (0.042)

Circulating cytokines are integral modulators of the immune system and allow for crosstalk between the gut and the brain.[47] Therefore, we investigated how RmTBI and microbiome depletion influenced circulating cytokine levels in adolescent and adult rats. Prior research has demonstrated that aging can induce a gradual decline in pathophysiological functioning, whereby increased blood brain barrier permeability,[48] changes to intestinal permeability and motility,[49] and impaired immune system functionality, all contribute to immunesenescence.[50] Together, this can induce chronic low-grade inflammation in aging populations, which is referred to as "inflammaging".[51] While the rodents in the present study would not be considered of 'old age', the process of "inflammaging" may actually commence earlier in adulthood than previously thought; especially when compared to adolescents. This may therefore explain our results, where we identified age-dependent changes in the majority of our cytokine panel, with adults exhibiting greater circulating cytokine levels than adolescents. In addition, we found more significant changes in cytokine levels for our male rats, when compared to females. Male circulating cytokine levels in both age groups, were more responsive to microbiome depletion and RmTBI manipulations, while the females exhibited a more blunted response to these exposures. This was similarly demonstrated in a previous study conducted by Salberg et al.,[52] where the male neuroimmune response was exacerbated following exposure to early life manipulations, but the females were largely unaffected. Previous studies have also suggested that prepubescent males have a greater innate immune response and therefore exhibit increased inflammatory reactions, which may have contributed to our differences within the adolescent age groups.[53] Furthermore, the cytokine response in adult males, may have been associated with increased testosterone, as testosterone has also been linked to anti-inflammatory effects.[54] Supplementation of testosterone has been shown to improve recovery, inflammation, and oxidative stress induced by injury.[55] Therefore, given that both RmTBI and microbial dysbiosis can lead to reduced testosterone in males, rats in these groups may have initiated a greater proinflammatory response. Contrary to our hypothesis that adolescents would be more susceptible to RmTBI-induced impairments, adult levels of circulating proinflammatory cytokines (IL2, IL5, IP10, leptin and MCP1) were more substantially elevated in response to injury. This may be a result of the aging process, or could be attributed to a greater compensatory/resilience capacity of the adolescent neuroimmune response, as it is undergoing substantial development allowing for adaptation and increases in neuroprotection.[56,57] On the contrary, the adult immune system is highly specialized and may therefore mount a stronger and prolonged inflammatory response to the RmTBIs.[58]

Given the bidirectional relationship with the immune system, microbial dysbiosis is also capable of inducing changes to the peripheral cytokine response.[59] In this study, microbiome depletion reduced leptin and IP-10 expression across sexes and in both age groups. Certain gut bacteria involved in the production of SCFAs (butyrate), such as *Lactobacilli*, have been linked to the metabolism and production of leptin.[60] Therefore, reductions in gut bacteria associated with the microbiome depletion may have reduced SCFA production and subsequently reduced leptin concentrations.[60] Moreover, as gut bacteria such as *Lactobacilli*, are also involved in the regulation of IP-10 production, via stimulation of IFN- γ production,[61,62] microbiome depletion may have modulated circulating levels of IP-10.

Adolescence encompasses a time of exponential neuroplasticity, whereby regions within the SBN including the mPFC and OFC undergo significant development. Specialised extracellular matrix structures, known as PNNs, envelop fast-spiking PV GABAergic interneurons soma, dendrites, and axonal segments[63] whereby they regulate neuroplasticity across the lifespan.[64] The dynamic structural composition of PNNs regulates neuroplasticity and neuroprotection within the SBN via the regulation of inhibitory/excitatory signaling,[21] neuronal connectivity,[65] synaptic formation,[66] and the motility of receptors at synapses.[67] The structural density of PNNs within the mPFC and OFC increases over the lifespan, with increased density characterising the closure of critical neurodevelopmental windows.[68] Some of the primary functions of the mPFC are to regulate impulse control, decision making, and coordination of complex movements,[69] whereas the OFC assists in the distinguishing between social partners, social play behavior fluidity, and appropriate play architecture.[70] Given these differences, region-specific alterations to PNN structure, number, and functionality have the potential to differentially modify social behavior.[71]

PNNs within the mPFC and OFC are highly vulnerable to CNS injury.[72] However, there have been minimal studies investigating the influence of mTBI on PNN numbers and structure, and the mechanistic role they play in post-injury symptomology. To our knowledge, this is the first study that has investigated PNN branching structure within this context. Our findings show that 11-days post-RmTBI, PNN branch length increased, along with the number of branches and terminal branch points in the mPFC. The increase in branching may have occurred to protect PV+ neurons and their synapses from RmTBI-induced oxidative stress and secondary injury cascades.[72] Similarly, glycosaminoglycan and

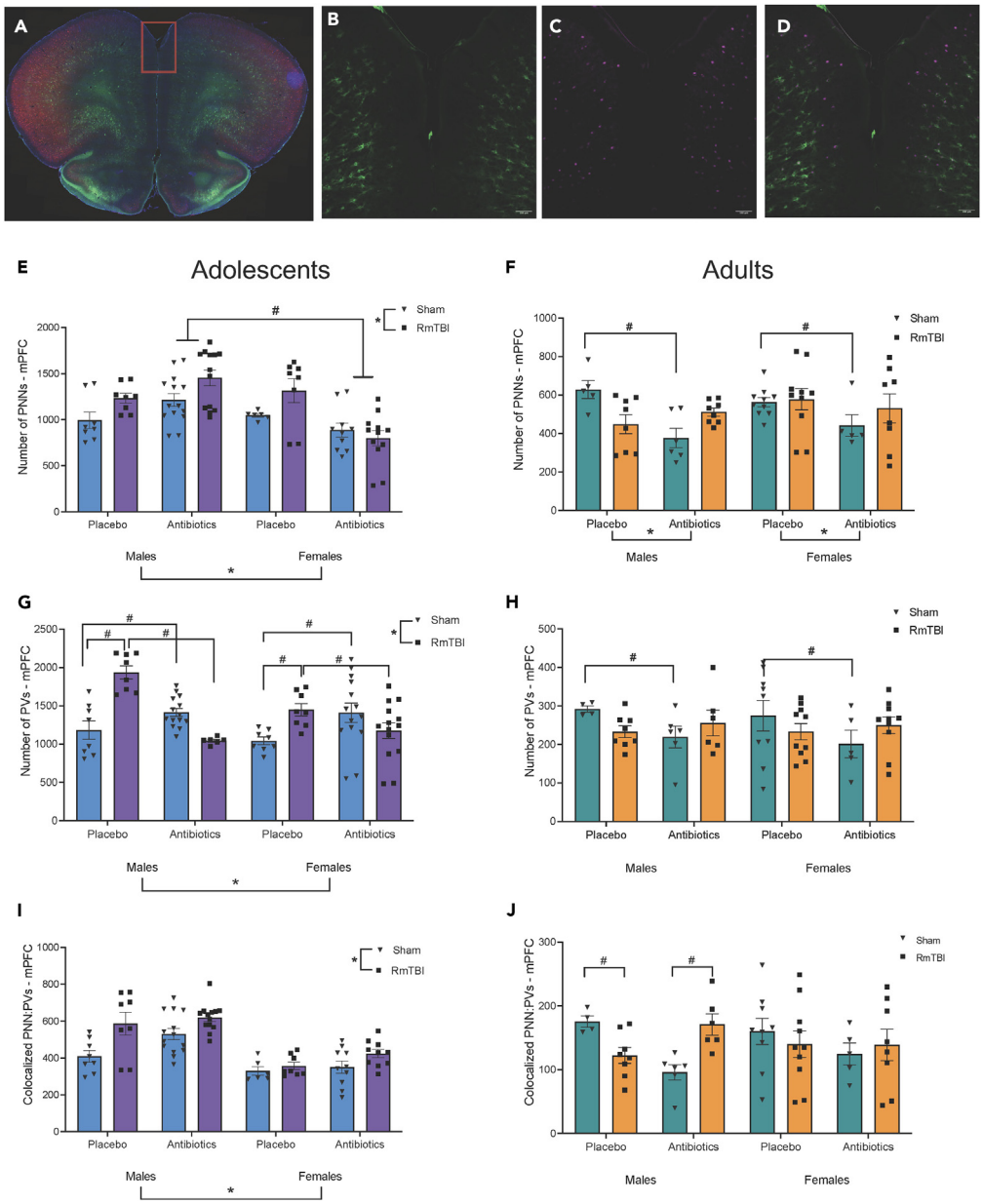


Figure 8. Number of PNN and PV cells within the mPFC in adolescent and adult rats

(A) Whole brain image demonstrating the location of mPFC sampling – PNNs are labeled green and PVs are labeled purple; (B) Increased magnification display of representative PNN staining; (C) zoomed display of representative PV staining; (D) Increased magnification display of representative PNN and PV staining; (E) Number of PNNs within the mPFC in males and (F) females; (G) Number of PVs within the mPFC in males and (H) females; (I) Number of colocalized PNN and PV cells within the mPFC in males and (J) females. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

chondroitin sulfate proteoglycans (CSPGs) are integral components of PNNs that have been implicated in injury recovery.[73] Specifically, following CNS injury, research has demonstrated an upregulation of CSPGs, which subsequently increases PNN density[74] and may explain the greater PNN branch length and arborization we identified.

Alternatively, under homeostatic conditions, microglia are involved in the pruning synapses of PV+ interneurons and structurally modify their associated PNNs.[75] Therefore, when microglia are depleted and not available to interact with PNNs, there is a reduction in pruning which results in greater spine density, terminal branch points, length, and increases in PNN density.[75] Chronic activation of microglia, which commonly occurs post-RmTBI,[76] may dysregulate their homeostatic functioning, leading to reduced pruning and increased arborization.[75] It is important to note

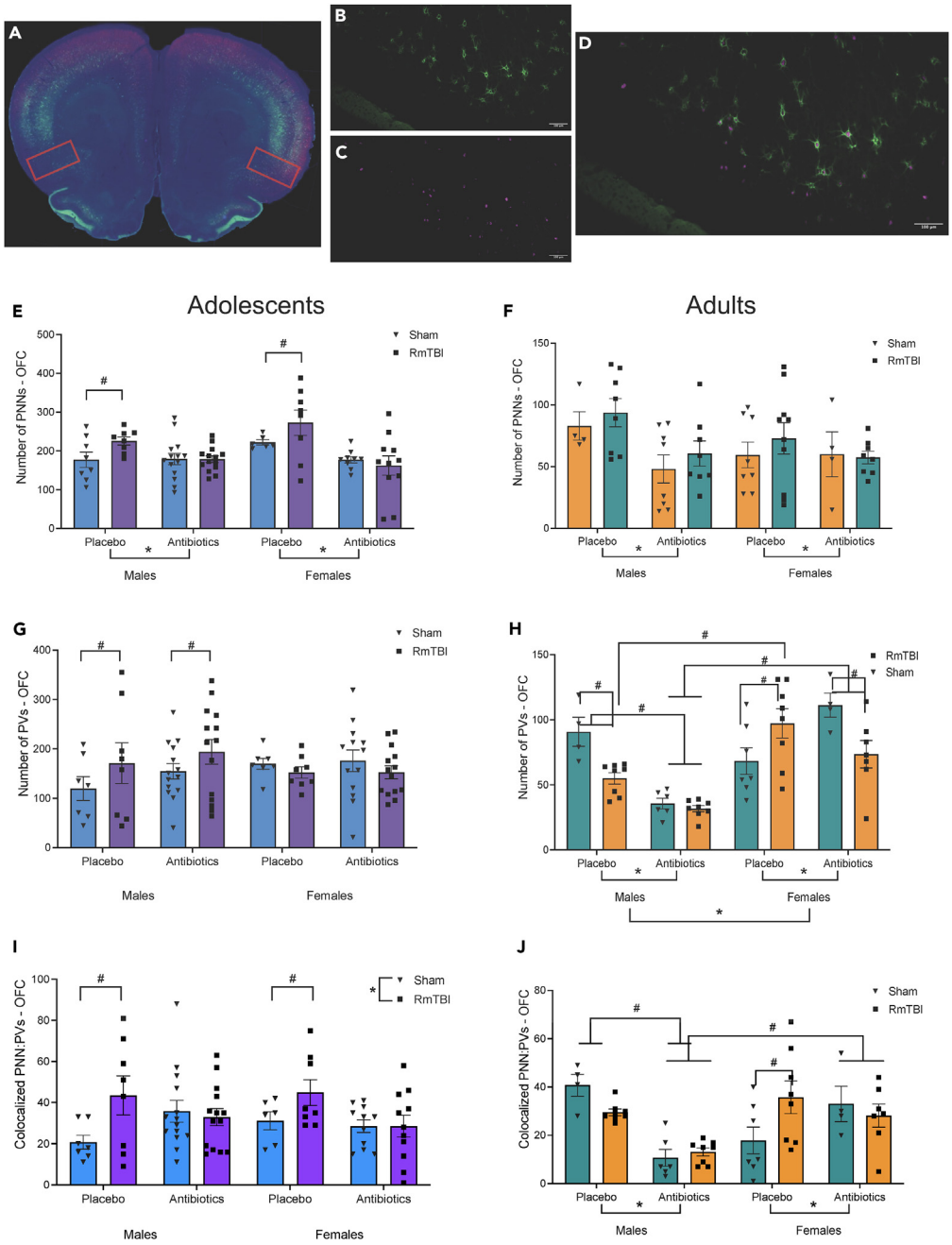


Figure 9. Number of PNN and PV cells within the OFC in adolescent and adult rats

(A) Whole brain image demonstrating the location of OFC sampling – PNNs are labeled green and PVs are labeled purple; (B) Increased magnification display of representative PNN staining; (C) zoomed display of representative PV staining; (D) Increased magnification display of representative PNN and PV staining; (E) Number of PNNs within the OFC in males and (F) females; (G) Number of PVs within the OFC in males and (H) females; (I) Number of colocalized PNN and PV cells within the OFC in males and (J) females. Graphs are shown as means ± SEM, * represents significant interaction, # represent significant difference, $p < 0.05$.

that increased branching is not always associated with improved functionality, connectivity, and processing; rather it is often associated with improper/unreliable connections and has been implicated in the development of neuropsychiatric disorders, including those associated with altered social function.[77,78] Not only was the structure of PNN-PV+ cells altered following RmTBI, but the number of PVs within the mPFC and OFC that were colocalised was also affected. In adolescents there were increases in the number of PVs, PNNs, and those that were colocalized, whereas adults exhibited the opposite effect, and cell quantity was reduced. Considering TBI events often cause an imbalance in

Table 4. Summary of three-way ANOVA statistical results for PNN and PV cell counts for adolescents and adults

Age	Brain Region	Cell Count	Main Effect Sex F(p)	Main Effect Antibiotics F(p)	Main Effect Injury F(p)	Significant Interactions
Adolescent	mPFC	PNN	11.70 (0.001)	0.873 (0.353)	7.130 (0.009)	Sex x Antibiotics: 20.218 (<0.001)
		PV	21.004 (<0.001)	0.454 (0.502)	23.066 (<0.001)	Sex x Injury: 12.395 (<0.001) Antibiotics x Injury: 13.886 (<0.001)
		Co-localized	53.303 (<0.001)	6.648 (0.012)	15.324 (<0.001)	None
	OFC	PNN	2.743 (0.102)	13.316 (<0.001)	1.924 (0.170)	Antibiotics x Injury: 5.978 (0.017)
		PV	0.029 (0.865)	0.971 (0.328)	0.590 (0.445)	Sex x Injury: 4.198 (0.046)
		Co-localized	0.000 (0.990)	0.810 (0.371)	4.389 (0.040)	Antibiotics x Injury: 6.008 (0.017)
Adult	mPFC	PNN	0.907 (0.346)	5.192 (0.027)	0.075 (0.785)	Antibiotics x Injury: 6.187 (0.011)
		PV	0.137 (0.713)	1.280 (0.263)	0.081 (0.777)	Antibiotics x Injury: 4.130 (0.048)
		Co-localized	0.005 (0.946)	1.076 (0.305)	0.030 (0.863)	Antibiotics x Injury: 6.795 (0.012)
	OFC	PNN	1.090 (0.302)	5.592 (0.022)	0.993 (0.324)	None
		PV	24.919 (<0.001)	4.343 (0.043)	3.203 (0.081)	Sex x Antibiotics: 12.710 (<0.001) Three-way Interaction: 12.931 (<0.001)
		Co-localized	2.572 (0.116)	5.138 (0.029)	0.000 (0.987)	Sex x Antibiotics: 14.169 (<0.001) Three-way Interaction: 6.896 (0.012)

excitatory and inhibitory signaling,[79] the number of PNNs and PVs may have increased in adolescents to compensate for this imbalance and to reduce excitotoxicity, whereas this excitation may have been exacerbated in adults owing to their heightened levels of inflammation.

Contrary to our hypothesis, microbiome depletion prior to RmTBI increased PNN branch length and number of branches in the mPFC of adult males and the OFC of adult females. Moreover, in the OFC, microbiome depleted sham adult males had the greatest changes to PNN arborization. Recent studies have demonstrated that sex hormones can influence the myelination and transmission within the OFC and mPFC in adults.[80] Furthermore, it is well established that the gut microbiome is involved in neurodevelopmental plasticity[81] and regulates sex hormones.[82] Therefore, it is not surprising that it may also play a role in the organization of PNN structure and proliferation in a sex and region dependant manner. Interestingly, microbiome depletion reduced the number of PNNs and PVs within the mPFC and OFC, and when combined with RmTBI this was blunted, whereby their quantity and colocalization were normalized, potentially suggesting that depletion of the gut microbiota prior to RmTBI may be neuroprotective in the context of PNN neuroplasticity. In fact, various gram-positive bacteria produce hyaluronidase, which can break down the backbone of the extracellular matrix.[83] Therefore, it is possible that following microbiome depletion, the hyaluronidase-producing bacteria were reduced, and consequently PNNs had the ability to grow and branch at greater rates. SCFAs also influence the functionality and inflammatory state of microglia,[84] with reductions in SCFAs causing microglia malformation and induction of pro-inflammatory states.[85] Hence, microbiome depletion-induced reductions in SCFA producing bacteria, may have combined with the RmTBI, to further influence microglia functionality; whereby they were diverted from pruning and refinement of neuronal architecture to the removal of injury-induced neurotoxic waste.[86] Our results provide support for this hypothesis, as we demonstrated a greater inflammatory profile following microbiome depletion in males (which would result in increased neurotoxic waste). Moreover, males exhibit earlier onset and completion of PNN maturation,[87] suggesting they would have established roles in both processes. Our findings support this idea, as adult males exhibited the greatest change in quantity of PVs, PNNs, and their colocalization, in response to the manipulations. Therefore, our findings highlight the important role that PNNs play in regulating neuroplasticity of the adult brain. Importantly, we also demonstrate that the exponential development and neuroplasticity that occurs during adolescence may actually aid in recovery and protection from chronic post-injury changes to the functionality and structure of PNNs within the components of the SBN.

Conclusion

To further our understanding of the potential involvement of the gut microbiome in recovery from RmTBI, we depleted the gut microbiome prior to injury and investigated its effects on social behavior and neuroplasticity within the mPFC and OFC of the SBN. To our knowledge, this is the first study that has demonstrated that the microbiome may mediate RmTBI-induced social deficits and alter neuroplasticity differentially, dependent upon age and sex. We demonstrate that microbiome depletion prior to RmTBI improved social behavior deficits and its comorbidities and did not exacerbate injury-induced pro-inflammatory cytokine release. However, compared to adolescents, adults demonstrated the most significant changes to socio-emotional behaviors and exhibited greater pro-inflammatory responses post-RmTBI. In the mPFC and OFC, microbiome depletion prior to RmTBI resulted in abnormal arborization and increased colocalization of PNN-PV+ in adults, particularly in males. In comparison, adolescents presented as more resilient to these manipulations, possibly due to their heightened window of plasticity. Our results suggest that adult age and male sex may be a risk factor for social deficits following injury, or at the least these cohorts may be more vulnerable to microbiome manipulations. Importantly, neuroplastic changes associated with RmTBI continued throughout adulthood and it appears that the

gut microbiome is involved in this process, at least within the mPFC and OFC. To further elucidate the mechanistic action underpinning these changes to neuroplasticity following RmTBI and its effect on social deficits, future studies could employ metabolomic analysis to determine which gut microbiota specific pathways are contributing to the abnormal arborization of PNNs within the SBN. Moreover, future studies should focus on elucidating the mechanistic underpinnings that actually drive the behavioral changes associated with differences in microbiome composition and functionality. This would provide further understanding of the important findings generated in the present study. To improve recovery post-RmTBI, it is also imperative that we gain a better understanding of the role of PNN structure and function in the context of other neuronal cell types, such as microglia. Finally, longitudinal studies investigating the chronic effects of gut-brain-immune axis changes in adolescence should be explored, as the long-term consequences of these manipulations are currently unknown.

Limitations of the study

Although this study provided a comprehensive examination of the changes in neuroplasticity associated with microbiome depletion and RmTBI, it was limited by the number of brain regions we were able to examine. Given the multitude of structures that make up the SBN, it would have been ideal to examine all of them. In addition, the relationship identified between social behavior and changes in PNN structure and function was correlational, and future studies should investigate the mechanisms driving this association.

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 STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Injury induction
  - Behavioral testing
  - Tissue collection
  - Immunohistochemical staining, imaging, and analyses
  - Perineuronal net imaging and Imaris branching analysis – adolescents and adults
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109395.

ACKNOWLEDGMENTS

The authors acknowledge the facilities and scientific assistance of Crux Biolabs for their assistance with the cytokine panel. Moreover, the authors would like to also acknowledge the facilities, scientific, and technical assistance of Monash Micro Imaging (MMI) Platform for their assistance with the imaging and image analysis. The authors would like to thank the National Health and Medical Research Council (NHMRC; RM-AP1173565) for their financial support and contributions.

AUTHOR CONTRIBUTIONS

M.S., G.Y., and R.M. were responsible for study design and conception. M.S., Z.K., C.L., I.C., S.W., A.R., G.Y., and R.M. were involved with data collection and analyses. M.S., G.Y., and R.M. were responsible for data verification and management. M.S. and R.M. wrote the first draft of the manuscript. All authors have edited and approved the final version.

DECLARATION OF INTERESTS

The authors have no conflicts of interest.

Received: October 2, 2023
Revised: November 21, 2023
Accepted: February 28, 2024
Published: March 2, 2024

REFERENCES

1. Gardner, R.C., and Yaffe, K. (2015). Epidemiology of mild traumatic brain injury and neurodegenerative disease. Mol. Cell. Neurosci. *66*, 75–80.
2. Maas, A.I.R., Menon, D.K., Adelson, P.D., Andelic, N., Bell, M.J., Belli, A., Bragge, P., Brazinova, A., Büki, A., Chesnut, R.M., et al. (2017). Traumatic brain injury: integrated approaches to improve prevention, clinical care, and research. Lancet Neurol. *16*, 987–1048.
3. Mychasiuk, R., Hehar, H., Candy, S., Ma, I., and Esser, M.J. (2016). The direction of the acceleration and rotational forces associated with mild traumatic brain injury in rodents effect behavioural and molecular outcomes. J. Neurosci. Methods *257*, 168–178.
4. Halstead, M.E., and Walter, K.D.; Council on Sports Medicine and Fitness (2010). American Academy of Pediatrics. Clinical report–sport-related concussion in children and adolescents. Pediatrics *126*, 597–615.
5. Curry, A.E., Arbogast, K.B., Metzger, K.B., Kessler, R.S., Breiding, M.J., Haarbauer-Krupa, J., DePadilla, L., Greenspan, A., and Master, C.L. (2019). Risk of Repeat Concussion Among Patients Diagnosed at a Pediatric Care Network. J. Pediatr. *210*, 13–19.e2.
6. Daugherty, J., DePadilla, L., Sarmiento, K., and Breiding, M.J. (2020). Self-Reported Lifetime Concussion Among Adults: Comparison of 3 Different Survey Questions. J. Head Trauma Rehabil. *35*, E136–E143.
7. Badea, A., Kamnaksh, A., Anderson, R.J., Calabrese, E., Long, J.B., and Agoston, D.V. (2018). Repeated mild blast exposure in young adult rats results in dynamic and persistent microstructural changes in the brain. Neuroimage. Clin. *18*, 60–73.
8. Eyolfson, E., Yamakawa, G.R., Griep, Y., Collins, R., Carr, T., Wang, M., Lohman, A.W., and Mychasiuk, R. (2020). Examining the Progressive Behavior and Neuropathological Outcomes Associated with Chronic Repetitive Mild Traumatic Brain Injury in Rats. Cereb. Cortex Commun. *1*, tgaa002.
9. Smith, A.R., Chein, J., and Steinberg, L. (2013). Impact of socio-emotional context, brain development, and pubertal maturation on adolescent risk-taking. Horm. Behav. *64*, 323–332.
10. Milders, M. (2019). Relationship between social cognition and social behaviour following traumatic brain injury. Brain Inj. *33*, 62–68.
11. Mollayeva, T., Pratt, B., Mollayeva, S., Shapiro, C.M., Cassidy, J.D., and Colantonio, A. (2016). The relationship between insomnia and disability in workers with mild traumatic brain injury/concussion: Insomnia and disability in chronic mild traumatic brain injury. Sleep Med. *20*, 157–166.
12. Averbeck, B.B. (2022). Pruning recurrent neural networks replicates adolescent changes in working memory and reinforcement learning. Proc. Natl. Acad. Sci. USA *119*, e2121331119.
13. Thompson, D.K., Yang, J.Y.M., Chen, J., Kelly, C.E., Adamson, C.L., Alexander, B., Gilchrist, C., Matthews, L.G., Lee, K.J., Hunt, R.W., et al. (2022). Brain White Matter Development Over the First 13 Years in Very Preterm and Typically Developing Children Based on the T 1-w/T 2-w Ratio. Neurology *98*, e924–e937.
14. Sgro, M., and Mychasiuk, R. (2020). Playful genes: what do we know about the epigenetics of play behaviour? Int. J. Play *9*, 25–38.
15. Himmler, B.T., Mychasiuk, R., Nakahashi, A., Himmler, S.M., Pellis, S.M., and Kolb, B. (2018). Juvenile social experience and differential age-related changes in the dendritic morphologies of subareas of the prefrontal cortex in rats. Synapse *72*, e22022.
16. Barlow, K.M., Crawford, S., Stevenson, A., Sandhu, S.S., Belanger, F., and Dewey, D. (2010). Epidemiology of postconcussion syndrome in pediatric mild traumatic brain injury. Pediatrics *126*, e374–e381.
17. Johnson, M.H., Griffin, R., Csibra, G., Halit, H., Farroni, T., de Haan, M., Tucker, L.A., Baron-Cohen, S., and Richards, J. (2005). The emergence of the social brain network: evidence from typical and atypical development. Dev. Psychopathol. *17*, 599–619.
18. Pellis, S., Pellis, V., and Broccard-Bell, H. (2011). The Function of Play in the Development of the Social Brain. Am. J. Play *2*, 278–296.
19. Amegandjin, C.A., Choudhury, M., Jadhav, V., Carriço, J.N., Quintal, A., Berryer, M., Snapyan, M., Chattopadhyaya, B., Saghatelyan, A., and Di Cristo, G. (2021). Sensitive period for rescuing parvalbumin interneurons connectivity and social behavior deficits caused by TSC1 loss. Nat. Commun. *12*, 3653.
20. Ueno, H., Suemitsu, S., Murakami, S., Kitamura, N., Wani, K., Matsumoto, Y., Okamoto, M., Aoki, S., and Ishihara, T. (2018). Juvenile stress induces behavioral change and affects perineuronal net formation in juvenile mice. BMC Neurosci. *19*, 41.
21. Cabungcal, J.H., Steullet, P., Morishita, H., Kraftsik, R., Cuenod, M., Hensch, T.K., and Do, K.Q. (2013). Perineuronal nets protect fast-spiking interneurons against oxidative stress. Proc. Natl. Acad. Sci. USA *110*, 9130–9135.
22. Wöhr, M., Orduz, D., Gregory, P., Moreno, H., Khan, U., Vörckel, K.J., Wolfer, D.P., Welzl, H., Gall, D., Schiffmann, S.N., and Schwaller, B. (2015). Lack of parvalbumin in mice leads to behavioral deficits relevant to all human autism core symptoms and related neural morphofunctional abnormalities. Transl. Psychiatry *5*, e525.
23. Xenos, D., Kamceva, M., Tomasi, S., Cardin, J.A., Schwartz, M.L., and Vaccarino, F.M. (2018). Loss of TrkB Signaling in Parvalbumin-Expressing Basket Cells Results in Network Activity Disruption and Abnormal Behavior. Cereb. Cortex *28*, 3399–3413.
24. Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R.D., Cotter, P.D., Dinan, T.G., and Cryan, J.F. (2015). Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. Brain Behav. Immun. *48*, 165–173.
25. Jin, W., Wang, H., Ji, Y., Hu, Q., Yan, W., Chen, G., and Yin, H. (2008). Increased intestinal inflammatory response and gut barrier dysfunction in Nrf2-deficient mice after traumatic brain injury. Cytokine *44*, 135–140.
26. Fowlie, G., Cohen, N., and Ming, X. (2018). The Perturbance of Microbiome and Gut-Brain Axis in Autism Spectrum Disorders. Int. J. Mol. Sci. *19*, 2251. https://doi.org/10.3390/ijms19082251.
27. Wong, K.R., Sgro, M., Yamakawa, G.R., Li, C., McDonald, S.J., Sun, M., Shultz, S.R., Brady, R.D., and Mychasiuk, R. (2021). Gut microbiome depletion and repetitive mild traumatic brain injury differentially modify bone development in male and female adolescent rats. Bone Rep. *15*, 101123.
28. Sgro, M., Iacono, G., Yamakawa, G.R., Kodila, Z.N., Marsland, B.J., and Mychasiuk, R. (2022). Age matters: Microbiome depletion prior to repeat mild traumatic brain injury differentially alters microbial composition and function in adolescent and adult rats. PLoS One *17*, e0278259.
29. Wang, S., Qu, Y., Chang, L., Pu, Y., Zhang, K., and Hashimoto, K. (2020). Antibiotic-induced microbiome depletion is associated with resilience in mice after chronic social defeat stress. J. Affect. Disord. *260*, 448–457.
30. Smeester, B.A., Lee, J.-H., and Beitz, A.J. (2017). Influence of social interaction on nociceptive-induced changes in locomotor activity in a mouse model of acute inflammatory pain: Use of novel thermal assays. Brain Res. Bull. *134*, 47–54.
31. Johnson, K.V.A. (2020). Gut microbiome composition and diversity are related to human personality traits. Hum. Microb. J. *15*. None-None.
32. Salberg, S., Noel, M., Burke, N.N., Vinall, J., and Mychasiuk, R. (2020). Utilization of a rodent model to examine the neurological effects of early life adversity on adolescent pain sensitivity. Dev. Psychobiol. *62*, 386–399.
33. Xie, S., Zhang, X., Cheng, W., and Yang, Z. (2021). Adolescent anxiety disorders and the developing brain: comparing neuroimaging findings in adolescents and adults. Gen. Psychiatr. *34*, e100411.
34. Salokangas, R.K.R., Hietala, J., Armio, R.L., Laurikainen, H., From, T., Borgwardt, S., Riecher-Rössler, A., Brambilla, P., Bonivento, C., Meisenzahl, E., et al. (2021). Effect of childhood physical abuse on social anxiety is mediated via reduced frontal lobe and amygdala-hippocampus complex volume in adult clinical high-risk subjects. Schizophr. Res. *227*, 101–109.
35. Blakemore, S.J., and Mills, K.L. (2014). Is adolescence a sensitive period for sociocultural processing? Annu. Rev. Psychol. *65*, 187–207.
36. Domonkos, E., Hodosy, J., Ostatníková, D., and Celec, P. (2018). On the Role of Testosterone in Anxiety-Like Behavior Across Life in Experimental Rodents. Front. Endocrinol. *9*, 441.
37. Hazari, A., Salberg, S., Griep, Y., Yamakawa, G.R., and Mychasiuk, R. (2020). Examining changes in rodent temperament following repetitive mild traumatic brain injury in adolescence. Behav. Neurosci. *134*, 384–393.
38. Zhang, J., Song, L., Wang, Y., Liu, C., Zhang, L., Zhu, S., Liu, S., and Duan, L. (2019). Beneficial effect of butyrate-producing Lachnospiraceae on stress-induced visceral hypersensitivity in rats. J. Gastroenterol. Hepatol. *34*, 1368–1376.
39. Chong, P.P., Chin, V.K., Looi, C.Y., Wong, W.F., Madhavan, P., and Yong, V.C. (2019). The Microbiome and Irritable Bowel Syndrome - A Review on the Pathophysiology, Current Research and Future Therapy. Front. Microbiol. *10*, 1136.

40. Tong, H., Maloney, T.C., Payne, M.F., King, C.D., Ting, T.V., Kashikar-Zuck, S., Coghill, R.C., and López-Solà, M. (2022). Processing of pain by the developing brain: evidence of differences between adolescent and adult females. Pain *163*, 1777–1789.
41. Arakawa, H. (2005). Interaction between isolation rearing and social development on exploratory behavior in male rats. Behav. Processes *70*, 223–234.
42. Mychasiuk, R., Hehar, H., Farran, A., and Esser, M.J. (2014). Mean girls: sex differences in the effects of mild traumatic brain injury on the social dynamics of juvenile rat play behaviour. Behav. Brain Res. *259*, 284–291.
43. Milders, M., Ietswaart, M., Crawford, J.R., and Currie, D. (2008). Social behavior following traumatic brain injury and its association with emotion recognition, understanding of intentions, and cognitive flexibility. J. Int. Neuropsychol. Soc. *14*, 318–326.
44. Bonaz, B., Sinniger, V., and Pellissier, S. (2016). Anti-inflammatory properties of the vagus nerve: potential therapeutic implications of vagus nerve stimulation. J. Physiol. *594*, 5781–5790.
45. Simon, D.W., Rogers, M.B., Gao, Y., Vincent, G., Firek, B.A., Janesko-Feldman, K., Vagni, V., Kochanek, P.M., Ozolek, J.A., Mollen, K.P., et al. (2020). Depletion of gut microbiota is associated with improved neurologic outcome following traumatic brain injury. Brain Res. *1747*, 147056.
46. Wang, S., Zhu, K., Hou, X., and Hou, L. (2021). The association of traumatic brain injury, gut microbiota and the corresponding metabolites in mice. Brain Res. *1762*, 147450.
47. Celorrio, M., Abellanas, M.A., Rhodes, J., Goodwin, V., Moritz, J., Vadivelu, S., Wang, L., Rodgers, R., Xiao, S., Anabayan, I., et al. (2021). Gut microbial dysbiosis after traumatic brain injury modulates the immune response and impairs neurogenesis. Acta Neuropathol. Commun. *9*, 40.
48. Montagne, A., Barnes, S.R., Sweeney, M.D., Halliday, M.R., Sagare, A.P., Zhao, Z., Toga, A.W., Jacobs, R.E., Liu, C.Y., Amezcua, L., et al. (2015). Blood-brain barrier breakdown in the aging human hippocampus. Neuron *85*, 296–302.
49. Dumic, I., Nordin, T., Jecmenica, M., Stojkovic Lalosevic, M., Milosavljevic, T., and Milovanovic, T. (2019). Gastrointestinal Tract Disorders in Older Age. Can. J. Gastroenterol. Hepatol. *2019*, 6757524.
50. Nikolich-Žugich, J. (2018). The twilight of immunity: emerging concepts in aging of the immune system. Nat. Immunol. *19*, 10–19.
51. Franceschi, C., Garagnani, P., Parini, P., Giuliani, C., and Santoro, A. (2018). Inflammaging: a new immune-metabolic viewpoint for age-related diseases. Nat. Rev. Endocrinol. *14*, 576–590.
52. Salberg, S., Yamakawa, G.R., Beveridge, J.K., Noel, M., and Mychasiuk, R. (2023). A high-fat high-sugar diet and adversity early in life modulate pain outcomes at the behavioural and molecular level in adolescent rats: The role of sex. Brain Behav. Immun. *108*, 57–79.
53. Klein, S.L., and Flanagan, K.L. (2016). Sex differences in immune responses. Nat. Rev. Immunol. *16*, 626–638.
54. Papadopoulos, A.D., and Wardlaw, S.L. (2000). Testosterone suppresses the response of the hypothalamic-pituitary-adrenal axis to interleukin-6. Neuroimmunomodulation *8*, 39–44.
55. Carteri, R.B., Kopczynski, A., Rodolphi, M.S., Strogulski, N.R., Sartor, M., Feldmann, M., De Bastiani, M.A., Duval Wannmacher, C.M., de Franceschi, I.D., Hansel, G., et al. (2019). Testosterone Administration after Traumatic Brain Injury Reduces Mitochondrial Dysfunction and Neurodegeneration. J. Neurotrauma *36*, 2246–2259.
56. Sgro, M., Kodila, Z.N., Brady, R.D., Reichelt, A.C., Mychaisuk, R., and Yamakawa, G.R. (2021). Synchronizing our clocks as we age: the influence of the brain-gut-immune axis on the sleep-wake cycle across the lifespan. Sleep *45*, zsab268.
57. Cryan, J.F., O'Riordan, K.J., Cowan, C.S.M., Sandhu, K.V., Bastiaanssen, T.F.S., Boehme, M., Codagnone, M.G., Cussotto, S., Fulling, C., Golubeva, A.V., et al. (2019). The Microbiota-Gut-Brain Axis. Physiol. Rev. *99*, 1877–2013.
58. Valiathan, R., Ashman, M., and Asthana, D. (2016). Effects of Ageing on the Immune System: Infants to Elderly. Scand. J. Immunol. *83*, 255–266.
59. Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., Horst, R.T., Jansen, T., Jacobs, L., Bonder, M.J., et al. (2016). Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. Cell *167*, 1125–1136.e8.
60. Xiong, Y., Miyamoto, N., Shibata, K., Valasek, M.A., Motoike, T., Kedzierski, R.M., and Yanagisawa, M. (2004). Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc. Natl. Acad. Sci. USA *101*, 1045–1050.
61. von Schillde, M.-A., Hörmannsperger, G., Weiher, M., Alpert, C.-A., Hahne, H., Bäuerl, C., van Huynegem, K., Steidler, L., Hrncir, T., Pérez-Martínez, G., et al. (2012). Lactocepin Secreted By Lactobacillus Exerts Anti-Inflammatory Effects By Selectively Degrading Proinflammatory Chemokines. Cell Host Microbe *11*, 387–396.
62. Hoermannsperger, G., Clavel, T., Hoffmann, M., Reiff, C., Kelly, D., Loh, G., Blaut, M., Hölzlwimmer, G., Laschinger, M., and Haller, D. (2009). Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. PLoS One *4*, e4365.
63. Dityatev, A., Brückner, G., Dityateva, G., Grosche, J., Kleene, R., and Schachner, M. (2007). Activity-dependent formation and functions of chondroitin sulfate-rich extracellular matrix of perineuronal nets. Dev. Neurobiol. *67*, 570–588.
64. Brückner, G., Grosche, J., Schmidt, S., Härtig, W., Margolis, R.U., Delpech, B., Seidenbecher, C.I., Czaniera, R., and Schachner, M. (2000). Postnatal development of perineuronal nets in wild-type mice and in a mutant deficient in tenascin-R. J. Comp. Neurol. *428*, 616–629.
65. Barritt, A.W., Davies, M., Marchand, F., Hartley, R., Grist, J., Yip, P., McMahon, S.B., and Bradbury, E.J. (2006). Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. J. Neurosci. *26*, 10856–10867.
66. Deepa, S.S., Carulli, D., Galtrey, C., Rhodes, K., Fukuda, J., Mikami, T., Sugahara, K., and Fawcett, J.W. (2006). Composition of Perineuronal Net Extracellular Matrix in Rat Brain: A DIFFERENT DISACCHARIDE COMPOSITION FOR THE NET-ASSOCIATED PROTEOGLYCANS* *The work at Cambridge University was supported by the Wellcome Trust, the UK Medical Research Council, the Christopher Reeve Foundation, and the Henry Smith Charity. The work at Kobe Pharmaceutical University was supported in part by HAITEKU from the Japan Private School Promotion Foundation, Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology (JST) Corporation, and grantsin-aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports of Japan (MEXT). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. J. Biol. Chem. *281*, 17789–17800.
67. Frischknecht, R., Heine, M., Perrais, D., Seidenbecher, C.I., Choquet, D., and Gundelfinger, E.D. (2009). Brain extracellular matrix affects AMPA receptor lateral mobility and short-term synaptic plasticity. Nat. Neurosci. *12*, 897–904.
68. Mauney, S.A., Athanas, K.M., Pantazopoulos, H., Shaskan, N., Passeri, E., Berretta, S., and Woo, T.U.W. (2013). Developmental pattern of perineuronal nets in the human prefrontal cortex and their deficit in schizophrenia. Biol. Psychiatry *74*, 427–435.
69. Bell, H.C., Pellis, S.M., and Kolb, B. (2010). Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. Behav. Brain Res. *207*, 7–13.
70. Himmler, B.T., Pellis, V.C., and Pellis, S.M. (2013). Peering into the dynamics of social interactions: measuring play fighting in rats. J. Vis. Exp. *71*, e4288.
71. Vazquez-Sanroman, D.B., Arlington Wilson, G., and Bardo, M.T. (2021). Effects of Social Isolation on Perineuronal Nets in the Amygdala Following a Reward Omission Task in Female Rats. Mol. Neurobiol. *58*, 348–361.
72. Vita, S.M., Grayson, B.E., and Grill, R.J. (2020). Acute damage to the blood–brain barrier and perineuronal net integrity in a clinically-relevant rat model of traumatic brain injury. Neuroreport *31*, 1167–1174.
73. Corvetti, L., and Rossi, F. (2005). Degradation of chondroitin sulfate proteoglycans induces sprouting of intact purkinje axons in the cerebellum of the adult rat. J. Neurosci. *25*, 7150–7158.
74. Harris, N.G., Mironova, Y.A., Hovda, D.A., and Sutton, R.L. (2010). Pericontusion Axon Sprouting Is Spatially and Temporally Consistent With a Growth-Permissive Environment After Traumatic Brain Injury. J. Neuropathol. Exp. Neurol. *69*, 139–154.
75. Liu, Y.J., Spangenberg, E.E., Tang, B., Holmes, T.C., Green, K.N., and Xu, X. (2021). Microglia Elimination Increases Neural Circuit Connectivity and Activity in Adult Mouse Cortex. J. Neurosci. *41*, 1274–1287.
76. Corrigan, F., Arulsamy, A., Collins-Praino, L.E., Holmes, J.L., and Vink, R. (2017). Toll like receptor 4 activation can be either detrimental or beneficial following mild repetitive traumatic brain injury depending on timing of activation. Brain Behav. Immun. *64*, 124–139.
77. Mychasiuk, R., Hehar, H., Ma, I., Kolb, B., and Esser, M.J. (2015). The development of lasting impairments: a mild pediatric brain injury alters gene expression, dendritic morphology, and synaptic connectivity in the prefrontal cortex of rats. Neuroscience *288*, 145–155.
78. Eyolfson, E., Khan, A., Mychasiuk, R., and Lohman, A.W. (2020). Microglia dynamics in

adolescent traumatic brain injury. J. Neuroinflammation *17*, 326.
79. Carron, S.F., Alwis, D.S., and Rajan, R. (2016). Traumatic Brain Injury and Neuronal Functionality Changes in Sensory Cortex. Front. Syst. Neurosci. *10*, 47.
80. Knouse, M.C., McGrath, A.G., Deutschmann, A.U., Rich, M.T., Zallar, L.J., Rajadhyaksha, A.M., and Briand, L.A. (2022). Sex differences in the medial prefrontal cortical glutamate system. Biol. Sex Differ. *13*, 66.
81. Cowan, C.S.M., Dinan, T.G., and Cryan, J.F. (2020). Annual Research Review: Critical windows - the microbiota-gut-brain axis in neurocognitive development. J. Child Psychol. Psychiatry *61*, 353–371.
82. Shin, J.H., Park, Y.H., Sim, M., Kim, S.A., Joung, H., and Shin, D.M. (2019). Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome. Res. Microbiol. *170*, 192–201.
83. Hynes, W.L., and Walton, S.L. (2000). Hyaluronidases of Gram-positive bacteria. FEMS Microbiol. Lett. *183*, 201–207.
84. Caetano-Silva, M.E., Rund, L., Hutchinson, N.T., Woods, J.A., Steelman, A.J., and Johnson, R.W. (2023). Inhibition of inflammatory microglia by dietary fiber and short-chain fatty acids. Sci. Rep. *13*, 2819.
85. Wenzel, T.J., Gates, E.J., Ranger, A.L., and Klegeris, A. (2020). Short-chain fatty acids (SCFAs) alone or in combination regulate select immune functions of microglia-like cells. Mol. Cell. Neurosci. *105*, 103493.
86. Reichelt, A.C., Lemieux, C.A., Princz-Lebel, O., Singh, A., Bussey, T.J., and Saksida, L.M. (2021). Age-dependent and region-specific alteration of parvalbumin neurons, perineuronal nets and microglia in the mouse prefrontal cortex and hippocampus following obesogenic diet consumption. Sci. Rep. *11*, 5593.
87. Griffiths, B.B., Madden, A.M.K., Edwards, K.A., Zup, S.L., and Stary, C.M. (2019). Age-dependent sexual dimorphism in hippocampal cornu ammonis-1 perineuronal net expression in rats. Brain Behav. *9*, e01265.
88. Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., et al. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. *18*, e3000410.
89. Hoban, A.E., Moloney, R.D., Golubeva, A.V., McVey Neufeld, K.A., O'Sullivan, O., Patterson, E., Stanton, C., Dinan, T.G., Clarke, G., and Cryan, J.F. (2016). Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. Neuroscience *339*, 463–477.
90. Eyolfson, E., Carr, T., Fraunberger, E., Khan, A., Clark, I., Mychasiuk, R., and Lohman, A.W. (2022). Repeated mild traumatic brain injuries in mice cause age- and sex-specific alterations in dendritic spine density. Exp. Neurol. *357*, 114172.
91. Whishaw, I., and Kolb, B. (2005). The Behavior of the Laboratory Rat: A Handbook with Tests (Oxford University Press).
92. Pellis, S.M., and Pellis, V.C. (1990). Differential rates of attack, defense and counterattack during development decrease in play fighting by male and female rats. Dev. Psychobiol. *23*, 215–231.
93. Fuzesi, T., Daviu, N., Wamsteeker Cusulin, J., Bonin, R., and Bains, J. (2016). Hypothalamic CRH neurons orchestrate complex behaviors after stress. Nat. Commun. *16*, 11937.
94. Barrot, M. (2012). Tests and models of nociception and pain in rodents. Neuroscience *211*, 39–50.
95. Zilles, K. (1985). The Cortex of the Rat: A Stereotaxis Atlas (Springer-Verlag).
96. Zhang, N., Yan, Z., Liu, H., Yu, M., He, Y., Liu, H., Liang, C., Tu, L., Wang, L., Yin, N., et al. (2021). Hypothalamic Perineuronal Nets Are Regulated by Sex and Dietary Interventions. Front. Physiol. *12*, 714104.
97. Brewton, D.H., Kokash, J., Jimenez, O., Pena, E.R., and Razak, K.A. (2016). Age-Related Deterioration of Perineuronal Nets in the Primary Auditory Cortex of Mice. Front. Aging Neurosci. *8*, 270.
98. Cohen, J. (1998). Statistical Power Analysis for the Behavioral Sciences, ed. 2nd (Routledge).

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin conjugate Wisteria floribunda agglutinin (WFA) lectin	Thermofisher	Cat# L32481
Polyclonal rabbit anti-parvalbumin	Abcam	Cat# Ab11427; RRID: AB_298032
Streptavidin AF488	Thermofisher	Cat# S11223
Donkey anti-rabbit AF647	Thermofisher	Cat# A-31573; RRID: AB_2536183
Critical commercial assays		
QIAamp Fast DNA Stool Mini Kit	Qiagen	Cat# 51604
Experimental models: Organisms/strains		
Rats / Sprague Dawley	Monash Animal Platform	
Software and algorithms		
Fiji ImageJ	FIJI	https://fiji.sc/
Imaris – FilamentTracer (ver. 9.1.2)	IMARIS	https://imaris.oxinst.com/
Graphpad Prism 8	Monash University	N/A
SPSS Statistics 27	Monash University	N/A
Temperament Analysis Software	Microsoft Excel Plugin	N/A

RESOURCE AVAILABILITY

Lead contact

Professor Richelle Mychasiuk (richelle.mychasiuk@monash.edu) is the lead contact for this manuscript.

Materials availability

This study did not generate any new materials or reagents.

Data and code availability

- All raw data can be found at the open source framework site – https://osf.io/hp43a/?view_only=0d51d293cf5b426bb627343a7116a475.
- This paper does not report original code or other original omics, sequencing, or high-throughput related data. Reagents and antibodies used can be found in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

A total of 172 Sprague Dawley rats were used for this study: 80 adolescents (P21-P65; 40 male and 40 female) and 92 adults (P100-P150; 44 females and 48 males). All animals were obtained from the Monash Animal Research Platform (Clayton, Victoria, Australia). All procedures were approved by the Alfred Medical Research and Educational Precinct (AMREP) Animal Ethics Committee and carried out in accordance with ethics number E/1992/2020/M, and the ARRIVE guidelines.[88] Rats were group housed with animals from the same sex, treatment, and injury – adolescents were housed in groups of four while adults were housed in pairs or threes. Rats of each sex were randomly assigned to one of four groups: Adolescents (Placebo + Sham; $n = 8$), (Placebo + RmTBI; $n = 8$), (Antibiotics + Sham; $n = 12$), (Antibiotics + RmTBI; $n = 12$); Adults (Placebo + Sham; $n = 12$), (Placebo + RmTBI; $n = 12$), (Antibiotics + Sham; $n = 11$ or 12), (Antibiotics + RmTBI; $n = 11$ or 12). Rats were maintained on a standard 12:12 light:dark cycle in a temperature-controlled environment with access to food *ad libitum*.

METHOD DETAILS

At P21 or P100, the rats within the antibiotic treatment groups began supplementation with an antibiotic cocktail in their drinking water comprised of ampicillin (1g/L), vancomycin (500mg/L), imipenem (250mg/L), metronidazole (1g/L) and ciprofloxacin HCL (20mg/L). This 14-day antibiotic treatment regimen has previously demonstrated effective depletion of the gut microbiota.[89] Antibiotic water was administered in place of normal drinking water for a two-week period (P21-P35 for adolescents and P100-P114 for adults). Rats in the placebo groups

were maintained on standard drinking water. Fecal samples were collected from onset of antibiotic administration until completion of the experiment for all cohorts. Fecal samples were frozen at −80°C until needed for analyses. See Figure 1 for overview of experimental designs.

A portion of the fecal samples were thawed, and bacterial DNA was extracted using QIAamp Fast DNA Stool Mini Kit according to the manufacturer protocols (Qiagen, Hilden Germany). The QIAxpert Spectrophotometer was used at an absorbance ratio of A260/280 to determine the quality and concentration of bacterial DNA in the stool samples (Qiagen, Hilden Germany). Figures 2A and 2B demonstrate the loss of bacterial DNA within fecal samples of adolescent and adult rats that consumed the antibiotic drinking water.

Injury induction

Rats were randomly assigned to receive three mTBIs or sham injuries which were induced with the lateral impact device.[3] In previous studies conducted by our laboratory, rats exposed to RmTBIs with this lateral impact device schedule demonstrated impaired motor, cognitive, and social behaviors that often differ between sexes.[42,90] Briefly, rats were lightly anaesthetised for 90 seconds via inhalation of 5% isoflurane in O_2 before being placed chest down on a Teflon® board. The left temporal lobe of the rat was aligned with a small metal helmet to prevent damage to the skull. Pneumatic pressure was used to propel a 50-gram metal projectile towards the helmet forcing the rat into a 180 °rotation. The average speed of impact was 8.39 $\pm$ 0.31 m/s which induced mTBIs at approximately 85.52 Gs. Rats were monitored in a clean cage and the amount of time to flip from a supine to a prone position (time-to-right) was recorded as a measure of a loss of consciousness. The three mTBIs were delivered at P36, P38, and P40 for adolescents and P115, P117, and P119 for adults. Sham rats underwent the same procedure excluding induction of the injury.

Behavioral testing

All behavioral testing occurred during the rat's light phase, between 0800 and 1500. All behavioral tests were administered and scored by individuals blinded to experimental groups.

Elevated plus maze (EPM)

The EPM is used as a measure of anxiety-like behavior.[91] It is performed in an apparatus that is raised 60cm from the ground and is constructed of two open arms (10cm x 44cm), two closed arms (10cm x 44cm x 10cm), and a centre area (12cm x 12cm). The rat is placed in the centre of the maze and allowed to freely explore the area for 300 seconds. The rat's movement is recorded using Topscan software and the amount of time spent in each area is recorded. The EPM was cleaned with 80% ethanol between each rat.

Play behavior and temperament

Play behavior and temperament analyses were used to measure social engagement and activity.[37,42] Prior to testing, rats were isolated from their cage mates for 24 hours. Half of the rats were tail-marked with a black Sharpie® so they could be identified upon reintroduction. The next day, rats were reunited with their cage mates of same sex, injury, and treatment (i.e., in pairs) within a clear Plexiglass box. Testing occurred under dim red-light conditions. Following reintroduction, all experimenters left the room, and the rats were video-recorded for a 15-minute period. The box was cleaned with ethanol between each pair. After testing, all rats were returned to their original cage arrangements for the remainder of the experiment.

For the play and temperament analyses, two separate researchers, blinded to the experimental manipulations, observed the videos. For the play analyses, the video recordings were scored frame by frame for the number of attacks they made to the other rat's nape, and their defensive response to an attack; complete rotation, partial rotation, horizontal rotation, evasion, and no-response, as described by Pellis et al.,.[92] For the temperament analyses the video recordings were reviewed in real time for the frequency and types of behaviors the rats engaged in. For the play and temperament analyses both partners in the dyad were scored for all measures. The specific behaviors analysed with the temperament paradigm are outlined in Table 2. The software designed by Füzesi et al.,[93] records the duration of specific behaviors when the corresponding symbol is selected on the keyboard, as well as the frequency with which the rats alternate between tasks. See Figure 1.

Hot/cold plate

The hot/cold plate (Ugo Basile®, Gemonio, Italy) is used as a measure of thermal nociceptive sensitivity.[94] A temperature regulated circular plate is enclosed within a clear plastic cylinder. The rats are habituated to the device for two days prior to testing, by being placed on the plate at room temperature for two minutes. On testing days, rats are placed initially on the hot plate (52°C). Following a significant recovery period (minimum of two hours), the rats are placed again on the plate, however this time it is set to the cold temperature (2°C). The latency to react (e.g., licks hind paw or jumps) is recorded and the rat is immediately removed from the apparatus and returned to its home cage. The hot/cold plate was cleaned with Virkon between each rat.

Tissue collection

Adolescent rats were euthanised at P65 and adult rats were euthanised at P150. Rats were administered an intraperitoneal injection of pentobarbitone (160mg/kg) and were then transcardially perfused with cold phosphate buffered saline solution and 4% paraformaldehyde (PFA) solution. The rats were decapitated, the brain was removed and weighed prior to being immersed in 4% PFA for storage at 5°C for 24 hours.

After 24 hours, the brains were transferred to 30% sucrose solution for long-term storage. Prior to perfusion, blood was collected from the left atrium in serum separator tubes and allowed to clot at room temperature for 30 minutes before being centrifuged (20 minutes at 1500g at 4°C). Serum was stored at -80°C until analysed by Crux Biolabs (Melbourne, Australia) with the Luminex Service: Milliplex Rat 27plex. Following optimization of dilution factors, all samples were run in duplicates at a 1:2 dilution.

Immunohistochemical staining, imaging, and analyses

The PFC of each brain was sliced into 20-micron coronal sections between bregma +4.20mm and -1.8mm before being mounted onto Superfrost Plus Slides. An immunohistochemical stain was used to label the PNNs and PV expressing interneurons within the mPFC and OFC. The tissue was circled with a hydrophobic pap pen to prevent run off during blocking and antibody treatments. See Table S1 for specific antibody protocols. A coverslip was applied to each slide and adhered to with 3-4 drops of Dako Fluorescent Mounting Media (S3023, Agilent Technologies, Santa Clara, USA). Slides were left in a light proof box at room temperature until dry.

Image collection for cell counts was carried out with Plan Apop 10/0.45 NA objective on Nikon Ti-E fluorescent microscope equipped with sCMOS Andor Zyla camera. Images of the mPFC and OFC were collected and identified as Cg3 and AID according to Zilles rat atlas.[95] Two different channels were used to capture the double label including FITC and Cy5. FITC was used for excitation and detection of emission of photons for Streptavidin 488 and Cy5 was for 647. PNNs, PVs, and PNNs colocalised with PV neurons were counted in ImageJ using the Cell Counter Plugin associated with Fiji by an experimenter blinded to the conditions. To ensure consistency and ensure counts of PNNs and not "loose" fragments of extracellular matrix structures, only PNNs that were complete and not degraded were counted.[96] Colocalised cells were counted using the same program.

Perineuronal net imaging and Imaris branching analysis – adolescents and adults

To investigate the differing effects of RmTBI and microbiome depletion on the PNN lattice structure and its neuroprotection of PV neurons, the arborization of PNNs were analysed. Image collection for visualisation and analysis of PNN branching was carried out with Plan Fluor 40x/1.30 oil objective on a Nikon AR1 Confocal microscope. Images of each adolescent and adult brains were collected in the mPFC and OFC regions identified as Cg3 and AID respectively, according to Zilles rat atlas.[95] The images were analysed in Imaris 9.1.2. To obtain the length of PNN branches, number of branches, and terminal branch points, the FilamentTracer tool in Imaris was used. Images were batch pre-processed by background subtracting both the 488 and 647 channels, as well as applying Gaussian filter to Alexa 488 antibody stain of all images. In FilamentTracer, the Alexa 488 antibody staining was selected to be traced. The centre of PNNs were used as the starting point for each cell. The largest diameter of these starting points was set to 7 μm. Maximum diameter of PNNs was calculated as an average size of all PNNs across images. The thinnest diameter was automatically set to 0.6 μm by Imaris AI. Seed points were automatically detected by Imaris AI. Diameter of sphere region by which Imaris removes seed points around the starting points was set to 15 μm. Disconnected segments were selected to be removed and smoothed with 0.600 μm which was automatically detected by Imaris AI. Absolute thresholding was manually allocated to each image. Dendrite threshold was set to 3.62 μm and max gap length between 488 AlexaFluor pixels set to 7 μm. Only PNNs colocalised with a PV (PNN:PV+) were selected to be analysed. In addition, to ensure consistency, only PNN:PV+ cells that were complete and not degraded were selected for analysis.[97] This ensured investigation of the connectivity and functionality of the PNN lattice structure of PNNs surrounding PVs following injury/treatment, rather than any fragmentation of the ECM lattice. Five PNN:PV+ were selected from both the left and right hemisphere in each brain region. The average length of the branches, number of branches, and terminal branch points for each PNN selected was calculated by Imaris FilamentTracer AI.

QUANTIFICATION AND STATISTICAL ANALYSIS

Power calculations were completed with G*Power software (version 3.1). Based upon Cohen's (1988) criteria[98] and a moderate effect size of 0.40, alpha of 0.05, a power of 0.80, the estimated sample size needed for four-way ANOVAs was N = 131; indicating we were adequately powered with a sample of 172. Therefore, four-way ANOVAs with age, (adolescent; adult), sex (male; female), injury (RmTBI; sham), and treatment (antibiotics; placebo), as factors were run for all behavioral and immunohistochemical measures, except PNN and PV cell counts. For PNN and PV cell counts, three-way ANOVAs were run separately for adults and adolescents with sex (male; female), injury (RmTBI; sham), and treatment (antibiotics; placebo), as factors. All analyses were conducted using SPSS 27.0 for MAC. Post-hoc Bonferroni pairwise comparisons were run when appropriate. All figures are displayed as means ± standard error and statistical significance was considered $p < .05$.