

Title: Investigating the “two-hit hypothesis”: effects of prenatal maternal immune activation and adolescent cannabis use on neurodevelopment in mice

Authors: Elisa Guma ^{1,2,3}, Lani Cupo ^{1,2}, Weiya Ma ⁵, Daniel Gallino ¹, Luc Moquin ⁵, Alain Gratton ^{2,4,5}, Gabriel A Devenyi ^{1,4,5}, M Mallar Chakravarty ^{1,2,4,5,6}

Affiliations

¹ Computational Brain Anatomy Laboratory, Cerebral Imaging Center, Douglas Mental Health University Institute, Montreal, Quebec, Canada

² Integrated Program in Neuroscience, McGill University, Montreal, Quebec, Canada

³ Developmental Neurogenomics Unit, Human Genetics Branch, National Institute of Mental Health, Bethesda, MD, USA

⁴ Department of Psychiatry, McGill University, Montreal Quebec, Canada

⁵ Douglas Mental Health University Institute, McGill University, Montréal, Québec, Canada

⁶ Department of Biological and Biomedical Engineering, McGill University, Montreal, Quebec, Canada

Abstract

Background: Prenatal exposure to maternal immune activation (MIA) and chronic adolescent cannabis use are both risk factors for neuropsychiatric disorders. However, exposure to a single risk factor may not result in major mental illness, indicating that multiple exposures may be required for illness onset. Here, we examine whether combined exposure to prenatal MIA and adolescent delta-9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis, lead to enduring neuroanatomical and behavioural changes in adulthood.

Methods: Mice were prenatally exposed to viral mimetic, poly I:C (5mg/kg), or vehicle at gestational day (GD)9, and postnatally exposed to chronic THC (5mg/kg, intraperitoneal) or vehicle during adolescence (postnatal day [PND]28-45). Longitudinal magnetic resonance imaging (MRI) was performed pre-treatment, PND25, post-treatment, PND50, and in adulthood, PND85, followed by behavioural tests for anxiety-like, social, and sensorimotor gating. Post-mortem assessment of cannabinoid (CB)1 and 2 receptor expressing cells was performed in altered regions identified by MRI (anterior cingulate and somatosensory cortices, striatum, and hippocampus).

Results: Subtle deviations in neurodevelopmental trajectory and subthreshold anxiety-like behaviours were observed in mice exposed to both risk factors. Sex-dependent effects were observed in patterns of shared brain-behaviour covariation, indicative of potential sex differences in response to MIA and THC. Density of CB1 and CB2 receptor positive cells was significantly decreased in all mice exposed to MIA, THC, or both.

Conclusions: These findings suggest that there may be a cumulative effect of risk factor exposure on gross neuroanatomical development, and that the endocannabinoid system may be sensitive to both prenatal MIA, adolescent THC, or the combination.

1. Introduction

Multiple lines of evidence suggest that exposure to environmental risk factors during sensitive periods of brain development alters neurodevelopmental trajectories, increasing risk for neuropsychiatric disorders (1,2). The fetal period is thought to be especially sensitive to environmental risk factors related to neurodevelopmental disorders (3). Exposure to maternal immune activation (MIA) *in utero* has been identified as one such strong risk factor in epidemiological studies, whereby the elevation of maternal proinflammatory cytokines (and not the pathogen itself) interfere with fetal brain development (4,5). However, most maternal infections do not lead to disorders in the offspring (4). While multiple exposures may increase risk for major mental illness, MIA appears to act as a “disease primer” making individuals more susceptible to the effects of other risk factors later in life (6). In support of this hypothesis, previous work from our group found that mice prenatally exposed to MIA in early gestation (gestational day [GD]9) exhibited altered neurodevelopment, characterized by the emergence of neuroanatomical and behavioural abnormalities in the adolescent period (7), a time in which many neuropsychiatric illnesses emerge (8). However, these alterations normalized in adulthood, strongly suggesting that MIA-exposure in isolation was insufficient to create neurodevelopmental abnormalities that persisted into adulthood (7).

Cumulative exposure to drugs of abuse, particularly during the critical neurobiological remodeling that occurs during adolescence, may increase the impact of risk factors (8). Prospective, longitudinal, epidemiological evidence suggests that adolescent cannabis use may increase risk for psychosis, amongst other neuropsychiatric disorders (9–11). This is particularly evident if use begins before the age of 16 years (12). The age of sensitivity corresponds to the developmental period of the endocannabinoid system (specifically the cannabinol [CB]-1 receptor), which plays a fundamental role in brain development (13). The main psychoactive component of cannabis is delta-9-tetrahydrocannabinol (THC), which activates the endocannabinoid system via cannabinoid (CB)1 (and to some extent CB2) receptors in the brain (14). Activation of CB-1 receptors is thought to create an imbalance in excitatory-inhibitory signaling in the brain by influencing the GABA-ergic, glutamatergic, and dopaminergic systems (8). Even if cannabis exposure in adolescence increases risk for psychosis, only a minority of cannabis users develop psychosis, suggesting that cannabis use may interact with pre-existing vulnerabilities, such as prenatal MIA-exposure, and increase the risk for neuropsychiatric illness (15,16).

In this study, we leverage the strategy of performing longitudinal assays of the brain using *in vivo* magnetic resonance imaging (MRI) throughout development, and multi-behavioural testing in adulthood to investigate the potential synergistic effects of prenatal MIA-exposure in early gestation (GD9), followed by chronic adolescent THC exposure (postnatal day [PND] 28-45) in mice. Our previous work demonstrated that

exposure to MIA alone leads to neuroanatomical remodeling in fetal stages (17) persisting to neurodevelopmental and behavioural abnormalities that appeared in adolescence and normalized by adulthood (7). Based on these findings, we hypothesized that exposure to each risk factor alone would result in subthreshold neuroanatomical and behavioural alterations in offspring, but that the combined exposure would lead to enduring neuroanatomical and behavioural changes in adulthood. Our neuroanatomical results partially support this hypothesis, however, investigation of CB1 and 2 receptor density suggests that there may be similar susceptibility to each risk factor.

2. Methods

2.1 Animals

Timed-mating was used to generate pregnant dams (injected intraperitoneally (i.p.) with poly I:C (POL; P1530-25MG sodium salt TLR ligand tested; Sigma Aldrich; 5mg/kg) or vehicle (SAL; 0.9% NaCl) at GD 9 (10 POL and 8 SAL dams; breeding details in **supplement 1.1**). Offspring were then randomly assigned to receive daily injections (i.p.) of THC (1:1:18 THC 5 mg/kg [Cayman Chemicals], cremophor [Sigma Aldrich], saline solution) or vehicle (1:18 cremophor:saline) throughout the adolescent period (PND 28-45). The dosage was chosen to model moderate daily use, with a potency roughly equivalent to one average-sized cannabis cigarette ("joint") with 11% THC content, scaled to the weight of the mouse (18–20) (weight data in **supplement 1.5.1, 2.3 & supplementary figure S1**). This resulted in 4 groups: (SAL-SAL, SAL-THC, POL-SAL, POL-THC). In a separate group of dams, the immunostimulatory potential of our poly I:C was confirmed (n=4 GD9-POL, n=5 GD9-SAL; **supplement 1.2 for methods, 2.1 and supplementary table 1 for results**).

We validated our physiologically compatible THC solution (1:1:18 THC:cremophor:saline at 2.5, 5, or 10 mg/kg of THC; Cayman Chemicals, Ann Arbor, MI, USA) by measuring plasma levels of THC and two THC metabolites (11-hydroxy-delta-9-THC and 11nor-9carboxy delta-9-THC) in a separate cohort of mice using gas-chromatography mass spectrometry (GCMS) (**supplement 1.3 for methods, 2.2 and supplementary table 2 for results**). A license to possess cannabis for research purposes (LIC-ZULO6PJ7NH-2019) and an import permit (IMP-0257-2019) to purchase cannabis were obtained from Health Canada. All procedures were approved by McGill University's Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

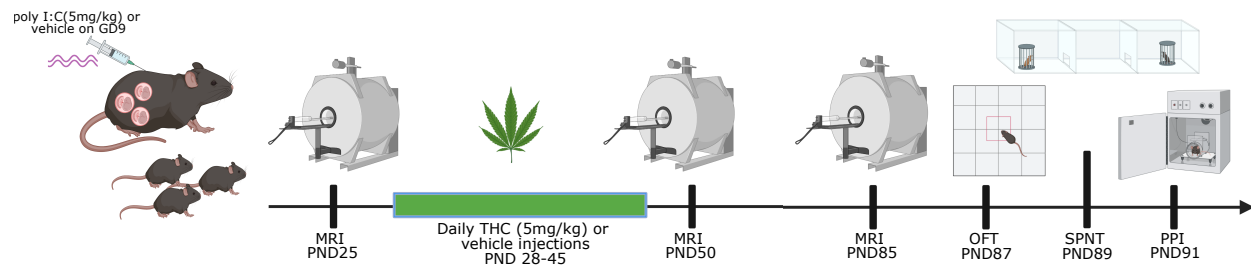


Figure 1. Experimental timeline. Pregnant dams were injected (i.p.) with poly I:C (5mg/kg) or vehicle (0.9% sterile NaCl solution) at gestational day (GD) 9. Offspring were weaned and sexed on postnatal day (PND) 21. Longitudinal structural magnetic resonance imaging (MRI) was performed at PND 25, 50, and 85 (denoted by the MRI image). Following the first scan on PND 25, offspring were randomly assigned to receive daily injections (i.p.) of delta-9-tetrahydrocannabinol (THC; 5 mg/kg) or vehicle (1:18 cremophor:saline) from PND 28-45. Two days following the final scan on PND85, mice were assessed in the open field test (OFT), the social preference/novelty test (SNPT), and the prepulse inhibition task (PPI), with a 2-day rest between tests.

2.4 Magnetic resonance imaging

2.4.1 Acquisition and image processing

Longitudinal T1-weighted structural MRIs were acquired *in vivo* at postnatal day (PND) 24-26 (~childhood; pretreatment), 47-51 (~adolescence; post-treatment), 80-86 (~adulthood) (21,22) in anesthetized offspring (7,23-26). Anesthesia was induced with 3% isoflurane in oxygen and a (0.075 mg/kg bolus) dexmedetomidine injection. Anesthesia was maintained during the scan between 1.5-0.5% isoflurane, and a constant infusion of dexmedetomidine (0.05mg/kg/h continuous mg/kg during scan). Scans were conducted in a 7 Tesla Bruker, 30 cm bore magnet with AVANCE electronics, using a cryogenically cooled surface coil. A 3D FLASH (Fast, Low Angle SHot) sequence was used with a flip angle of 20 degrees and TE/TR of 4.5/20 ms (2 averages, ~14 minutes). Voxel size was 100 μ m, isotropic, in a matrix of 180 by 160 by 90. Structural images were visually inspected for quality (3 removed due to motion or hydrocephalus, yielding n=242 images). Images were processed using a longitudinal two-level deformation based morphometry technique using the ANTs toolkit (<http://stnava.github.io/ANTs/>) for linear and non-linear registration (27) as described in our previous work (28) (**Supplement 1.4**).

Table 1. Sample size per timepoint following quality control. Postnatal day (PND); poly I:C (POL); saline (SAL); delta-9-tetrahydrocannabinol (THC); male (M); female (F).

	PND 25		PND 50		PND 85		
	Males	Females	Males	Females	Males	Females	Litters
SAL-SAL	10	9	10	9	10	9	7
POL-SAL	10	10	11	10	11	10	9
SAL-THC	10	8	10	8	9	9	7
POL-THC	12	9	11	11	11	11	8

2.4.2 Statistical Analyses

A voxel-wise linear mixed-effects model (R-3.5.1, RMINC-1.5.2.2, lme4 1.1-21) was used to examine a group by age (age as a natural second order spline) interaction covarying for sex (with subject and litter as random intercepts). The False Discovery Rate (FDR)(29) correction was applied to control for multiple comparisons across voxels. First, we examined whether each risk factor alone had significant effects on neurodevelopment. We performed pairwise comparisons to determine the impact of individual or combined risk factors while holding SAL-SAL as the reference group. Given evidence of sex-differences in response to MIA and THC, a sex-by group-by-age interaction was investigated post-hoc (**Supplement 1.6.2**) (30,31).

2.5 Behavioural testing

2.5.1 Data acquisition

Following the final scan at PND ~85, testing was performed to assay several behaviours relevant to neurodevelopmental and neuropsychiatric disorders. These included the open field test to assess exploratory and anxiety-like behaviours, wherein the distance traveled in the anxiogenic center zone was examined relative to the total distance traveled. Next, the three chambered social task was performed to assay both social preference (i.e., preference for a novel mouse relative to a nonsocial object), and social novelty (i.e., preference for a novel mouse relative to a familiar mouse) behaviours. Finally, sensorimotor gating to an acoustic startle was assessed with the prepulse

inhibition (PPI) task, wherein a prepulse tone is presented (at various levels, across multiple trials) to determine whether it is successful at dampening the startle reaction to the acoustic stimulation. A 2-day resting phase was allowed between tests; details for all behavioural tests are provided in **supplement 1.5**.

2.5.2 Statistical Analyses

We used linear mixed-effects models for adult behavioural data, with group and sex as fixed effects, and litter as a random intercept. A Bonferroni correction was applied (4 tests: $\alpha = 0.05/4 = 0.0125$ set as significance threshold, uncorrected p-values, and corrected p-values reported). Sex differences were investigated post-hoc (**supplement 1.6.2**).

2.6 Partial Least Squares Analysis

To assess brain-behaviour relationships in our mice, we used a partial least squares (PLS) analysis. This multivariate technique allows us to relate two sets of variables by finding the optimal weighted linear combinations of variables that maximally covary with each other (32–34) (details in **supplement 1.7**). We performed two analyses: for the first analysis we used the final MRI timepoint cross-sectional volume data (PND 85; brain matrix); for the second analysis we calculated the within-subject voxel-wise brain volume change from PND 50 to 85 (i.e. immediately post-treatment, to the final timepoint; brain matrix; [https://github.com/CoBrALab/documentation/wiki/Create-a-nifti-of-within-subject-change-\(2-timepoints,-output-from-dbm\)](https://github.com/CoBrALab/documentation/wiki/Create-a-nifti-of-within-subject-change-(2-timepoints,-output-from-dbm))) to use as the input brain data. Both were evaluated against behavioural metrics derived from the three tests described above (**2.5**).

2.7 Immunohistochemistry

Two days following the last behavioural test, mice were perfused (as described in **Supplement 1.8.1**), brains were extracted, sucrose protected, and sectioned coronally at 50 μm -thickness in preparation for immunofluorescent staining [SAL-SAL (n=5), POL-SAL (n=6), POL-THC (n=6) and SAL-THC (n=6)]. Regions of interest, selected based on MRI results and MIA/THC literature, included the primary somatosensory cortex and striatum, two regions that do not show normalization in adulthood in the POL-THC group, and have been associated with sensitivity to these risk factors (7,35). Additionally, we investigated the anterior cingulate cortex (ACC), which shows post treatment effects and a normalization in adulthood. Finally, we focused on the hippocampus, a region shown to be affected following MIA-exposure by our group and others, as well as by THC exposure (7,36–38).

We examined CB1- and CB2-immunoreactive (IR) cells in our ROIs and captured average optical intensity of axon terminals per counting area (Fiji ImageJ (39); values ranged from 1-256) in all regions of interest (striatum Bregma +0.38 mm, ACC Bregma -0.94 mm, somatosensory cortex Bregma -0.94 mm, dentate gyrus (DG) and CA1 molecular layers of the hippocampus Bregma -2.80 mm). In all regions (except the striatum and CA1), cell (neuron) density (cell number/area) as well as fiber density (optical intensity/area) were quantified (details in **Supplement 1.8.2**). Although we did not co-stain for a neuron marker, such as NeuN, based on the morphology of stained cells and the location it is likely that the cells stained were neurons (ex: pyramidal shape in dentate gyrus) (40,41). Differences in CB1- and CB2-IR cell (neuron) density and/or fiber density due to pre- and postnatal exposures were assessed with a two-way ANCOVA, with a prenatal [SAL/POL] by adolescent [SAL/THC] treatment interaction (sex [M/F] and hemisphere [L/R] were included as covariates). Post-hoc, pairwise testing was performed using Tukey HSD (significance level was set at $p < 0.05$).

3. Results

3.1. Alterations in neurodevelopmental trajectories

3.1.1 Prenatal MIA-exposure

Overall differences between groups are summarized in **supplementary figure S2 & supplement 2.4**. Effects of MIA exposure on offspring development were assessed by comparing POL-SAL to SAL-SAL. The group by age interaction modeled with a second-order natural spline (ns) ($group:ns(age,2)1$) interaction was significant in a limited number of voxels in the cerebellum, at a lenient 20% FDR threshold ($t=4.662$; **Figure 2AB**). To determine whether some of the changes we observed in our previous work (7) were recapitulated here at a subthreshold level, we investigated changes in trajectory at a threshold of uncorrected $p < 0.01$. At this exploratory level, we observed that POL exposed offspring had a smaller volume at PND 25, relative to saline, which overshot (was relatively larger) at PND 50, and then normalized at PND 85 (7). This was observed in regions such as the lateral septum, striatum, hippocampus, and somatosensory cortex. Post-hoc investigation of sex differences revealed no effects ($<20\%$ FDR).

3.1.2 THC

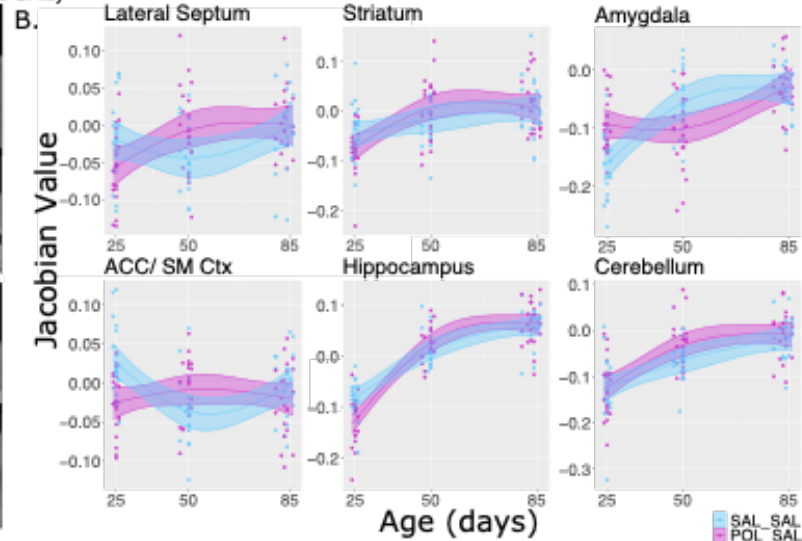
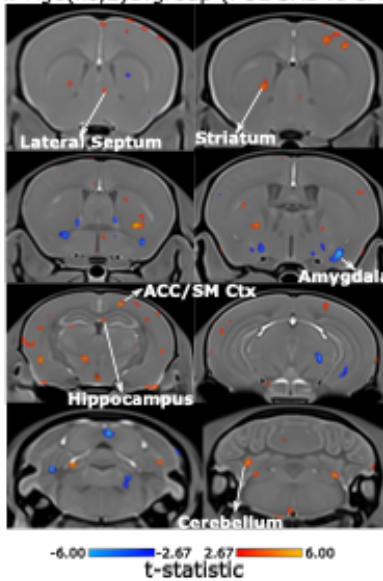
We observed a significant group by age ($group:ns(age,2)1$) interaction ($t=5.16$, 5%FDR; **Figure 2CD**) between SAL-SAL and SAL-THC groups. To evaluate more subtle neuroanatomical changes, brain maps were investigated at a more lenient threshold of 20% FDR. We observed the greatest deviations in SAL-THC mouse trajectory relative to

SAL-SAL at the PND 50, or post-treatment timepoint. This suggests that the chronic THC exposure was inducing some brain remodeling, however, we see that the trajectories normalize in later adulthood (PND 85), which may indicate that the brain had a chance to self-correct following a washout period from THC. In the medial preoptic area, somatosensory cortex, globus pallidus, third ventricle, and reticular nucleus, the SAL-THC offspring had larger volumes at PND50, whereas in the CA1 region of the hippocampus, they had smaller volumes. Post-hoc investigation of sex differences revealed no significant sex by group by age interactions.

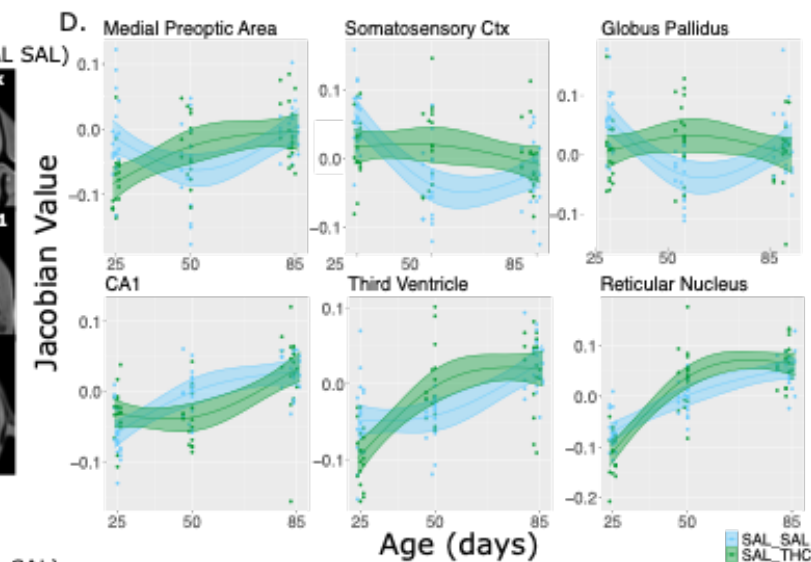
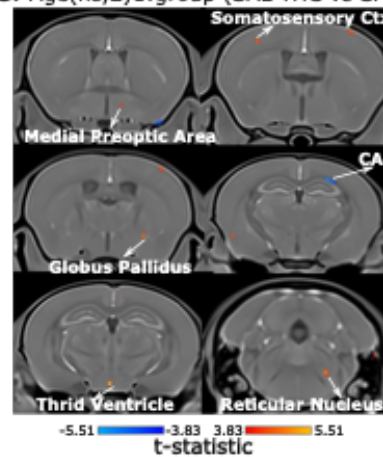
3.1.3 Combined MIA-THC

A significant group by age (*group:ns(age,2)1*) interaction ($t=3.63$, 5%FDR) was observed when comparing POL-THC to SAL-SAL trajectories. Again, we observed a significant deviation in trajectory in the post-treatment, PND50 timepoint wherein increased volume in the POL-THC group was observed in the nucleus accumbens, striatum, somatosensory, anterior cingulate, and entorhinal cortices, while decreases were observed in the subiculum. Interestingly, this deviation in trajectory normalized in several regions, including the nucleus accumbens, anterior cingulate and entorhinal cortices, while the deviation was sustained in later adulthood (PND85) in the striatum, somatosensory cortex, and subiculum. This suggests that some regions may recover following combined exposure, while others remain affected throughout development (**Figure 2 EF**). Investigation of the effects of POL-THC relative to POL-SAL are summarized in **supplement 2.5 and supplementary figure S3**. Post-hoc investigation of sex differences in this group revealed a significant three way interaction (*group:ns(age,2)1:sex*) ($t=4.238$, 5%FDR) (described in **supplement 2.7.1, supplementary figure S5**).

A. Age(ns,2)1:group (POL SAL vs SAL SAL)



C. Age(ns,2)1:group (SAL THC vs SAL SAL)



E. Age(ns,2)1:group (POL THC vs SAL SAL)

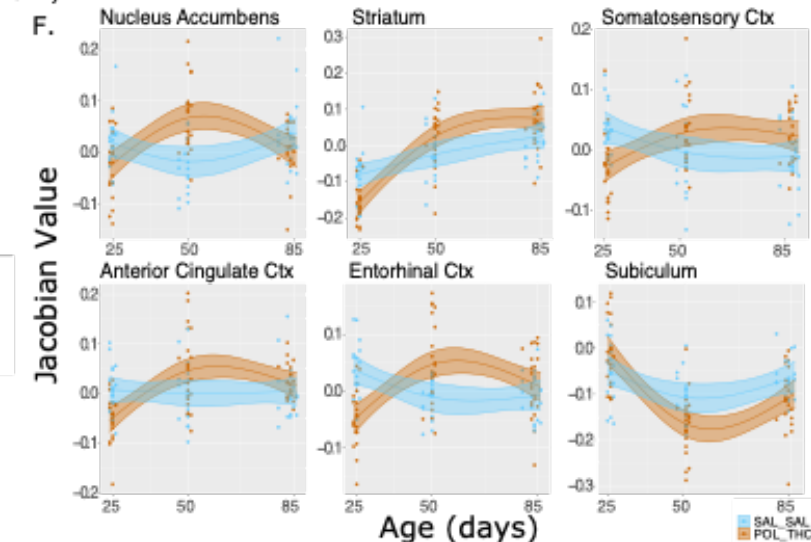
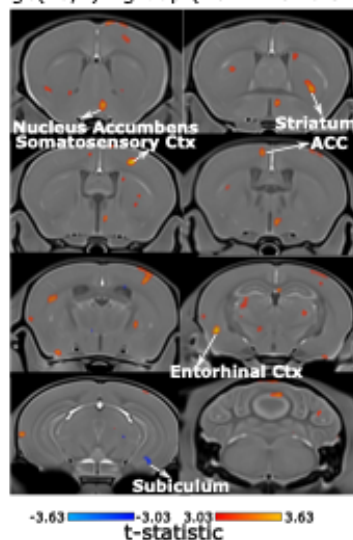


Figure 2. Neuroanatomical alterations due to pre- and/or postnatal risk factor exposure from pre-adolescence to adulthood. **A.** Exploration of neuroanatomical alteration due to prenatal MIA-exposure alone at an uncorrected threshold ($p < 0.01$). T-statistic map of group (POL-SAL vs SAL-SAL) by age (first order natural spline (ns) of age) ($t = 2.67$, $p = 0.01$). **B.** Plot of peak voxels selected from regions of interest highlighted in **A**, wherein age is plotted on the x-axis, and the absolute Jacobian determinants plotted on the y-axis. Here a value of 1 means the voxel is no different than the average, anything above one is relatively larger, and below 0 is relatively smaller. Trajectories reflect the statistical model used (see **2.4.2**). **C.** Neuroanatomical alteration due to adolescent THC-exposure. T-statistic map of group (SAL-THC vs SAL-SAL) by age (first order natural spline of age) thresholded between 5% FDR (top, $t = 5.51$) and 20% FDR (bottom, $t = 3.83$). **D.** Plot of peak voxels selected from regions of interest highlighted in **C**, wherein age is plotted on the x-axis, and the absolute Jacobian determinants plotted on the y-axis. Trajectories reflect the statistical model used (see **2.4.2**). **E.** Neuroanatomical alteration due to combined prenatal MIA-exposure and adolescent THC-exposure. t-statistic map of group (SAL-THC vs SAL-SAL) by age (first order natural spline of age) thresholded between 5% FDR (top, $t = 3.63$) and 10% FDR (bottom, $t = 3.03$). **F.** Plot of peak voxels selected from regions of interest highlighted in **E**, wherein age is plotted on the x-axis, and the absolute Jacobian determinants plotted on the y-axis. Trajectories reflect the statistical model used (see **2.4.2**).

3.4 Alteration in behavior

Overall, none of the behavioural effects observed survived multiple comparisons correction. No significant effects due to prenatal MIA exposure (POL-SAL vs. SAL-SAL) or THC exposure (SAL-THC vs. SAL-SAL) were observed in the open field test, PPI, or social preference or novelty behaviours ($q > 0.0125$). Finally, for the combined exposure group (POL-THC vs. SAL-SAL), a subthreshold increase in the relative distance traveled in the center zone of the open field was observed ($t = 2.344$, $p = 0.041$, $q = 0.164$). No significant effects were observed in sensorimotor gating overall, however, when investigating effects over increasing prepulse level, there was a subthreshold interaction ($t = 2.239$, $p = 0.027$, $q = 0.108$) wherein POL-THC offspring were impaired at across prepulse tones relative to SAL-SAL offspring. Again, no social deficits were observed for either preference or novelty (**Supplementary figure 4**). Finally, investigating THC effects within the prenatal POL groups (POL-SAL vs. POL-THC) revealed no significant differences, other than similar relationships in PPI reported above ($t = 2.239$, $p = 0.027$, $q = 0.108$). Comparison between POL-THC and POL-SAL detailed in **Supplement 2.6.1, Supplementary figure S4, Supplementary table 3**). Behavioural results are summarized in **Supplementary table 3**. No significant sex-by-group interactions were observed (**Supplement 2.6.2, Supplementary figure S6; supplementary table 4**).

3.5 Brain-behaviour covariation

3.5.1 PND85 brain-behaviour

Using PLS to examine adult, whole-brain, voxel-wise volume differences and 18 behavioural metrics across our 3 main tests (including sex and litter size), we identified one significant LV ($p < 0.00001$, percent-covariance=35%; **Figure 3A**). The observed pattern identified increased pancortical, striatal, thalamic, and cerebellar volumes to be associated with decreased interactions in the social preference test, decreased sensorimotor gating abilities, highly expressed in females (**Figure 3BC**). Correlations between brain-behaviour scores suggests that all female offspring heavily express the brain-behaviour pattern, as do the SAL-SAL male offspring, but all male offspring exposed to either a single (prenatal MIA, adolescent THC) or combined risk factors (both MIA and THC) do not express the pattern. This is indicative of potential sex differences in response to risk factor exposure, with greater group differences in male offspring (**Figure 3D**).

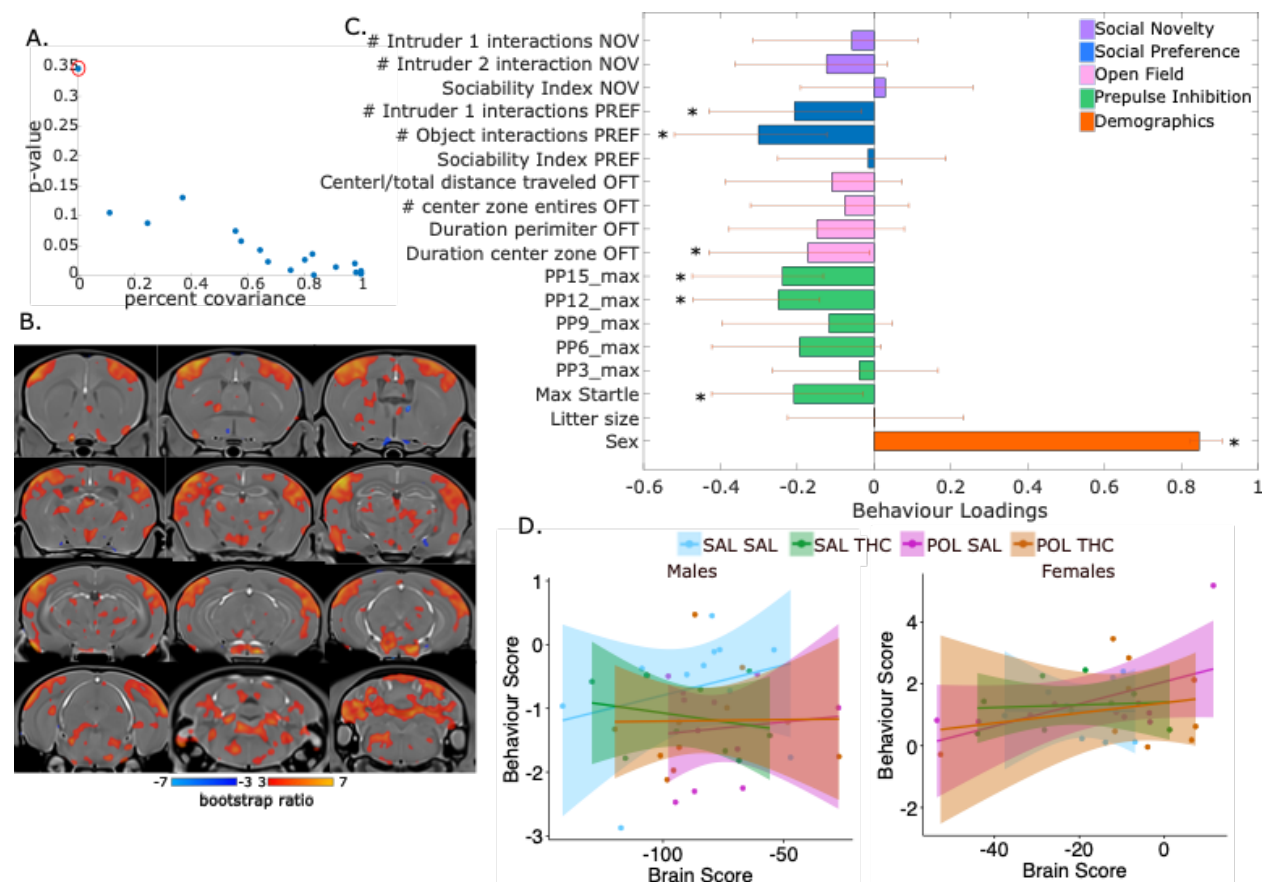


Figure 3. Partial least squares (PLS) analysis results for the first latent variable (LV1) comparing adult (PND85) brain and behaviour. **A.** Covariance explained (y-axis) and permutation p-values

(x-axis) for all 18 LVs in the PLS analysis. LV1 is circled in red ($p < 0.00001$, % covariance=35%) and was chosen for subsequent investigation based on the covariance explained and behavioural relevance of results. **B.** Brain loading bootstrap ratios for the LV1 deformation pattern overlaid on the population average, with positive bootstrap ratios in orange-yellow (indicative of larger volume), and negative in blue (indicative of smaller volume). Colored voxels make significant contributions to LV1. **C.** Behaviour weight for each behavioural measure included in the analysis showing how much they contribute to the pattern of LV1. Singular value decomposition estimates the size of the bars whereas confidence intervals are estimated by bootstrapping. Bars with error bars that cross the 0 line should not be considered significant, marked by *. **D.** Correlation of individual mouse brain and behaviour scores, colour coded by treatment group with a trend line per group split by sex. Male SAL-SAL offspring, and all female offspring express the pattern more strongly; male offspring exposed to any of the risk factors express the pattern less strongly. SAL-SAL (cyan), POL-SAL (magenta), SAL-THC (green), POL-THC (orange).

3.5.2 Association between volume change from post-treatment to adulthood and adult behaviour

Next, we used PLS to examine within-subject volume change (value representing difference in volume between timepoints) from the post-treatment timepoint (PND50) to the adult timepoint (PND85) at a voxel level across the brain and the same 18 behavioural and demographic metrics as in **3.5.1**. We identified two significant LVs (LV1: $p < 0.00001$, %covariance=24%; LV2: $p = 0.01$, %covariance=19%; **Supplementary figure S7 & supplement 2.7**). For LV1, the observed pattern identified increased somatomotor, striatal, hippocampal, and cerebellar volumes to be associated with fewer interactions in the social preference test, greater impairments in sensorimotor gating, and to be more expressed in females, similar to the pattern observed in **3.5.1 (Figure 4AB)**. Correlations between brain-behaviour scores show that females express this pattern, while males show some group differences, with greater loading in SAL-SAL and POL-THC groups (left two plots **Figure 4C**). Removal of outliers (right two plots **Figure 4C**) suggests that male offspring express this pattern, as do females, except for the SAL-THC female offspring, who seem to express this pattern less, indicative of potential sex differences.

The pattern captured by LV2 reflects decreased cortical (somatomotor) and cerebellar volume and increased thalamic volume to associate with increased social novelty interactions, increased locomotion in the open field test, and to be more associated with male mice from smaller litters (**Figure 4EF**). Brain-behaviour score correlations suggest that all offspring express this pattern, with a potential difference in the POL-THC exposed female offspring (left two plots **Figure 4G**). Removal of outliers (right two plots **Figure 4G**) confirms this association.

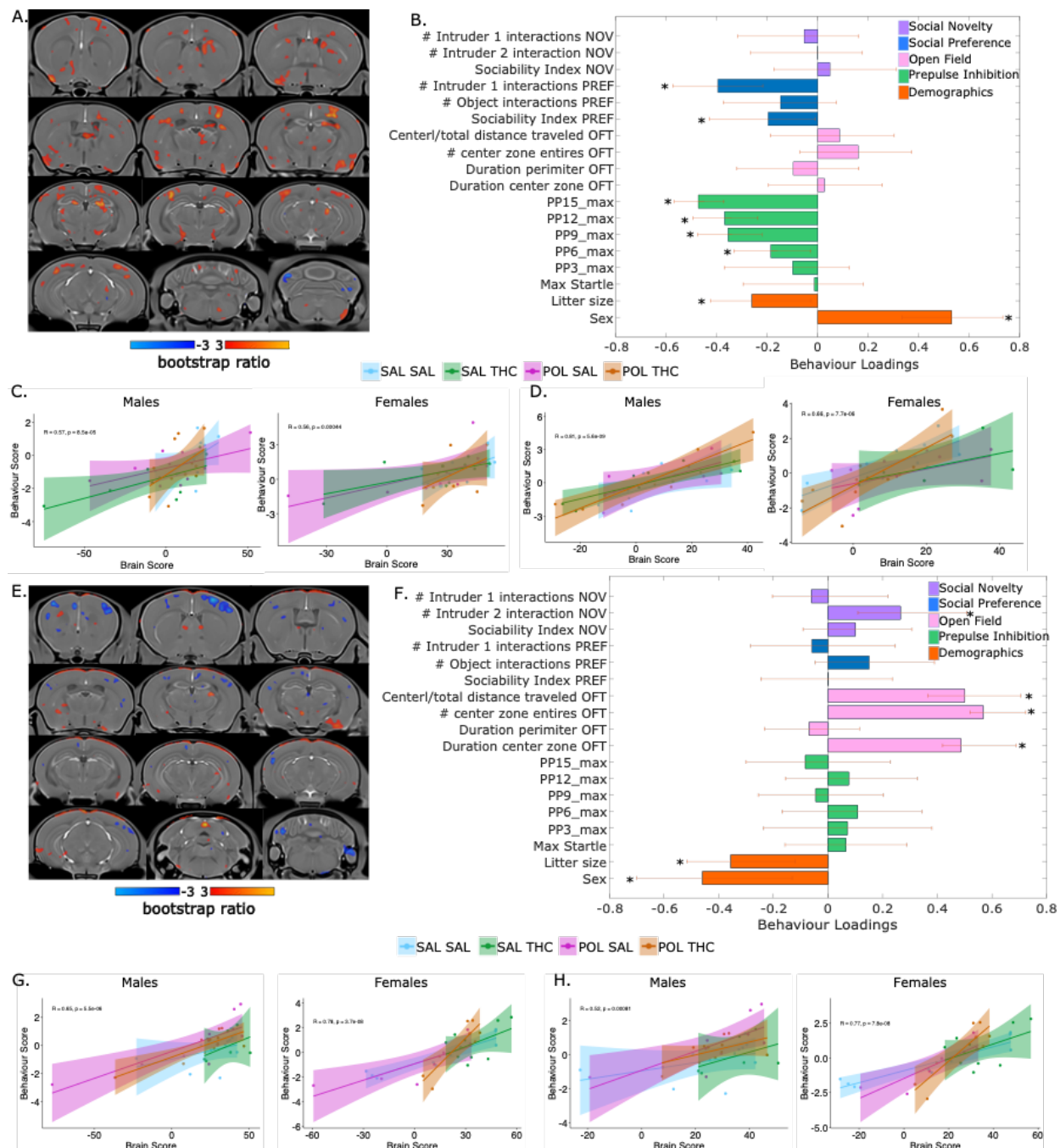


Figure 4. Partial least squares (PLS) analysis results for the first and second latent variables (LV) comparing the difference between PND50 and PND85 voxel-wise brain volume, and behaviour. **A.** Brain loading bootstrap ratios for the LV1 deformation pattern overlaid on the population average, with positive bootstrap ratios in orange-yellow (indicative of positive change in volume), and negative in blue (indicative of negative change in volume). Colored voxels make significant contributions to LV1. **B.** Behaviour weight for each behavioural measure included in the analysis showing how much they contribute to the pattern of LV1. Singular value decomposition estimates the size of the bars whereas confidence intervals are estimated by bootstrapping. Bars with error bars that cross the 0 line should not be considered significant and are marked by *. **C.** Correlation

of individual mouse brain and behaviour scores, color coded by treatment group with a trend line per group split by sex. **D.** Correlation of individual brain-behaviour scores with the exclusion of outliers (determined using the following cut offs: minimum (quartile 1 - 1.5x interquartile range) and maximum (quartile3 + 1.5x interquartile range). **E.** Brain loadings for LV2 (as in **A**). **F.** Behaviour loadings for LV2. **G.** Correlation for individual mouse brain and behaviour score, coloured by treatment and split by sex. **H.** Correlation of individual brain-behaviour scores with the exclusion of outliers. SAL-SAL (cyan), POL-SAL (magenta), SAL-THC (green), POL-THC (orange).

3.6 CB1- and CB2-IR neurons and axonal fiber and density are decreased across all brain regions due to exposure to any risk factor

Neuron cell density and axonal fiber density for CB1- and CB2-IR cells were significantly reduced across all brain regions for any of the three risk-factor exposed groups, relative to the SAL-SAL controls. A significant pre- by postnatal treatment interaction was observed for CB1-IR ($F(5,40)=12.651$, $p=0.00098$) and CB2-IR density ($F(5,40)=6.051$, $p=0.01832$). Similar effects were observed in the somatosensory cortex for CB1- ($F(5,40)=41.537$, $p=1e-06$) and CB2-IR density ($F(5,40)=14.823$, $p=0.000416$). In both these regions, Tukey's post-hoc pairwise testing confirmed that POL-SAL, SAL-THC, and POL-THC all had significantly decreased CB1- and CB2-IR density relative to the SAL-SAL controls ($p>0.05$). In the striatum, the same pattern was observed for fiber density measures (cell density not quantified), with a significant pre- by postnatal treatment interaction for both CB1($F(5,40)=4.632$, $p=3.80e-05$) and CB2-IR ($F(5,40)=4.478$). As with the ACC and somatosensory cortex, in the striatum, all three risk factor groups also had significantly reduced CB1-IR and CB2-IR fiber density relative to the SAL-SAL controls ($p<0.01$) (**Figure 5**).

In the DG molecular layer, a subthreshold effect for CB1-IR ($F(5,40)= 4.027$, $p=0.052$) and significant effect for CB2-IR ($F(5,40)=17.807$, $p=0.000136$) cell density was observed. Tukey's post hoc pairwise test revealed a significant decrease for POL-SAL relative to SAL-SAL for CB1-IR density, ($p=0.0162$), and a significant decrease in CB2-IR density for all 3 risk factor groups relative to SAL-SAL ($p<0.001$) (**Figure 5**). In these regions, similar patterns were observed for fiber density measures (presented in **Supplement 2.8**). Similarly, there was a significant interaction in CA1 molecular layer of the hippocampus for fiber density (cell density not quantified) for CB1-IR ($F(5,40)= 6.184$, $p=0.0172$) and CB2-IR ($F(5,40)= 6.191$, $p=2.55e-07$). CB1-IR fiber density was significantly reduced in POL-SAL relative to SAL-SAL ($p<0.05$), while all three risk factor groups had reduced CB2 fiber density relative to SAL-SAL ($p<0.00001$) (**Figure 5**). Summary statistics available in **Supplementary tables 5-20**.

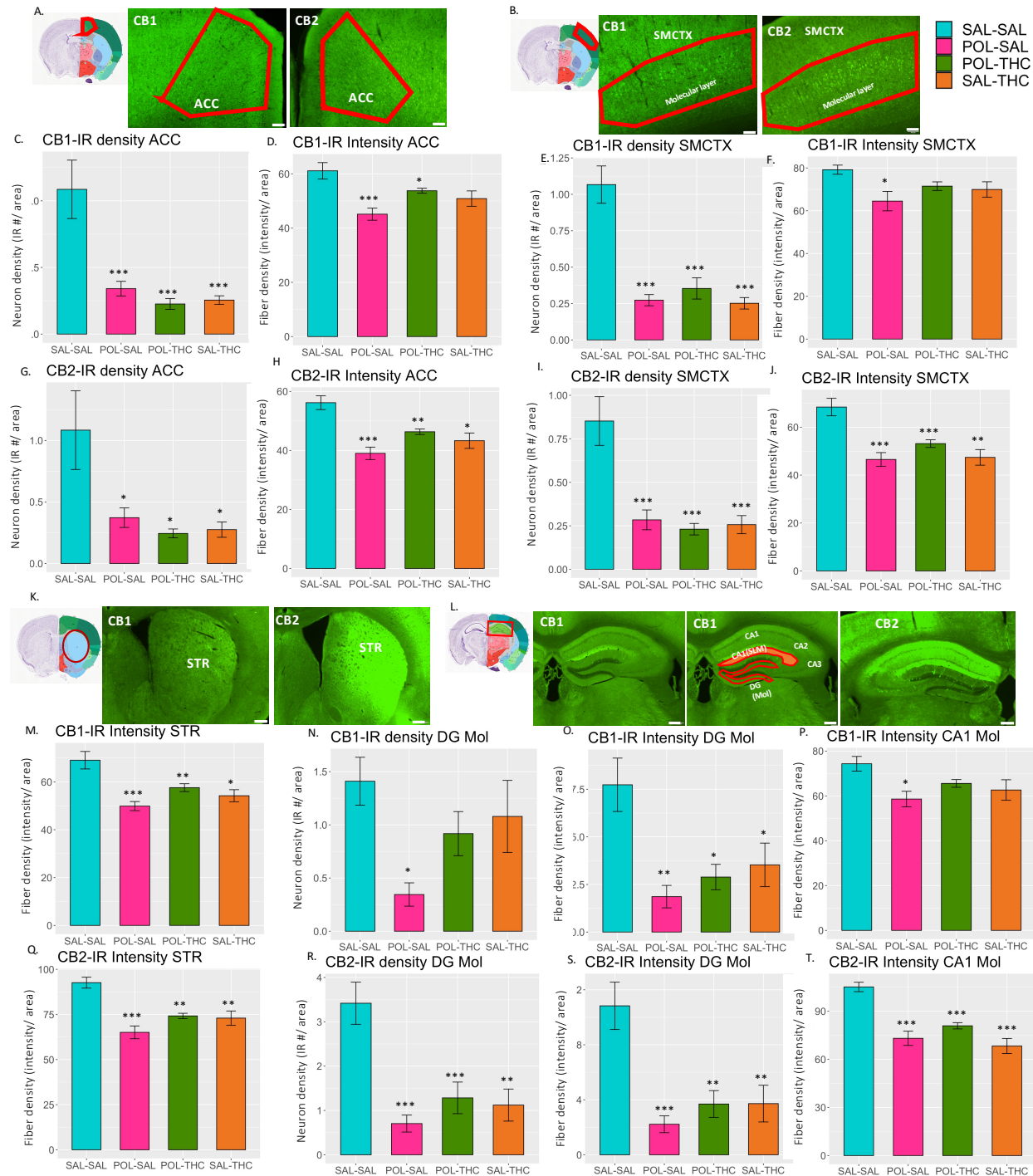


Figure 5. Decreases in the neuron cell and axonal fiber density of cells expressing CB1 and CB2 receptors due to risk factor exposure. **A.** The reference slice from the Allen Brain mouse reference atlas (<https://mouse.brain-map.org/static/atlas>), plus representative images of CB1 and CB2 stains are presented for the anterior cingulate cortex (ACC), with neuron cell density (**C**) and fiber density (**D**) for CB1 expression and CB2 expression (**G** neuron cell density and **H** fiber density). The same pattern is used for the somatosensory cortex (SMCTX; **B**) with CB1 and CB2 neuron cell density and fiber density (**E** CB1, **I** CB2). For the striatum (STR; **K**) CB1 and CB2 fiber

densities are represented in **M** and **Q**, respectively. Finally, for the hippocampus (**L**), results for the dentate gyrus molecular layer CB1 neuron cell density (**N**) and fiber density (**O**) are available for CB1 (**NO**) and CB2 (**RS**), while results for the CA1 molecular layer CB1 and CB2 fiber density are represented in **P** and **T**, respectively. Scale bars represent 100 μm for all regions except the hippocampus, where they represent 200 μm . * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ for Tukey's HSD.

4. Discussion

Converging lines of evidence across epidemiological and experimental studies have identified prenatal MIA-exposure and adolescent THC exposure as risk factors for neuropsychiatric illness. However, most individuals exposed to either risk factor alone do not develop psychiatric or neurodevelopmental disorders, which suggests that multiple exposures within an individual are required for disease onset (4,42). The work presented here is aimed at experimentally testing the multiple exposure hypothesis, by exposing MIA-primed offspring to a second hit: chronic adolescent THC exposure. Our results suggest that exposure to either prenatal MIA or adolescent THC were not sufficient to induce enduring neuroanatomical or behavioural alterations in adulthood, while combined exposure was, in some regions but not others. Behaviourally, only subtle, subthreshold alterations in anxiety-like and sensorimotor gating behaviours were observed in the group exposed to both risk factors. Interestingly, sex-differences were observed in patterns of brain-behaviour covariation, both for adult brain volume and for the within-subject brain volume change from post-treatment to adulthood, and adult behaviour. In several brain regions showing susceptibility to risk factor exposure (anterior cingulate and somatosensory cortices, striatum, hippocampus), striking decreases in the expression of CB1- and CB2-IR cells were observed in offspring exposed to either prenatal MIA, adolescent THC, or both, indicating that the effects of these risk factors may converge on the endocannabinoid system, amongst others. Our results show that a single risk factor may not be sufficient to cause enduring changes in gross neuroanatomy and behaviour, but that a combined exposure may be. Further, a single risk factor may affect the brain at finer scales of resolution.

Preclinical investigation of MIA-exposure has been critical to our understanding of neurodevelopmental disorders (6,43,44) identifying behavioural, neuroanatomical, and transcriptional alterations relevant to ASD and schizophrenia (44–49). Mice prenatally exposed to MIA had no statistically significant deviations in developmental trajectories, nor in anxiety-like, social, or sensorimotor gating behaviours. The lack of statistically significant effects was somewhat surprising, and not consistent with previous reports of significantly altered brain and behavioural development from our group (7) and others (37,49). However, exploration of the findings at an uncorrected ($p < 0.05$) threshold identified altered trajectories in the striatum, hippocampus, lateral septum, and

somatosensory cortex, wherein the MIA-exposed mice had an overshoot in the adolescent/early-adult period followed by a normalization in later adulthood (relative to SAL-SAL), in line with our previously published findings (7). The lack of behavioural alterations in adulthood is consistent with our previous findings; however, there are many reports of altered behaviour in adult MIA-exposed offspring across multiple domains (44). Some methodological differences may explain why we do not recapitulate our findings at the same level of statistical significance between this study and our previous one (7); most importantly, mice (lower n) were scanned only three times (compared to four in our previous work), which may have reduced our sensitivity to detect alterations in developmental trajectories (50). Additionally, the ages selected for MRI in this study did not perfectly match those of our previous work, which may have decreased our ability to capture the stages of greatest change across development. Significant brain structure alterations have been replicated by our group in adolescent/young adult (PND 35 and 60) MIA-exposed offspring indicating that we may have missed the vulnerable window in the current study (51). Finally, structural imaging was performed slightly differently between studies; here we used a cryogenically cooled surface coil with no contrast enhancement (manganese chloride administration 24 hours prior to scan), whereas in the previous study we used a quadrature volumetric coil, with contrast enhancement; perhaps differences in structural MRI acquisition could contribute to the lack of consistency in our findings.

Mice chronically exposed to THC in adolescence similarly had transient deviations in neurodevelopmental trajectories, with a peak difference in the adolescent/early-adult period. In various regions including the somatosensory cortex, medial preoptic area, globus pallidus, third ventricle, and reticular nucleus, THC exposed mice experienced a volume increase at the post-treatment timepoint (PND50), followed by a normalization at the later adult timepoint. In contrast, hippocampal volume decreased at the post-treatment timepoint, but also normalized. These results indicate that the chronic THC treatment may have induced neuroanatomical remodeling, but that given a few weeks without THC exposure, the brain normalizes relative to the control (SAL-SAL) exposed offspring. In agreement with the lack of neuroanatomical differences by the adult timepoint (PND85), we did not detect any behavioural alterations; had we tested these mice earlier in development or across other behavioural domains, we may have detected differences.

To our knowledge, no longitudinal rodent MRI studies exist examining the effects of chronic adolescent THC exposure on brain structure. Studies using other modalities do report neuroanatomical, neurochemical, and behavioural abnormalities relevant to the psychosis spectrum (36,38,52). Positron emission tomography studies of rats exposed to chronic THC report increased D2-like receptor availability in the dorsal striatum (53). Furthermore, chronic THC exposure in adolescence has been associated with decreased synaptic arborization in the rat hippocampus (36), and the prefrontal cortex (38).

Impairments in cognitive flexibility, sensorimotor gating, and memory have all been identified in rodents chronically exposed to THC in adolescence (54–56).

Greater alterations to neurodevelopmental trajectories were observed in offspring exposed to both prenatal MIA and chronic adolescent THC. In the nucleus accumbens, anterior cingulate cortex, and entorhinal cortex, significant deviations in trajectory were observed, defined by enlarged volume in the POL-THC group relative to SAL-SAL at the post-treatment (PND50) scan. Similarly, to the SAL-THC group, these brain regions normalized in the absence of continued THC exposure by later adulthood (PND85). However, in the striatum and somatosensory cortex, the volume increase observed following the post-treatment scan was sustained into later adulthood, indicative of a more lasting neuroanatomical change. Similarly, in the subiculum, a volume decrease was observed following treatment, which was sustained into later adulthood. These regions may be more sensitive to the combined risk factor exposure; interestingly, they have been previously implicated in neurodevelopmental and neuropsychiatric disorders (57–62). Finally, investigation of the effects of THC in addition to MIA (POL-THC vs POL-SAL groups) revealed that mice exposed to both risk factors experience sustained growth of the bed nucleus of the stria terminalis, ventromedial thalamus, reticular nucleus and CA1, relative to mice exposed to MIA and adolescent SAL.

Behaviourally, we only observed subthreshold decreases in anxiety-like behaviours in our combined POL-THC group relative to SAL-SAL, potentially reflective of increased risk-taking behaviours. A subthreshold difference was also observed in sensorimotor gating behaviour, wherein the POL-THC mice performed worse at lower prepulse tones, but better at louder tones. Thus, although the combined risk factors were sufficient to induce neuroanatomical changes, they may not be sufficient to affect the behaviours we tested.

Strikingly, we observed dramatic decreases in CB-1 and CB-2 cell and fiber density in our regions of interest. Most pronounced were the decreases in the somatosensory cortex and ACC for both CB1 and 2. The somatosensory cortex showed consistent neuroanatomical alteration across the three risk factor groups, while the ACC showed sensitivity to both POL and POL-THC exposure. We observed a similar decrease in the fiber density of both CB1 and 2 staining in the striatum, another region affected by POL and POL-THC exposure. Finally, in the hippocampus we observed the most striking decreases in both CB1 and 2 cell and fiber density in the mice prenatally exposed to POL, while those exposed to adolescent THC (either prenatal SAL or POL) also had decreased density relative to SAL-SAL, but higher density relative to POL-SAL. Decreased CB1 receptor expression has been previously reported in the rodent brain (hippocampus and striatum) following both acute (63) and chronic THC exposure (64). Decreased sensitivity has also been observed in a number of rodent brain areas (cortex, hippocampus, and periaqueductal gray) following chronic use (31), as well as in chronic human users (temporal lobe, cingulate cortex, and nucleus accumbens (65). In contrast, increased

expression has also been reported following short exposure to THC (66), indicating that the duration of exposure may modulate the directionality of changes to CB1 expression. Interestingly, previous work using PET imaging to longitudinally assess CB1 receptor expression in the offspring of MIA-exposed offspring (GD 15) found that MIA-exposed rats had lower CB1 receptor standard uptake values in the globus pallidus in adolescence, and lower values in the sensory cortex and hypothalamus in adulthood, relative to controls; these findings indicate that MIA may alter the cannabinoid system in adolescence and adulthood, potentially leading to greater sensitivity to drug use in this period (67).

The endocannabinoid system plays a crucial role in early brain development (68), and undergoes dynamic changes during the adolescent period, indicating that these two developmental windows may be particularly vulnerable to risk factors affecting this system (69). THC activates the endocannabinoid system precisely through the CB1 and CB2 receptors. The CB1 receptors are expressed in high concentrations in the hippocampus, amygdala, basal ganglia, many regions of the cerebral cortex and cerebellum (70,71). Activation of presynaptic CB1 receptors (via THC or endogenous endocannabinoids) suppresses GABA release (72), which can disrupt the development of pyramidal and parvalbumin-containing basket cell circuitry, creating an imbalance in excitatory-inhibitory signaling in the brain. Similar imbalances are thought to underlie psychosis (73), and have been identified in MIA-exposed offspring (74). Cannabis has also been shown to increase dopamine release acutely, which may underlie positive psychotic symptoms (hallucinations, delusions) (8). CB-1 receptors may also play a critical role in synaptic plasticity (75), and neuroinflammation, as CB1 receptors have been identified at low levels within microglial populations (76), and have been observed to affect glial cell function in response to cellular injury (77). CB2 receptors are highly expressed in the peripheral immune system and thought to have potent anti-inflammatory effects, making them interesting candidates for managing neuroinflammation as well (78). More recently, CB2 receptor expression has been identified in the cerebral cortex, hippocampus, striatum, amygdala, thalamic nuclei, periaqueductal grey, cerebellum and several brain stem nuclei of the rodent brain (40,78). These receptors may also play a functionally relevant role in the central nervous system, primarily through microglia (76). These may be interesting avenues for further investigation, as microglial activation has been implicated in many of the downstream neurodevelopmental aberrations due to MIA-exposure.

Few studies exist investigating the combined effects of the two risk factors studied here on offspring development. Previous work has identified decreased CB1 receptor availability due to prenatal MIA-exposure and adolescent cannabinoid exposure in the hypothalamus and sensory cortex, which are regions where we detect sustained alterations due to MIA and THC (79). Alterations to small non-coding microRNAs in the entorhinal cortex, associated with neurotransmission, cellular signaling, and

schizophrenia, have been identified in rats exposed to both risk factors (80,81). Finally, alterations to the serotonergic (15) and dopaminergic systems (16) have both been identified in rats exposed to both risk factors; these neurotransmitter systems are relevant to the disease pathology of a number of neuropsychiatric disorders (82,83). These findings suggest that pathological changes may be occurring in response to the combined risk factors in some of the regions where we observe volume alterations. MIA-exposure has been shown to potentiate the effects of other environmental risk factors such as adolescent stress (84) or exposure to drugs of abuse (15), as well as genetic risk factors such as *DISC1* (85,86), *NRG1* (87), *NR4A2*, *TSC2* (88) causing greater deficits than either exposure alone (5,6).

Finally, our investigation of brain-behaviour covariation identified some underlying sex differences, not clearly uncovered in our univariate analyses. The pattern identifying in adulthood differentiated the male SAL-SAL offspring from all other male offspring exposed to either a single or a combined risk factor, whereas it did not differentiate the female offspring. This was defined by increased cortical, thalamic and cerebellar volume associated with decreased social interactions and sensorimotor gating. The assessment of within subject volume change post-treatment to adulthood with behaviour identified a similar behavioural pattern, associated with the somatosensory cortex striatum, hippocampus, and cerebellum; in this case, male mice were more similar in their expression of the pattern, whereas female SAL-THC offspring different from the other three groups. Overall, this analysis suggests that there may be interesting sex differences underlying the neurodevelopmental abnormalities associated MIA and THC exposure. This dimensional approach may parse variability better than categorical approaches (89) and has proven successful in our previous work (7,17,28)

Sex differences in response to MIA-exposure have been identified in human birth cohorts, showing that males are more likely to develop neurodevelopmental disorders following MIA-exposure to bacterial infection (90). Rodent studies also provide evidence for increased susceptibility in males, with reports of earlier deviations in neuroanatomy and behaviour based on longitudinal studies (49), as well as male-biased deficits in spatial working memory, PPI, and locomotion (91). Finally, altered synaptic function in the hippocampus and abnormal microglia activation have been reported in male MIA-exposed offspring, at a greater level than in females (30,92). Sex-biases have also been observed in response to cannabinoids; some evidence from human studies suggest that males may be more susceptible to the effects of cannabis use if they carry genetic risk for schizophrenia (12), and are also more likely to initiate use earlier than females (93). Rodent studies suggest that females may be more likely to develop behavioural despair, anhedonia, and catalepsy in response to THC treatment (94,95); these behavioural differences may, in part, be explained by differences in THC metabolism (96). Higher CB1 receptor density in males relative to females has been reported in regions such as the striatum, limbic system, and pituitary (97). Underlying sex-differences in several

neurobiological systems including microglia and synaptic function associated with MIA-exposures, as well as CB1 receptor density and THC metabolism, may result in the sex-differences in the brain-behaviour alterations we observe.

Based on our multivariate results, we believe that future studies should be performed to specifically investigate sex differences in response to both risk factors, ensuring sufficient power to detect these complex statistical interactions. Further, acquiring MRI and behavioural data more frequently throughout the development of the offspring may allow us to fully investigate subtle changes. This may, in part, explain why we did not detect significant alteration in the MIA-exposed mice, and only detected subtle changes due to THC exposure. Furthermore, performing behavioural assays more proximal to the chronic THC exposure would allow us to uncover more acute behavioral alteration in response to treatment.

We find that exposure to a single risk factor, either prenatal MIA-exposure or chronic adolescent THC exposure are not sufficient to induce lasting neuroanatomical or behavioural changes in adulthood, although transient alterations were observed in adolescent/early-adult neuroanatomy. In contrast, exposure to both risk factors may induce lasting neuroanatomical deviations in some regions, but not others, while only inducing subthreshold behavioural alterations. Interestingly, exposure to either prenatal MIA or adolescent THC, or both, significantly decreases the expression of CB1 and 2 receptors in the brain. This points to the central role of the endocannabinoid system in both prenatal and adolescent brain development, as well as its sensitivity to disruptions due to risk factor exposure. In conclusion, our findings show that exposure to multiple risk factors may have greater effects on offspring brain volume throughout development than exposure to a single risk factor, but that CB1 and 2 receptor density is similarly decreased following exposure to a single or multiple risk factors. Finally, exposure to either one or two risk factors did not alter adult behaviour. These findings may further our understanding of how exposure to risk factors throughout development could increase the likelihood of developing neuropsychiatric illnesses.

Acknowledgements

The authors would like to thank Dr. Joseph Rochford for sharing his expertise in data collection and analysis of the prepulse inhibition behavioural task, and Dr's Bruno Giros and Salah El Mestikawy for lending us their centrifuge. Data will be freely available for download on the zenodo platform (10.5281/zenodo.6373191). The authors would like to acknowledge their funding bodies, including the Canadian Institute of Health Research and Healthy Brains for Healthy Lives for providing support for this research. Additionally, we would like to thank the Fonds de Recherche du Québec en Santé for providing salary support for EG, KP, and MMC, as well as the Kappa Kappa Gamma Foundation of Canada for supporting EG's salary.

References

1. Shah JL, Chakravarty MM, Joober R, Lepage M. Dynamic endophenotypes and longitudinal trajectories: capturing changing aspects of development in early psychosis. *J Psychiatry Neurosci*. 2016 Apr;41(3):148–51.
2. van Os J, Kenis G, Rutten BPF. The environment and schizophrenia. *Nature*. 2010 Nov 11;468(7321):203–12.
3. Monk C, Lugo-Candelas C, Trumpff C. Prenatal Developmental Origins of Future Psychopathology: Mechanisms and Pathways. *Annu Rev Clin Psychol*. 2019 May 7;15:317–44.
4. Estes ML, McAllister AK. Maternal immune activation: Implications for neuropsychiatric disorders. *Science*. 2016 Aug 19;353(6301):772–7.
5. Meyer U. Prenatal poly(i:C) exposure and other developmental immune activation models in rodent systems. *Biol Psychiatry*. 2014 Feb 15;75(4):307–15.
6. Reisinger S, Khan D, Kong E, Berger A, Pollak A, Pollak DD. The Poly(I:C)-induced maternal immune activation model in preclinical neuropsychiatric drug discovery. *Pharmacol Ther*. 2015 May 1;149:213–26.
7. Guma E, Bordignon P do C, Devenyi GA, Gallino D, Anastassiadis C, Cvetkovska V, et al. Early or late gestational exposure to maternal immune activation alters neurodevelopmental trajectories in mice: an integrated neuroimaging, behavioural, and transcriptional study. *Biol Psychiatry* [Internet]. 2021 Mar 22; Available from: <https://doi.org/10.1016/j.biopsych.2021.03.017>
8. Keshavan MS, Giedd J, Lau JYF, Lewis DA, Paus T. Changes in the adolescent brain and the pathophysiology of psychotic disorders. *Lancet Psychiatry*. 2014 Dec;1(7):549–58.
9. Volkow ND, Swanson JM, Evins AE, DeLisi LE, Meier MH, Gonzalez R, et al. Effects of Cannabis Use on Human Behavior, Including Cognition, Motivation, and Psychosis: A Review. *JAMA Psychiatry*. 2016 Mar;73(3):292–7.
10. Zammit S, Allebeck P, Andreasson S, Lundberg I, Lewis G. Self reported cannabis use as a risk factor for schizophrenia in Swedish conscripts of 1969: historical cohort study. *BMJ*. 2002 Nov 23;325(7374):1199.
11. Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE. Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. *BMJ*. 2002 Nov 23;325(7374):1212–3.
12. French L, Gray C, Leonard G, Perron M, Pike GB, Richer L, et al. Early Cannabis Use, Polygenic Risk Score for Schizophrenia and Brain Maturation in Adolescence. *JAMA Psychiatry*. 2015 Oct;72(10):1002–11.
13. Battistella G, Fornari E, Annoni J-M, Chtioui H, Dao K, Fabritius M, et al. Long-term effects of cannabis on brain structure. *Neuropsychopharmacology*. 2014 Aug;39(9):2041–8.
14. Campolongo P. The endocannabinoid system: a key modulator of emotions and cognition.

Frontiers E-books; 142 p.

15. Dalton VS, Verdurand M, Walker A, Hodgson DM, Zavitsanou K. Synergistic Effect between Maternal Infection and Adolescent Cannabinoid Exposure on Serotonin 5HT1A Receptor Binding in the Hippocampus: Testing the “Two Hit” Hypothesis for the Development of Schizophrenia [Internet]. Vol. 2012, ISRN Psychiatry. 2012. p. 1–9. Available from: <http://dx.doi.org/10.5402/2012/451865>
16. Lecca S, Luchicchi A, Scherma M, Fadda P, Muntoni AL, Pistis M. Δ^9 -Tetrahydrocannabinol During Adolescence Attenuates Disruption of Dopamine Function Induced in Rats by Maternal Immune Activation. *Front Behav Neurosci*. 2019 Sep 6;13:202.
17. Guma E, Bordeleau M, González Ibáñez F, Picard K, Snook E, Desrosiers-Grégoire G, et al. Differential effects of early or late exposure to prenatal maternal immune activation on mouse embryonic neurodevelopment. *Proc Natl Acad Sci U S A*. 2022 Mar 22;119(12):e2114545119.
18. Campolongo P, Trezza V, Ratano P, Palmery M, Cuomo V. Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents. *Psychopharmacology* . 2011 Mar;214(1):5–15.
19. DiNieri JA, Hurd YL. Rat Models of Prenatal and Adolescent Cannabis Exposure. In: Kobeissy FH, editor. *Psychiatric Disorders: Methods and Protocols*. Totowa, NJ: Humana Press; 2012. p. 231–42.
20. Harkany T, Guzmán M, Galve-Roperh I, Berghuis P, Devi LA, Mackie K. The emerging functions of endocannabinoid signaling during CNS development. *Trends Pharmacol Sci*. 2007 Feb;28(2):83–92.
21. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species [Internet]. Vols. 106-107, *Progress in Neurobiology*. 2013. p. 1–16. Available from: <http://dx.doi.org/10.1016/j.pneurobio.2013.04.001>
22. Clancy B, Finlay BL, Darlington RB, Anand KJS. Extrapolating brain development from experimental species to humans. *Neurotoxicology*. 2007 Sep;28(5):931–7.
23. Kong V, Devenyi GA, Gallino D, Ayranci G, Germann J, Rollins C, et al. Early-in-life neuroanatomical and behavioural trajectories in a triple transgenic model of Alzheimer’s disease. *Brain Struct Funct* [Internet]. 2018 Jun 13; Available from: <http://dx.doi.org/10.1007/s00429-018-1691-4>
24. Gallino D, Devenyi GA, Germann J, Guma E, Anastassiadis C, Chakravarty MM. Longitudinal assessment of the neuroanatomical consequences of deep brain stimulation: Application of fornical DBS in an Alzheimer’s mouse model. *Brain Res*. 2019 Jul 15;1715:213–23.
25. Rollins CPE, Gallino D, Kong V, Ayranci G, Devenyi GA, Germann J, et al. Contributions of a high-fat diet to Alzheimer’s disease-related decline: A longitudinal behavioural and structural neuroimaging study in mouse models. *NeuroImage: Clinical*. 2019 Jan 1;21:101606.

26. Guma E, Rocchetti J, Devenyi GA, Tanti A, Mathieu A, Lerch JP, et al. Regional brain volume changes following chronic antipsychotic administration are mediated by the dopamine D2 receptor. *Neuroimage*. 2018 Apr 26;176:226–38.
27. Avants BB, Tustison NJ, Song G, Cook PA, Klein A, Gee JC. A reproducible evaluation of ANTs similarity metric performance in brain image registration. *Neuroimage*. 2011 Feb 1;54(3):2033–44.
28. Guma E, Snook E, Spring S, Lerch JP, Nieman BJ, Devenyi GA, et al. Subtle alterations in neonatal neurodevelopment following early or late exposure to prenatal maternal immune activation in mice. *Neuroimage Clin*. 2021 Oct 29;32:102868.
29. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Series B Stat Methodol*. 1995;57(1):289–300.
30. Hui CW, Vecchiarelli HA, Gervais É, Luo X, Michaud F, Scheefhals L, et al. Sex Differences of Microglia and Synapses in the Hippocampal Dentate Gyrus of Adult Mouse Offspring Exposed to Maternal Immune Activation. *Front Cell Neurosci*. 2020 Oct 15;14:558181.
31. Burston JJ, Wiley JL, Craig AA, Selley DE, Sim-Selley LJ. Regional enhancement of cannabinoid CB₁ receptor desensitization in female adolescent rats following repeated Delta-tetrahydrocannabinol exposure. *Br J Pharmacol*. 2010 Sep;161(1):103–12.
32. Zeighami Y, Fereshtehnejad S-M, Dadar M, Collins DL, Postuma RB, Mišić B, et al. A clinical-anatomical signature of Parkinson's disease identified with partial least squares and magnetic resonance imaging. *Neuroimage*. 2019 Apr 15;190:69–78.
33. McIntosh AR, Mišić B. Multivariate statistical analyses for neuroimaging data. *Annu Rev Psychol*. 2013;64:499–525.
34. McIntosh AR, Lobaugh NJ. Partial least squares analysis of neuroimaging data: applications and advances. *Neuroimage*. 2004;23 Suppl 1:S250–63.
35. Shin Yim Y, Park A, Berrios J, Lafourcade M, Pascual LM, Soares N, et al. Reversing behavioural abnormalities in mice exposed to maternal inflammation. *Nature*. 2017 Sep 28;549(7673):482–7.
36. Rubino T, Realini N, Braidà D, Guidi S, Capurro V, Viganò D, et al. Changes in hippocampal morphology and neuroplasticity induced by adolescent THC treatment are associated with cognitive impairment in adulthood. *Hippocampus*. 2009 Aug;19(8):763–72.
37. Crum WR, Sawiak SJ, Chege W, Cooper JD, Williams SCR, Vernon AC. Evolution of structural abnormalities in the rat brain following in utero exposure to maternal immune activation: A longitudinal in vivo MRI study. *Brain Behav Immun*. 2017 Jul;63:50–9.
38. Miller ML, Chadwick B, Dickstein DL, Purushothaman I, Egervari G, Rahman T, et al. Adolescent exposure to Δ9-tetrahydrocannabinol alters the transcriptional trajectory and dendritic architecture of prefrontal pyramidal neurons [Internet]. Vol. 24, *Molecular Psychiatry*. 2019. p. 588–600. Available from: <http://dx.doi.org/10.1038/s41380-018-0243-x>
39. Abramoff MD. ImageJ as an Image Processing Tool and Library [Internet]. Vol. 13, *Microscopy and Microanalysis*. 2007. Available from:

<http://dx.doi.org/10.1017/s1431927607079652>

40. Chen D-J, Gao M, Gao F-F, Su Q-X, Wu J. Brain cannabinoid receptor 2: expression, function and modulation. *Acta Pharmacol Sin.* 2017 Mar;38(3):312–6.
41. Marsicano G, Lutz B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci.* 1999 Dec;11(12):4213–25.
42. Rapoport JL, Addington AM, Frangou S, Psych MRC. The neurodevelopmental model of schizophrenia: update 2005. *Mol Psychiatry.* 2005 May;10(5):434–49.
43. Brown AS, Meyer U. Maternal Immune Activation and Neuropsychiatric Illness: A Translational Research Perspective. *Am J Psychiatry.* 2018 Nov 1;175(11):1073–83.
44. Gumusoglu SB, Stevens HE. Maternal Inflammation and Neurodevelopmental Programming: A Review of Preclinical Outcomes and Implications for Translational Psychiatry. *Biol Psychiatry.* 2019 Jan 15;85(2):107–21.
45. Boksa P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain Behav Immun.* 2010 Aug;24(6):881–97.
46. Guma E, Plitman E, Chakravarty MM. The role of maternal immune activation in altering the neurodevelopmental trajectories of offspring: A translational review of neuroimaging studies with implications for autism spectrum disorder and schizophrenia. *Neurosci Biobehav Rev* [Internet]. 2019; Available from: <https://www.sciencedirect.com/science/article/pii/S0149763419302088>
47. Haddad FL, Patel SV, Schmid S. Maternal Immune Activation by Poly I:C as a preclinical Model for Neurodevelopmental Disorders: A focus on Autism and Schizophrenia. *Neurosci Biobehav Rev.* 2020 Jun;113:546–67.
48. Kreitz S, Zambon A, Ronovsky M, Budinsky L, Helbich TH, Sideromenos S, et al. Maternal immune activation during pregnancy impacts on brain structure and function in the adult offspring. *Brain Behav Immun.* 2020 Jan;83:56–67.
49. Piontkewitz Y, Arad M, Weiner I. Abnormal trajectories of neurodevelopment and behavior following in utero insult in the rat. *Biol Psychiatry.* 2011 Nov 1;70(9):842–51.
50. Lerch JP, Gazdzinski L, Germann J, Sled JG, Henkelman RM, Nieman BJ. Wanted dead or alive? The tradeoff between in-vivo versus ex-vivo MR brain imaging in the mouse. *Front Neuroinform.* 2012 Mar 23;6:6.
51. Cupo L, Guma E, Gallino D, Mar K, Fowler C, Dehghani M, et al. Impact of Maternal Immune Activation Early in Pregnancy on Brain Development of Offspring: A Combined Morphological, Spectroscopic, and Behavioral Study. 2022; Available from: <https://www.preprints.org/manuscript/202203.0136>
52. Renard J, Szkudlarek HJ, Kramar CP, Jobson CEL, Moura K, Rushlow WJ, et al. Adolescent THC Exposure Causes Enduring Prefrontal Cortical Disruption of GABAergic Inhibition and Dysregulation of Sub-Cortical Dopamine Function. *Sci Rep.* 2017 Sep 12;7(1):11420.
53. Ginovart N, Tournier BB, Moulin-Sallanon M, Steimer T, Ibanez V, Millet P. Chronic $\Delta 9$ -

- Tetrahydrocannabinol Exposure Induces a Sensitization of Dopamine D2/3 Receptors in the Mesoaccumbens and Nigrostriatal Systems [Internet]. Vol. 37, Neuropsychopharmacology. 2012. p. 2355–67. Available from: <http://dx.doi.org/10.1038/npp.2012.91>
54. Verrico CD, Gu H, Peterson ML, Sampson AR, Lewis DA. Repeated $\Delta 9$ -tetrahydrocannabinol exposure in adolescent monkeys: persistent effects selective for spatial working memory. *Am J Psychiatry*. 2014 Apr;171(4):416–25.
55. Gomes FV, Guimarães FS, Grace AA. Effects of pubertal cannabinoid administration on attentional set-shifting and dopaminergic hyper-responsivity in a developmental disruption model of schizophrenia. *Int J Neuropsychopharmacol* [Internet]. 2014 Dec 13;18(2). Available from: <http://dx.doi.org/10.1093/ijnp/pyu018>
56. Gleason KA, Birnbaum SG, Shukla A, Ghose S. Susceptibility of the adolescent brain to cannabinoids: long-term hippocampal effects and relevance to schizophrenia. *Transl Psychiatry*. 2012 Nov 27;2:e199.
57. Piontkewitz Y, Arad M, Weiner I. Tracing the development of psychosis and its prevention: what can be learned from animal models. *Neuropharmacology*. 2012 Mar;62(3):1273–89.
58. Langen M, Bos D, Noordermeer SDS, Nederveen H, van Engeland H, Durston S. Changes in the development of striatum are involved in repetitive behavior in autism. *Biol Psychiatry*. 2014 Sep 1;76(5):405–11.
59. Grace AA, Fox KM, Lipski WJ, Valenti O, Behrens MM, Lodge DJ. STRESS AND THE HIPPOCAMPUS SUBICULUM: KEY SITE FOR INTERVENTION IN THE PREVENTION AND TREATMENT OF DOPAMINE HYPER-RESPONSIVITY IN PSYCHOSIS [Internet]. Vol. 117, Schizophrenia Research. 2010. p. 156. Available from: <http://dx.doi.org/10.1016/j.schres.2010.02.158>
60. Schobel SA, Chaudhury NH, Khan UA, Paniagua B, Styner MA, Asllani I, et al. Imaging patients with psychosis and a mouse model establishes a spreading pattern of hippocampal dysfunction and implicates glutamate as a driver. *Neuron*. 2013 Apr 10;78(1):81–93.
61. Khan S, Michmizos K, Tommerdahl M, Ganesan S, Kitzbichler MG, Zetino M, et al. Somatosensory cortex functional connectivity abnormalities in autism show opposite trends, depending on direction and spatial scale. *Brain*. 2015 May;138(Pt 5):1394–409.
62. Daskalakis AA, Zomorodi R, Blumberger DM, Rajji TK. Evidence for prefrontal cortex hypofunctioning in schizophrenia through somatosensory evoked potentials. *Schizophr Res*. 2020 Jan;215:197–203.
63. Bonilla-Del Río I, Puente N, Mimenza A, Ramos A, Serrano M, Lekunberri L, et al. Acute $\Delta 9$ -tetrahydrocannabinol prompts rapid changes in cannabinoid CB1 receptor immunolabeling and subcellular structure in CA1 hippocampus of young adult male mice. *J Comp Neurol*. 2021 Jun;529(9):2332–46.
64. Zhuang S-Y, Kittler J, Grigorenko EV, Kirby MT, Sim LJ, Hampson RE, et al. Effects of long-term exposure to $\Delta 9$ -THC on expression of cannabinoid receptor (CB1) mRNA in different rat brain regions. *Molecular Brain Research*. 1998 Nov 26;62(2):141–9.

65. Ceccarini J, Kuepper R, Kemels D, van Os J, Henquet C, Van Laere K. [18F]MK-9470 PET measurement of cannabinoid CB1 receptor availability in chronic cannabis users. *Addict Biol.* 2015 Mar;20(2):357–67.
66. Romero J, Garcia-Palomero E, Castro JG, Garcia-Gil L, Ramos JA, Fernandez-Ruiz JJ. Effects of chronic exposure to Δ^9 -tetrahydrocannabinol on cannabinoid receptor binding and mRNA levels in several rat brain regions. *Molecular Brain Research.* 1997 Jun 1;46(1):100–8.
67. Verdurand M, Dalton VS, Nguyen V, Grégoire M-C, Zahra D, Wyatt N, et al. Prenatal poly I: C age-dependently alters cannabinoid type 1 receptors in offspring: a longitudinal small animal PET study using [18F] MK-9470. *Exp Neurol.* 2014;257:162–9.
68. Fride E, Gobshtis N, Dahan H, Weller A, Giuffrida A, Ben-Shabat S. The endocannabinoid system during development: emphasis on perinatal events and delayed effects. *Vitam Horm.* 2009;81:139–58.
69. Meyer HC, Lee FS, Gee DG. The Role of the Endocannabinoid System and Genetic Variation in Adolescent Brain Development. *Neuropsychopharmacology.* 2018 Jan;43(1):21–33.
70. DeLisi LE. The effect of cannabis on the brain: can it cause brain anomalies that lead to increased risk for schizophrenia? *Curr Opin Psychiatry.* 2008 Mar;21(2):140–50.
71. Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study [Internet]. Vol. 11, *The Journal of Neuroscience.* 1991. p. 563–83. Available from: <http://dx.doi.org/10.1523/jneurosci.11-02-00563.1991>
72. Malone DT, Hill MN, Rubino T. Adolescent cannabis use and psychosis: epidemiology and neurodevelopmental models. *Br J Pharmacol.* 2010 Jun;160(3):511–22.
73. Bloomfield MAP, Hindocha C, Green SF, Wall MB, Lees R, Petrilli K, et al. The neuropsychopharmacology of cannabis: A review of human imaging studies. *Pharmacol Ther.* 2019 Mar;195:132–61.
74. Canetta S, Bolkan S, Padilla-Coreano N, Song LJ, Sahn R, Harrison NL, et al. Maternal immune activation leads to selective functional deficits in offspring parvalbumin interneurons. *Mol Psychiatry.* 2016 Jul;21(7):956–68.
75. Shrivastava A, Johnston M, Terpstra K, Bureau Y. Cannabis and psychosis: Neurobiology. *Indian J Psychiatry.* 2014 Jan;56(1):8–16.
76. Cabral GA, Raborn ES, Griffin L, Dennis J, Marciano-Cabral F. CB2 receptors in the brain: role in central immune function. *Br J Pharmacol.* 2008 Jan;153(2):240–51.
77. Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, et al. Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *J Neurosci.* 2003 Feb 15;23(4):1398–405.
78. Roche M, Finn DP. Brain CB₂ Receptors: Implications for Neuropsychiatric Disorders. *Pharmaceuticals .* 2010 Aug 10;3(8):2517–53.

79. Verdurand M, Dalton VS, Nguyen V, Grégoire M-C, Zahra D, Wyatt N, et al. Prenatal poly I:C age-dependently alters cannabinoid type 1 receptors in offspring: a longitudinal small animal PET study using [(18)F]MK-9470. *Exp Neurol*. 2014 Jul;257:162–9.
80. Hollins SL, Zavitsanou K, Walker FR, Cairns MJ. Alteration of imprinted Dlk1-Dio3 miRNA cluster expression in the entorhinal cortex induced by maternal immune activation and adolescent cannabinoid exposure. *Transl Psychiatry*. 2014 Sep 30;4:e452.
81. Hollins SL, Zavitsanou K, Walker FR, Cairns MJ. Alteration of transcriptional networks in the entorhinal cortex after maternal immune activation and adolescent cannabinoid exposure. *Brain Behav Immun*. 2016 Aug;56:187–96.
82. Daubert EA, Condrion BG. Serotonin: a regulator of neuronal morphology and circuitry. *Trends Neurosci*. 2010 Sep 1;33(9):424–34.
83. Ali S, Pereira F. Dopamine: Neuropsychiatric disorders and neurotoxicity [Internet]. Vol. 280, *Toxicology Letters*. 2017. p. S62. Available from: <http://dx.doi.org/10.1016/j.toxlet.2017.07.156>
84. Giovanoli S, Engler H, Engler A, Richetto J, Voget M, Willi R, et al. Stress in Puberty Unmasks Latent Neuropathological Consequences of Prenatal Immune Activation in Mice [Internet]. Vol. 339, *Science*. 2013. p. 1095–9. Available from: <http://dx.doi.org/10.1126/science.1228261>
85. Abazyan B, Nomura J, Kannan G, Ishizuka K, Tamashiro KL, Nucifora F, et al. Prenatal interaction of mutant DISC1 and immune activation produces adult psychopathology. *Biol Psychiatry*. 2010 Dec 15;68(12):1172–81.
86. Lipina TV, Zai C, Hlousek D, Roder JC, Wong AHC. Maternal immune activation during gestation interacts with Disc1 point mutation to exacerbate schizophrenia-related behaviors in mice. *J Neurosci*. 2013 May 1;33(18):7654–66.
87. Vuillermot S, Joodmardi E, Perlmann T, Ögren SO, Feldon J, Meyer U. Prenatal immune activation interacts with genetic Nurr1 deficiency in the development of attentional impairments. *J Neurosci*. 2012 Jan 11;32(2):436–51.
88. Crawley JN. What's Wrong With My Mouse?: Behavioral Phenotyping of Transgenic and Knockout Mice. John Wiley & Sons; 2007. 544 p.
89. Chakravarty MM, Guma E. Small animal imaging presents an opportunity for improving translational research in biological psychiatry. *J Psychiatry Neurosci*. 2021 Sep;46(5):E579–82.
90. Lee YH, Cherkerzian S, Seidman LJ, Papandonatos GD, Savitz DA, Tsuang MT, et al. Maternal Bacterial Infection During Pregnancy and Offspring Risk of Psychotic Disorders: Variation by Severity of Infection and Offspring Sex. *Am J Psychiatry*. 2020 Jan 1;177(1):66–75.
91. Gogos A, Sbisà A, Witkamp D, van den Buuse M. Sex differences in the effect of maternal immune activation on cognitive and psychosis-like behaviour in Long Evans rats. *Eur J Neurosci*. 2020 Jul;52(1):2614–26.

92. Haida O, Al Sagheer T, Balbous A, Francheteau M, Matas E, Soria F, et al. Sex-dependent behavioral deficits and neuropathology in a maternal immune activation model of autism. *Transl Psychiatry*. 2019 Mar 28;9(1):124.
93. Kohn L, Kittel F, Piette D. Peer, family integration and other determinants of cannabis use among teenagers. *Int J Adolesc Med Health*. 2004 Oct;16(4):359–70.
94. Rubino T, Vigano' D, Realini N, Guidali C, Braidà D, Capurro V, et al. Chronic delta 9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. *Neuropsychopharmacology*. 2008 Oct;33(11):2760–71.
95. Biscaia M, Marín S, Fernández B, Marco EM, Rubio M, Guaza C, et al. Chronic treatment with CP 55,940 during the peri-adolescent period differentially affects the behavioural responses of male and female rats in adulthood. *Psychopharmacology* . 2003 Nov;170(3):301–8.
96. Tseng AH, Craft RM. CB(1) receptor mediation of cannabinoid behavioral effects in male and female rats. *Psychopharmacology* . 2004 Feb;172(1):25–30.
97. González S, Bisogno T, Wenger T, Manzanares J, Milone A, Berrendero F, et al. Sex Steroid Influence on Cannabinoid CB1 Receptor mRNA and Endocannabinoid Levels in the Anterior Pituitary Gland [Internet]. Vol. 270, *Biochemical and Biophysical Research Communications*. 2000. p. 260–6. Available from: <http://dx.doi.org/10.1006/bbrc.2000.2406>