Sodium Butyrate Rescues Neurodevelopmental Deficits Following Perinatal Methadone Exposure

Isobel A. R. Williams, BPsychSc(Hons) (1), Josie van Dorst, PhD (2), Sarah-Jane Leigh, PhD (1), Sarah. J. Baracz, PhD (1), Abigail Marus, BAdvSci(Hons) (1), Delyse McCaffrey BSc(Hons) (3,4) Adam Walker, PhD (3,4,5), Chee Y. Ooi, PhD (2), Meredith C. Ward, PhD (6,7), Ju-Lee Oei, MBBS (6,7), Kelly J. Clemens, PhD (1).

(1) School of Psychology, Faculty of Science, University of New South Wales, (2) Department of Gastroenterology, Sydney Children's Hospital Randwick, Sydney, NSW, Australia, (3) Neuroscience Research Australia, University of New South Wales, (4) Discipline of Psychiatry and Mental Health, Faculty of Medicine, University of New South Wales, (5) Drug Discovery Biology Theme, Monash Institute of Pharmaceutical Sciences, Monash University, (6) School of Women's and Children's Health, Faculty of Medicine, University of New South Wales, (7) Department of Newborn Care, Royal Hospital for Women, Randwick, NSW, Australia

Corresponding author

Kelly Clemens, PhD, UNSW School of Psychology, Randwick, NSW 2033, 02 9385 8250, k.clemens@unsw.edu.au

Short/running title

Sodium butyrate mediates prenatal opioid exposure

Abstract – must be 250 words or less

Prenatal opioid exposure (POE) induces long-term neurodevelopmental, behavioral and cognitive deficits that currently have no treatment options. The mechanisms underlying these symptoms are poorly understood, but have been linked to a range of central, peripheral, and enteric nervous system changes. Emerging evidence indicates that maternal microbiota changes may contribute to these long-term deficits in offspring. Here we test the efficacy of the short-chain fatty acid sodium butyrate (NaB), known to be reduced with chronic opioid use, in mitigating POE-related deficits. Both methadone and sodium butyrate treatments disrupted dam and offspring microbiota composition and function: notably methadone altered dam microbial gene expression of critical enzymes for butyric acid production. Similar disruptions were observed in pups at postnatal day 21 but resolved in adulthood. POE induced anxiety in adolescence on the open-field test, and adult deficits in working spatial memory (TUNL task) and attentional processing (5-CSRTT), which were partially rescued in rats that had received prenatal NaB. NaB treatment mediated the impact of POE on reduced myelination. In summary, these results indicate that early treatment with NaB could significantly improve the outcomes of children born with POE. Furthermore, they highlight the link between maternal microbiota health and offspring cognition in animal models of POE.

Introduction

Opioid use is at an all-time high, with over 16 million people around the world reporting opioid use disorder[1]. Of particular concern is use among pregnant women - opioid use disorder during pregnancy increased over 400% between 1999 and 2014[2]. Use of prescription (e.g. oxycodone) and illicit (e.g. heroin) opioids is highly problematic in pregnant women as opioids readily cross the placenta, creating dependence in the unborn child[3]. Acutely, this presents a high risk of opioid withdrawal at birth, resulting in neurological, physical and gastrointestinal symptoms, including respiratory depression, seizures, and even death[4]. Although less well characterized, the long-term effects also significantly impact quality-of-life. Exposed infants exhibit deficits in gross motor development and cognitive functioning, and increased anxiety and impulsivity[5–11], particularly in males[12–14]. These developmental and behavioral issues are associated with neurological and physiological disruptions. At birth this includes decreased brain size[5], reduced white matter connectivity[5,15], increased expression of inflammatory genes correlating with impaired myelination[16], and decreased peripheral immune markers[17]. These infants are at increased risk of infection and immune conditions[18,19], as well as altered gastrointestinal motility for up to two months after birth[20–22]. However, interpretation of these findings is challenging given the wide range of external factors that can influence outcomes[23–25].

Rodent models of prenatal opioid exposure (POE) offer insight into the mechanisms underlying the adverse long-term outcomes, recapitulating many of the core behavioral and neurological features of human POE. This includes impaired cognitive functioning[26–28] (particularly recognition and spatial memory[27,29–31]), and increased anxiety-like behaviour[26,32]. As in humans, POE rodents show evidence of less mature and organized white matter tracts[29], changes to innate and adaptive immune system function in the brain and periphery[28,29,33,34], and altered gut-microbiota composition[35,36]. For this reason, the rodent model offers a unique

opportunity to develop potential treatments across an abbreviated timeframe and with a view to assess long-term outcomes.

One potential therapeutic target comes from recent evidence for gut microbiome dysregulation in the symptomology and pathology of POE[35–37]. Both adult opioid users and POE rodent models indicate that opioid exposure results in altered gut microbial diversity and abundance[35–38]. Recently a rodent POE model demonstrated that probiotic supplementation can reverse POE induced pain sensitivity[39], highlighting the potential for microbiota modification as a treatment strategy in POE. In particular, chronic opioid exposure has been linked to decreased production of short-chain fatty acids (SCFA), the major metabolites of fiber, in both adult humans and rodent models of POE[35,38]. This is noteworthy, as decreased SCFA availability during development has been linked to impaired myelination and immune function in both rodent models of neonatal antibiotic exposure and germ-free animals[40–43]. These short-chain fatty acids are produced by a wide range of microbes in the mammalian gut through the fermentation of fiber, as well as amino acids glutarate and lysine[44]. Dietary supplementation with the specific SCFA sodium butyrate (NaB) can reduce peripheral inflammation in human metabolic disorders[45], and restore healthy gut bacteria and reduce peripheral inflammation in mice following gut microbiome disruption[40,41,46]. Furthermore, NaB has been shown to rescue deficits in myelination associated with neonatal antibiotic exposure[43], and improve white matter formation in a rat model of neonatal hypoxia-ischemia[47,48].

Therefore, the aim of this study is to assess the efficacy of NaB to rescue impairments induced by prenatal exposure to the opioid methadone. We hypothesized that NaB treatment would mitigate the negative impacts of methadone on rat pups' anxiety-like behavior, attention and cognition, CNS development, peripheral inflammation, and gut-microbiome composition. To test this hypothesis, dams were treated with methadone or received sham treatment across the perinatal period. Methadone was selected here due to its widespread use as a replacement

therapy in pregnant women with opioid use disorder and its use in treating infants born with neonatal opioid withdrawal syndrome[4,49]. Half of the dams concurrently received NaB via their drinking water. We then performed a battery of developmental, behavioral, microbiome and neurobiological assessments that revealed NaB effectively rescued specific deficits associated with POE.

Method

Subjects

Animals

Dams: Twenty-six time-mated female Sprague Dawley rats (223-332g) were purchased from Animal Resource Centre, Perth, Australia, at gestational day 3 (GD3). All animals were maintained on a 12-hour day/night light cycle (lights on at 0700h) in a temperature- and humidity-controlled colony room (22°C±1) with *ad libitum* access to irradiated rat and mouse diets SF00-100 (Specialty Feeds, Western Australia, Australia) and potable water. All dams gave birth within 24 hours of GD22 (designated postnatal day 0 [P0]).

Pups: On P0 litter sizes were standardized to 10 pups (5 male, 5 female where possible). Pups were marked with non-toxic marker for developmental tracking from P3, as previously reported[50,51]. On P22 pups were weaned into cages of 2-5 rats, matching for sex and experimental condition.

All procedures were approved by the University of New South Wales Animal Care and Ethics Committee (protocol number: 20/51B) and are in accordance with the NHMRC Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition).

Drugs:

Methadone hydrochloride (National Institute of Measurement, North Ryde, Australia) was dissolved in sterile saline and administered to dams via mini osmotic pumps (2ML2, Alzet CA, USA) at a dose of 9 mg/kg/day[52,53] from GD10 until P17 (Figure 1A). Minipumps were

used to minimize the impact on the dams and disruption to pups, as repeated injections can impact outcomes of interest (e.g. behavior and systemic inflammation[54–56]). GD10 onset was selected as opioid receptor mRNA is not expressed in the brain until GD11.5[57–59]. The dose was chosen as it results stable concentration in dams blood, accumulation in pups brain at GD22[60], and in physiologically-relevant concentrations in pups urine at P12[29].

3% w/v sodium butyrate (Sigma-Aldrich, Missouri, USA) was administered via drinking water (refreshed every 48-72 hours) across GD10-P12. This concentration is known to be tolerated by rats and result in increased faecal levels of butyric acid[61].

Procedures

On GD9 dams were randomly allocated to one of four experimental conditions: sham surgery/water (Sham/Veh), methadone pump/water (Met/Veh), sham surgery/sodium butyrate (Sham/NaB) and methadone pump/sodium butyrate (Met/NaB).

Surgery to implant the mini-osmotic pump was performed according to manufacturer's instructions[62] and as reported previously[29,30,34,50,63]. Control animals received an identical procedure, however no minipump was inserted (Sham), as sham surgery and saline-filled mini pumps insertion produce the same effects, and as previously reported[51,64]. This surgery was performed at GD10 and P3 to permit continuous methadone exposure across gestation and the early postnatal period (GD10 until P17), see Figure 1A.

Dam body weight was recorded daily between GD10-21 and P2-20. Their fluid intake was monitored daily from G10-P12.

Maternal care (P1-10)

As the type and quantity of maternal care can impact pup development[65], across P1 – 10 maternal behavior was observed at 2-minute intervals for 30 minutes a day in the dams' home cages. Behaviors recorded were arched back nursing, licking and grooming pups, blanket nursing and passive nursing (see [66] for behavioural definitions).

Physical development

On P0 the sex and number of live and stillborn pups was recorded. As prenatal opioid exposure has been linked to delayed physical development in humans[67] and rodents[68,69], pups were weighed daily from P3 – 20, and the percentage of pups in each litter with both eyes open was recorded across P10 – 15.

Open-Field Test (P24)

To assess anxiety-like behavior one female and one male from each litter underwent the open-field test on P24. Each pup was placed in the center of large Perspex arena (L 60cm x W 60cm x H 50cm) and recorded for 5 minutes. Behavior analysis was conducted using Deeplabcut 2.3[70] for tracking pose estimation while locomotion, and time and entries in regions of interest were scored using SimBA 1.5[71]. Rearing duration and frequency using behavioral classifiers in SimBA.

Adult Behavior and Cognition testing (~P65)

At ~P65 a female and male pup from each litter were allocated for testing either on the Trial Unique Non-Matching Location (TUNL) task or the Five Choice Serial Reaction Time Task (5-CSRTT), to give an indication of spatial and working memory[72], or motor impulsivity and sustained attention respectively.

Trial Unique Non-Matching to Location Task

The TUNL task was performed in CANTAB boxes (Campden Instruments, Leicestershire, UK), which featured one wall with a touch sensitive screen and a magazine and pellet delivery (45MG Dustless Precision Sucrose Pellet, Able Scientific, AUS) on the wall opposite. The protocol was consistent with past studies[73] and is described in full in Supplementary Methods i. Briefly, during TUNL pretraining rats learnt to touch one of 15 locations illuminated on the screen and withhold responses to any other location. Following pretraining rats progressed to the TUNL task. Each trial started with phase one, where one of

the 15 locations was illuminated (S1). Once the rat touched this location phase two began, where two locations were illuminated, one in the initial location (S1) and the other in a novel location (S2). Rats learnt to touch only the novel S2 location when two locations were illuminated. The distance between the two locations was pseudo randomly varied to modify task difficulty, as reduced stimulus separation is associated with reduced performance in healthy rats[74]. Once the rat could perform the spatial component of the task to criterion (see Supplementary Methods Table i for details) the delay between phase one and phase two was gradually increased across sessions, resulting in two session each with 2-, 4-, 6-, and 8-second delays, further increasing task difficulty. The primary dependent variables considered were accuracy (correct responses/correct + incorrect responses) and premature responses.

Five Choice Serial Reaction Time Task

The procedures for the Five Choice Serial Reaction Time Task (5-CSRTT) were based on Bari et. al 2008[75] and are described in full in Supplementary Methods ii. Briefly, the 5-CSRTT was performed in a modified operant chamber (MED Associates, VA, US) with five nose-poke holes on the left wall and a food magazine and pellet delivery (45MG Dustless Precision Sucrose Pellets, Able Scientific, AUS) on the right wall. Rats learn to nose-poke one of the five holes on the left wall when a light (stimulus) appeared in that hole, and withhold responding during the inter-trial interval (ITI) when no light was present. As training progresses, the difficulty of the task was increased by decreasing stimulus duration (SD), requiring greater levels of attention from the rat, and increasing the ITI, requiring greater motor impulse control. Finally, rats were tested with pseudo-randomly varying stimulus durations of 0.25-, 0.5-, 1-, 1.25- or 2.5-seconds within one multiple stimulus duration (MSD) session, and pseudo-randomly varying inter-trial intervals of 1-, 3-, 5- or 7-seonds within one multiple inter-trial interval (MITI) session, with one session at stage 7 in-between. For full details of each stage of testing see Supplementary Methods Table ii.

Tissue processing and immunofluorescence (P21)

On P21 a female and male pup from each litter were anaesthetized with a lethal injection of sodium pentobarbital (325mg/mL; Clifford Hallam Healthcare, NSW, Australia) and were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde solution in PBS. Brains were extracted and post-fixed for 24 hours in 4% paraformaldehyde at 4° Celsius, transferred to a 70% ethanol solution and then paraffin-embedded (Biospecimen Preparation Laboratory, Wallace Wurth Building, UNSW). Brains were sectioned (5µM) in the coronal plane using a microtome (Leica, Australia) and mounted. Immunofluorescence was performed to detect microglia (rabbit polyclonal anti-iba1 antibody 1:100, 109-19741; Novachem, Victoria, Australia), astrocytes (mouse monoclonal anti-GFAP antibody 1:200; MAB360, Merck Millipore, Darmstadt, Germany), and myelin basic protein (1:500, M3821; Merck Millipore) as previously reported[51] with some modifications. Full details of the protocol are in Supplementary Methods iii.

Microscopy and Image Analysis

Sections were imaged at 20x magnification using a slide scanning epifluorescence microscope (Axio Scan.Z1, Carl Zeiss, Germany) with ZenPro Software (Carl Zeiss). ImageJ (National Institutes of Health, Bethesda, USA) software was used for quantification. Optical density of MBP+ cell expression was measured in the anterior corpus callosum, posterior corpus callosum and dentate gyrus, whilst the in the dorsal medial and dorsal lateral striatum the average size of MBP+ fiber bundles was quantified using the analyze particles function[76]. Optical density of GFAP+ staining was quantified in the anterior corpus callosum, dentate gyrus, CA1 and CA3. The number of Iba1+ cells was counted using the analyze particles function in the anterior corpus callosum, dorsal medial striatum, dorsal lateral striatum, dentate gyrus, CA1 and CA3. The regions used are represented in Supplementary Methods Figure ii.

Real-time quantitative polymerase chain reaction (RT-qPCR) (P21)

At P21 an additional female and male pup from each litter were euthanized with pentobarbital, brains were rapidly removed and frozen in liquid nitrogen before storage at -80°C until dissection. Brain tissue was collected in a cryostat maintained at -20. The PFC was isolated and collected, punches were taken from the striatum (2 mm diameter) and dorsal hippocampus (1 mm diameter). Regions collected are represented in Supplementary Methods Figure iii. Upon collection samples were submerged in TRIzol Reagent (ThermoFisher Scientific, USA).

Total RNA was isolated using the TRIzol Reagent protocol according to the manufacturer's instructions and concentrations were quantified using NanoDrop Lite (ThermoFisher Scientific, Massachusetts, USA). RNA was treated with DNAse1 (Merck, Australia) and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, California, USA), in a T100 Thermal Cycler (Bio-Rad Laboratories, California, USA) according to the kits protocol.

RT-qPCR gene expression analyses were performed in a StepOnePlus™ System (Applied Biosystems, Massachusetts, USA) using gene-specific TaqMan assays, summarized in Supplementary Methods Table iii and according to manufacturer's instructions. All samples were run in triplicate and the expression of target genes normalized against the mean expression level of house keeper genes Gapdh and UBC. Statistics were performed on expression levels and data shown as fold change relative to Sham/Veh.

Cytokine Multiplex

At P7 and 21 blood was collected from the perfusion circuit and serum collected. Serum was used to assess levels of 23 cytokines and chemokines, listed in Supplementary Methods Table iv, using a magnetic bead immunoassay (Bio-Plex Pro™ Rat Cytokine 23-Plex Assay

#12005641, Bio-Rad Laboratories, California, USA) following manufacturer's instructions, see Supplementary Methods iv for details.

Microbiome analysis

Faecal samples were collected from dams at GD10, P3 and P22, and from pups at P22, and at the conclusion of adult behavioural testing (approx. P120). For taxonomic and functional profiling DNA was extracted using the QIAamp Fast DNA Stool Mini Kit and QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Metagenome library prep with Illumina library prep, and sequencing on the NovaSeq X Plus platform (Illumina, San Diago, USA) was performed at the Ramaciotti Center for Genomics (UNSW, Sydney, Australia). Sequencing adaptors and low-quality sequences (phread quality ³ 20) were trimmed using fastp v0.20.0[77]. PolyG tail trimming and base correction by overlap analysis was also performed using fastp with options --trim_poly_g and --correction, respectively. Trimmed reads were mapped to the rat genome (GRCr8) using bowtie2 v2.5.2[78] and the unmapped paired-end reads were extracted using samtools v1.19.2[79]. Cleaned non-host reads were used in taxonomic and function profiling. Taxonomic profiling of the bacteria was performed with MetaPhlAn4 following read alignment to the MetaPhlan4 database[80].

Functional profiling was conducted with HUMAnN 3.0 [81]using default parameters. QC'ed reads were mapped to UniRef90 gene families and MetaCyc metabolic pathways. UniRef90 gene-families were regrouped into KEGG orthology (KO) groups using the command 'humann_regroup_table'. All functional annotations were normalized using the command "humann_renorm_table" to copies per million (--units cpm). To establish if the butyrate treatment was supplementing existing butyrate levels, or replacing diminished or disrupted butyrate production, we evaluated the distribution of genes involved in the four known butyrate pathways: Acetyl-CoA, 4-aminobutyrate/Succinate, glutarate and lysine.

Data Analysis

To avoid litter effects[82] the unit of analysis for all statistical tests was the dam, with one male and one female pup from each litter included in each analysis unless otherwise stated (sex treated as a within-subjects factor). When only one male or female pup was present in a litter that pup was used for multiple measures (See Supplementary Methods Table v for litter sex distribution and Supplementary Methods Table vi for allocation). All physical, and behavioural data were analyzed using mixed model ANOVA with between subject factors of treatment (sham or methadone, vehicle or NaB) and within subjects' factors of sex (1 male, 1 female pup/litter), time (day or session) or spatial separation (TUNL task) where appropriate. Where an effect of sex was detected males and females were analyzed separately, where not reported there was no impact of sex. Serum data was log transformed before analysis. Data was inspected for outliers. Values were considered outliers if they were more than two standard deviations from the mean and were removed. One dam in the met/NaB group gave birth to only two pups who did not survive. As it was not possible to assess if this dam had always had a low number of pups or this was an effect of drug exposure her and her pups were excluded from all analysis.

For microbiome analysis, alpha diversity was assessed by calculating richness and the Shannon-Weaver diversity index in the vegan r package. Beta diversity was calculated using relative abundance data and Bray-Curtis dissimilarity metric to generate principal component analyses (PCA) plots. All microbiota measures were assessed using a linear mixed effects model approach, controlling for litter and cohort effects. Permutational multivariate analysis of variance (PERMANOVA) tests were conducted using the *adonis* function (*vegan* package) to determine if beta diversity was significantly different between treatments. Differential abundance analysis was calculated with the tjazi package in R[83] and corrected for multiple testing (false discovery rate (FDR) $q < 0.05$).Unless otherwise stated data were analyzed using IBM SPSS Statistics 26 software package. For all analyses rejection of the null hypothesis was

set at $\alpha = 0.05$. Partial eta squared (η_p^2) values were reported as a measure of effect size, where a η_p^2 of 0.14 was considered a large effect size. Data were graphed using GraphPad Prism10. All significant statistical results for the supplementary data are contained in Supplementary Statistics.

Results

Dams: We first assessed dam gut microbiota composition and function at the species-level by sequencing the faecal microbiome during exposure to methadone and NaB across gestation and lactation (Fig 1A). Microbial species richness exhibited a significant interaction (Fig 1B; pump by fluid; $F_{1,95}$=5.429, p=0.022), with Tukey-adjusted simple main effects demonstrating significant reductions in richness in both methadone-treated groups. Shannon's diversity (Fig 1C) was not significantly altered. Microbiota composition also exhibited a significant interaction effect (Fig1D; pump by fluid; $F_{1,75}$=1.371, p=0.008, R^2=0.013, ω_p^2=0.004) by PERMANOVA after controlling for litter and cohort. We assessed individual species differences and found 40 were significantly altered by Methadone, NaB or their interaction (Supplementary Results Figure i).

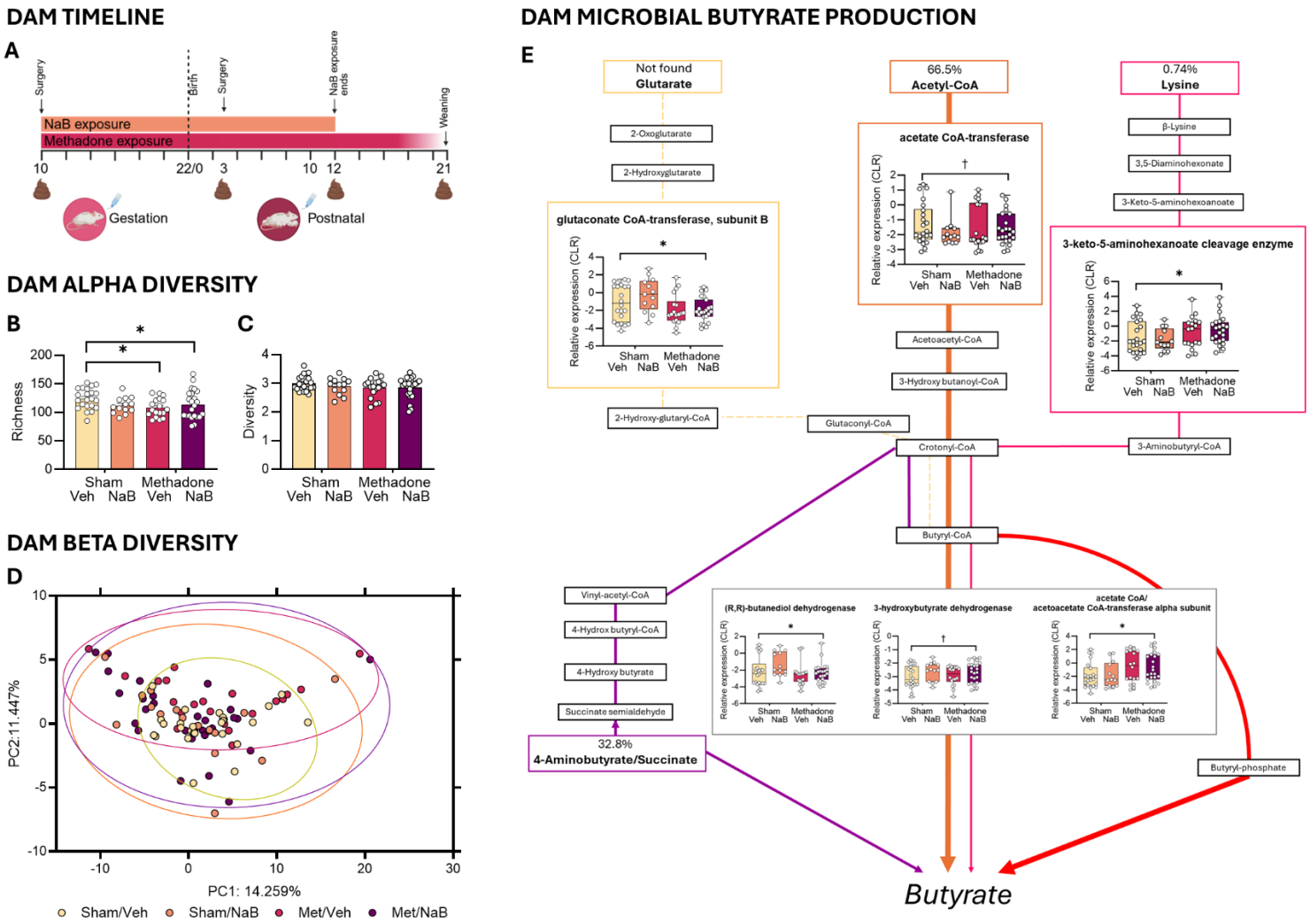
Since we hypothesized that methadone exposure would disrupt microbial butyrate production in the dam microbiome[84,85], we then examined functional microbial genes directly involved in butyrate production (Fig 1E). All but 2 of the 17 genes involved in the four butyrate synthesis pathways were identified. Of the 15 genes, 6 were significantly altered by Methadone, NaB or their interaction (K00004: drug effect: $F_{1,29.8}$=9.078, p=0.003; K00019: liquid effect: $F_{1,29.8}$=4.909, p=0.034; K01034: drug by day: $F_{1,77.5}$=2.912, p=0.027; K01040: drug effect: $F_{1,28.6}$=6.392, p=0.019; K18013: drug by day: $F_{1,81.3}$=2.900, p=0.027; K19709: drug by day: $F_{1,24.5}$=7.055, p=0.009). However, as most identified genes (63.9%) were terminal genes indistinguishable between butyrate synthesis pathways, we utilized the MetaCyc mapped pathways to quantify the distribution of butyrate synthesis potential

between pathways. A total of 6 intermediate pathways were identified: 2 from the Acetyl-CoA (66.5%), 3 from the 4-aminobutyrate/Succinate pathway (32.8%) and 1 from the lysine pathway (0.74%).

Following drug exposure (Supplementary Results Figure iiA) all dams gained weight during gestation and lactation (Supplementary Results Figure iiB), which was slightly reduced by NaB exposure during gestation and slightly increased by methadone during lactation. NaB administration slightly increased fluid consumption during and after gestation (Supplementary Results Figure iiC), resulting in an average daily dose of 6.221g/kg NaB. Both treatments impacted maternal behaviors: methadone reduced arched-back nursing while NaB reduced licking and grooming behaviors towards pups between P1 and 10 (Supplementary Results Figure iiD).

Pups' juvenile and adolescent period: Litter size, number of still born pups, initial weight, weight gain from P3-20, and eye opening was recorded, and is summarized in Supplementary Results Figure iii. Briefly, we observed that methadone reduced the number of pups born and the body weight of pups at P3, while accelerating the rate of eye opening. On P24 pups underwent the OFT, performance is summarized in Supplementary Results Figure ix. All methadone-exposed pups made fewer entries into the center of the field and spent less time in the center area, indicative of increased anxiety-like behavior. During the OFT (Supplementary Results Figure iv), pups that had been exposed to methadone spent less time rearing which was rescued with NaB exposure; rearing is a stress-sensitive measure[86] that is critical for spatial learning in young rodents[87]. No effects on locomotion were observed. In summary, while NaB did not rescue anxiety-like behavior, it may still promote better exploration and learning in a novel environment through increased rearing.

Fig. 1: Dam microbiome composition and inferred butyrate production

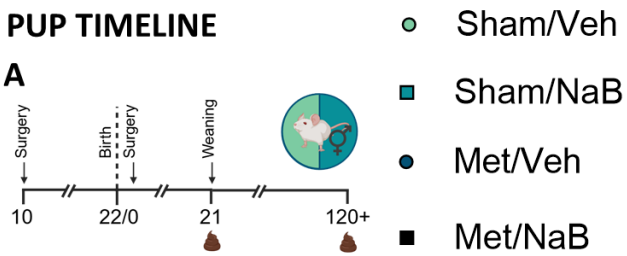


A Schematic of the experimental timeline indicating when fecal samples were collected from dams. **B** Microbial species richness by treatment group. **C** Shannon's diversity by treatment group. Data as mean ± SEM with individual points overlaid. Data was analyzed using linear mixed effects models controlling for litter and cohort. *$p<0.05$ Tukey-corrected simple main effects examining Drug*Liquid interaction. **D** Principal-component analysis displays microbiome composition differences by treatment group in terms of β-diversity (Aitchison distance; ellipses represent group 95% confidence intervals. **E** Targeted analysis of microbial genes involved in butyrate production. Data as mean ± interquartile range with individual points overlaid. Data was analyzed using linear mixed effects models controlling for litter and cohort. *: significant main effect of methadone; †: significant main effect of sodium butyrate; #: significant interaction effect. Percentages indicate observed distribution of 4 known pathways involved in microbial butyrate production.

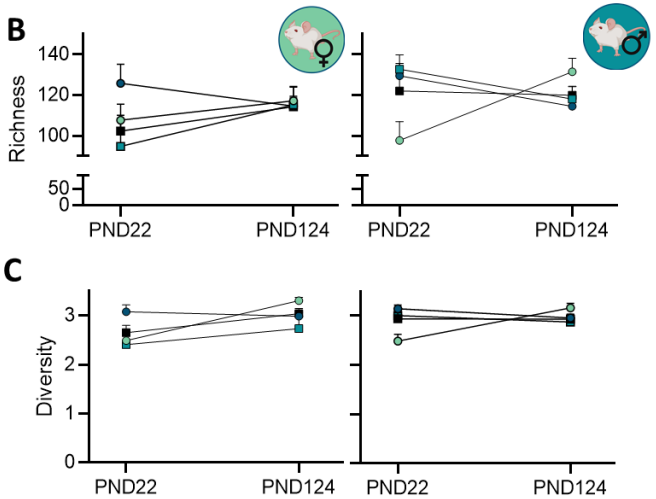
Pup microbiome: We also assessed pup microbiota composition and predicted butyrate production both at weaning and following adult behavioral testing (Fig 2A). Both microbial species richness (Fig 2B; Drug*liquid*day*sex: $F_{1,84.7}$=7.263, p=0.008) and shannon's diversity (Fig 2C; Drug*liquid*day: $F_{1,101.3}$=10.869, p=0.001; Drug*Liquid*Sex: $F_{1,85.3}$=6.408, p=0.013) were significantly altered by treatment, developmental day and pup sex. Notably, Shannon's diversity was significantly increased in male and female Sham/Veh offspring across development (Tukey-adjusted posthocs; p=0.021 and p=0.012 respectively) with no differences over time detected in the other treatment groups. At PND22, Sham/Veh and Met/Veh pups were significantly different (p=0.014). Offspring exhibited a significant interaction between treatments and developmental day in microbiota composition (Fig 2D; $F_{1,100}$=2.018, p=0.021, R^2=0.015, ω_p^2=0.009), although this may be due to differences in dispersion ($F_{7,108}$=2.423, p=0.024). We assessed individual species differences and found 79 were significantly altered by the interaction between development day and Methadone, NaB or both treatments (Supplementary Results Figure v).

Furthermore, we examined functional microbial genes directly involved in butyrate production (Fig 2E). Of the 15 genes we identified, 7 were significantly altered by Methadone, NaB or their interaction (K00634: drug by liquid by day effect: $F_{1,93.5}$=10.026, p=0.002; K00929: drug by liquid by day effect: $F_{1,105.1}$=9.003, p=0.003; K01844: drug effect: $F_{1,113.5}$ =4.313, p=0.040; K15736: drug effect: $F_{1,112.3}$=9.708, p=0.006; K17865: drug by liquid: $F_{1,114.2}$=7.440, p=0.007; K20903: drug by liquid by day effect: $F_{1,114.9}$ =5.052, p=0.027; K20904: drug by liquid effect: $F_{1,113.8}$ =5.224, p=0.040). Interestingly, while microbial butyrate production in both dams and pups were impacted by our interventions, in pups these disruptions appear to impact specific pathways, rather than universal, terminal enzymes as observed in the dam microbiota.

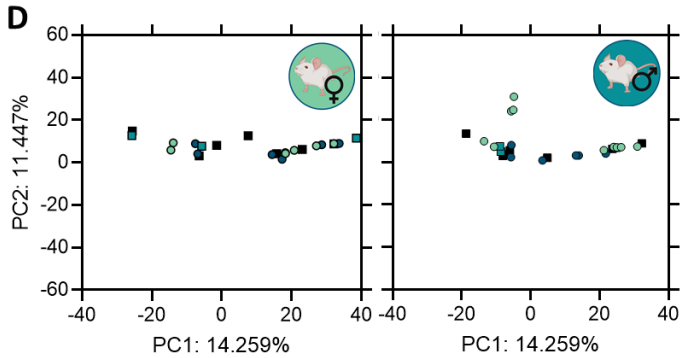
PUP TIMELINE



PUP ALPHA DIVERSITY



PUP BETA DIVERSITY – PND22



PUP MICROBIAL BUTYRATE PRODUCTION

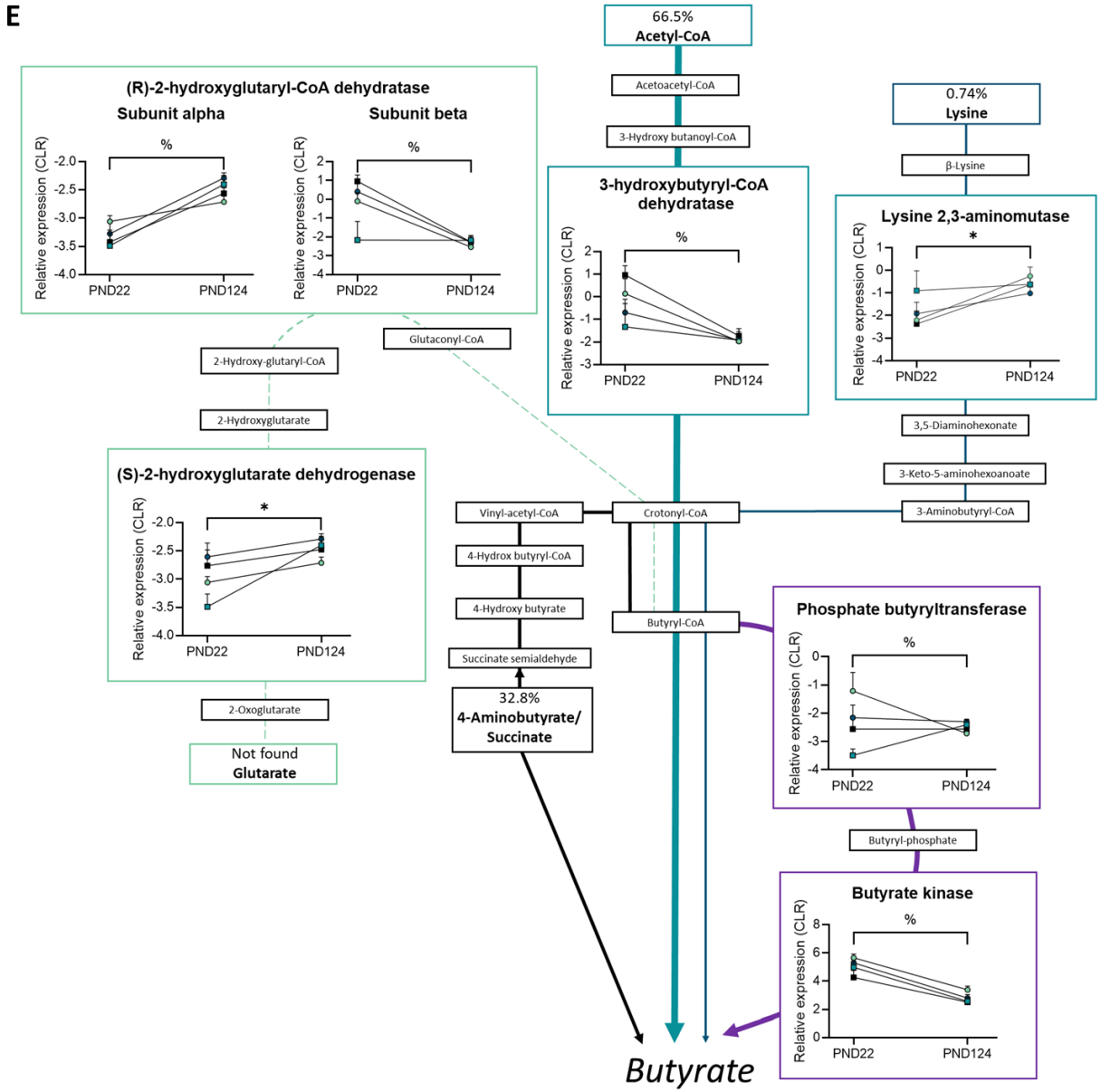


Figure 2: Offspring microbiome composition and inferred butyrate production. A Schematic of the experimental timeline indicating when fecal samples were collected from dams. **B** Microbial species richness by treatment group. **C** Shannon's diversity by treatment group. Data as mean ± SEM with individual points overlaid. Data was analyzed using linear mixed effects models controlling for litter and cohort. *$p<0.05$ Tukey-corrected simple main effects examining Drug*Liquid interaction. **D** Principal-component analysis displays microbiome composition differences by treatment group in terms of β-diversity (Aitchison distance; ellipses represent group 95% confidence intervals. **E** Targeted analysis of microbial genes involved in butyrate production. Data as mean ± interquartile range with individual points overlaid. Data was analyzed using linear mixed effects models controlling for litter and cohort. *: significant main effect of methadone; †: significant main effect of sodium butyrate; #: significant interaction effect. Percentages indicate observed distribution of 4 known pathways involved in microbial butyrate production.

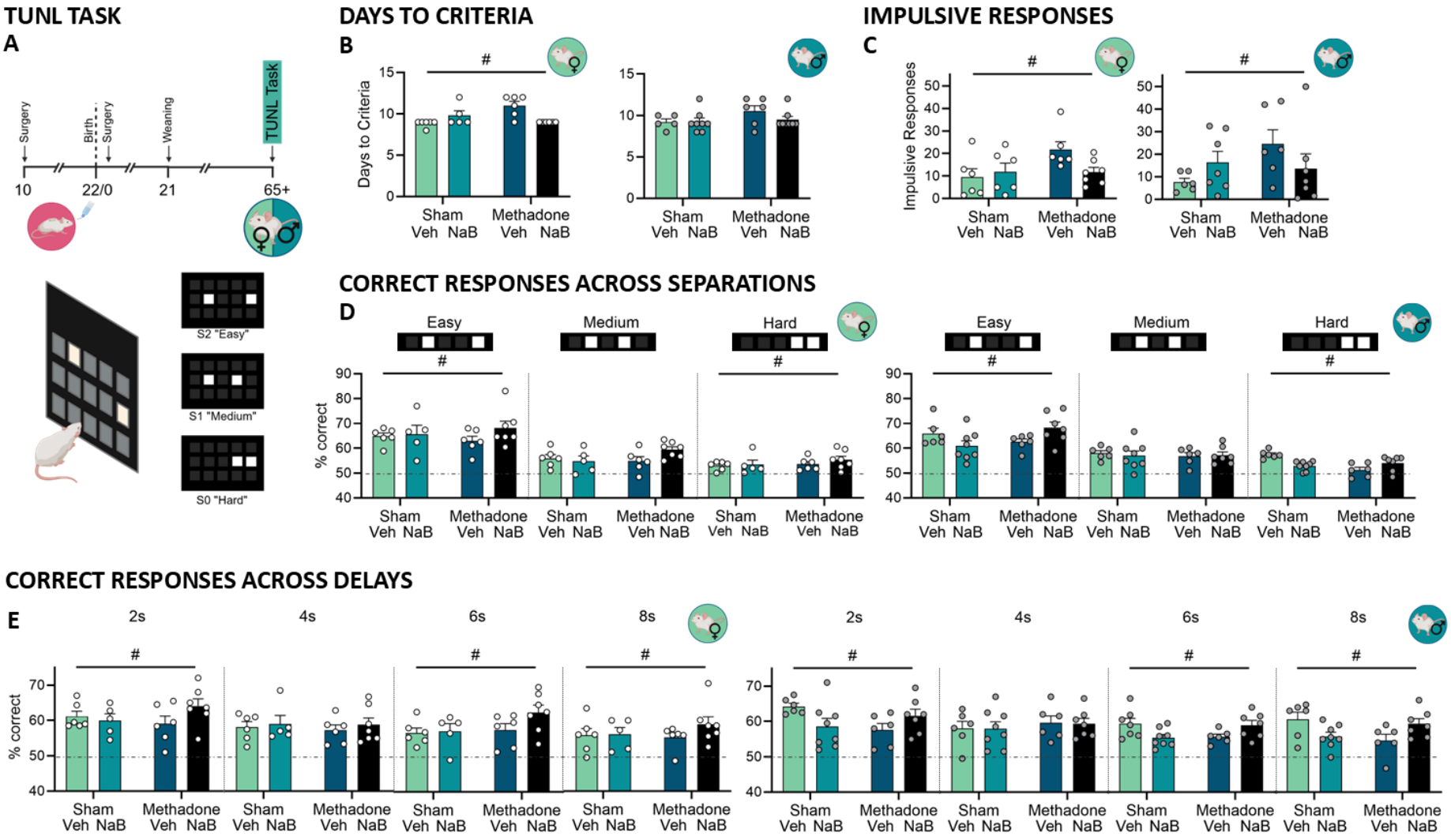
TUNL task: A female and male pup from each litter underwent the TUNL task in adulthood to assess working memory and pattern separation (Fig. 3A). Female rats who had been exposed to methadone took more days of pretraining to reach criteria for the TUNL task than females who had been exposed to methadone and NaB (Fig. 3B, interaction effect: $F_{1,19}$=5.307, p=0.034, η_p^2=0.238), indicative of slower associational learning. On the first day of the TUNL task female rats exposed to methadone or NaB made less correct responses than female rats who had been exposed to methadone and NaB together (Supplementary Results Figure viB). All groups took a similar number of days to reach delay testing criteria and made a similar number of correct responses in the final two sessions prior to the delays being introduced (Supplementary Results Figure viC). However, in these final sessions methadone-exposed animals touched the screen more during inter-trial intervals, a behavior shown in rats exposed to chronic mild stress, potentially indicative of increased impulsivity and habitual behavior[88], which was rescued in animals who had additional NaB exposure (Fig. 3C, interaction effect: $F_{1,19}$=4.880, p=0.040, η_p^2=0.204).

Once they had reached criteria rats progressed to the delay sessions, with increasing delays between displaying the initial location and the novel location to increase task difficulty. Responses on the "easy" (S2), "medium" (S1), and "hard" (S0) trials across all delay sessions were analyzed to identify the impact of spatial difficulty on performance. At both the "easy" and "hard" spatial difficulty level rats exposed to methadone made less correct responses than those exposed to methadone and NaB together (Fig 3D interaction effect; "easy": $F_{1,19}$=8.331, p=0.009, η_p^2=0.305, "hard": $F_{1,19}$=6.086, p=0.023, η_p^2=0.243).

In order to determine whether methadone impacted working memory performance, we then assessed how rats performed over various delay lengths (2, 4, 6 or 8 seconds). When the delay was 2-, 6-, or 8-seconds methadone or NaB exposed rats made fewer correct responses than rats that had been exposed to both methadone and NaB together (Fig. 3E, interaction

effect; 2s delay: $F_{1,19}$=4.797, p=0.041, η_p^2=0.202, 6s delay: $F_{1,19}$=9.223, p=0.007, η_p^2=0.327, 8s delay: $F_{1,19}$=5.284, p=0.033, η_p^2=0.218). An interaction between separation and delay across all sessions ($F_{6,114}$=2.519, p=0.025, η_p^2=0.117) indicated that delay and spatial difficulty interacted to impact performance. Performance at each spatial difficulty level during each type of delay session is summarized in Supplementary Results Figure vii. In summary, these results indicate that methadone impaired working memory and pattern separation, and that NaB treatment could rescue these effects in both male and female rats. The effects of methadone were not dependent on pattern difficulty or task delay, indicating that methadone likely impacts universal behavioral processes underlying the task.

Fig. 3: Performance on the trial unique non-matching location task



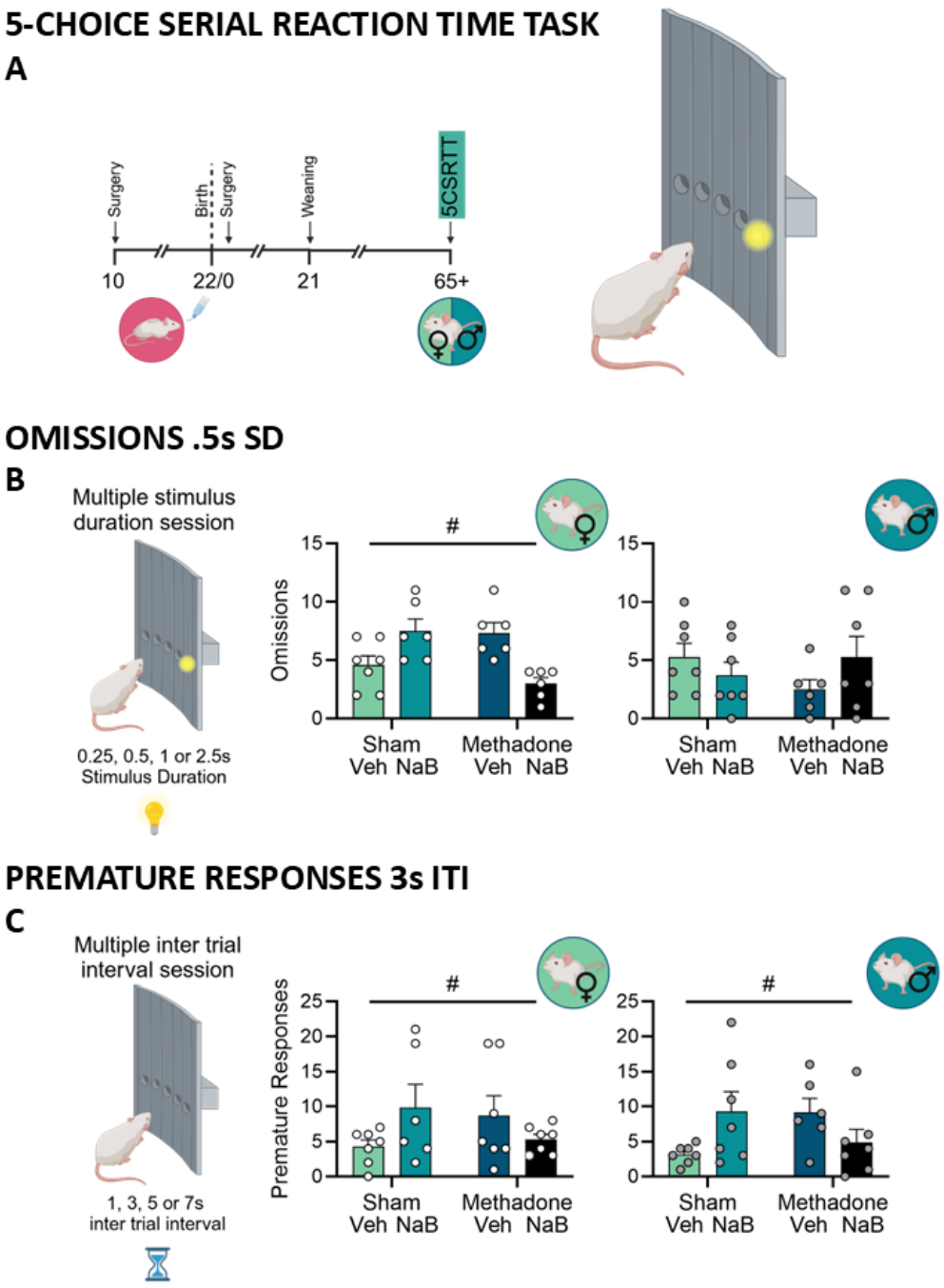
A Schematic of the TUNL task which one female and one male pup from each litter underwent from P65+. **B** The number of days taken for each rat to reach pretraining criteria and progress to the TUNL task. **C** The number of touches to the screen made during the inter-trial interval in the final TUNL training session before delays where introduced. **D** The percentage of correct responses made at "easy" (S2), "medium" (S1), and "hard" (S0) spatial difficulty trials across all delay sessions. **E** The percentage of correct responses made at each delay, 2 seconds, 4 seconds, 6 seconds or 8 seconds, across all spatial separations. Data points represent group mean ± SEM with 6/7 female (white points) and male (grey points) pups/group. The dashed horizontal line represents performance at chance, with no preference for the novel location. Hashtag indicates a significant interaction effect.

Five choice serial reaction time task: A further male and female pup from each litter underwent the 5CSRTT in adulthood to assess motor impulsivity and sustained attention (Fig. 4A). All rats reached criteria for MSD and MITI sessions at a similar rate, and performance was similar across all groups in the final session prior to the MSD and MITI sessions, summarized in Supplementary Results Figure viii.

Once rats had reached training criteria, they underwent the MSD session, with pseudo randomly varied SD of 0.25, 0.5, 1, 1.25 or 2.5 seconds, where a shorter SD requires higher levels of sustained attention. Performance at each SD was analyzed separately. When the SD was at its second shortest durations, 0.5s, female rats who had been exposed to methadone missed more trials than if they had additionally been exposed to NaB (Fig. 4B interaction effect: $F_{1,20}$ =15.556, p<.001, η_p^2=0.414), suggesting impaired sustained attention following methadone that is rescued with additional NaB exposure. Performance across other SDs was similar between groups and is summarized in Supplementary Results Figure ix.

Rats then progressed to the MITI sessions where the ITI varied between 1, 3, 5 and 7 seconds, with a longer ITI requiring rats to withhold a disadvantageous impulsive response for a longer period of time. Performance at each ITI length was analyzed separately. When the ITI was 3s rats that had been exposed to methadone made more premature responses than those who had been exposed to both methadone and NaB (Fig 4C, interaction effect: $F_{1,20}$=5.904, p=.024, η_p^2=0.212) suggesting that methadone exposure increased impulsive action, which was rescued by exposure to NaB. Performance across other ITIs was similar between groups and is summarized in Supplementary Results Figure ix. Together these results suggest subtle and sex-dependent negative effects of methadone on sustained attention and motor impulsivity that can be rescued with NaB treatment.

Fig. 4: Performance on the 5-choice serial reaction time task



A Schematic of the 5-choice serial reaction time task, which one female and one male pup from each litter underwent from P65+. **B** The number of trials where rats failed to respond (omissions) when the stimulus durations (SD) was .5 seconds in the multiple stimulus duration (MSD) session. **C** The number of responses inappropriately made before the stimulus was displayed (premature responses) when the intertrial interval (ITI) was 3 seconds

during the multiple inter trial interval (MITI) session. Data points represent group mean ± SEM with 6/7 female (white points) and male (grey points) pups/group. Hashtag indicate a significant interaction effect.

Myelination: On P21 one female and male rat from each litter were perfused (Fig 5A). Their brains were embedded in paraffin wax and stained for MBP in the striatum and dHPC (Supplementary Methods Figure iii) to give an indication of myelination in regions relevant to working and spatial memory[89], and motor impulsivity[7].

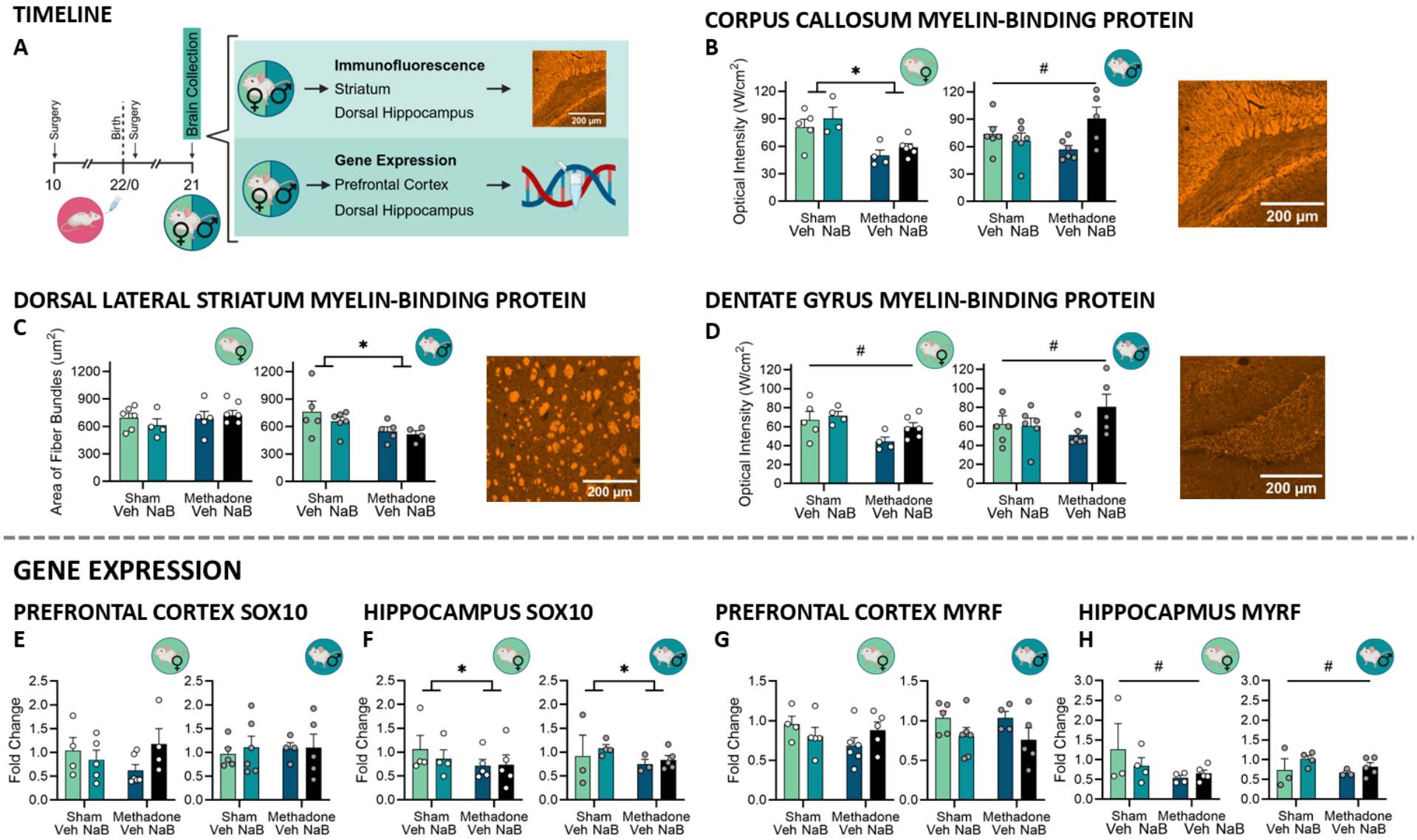
In the dentate gyrus, methadone and NaB exposed pups had greater staining intensity than those exposed to methadone alone (Fig. 5C interaction effect: $F_{1,14}$=7.955, p=.014, η_p^2=0.4362). A similar pattern was evident in the corpus callosum, where male pups exposed to methadone and NaB had greater MBP staining intensity than those exposed to methadone alone (Fig. 5B interaction effect: $F_{1,19}$=6.002, p=.024, η_p^2=0.240), while all females exposed to methadone had decreased staining intensity ($F_{1,14}$=17.335, p=.001, η_p^2=0.553). In the dorsal lateral striatum male pups exposed to methadone had smaller fiber bundles (Fig. 5D: $F_{1,16}$=5.815, p=.028, η_p^2=0.297). Overall these findings suggest that methadone exposure disrupts myelination in regions critical for working and spatial memory[89], and motor impulsivity[7]. Co-administration of NaB can partially rescue these effects in a sex-specific manner.

An additional female and male pup from each litter were euthanized and brains collected for gene expression analysis by RT qPCR to assess changes in oligodendrocyte proliferation that may have contributed to alterations in myelination (Fig 5A). In the PFC female pups that had been exposed to methadone and NaB had increased SOX10 expression compared to pups who had been exposed to methadone alone (Fig. 5E; sex by pump by fluid - $F_{1,13}$=6.252, p=.027, η_p^2=0.325). In the hippocampus methadone exposed pups had

decreased SOX10 expression (Fig. 5F; $F_{1,6}$=8.379, p=.032, η_p^2=0.563). In the hippocampus pups who had been exposed to methadone and NaB had increased MYRF expression compared to pups that had been exposed to methadone alone (Fig. 5G; $F_{1,6}$=20.765, p=.004, η_p^2=0.776). However, there were no differences in MYRF expression in the PFC (Fig. 5H). MBP expression and tissue from the striatum were also analyzed and showed no differences in expression between groups, summarized in Supplementary Results Figure xii, xiii and xiv. These results indicate region- and sex-specific effects of methadone and NaB exposure on oligodendrocyte related gene expression. They suggest methadone may disrupt myelination by altering oligodendrocyte proliferation, an effect that is partially recused when NaB has also been administered.

Inflammation: To assess potential impacts of methadone on immune function, central and peripheral inflammation was assessed using immunofluorescence staining for Iba1 and GFAP in the striatum and dHPC at P21, RT-qPCR assessing inflammatory gene expression in the PFC, striatum and dHPC at P21, and a cytokine multiplex assessment of blood serum on P7 and P21. These outcomes were minimally impacted by methadone exposure. However, NaB exposure increased the number of Iba1-positive cells in the corpus callosum, elevated CD68 and IL10 expression in the prefrontal cortex, reduced CD68 expression in the striatum, and altered cytokine serum levels. Together these finding suggests NaB mediated central and peripheral immune activity. These results are summarized in Supplementary Results Figures x, xi, xii, xiii and xiv, and Supplementary Results Table i and ii.

Fig. 5: Analysis of myelination on P21



A Timeline of brain collection for immunofluorescence and RT-qPCR. **B** Intensity of staining for myelin-basic protein (MBP) in the corpus callosum at the level of the dorsal hippocampus and a representative image of MBP staining in this region. **C** Intensity of staining for MBP in the dorsal lateral striatum and a representative image of MBP staining in this region. **D** Intensity of staining for MBP in the dorsal hippocampus and a representative image of MBP staining in this region. Fold change expression of **E** SOX10 in the prefrontal cortex, and **F** hippocampus, and of **G** MYRF in the prefrontal cortex, and **H** hippocampus. Data points represent group mean ± SEM with 3-6 female (white points) and male (grey points) pups/group. Asterisks and hashtag indicate a significant main effect of methadone or interaction effect respectively.

Discussion

Here, we demonstrate that methadone exposure during pregnancy and lactation disrupts both maternal and offspring microbiota composition and microbial butyrate production. These effects are associated with subtle impairments in anxiety-like behavior, pattern separation, working memory and attentional processing, as well as myelination changes in offspring. We show for the first time that NaB treatment rescued methadone-induced deficits in spatial and working memory, and reduced motor impulsivity. Molecular analysis suggests that the beneficial effects of NaB supplementation may be due to replacement of disrupted butyric acid supply in the maternal microbiota which supports offspring myelination.

The state of the evidence around the long-term neurodevelopmental and neuropsychiatric consequences of POE is mixed, indicating that there are likely subtle impairments associated with POE that are exacerbated by confounding factors, particularly poverty and maternal care, that are often poorly controlled in human studies[90]. On most outcomes infants appear to develop within normal limits, with differences emerging in school and language performance[91,92], as well as increased aggression, anxiety and incidence of

attention-deficit/hyperactivity disorder in childhood[93–95]. Of note, these neurodevelopmental and neuropsychiatric deficits are particularly pronounced in males[96]. These complex, often inconsistent findings are mirrored in preclinical work, most likely due to differences in route of administration, dosage, duration and choice of opioid, as well as the species and behavioural paradigms selected across studies[97]. Thus, the subtle constellation of behavioural deficits we observe with POE across multiple domains are unsurprising and indicate that this model translates to the human condition.

Notably, NaB rescued some of the methadone-induced deficits in spatial learning and working memory. In the TUNL task female pups exposed to methadone and NaB learnt the task faster, and both sexes exhibited a reversal of methadone-induced deficits during the more challenging working and spatial memory trials. While POE is known to impair recognition and spatial memory[27,30,31,51], and NaB has been shown to reverse deficits in recognition memory induced by neonatal antibiotic[43] or valproic acid[98] exposure, this is the first evidence of long-term spatial and working memory improvement following POE via NaB treatment.

This is the first research to demonstrate a direct impact of perinatal NaB on long-term deficits in sustained attention and impulsivity following POE[99]. In both the TUNL task and the 5-CSRTT NaB treated, methadone exposed rats showed a reduction in impulsive responses, suggesting decreased motor impulsivity. Additionally, NaB treatment reduced the number of missed trials in methadone exposed female rats, suggesting improved sustained attention. These data indicate that early life NaB has great potential as a therapy to mitigate POE-induced deficits likely to impair academic performance and quality-of-life.

The cognitive and behavioral improvements observed may be mediated, at least in part, by NaB's effects on POE-induced deficits in myelination. We found NaB rescued MBP staining intensity in the dentate gyrus in both sexes, a region critical for performance of the TUNL task[72], and in the corpus callosum in males. This aligns with previous studies showing

butyrate administration can reverse deficits in myelination induced by neonatal antibiotic exposure[43], and protect against demyelination while promoting remyelination in a rodent model of multiple sclerosis[47]. The altered myelination observed in our study also aligns with previous POE models, although outcomes seem to depend on opioid type and dose[29,51,104,105].

These changes in myelination may be due to opioids known disruption of oligodendrocyte differentiation[105], an essential part of the delicate and time sensitive myelination process. Conversely NaB appears to support this process by facilitating the differentiation of immature oligodendrocytes[47]. In this study we found downregulation of SOX10 following methadone exposure at P21, and a reversal of methadone-induced myrf suppression with NaB co-treatment. As SOX10 regulates myrf, and both are essential for successful oligodendrocyte maturation and myelination[106,107], these finding suggest a molecular mechanism for the observed changes in MBP staining. Given these interactions, our findings suggest the NaB's promyelinating effects via facilitation of oligodendrocyte differentiation likely contribute to the improved behavioral outcomes in this study.

Chronic opioid use has also been associated with disrupted gut microbiota composition and reduced short-chain fatty acid production in adult users[38]; here, we show for the first time that microbial functional genes required for butyrate production are dysregulated by maternal opioid exposure in both the maternal and early offspring microbiota. Butyric acid supplementation during pregnancy has been shown to confer resilience to colonic injury[108] and induce metabolic shifts[109–111] in offspring of healthy rodent dams. However, limited work has been conducted to examine the benefits of butyric acid supplementation in vulnerable pregnancies: in a model of rodent preterm birth, butyrate supplementation prolonged gestation and normalized immune development[6]. Our work indicates that maternal sodium butyrate administration partially restores attention, cognition and myelination in a model of POE and highlights that butyrate is likely to confer the most benefit when used as a targeted replacement.

While this work provides preliminary evidence linking perinatal NaB exposure to enhanced myelination and improved long-term cognitive outcomes following POE, the precise mechanisms underlying these effects remain unclear. One potential pathway involves NaB's ability to enhance both gut barrier and blood brain barrier integrity[43,46,100], systems disrupted with opioid use[38,101,102]. Previous research shows that in a model of neonatal antibiotic exposure NaB administration reverses deficits in gut permeability[43]. Improvement in barrier function may limit the translocation of pathogenic materials capable of triggering peripheral and CNS inflammation[103], supporting healthy CNS development.

Maternal care can impact long-term behavioral outcomes[66,112] and in extreme cases disrupt immune function[113,114]. The effect of opioid exposure on maternal care is poorly defined. Opioid using mothers are reported to be more irritable, ambivalent, and disinterested[115,116], however socioeconomic and environmental factors make it hard to determine if these are direct effects of opioid use[23,117]. Findings from animal models of POE are similarly mixed, with some studies reporting minimal or no effects on maternal care or behavioral outcomes[30,50,118], while others reported increased isolation of pups[119]. Here, methadone-exposed dams spend less time arch back nursing, while NaB dams exhibited the lowest levels of licking and grooming, suggesting the exposure may have been stressful[120,121]. Future experiments could consider a lower dose of NaB or intermittent access to normal drinking water to reduce potential maternal stress and better isolate the effects of NaB on offspring development. The lowest level of maternal care overall was in the methadone and NaB group. However, across multiple outcomes the Met/NaB group were most like control pups, suggesting maternal care had minimal impact, or the impact was mediated by exposure to both methadone and NaB.

One key limitation of this work is that we have assessed at single timepoints despite the fact that neurocognition and myelination are dynamic processes that continue to develop across adolescence and into adulthood in rats[122]. Therefore, MBP staining and gene expression at P21 may not reflect the final state of white matter development or contribute to the behavioral differences observed in adulthood. Future studies should incorporate longitudinal assessments of behavior and myelin integrity across developmental stages to better understand both the transient and enduring impacts of NaB and methadone.

Furthermore, NaB also exerts epigenetic effects as a histone deacetylase inhibitor[47]. Through this action NaB is thought to support myelination[47], enhance gut[123] and blood brain barrier function[100], and modulate immune function[124–127]. Given POE induces epigenetic modification[128,129], it is plausible that epigenetics could play a role in outcomes of this study. Understanding these pathways would not only clarify the mechanisms of NaB's therapeutic effects but could also help to identify novel targets and developmental windows for intervention, improving treatment outcomes for POE infants.

Across behavioral tasks, we found that maternal NaB exposure alone seemed to worsen performance. Additionally, NaB seemed to alter immune function, elevating cytokines at P7, altering microglial count in the striatum, and CD68 expression in the PFC and striatum at P21. While research around the use of NaB alone during pregnancy is limited, it's association with adverse outcomes such as increased intramuscular fat in offspring[109–111] and findings here suggest that NaB supplementation without opioid-induced pathology may negatively impact offspring development.

Identifying how specific pathways involved in NaB mediated improvements – supplementation of altered butyric acid production, altered gut microbiome, barrier integrity, immune modulation, and myelination – interact could enable more targeted therapeutic approaches. Importantly our findings highlight the need to include both sexes in studies of POE

and NaB treatment, as outcomes and underlying mechanism may differ. Clarifying the critical windows for treatment, concurrent with opioid exposure or following exposure to reverse deficits, is also essential when considering a clinical population whose access to care is likely to be sporadic. Together these insights will support the development of precise and effective treatment strategies.

Children born with POE represent a growing population with poor long-term outcomes and limited treatment options, placing an increasing strain on healthcare and social support systems[4–11]. Our findings position NaB as a serious candidate for future research, with potential to improve neurodevelopmental outcomes in this vulnerable group. More broadly this study provides compelling evidence linking gut-microbiome integrity to CNS development and cognition in rodent models of POE.

Acknowledgments

The authors acknowledge use of facilities in the Katharine Gaus Light Microscopy Facility at Mark Wainwright Analytical Centre for sample paraffin embedding. All timelines, schematics and explanatory figures were produced with Biorender.com. We would like to thank Melissa J. Bebbington for her invaluable assistance in teaching us immunofluorescence techniques, guiding us through the imaging process, and supporting our analysis using ImageJ. Her expertise and generous support greatly contributed to the success of this project.

Conflicts of Interest

All authors declare no conflicts of interests.

Funding

This work was supported by an NHMRC ideas grant to KJ.C. (APP2027709).

References

1. Chang, H.-Y., Kharrazi, H., Bodycombe, D., Weiner, J. P. & Alexander, G. C. Healthcare costs and utilization associated with high-risk prescription opioid use: a retrospective cohort study. *BMC Med* 16, 69 (2018).

2. Haight, S. C., Ko, J. Y., Tong, V. T., Bohm, M. K. & Callaghan, W. M. Opioid Use Disorder Documented at Delivery Hospitalization - United States, 1999-2014. *MMWR Morb Mortal Wkly Rep* 67, 845–849 (2018).

3. Gerdin, E., Rane, A. & Lindberg, B. Transplacental transfer of morphine in man. 18, 305–312 (1990).

4. Patrick, S. W. *et al.* Neonatal Abstinence Syndrome and Associated Health Care Expenditures: United States, 2000-2009. *JAMA* 307, 1934–1940 (2012).

5. Monnelly, V. J. *et al.* Prenatal methadone exposure is associated with altered neonatal brain development. *Neuroimage Clin* 18, 9–14 (2017).

6. Yeoh, S. L. *et al.* Cognitive and Motor Outcomes of Children With Prenatal Opioid Exposure: A Systematic Review and Meta-analysis. *JAMA Netw Open* 2, e197025–e197025 (2019).

7. Oei, J. L. *et al.* Neonatal Abstinence Syndrome and High School Performance. *Pediatrics* 139, e20162651 (2017).

8. Levine, T. A. & Woodward, L. J. Early inhibitory control and working memory abilities of children prenatally exposed to methadone. *Early Human Development* 116, 68–75 (2018).

9. Ornoy, A., Segal, J., Bar-Hamburger, R. & Greenbaum, C. Developmental outcome of school-age children born to mothers with heroin dependency: importance of environmental factors. *Developmental Medicine & Child Neurology* 43, 668–675 (2001).

10. Sherman, L. J., Ali, M. M., Mutter, R. & Larson, J. Mental Disorders Among Children Born With Neonatal Abstinence Syndrome. *PS* 70, 151–151 (2019).

11. Lee, S. J., Woodward, L. J. & Henderson, J. M. T. Educational achievement at age 9.5 years of children born to mothers maintained on methadone during pregnancy. *PLoS One* 14, e0223685–e0223685 (2019).

12. Nygaard, E., Moe, V., Slinning, K. & Walhovd, K. B. Longitudinal cognitive development of children born to mothers with opioid and polysubstance use. *Pediatric Research* 78, 330–335 (2015).

13. Hickey, J. E., Suess, P. E., Newlin, D. B., Spurgeon, L. & Porges, S. W. Vagal tone regulation during sustained attention in boys exposed to opiates in utero. *Addictive Behaviors* 20, 43–59 (1995).

14. Charles, M. K. *et al.* Male Sex Associated With Increased Risk of Neonatal Abstinence Syndrome. *Hospital Pediatrics* 7, 328–334 (2017).

15. Walhovd, K. B., Watts, R., Amlien, I. & Woodward, L. J. Neural Tract Development of Infants Born to Methadone-Maintained Mothers. *Pediatric Neurology* 47, 1–6 (2012).

16. Yen, E. *et al.* Sex-specific inflammatory and white matter effects of prenatal opioid exposure: a pilot study. *Pediatr Res* 93, 604–611 (2023).

17. Miller, N. W. *et al.* The impact of opioid exposure during pregnancy on the human neonatal immune profile. *Pediatr Res* 92, 1566–1574 (2022).

18. Arter, S., Lambert, J., Brokman, A. & Fall, N. Diagnoses during the First Three Years of Life for Children with Prenatal Opioid Exposure and Neonatal Abstinence Syndrome Using a Large Maternal Infant Data Hub. *Journal of Pediatric Nursing* 61, 34–39 (2021).

19. Kelty, E., Rae, K., Jantzie, L. L., Wyrwoll, C. S. & Preen, D. B. Prenatal Opioid Exposure and Immune-Related Conditions in Children. *JAMA Network Open* 7, e2351933 (2024).

20. Galinkin, J. *et al.* Recognition and Management of Iatrogenically Induced Opioid Dependence and Withdrawal in Children. *Pediatrics* 133, 152–155 (2014).

21. Maguire, D. & Gröer, M. Neonatal abstinence syndrome and the gastrointestinal tract. *Medical Hypotheses* 97, 11–15 (2016).

22. Pham, A., Johnson, M., Duvall, K. & Schetzina, K. Gastrointestinal Issues in Infants with Prenatal Substance Exposure. *Appalachian Student Research Forum* (2023).

23. Thaithumyanon, P., Limpongsanurak, S., Praisuwanna, P. & Punnahitanon, S. Perinatal effects of amphetamine and heroin use during pregnancy on the mother and infant. *J Med Assoc Thai* 88, 1506–1513 (2005).

24. Katz, C., El-Gabalawy, R., Keyes, K. M., Martins, S. S. & Sareen, J. Risk factors for incident nonmedical prescription opioid use and abuse and dependence: Results from a longitudinal nationally representative sample. *Drug and Alcohol Dependence* 132, 107–113 (2013).

25. Patrick, S. W., Kaplan, H. C., Passarella, M., Davis, M. M. & Lorch, S. A. Variation in treatment of neonatal abstinence syndrome in US Children's Hospitals, 2004–2011. *J Perinatol* 34, 867–872 (2014).

26. Zagon, I. S. & McLaughlin, P. J. PERINATAL METHADONE EXPOSURE AND BRAIN DEVELOPMENT: A BIOCHEMICAL STUDY1. *Journal of Neurochemistry* 31, 49–54 (1978).

27. Lum, J. S. *et al.* Prenatal methadone exposure impairs adolescent cognition and GABAergic neurodevelopment in a novel rat model of maternal methadone treatment. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 110, 110281 (2021).

28. Smith, B. L. *et al.* Perinatal Morphine Exposure Leads to Sex-Dependent Executive Function Deficits and Microglial Changes in Mice. *eNeuro* 9, ENEURO.0238-22.2022 (2022).

29. Jantzie, L. L. *et al.* Prenatal opioid exposure: The next neonatal neuroinflammatory disease. *Brain Behav Immun* 84, 45–58 (2020).

30. Kongstorp, M., Bogen, I. L., Stiris, T. & Andersen, J. M. Prenatal exposure to methadone or buprenorphine impairs cognitive performance in young adult rats. *Drug and Alcohol Dependence* 212, 108008 (2020).

31. Chen, H.-H. *et al.* Buprenorphine, methadone, and morphine treatment during pregnancy: behavioral effects on the offspring in rats. *Neuropsychiatr Dis Treat* 11, 609–618 (March 6, 2015a).

32. Daly, F. M., Hughes, R. N. & Woodward, L. J. Subsequent anxiety-related behavior in rats exposed to low-dose methadone during gestation, lactation or both periods consecutively. *Pharmacology Biochemistry and Behavior* 102, 381–389 (2012).

33. Gowen, A. M. *et al.* In utero and post-natal opioid exposure followed by mild traumatic brain injury contributes to cortical neuroinflammation, mitochondrial dysfunction, and behavioral deficits in juvenile rats. *Brain Behav Immun Health* 32, 100669 (2023).

34. Madurai, N. K. *et al.* Methadone alters the peripheral inflammatory and central immune landscape following prenatal exposure in rats. *Advances in Drug and Alcohol Research* 2, (2022).

35. Grecco, G. G., Gao, Y., Gao, H., Liu, Y. & Atwood, B. K. Prenatal opioid administration induces shared alterations to the maternal and offspring gut microbiome: A preliminary analysis. *Drug and Alcohol Dependence* 227, 108914 (2021).

36. Abu, Y. F. *et al.* Opioid-induced dysbiosis of maternal gut microbiota during gestation alters offspring gut microbiota and pain sensitivity. *Gut Microbes* 16, 2292224 (2024).

37. Lyu, Z. *et al.* Long-Term Effects of Developmental Exposure to Oxycodone on Gut Microbiota and Relationship to Adult Behaviors and Metabolism. *mSystems* 7, e00336-22 (2022).

38. Cruz-Lebrón, A. *et al.* Chronic opioid use modulates human enteric microbiota and intestinal barrier integrity. *null* 13, 1946368 (2021).

39. Singh, S. *et al.* Probiotic supplementation mitigates sex-dependent nociceptive changes and gut dysbiosis induced by prenatal opioid exposure. *Gut Microbes* 17, 2464942 (2025).

40. Erny, D. *et al.* Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 18, 965–977 (2015).

41. Erny, D. *et al.* Microbiota-derived acetate enables the metabolic fitness of the brain innate immune system during health and disease. *Cell Metabolism* 33, 2260-2276.e7 (2021).

42. Lynch, C. M., Nagpal, J., Clarke, G. & Cryan, J. F. Wrapping Things Up: Recent Developments in Understanding the Role of the Microbiome in Regulating Myelination. *Current Opinion in Physiology* 23, 100468 (2021).

43. Keogh, C. E. *et al.* Myelin as a regulator of development of the microbiota-gut-brain axis. *Brain, Behavior, and Immunity* 91, 437–450 (2021).

44. Singh, V. *et al.* Butyrate producers, "The Sentinel of Gut": Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. *Front Microbiol* 13, 1103836 (2023).

45. Cleophas, M. C. P. *et al.* Effects of oral butyrate supplementation on inflammatory potential of circulating peripheral blood mononuclear cells in healthy and obese males. *Scientific Reports* 9, 775 (2019).

46. Chen, G. *et al.* Sodium Butyrate Inhibits Inflammation and Maintains Epithelium Barrier Integrity in a TNBS-induced Inflammatory Bowel Disease Mice Model. *EBioMedicine* 30, 317–325 (2018).

47. Chen, T., Noto, D., Hoshino, Y., Mizuno, M. & Miyake, S. Butyrate suppresses demyelination and enhances remyelination. *Journal of Neuroinflammation* 16, 165 (2019).

48. Ziemka-Nalecz, M. *et al.* Sodium Butyrate, a Histone Deacetylase Inhibitor, Exhibits Neuroprotective/Neurogenic Effects in a Rat Model of Neonatal Hypoxia-Ischemia. *Mol Neurobiol* 54, 5300–5318 (2017).

49. Health NSW. Guidelines for the Management of Substance Use During Pregnancy Birth and the Postnatal Period. (2014).

50. Nyberg, H., Bogen, I. L., Nygaard, E. & Andersen, J. M. Effects of prenatal exposure to methadone or buprenorphine and maternal separation on anxiety-like behavior in rats. *Drug and Alcohol Dependence* 262, 111367 (2024).

51. Sahid, A. S. *et al.* Perinatal exposure to methadone or buprenorphine impairs hippocampal-dependent cognition and brain development in juvenile rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 137, 111255 (2025).

52. Ross, E. J., Graham, D. L., Money, K. M. & Stanwood, G. D. Developmental consequences of fetal exposure to drugs: what we know and what we still must learn. *Neuropsychopharmacology* 40, 61–87 (2015).

53. Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M. & Noble-Haeusslein, L. J. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology* 106–107, 1–16 (2013).

54. Drude, S. *et al.* Side effects of control treatment can conceal experimental data when studying stress responses to injection and psychological stress in mice. *Lab animal* 40, 119–28 (2011).

55. Du Preez, A. *et al.* The type of stress matters: repeated injection and permanent social isolation stress in male mice have a differential effect on anxiety- and depressive-like behaviours, and associated biological alterations. *Transl Psychiatry* 10, 325 (2020).

56. Aydin, C., Frohmader, K. & Akil, H. Revealing a latent variable: Individual differences in affective response to repeated injections. *Behav Neurosci* 129, 679–682 (2015).

57. Rius, R. A., Barg, J., Bem, W. T., Coscia, C. J. & Loh, Y. P. The prenatal developmental profile of expression of opioid peptides and receptors in the mouse brain. *Developmental Brain Research* 58, 237–241 (1991).

58. Tong, Y. *et al.* Ontogenic profile of the expression of the mu opioid receptor gene in the rat telencephalon and diencephalon: an in situ hybridization study. *Journal of Chemical Neuroanatomy* 18, 209–222 (2000).

59. Zhu, Y., Hsu, M.-S. & Pintar, J. E. Developmental Expression of the μ, κ, and δ Opioid Receptor mRNAs in Mouse. *J. Neurosci.* 18, 2538–2549 (1998).

60. Kongstorp, M., Bogen, I. L., Stiris, T. & Andersen, J. M. High Accumulation of Methadone Compared with Buprenorphine in Fetal Rat Brain after Maternal Exposure. *The Journal of Pharmacology and Experimental Therapeutics* 371, 130–137 (2019).

61. Freeman, H. J. Effects of differing concentrations of sodium butyrate on 1,2-dimethylhydrazine-induced rat intestinal neoplasia. *Gastroenterology* 91, 596–602 (1986).

62. Implantation & Explantation. *ALZET® Osmotic Pumps* https://www.alzet.com/guide-to-use/implementation_and_explanation/.

63. Williams, I. A. R. & Clemens, K. J. Emerging evidence of a link between inflammation and the neuropathology of prenatal opioid exposure. *Current Opinion in Neurobiology* 89, 102924 (2024).

64. Doucette, T. A., Ryan, C. L. & Tasker, R. A. Use of osmotic minipumps for sustained drug delivery in rat pups: effects on physical and neurobehavioural development. *Physiology & Behavior* 71, 207–212 (2000).

65. Caldji, C. *et al.* Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. *Proc Natl Acad Sci U S A* 95, 5335–5340 (1998).

66. Champagne, F., Francis, D., Mar, A. & Meaney, M. Champagne FA, Francis DD, Mar A, Meaney MJ. Variations in maternal care in the rat as a mediating influence for effects of environment on development. Physiol Behav 79: 359-371. *Physiology & behavior* 79, 359–71 (2003).

67. Wouldes, T. A. & Woodward, L. J. Neurobehavior of newborn infants exposed prenatally to methadone and identification of a neurobehavioral profile linked to poorer neurodevelopmental outcomes at age 24 months. *PLoS One* 15, e0240905–e0240905 (2020).

68. Ford, D. H. & Rhines, R. K. Prenatal exposure to methadone HCL in relationship to body and brain growth in the rat. *Acta Neurologica Scandinavica* 59, 248–262 (1979).

69. Robinson, S. E. & Wallace, M. J. Effect of perinatal buprenorphine exposure on development in the rat. *The Journal of pharmacology and experimental therapeutics* 298 2, 797–804 (2001).

70. Mathis, A. *et al.* DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nat Neurosci* 21, 1281–1289 (2018).

71. Goodwin, N. L. *et al.* Simple Behavioral Analysis (SimBA) as a platform for explainable machine learning in behavioral neuroscience. *Nat Neurosci* 27, 1411–1424 (2024).

72. Talpos, J. C., McTighe, S. M., Dias, R., Saksida, L. M. & Bussey, T. J. Trial-unique, delayed nonmatching-to-location (TUNL): A novel, highly hippocampus-dependent automated touchscreen test of location memory and pattern separation. *Neurobiology of Learning and Memory* 94, 341–352 (2010).

73. Oomen, C. A. *et al.* The touchscreen operant platform for testing working memory and pattern separation in rats and mice. *Nature Protocols* 8, 2006–2021 (2013).

74. Talpos, J. C., McTighe, S. M., Dias, R., Saksida, L. M. & Bussey, T. J. Trial-unique, delayed nonmatching-to-location (TUNL): A novel, highly hippocampus-dependent automated touchscreen test of location memory and pattern separation. *Neurobiology of Learning and Memory* 94, 341–352 (2010).

75. Bari, A., Dalley, J. & Robbins, T. The application of the 5-choice serial reaction time task for the assessment of visual attentional processes and impulse control in rats. *Nature protocols* 3, 759–67 (2008).

76. Papadopulos, F. *et al.* Common Tasks in Microscopic and Ultrastructural Image Analysis Using ImageJ. *Ultrastructural Pathology* 31, 401–407 (2007).

77. Chen, S. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta* 2, e107 (2023).

78. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9, 357–359 (2012).

79. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079 (2009).

80. Blanco-Míguez, A. *et al.* Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4. *Nat Biotechnol* 41, 1633–1644 (2023).

81. Beghini, F. *et al.* Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *Elife* 10, e65088 (2021).

82. Lazic, S. E. & Essioux, L. Improving basic and translational science by accounting for litter-to-litter variation in animal models. *BMC Neurosci* 14, 37–37 (2013).

83. Bugs as features (part 1): concepts and foundations for the compositional data analysis of the microbiome–gut–brain axis | Nature Mental Health. https://www.nature.com/articles/s44220-023-00148-3.

84. Grecco, G. G., Gao, Y., Gao, H., Liu, Y. & Atwood, B. K. Prenatal opioid administration induces shared alterations to the maternal and offspring gut microbiome: A preliminary analysis. *Drug and Alcohol Dependence* 227, 108914 (2021).

85. Cruz-Lebrón, A. *et al.* Chronic opioid use modulates human enteric microbiota and intestinal barrier integrity. *null* 13, 1946368 (2021).

86. Sturman, O., Germain, P.-L. & Bohacek, J. Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. *null* 21, 443–452 (2018).

87. Shan, X. *et al.* Rearing is critical for forming spatial representations in pre-weanling rats. *Behavioural Brain Research* 452, 114545 (2023).

88. Martis, L.-S., Brision, C., Holmes, M. C. & Wiborg, O. Resilient and depressive-like rats show distinct cognitive impairments in the touchscreen paired-associates learning (PAL) task. *Neurobiology of Learning and Memory* 155, 287–296 (2018).

89. Duda, W. & Węsierska, M. Spatial working memory in rats: Crucial role of the hippocampus in the allothetic place avoidance alternation task demanding stimuli segregation. *Behavioural Brain Research* 412, 113414 (2021).

90. Conradt, E. *et al.* Prenatal Opioid Exposure: Neurodevelopmental Consequences and Future Research Priorities. *Pediatrics* 144, e20190128 (2019).

91. Nelson, L. F. *et al.* Cognitive Outcomes of Young Children After Prenatal Exposure to Medications for Opioid Use Disorder: A Systematic Review and Meta-analysis. *JAMA Netw Open* 3, e201195 (2020).

92. Balalian, A. A. *et al.* Prenatal exposure to opioids and neurodevelopment in infancy and childhood: A systematic review. *Front Pediatr* 11, 1071889 (2023).

93. Sarfi, M., Eikemo, M., Welle-Strand, G. K., Muller, A. E. & Lehmann, S. Mental health and use of health care services in opioid-exposed school-aged children compared to foster children. *Eur Child Adolesc Psychiatry* 31, 495–509 (2022).

94. Kang, J. *et al.* Prenatal opioid exposure and subsequent risk of neuropsychiatric disorders in children: nationwide birth cohort study in South Korea. *BMJ* e077664 (2024) doi:10.1136/bmj-2023-077664.

95. Sherman, L. J., Ali, M. M., Mutter, R. & Larson, J. Mental Disorders Among Children Born With Neonatal Abstinence Syndrome. *PS* 70, 151–151 (2019).

96. Conradt, E. *et al.* Prenatal Opioid Exposure: Neurodevelopmental Consequences and Future Research Priorities. *Pediatrics* 144, e20190128 (2019).

97. Ferrante, J. R. & Blendy, J. A. Advances in animal models of prenatal opioid exposure. *Trends in Neurosciences* 47, 367–382 (2024).

98. Takuma, K. *et al.* Chronic treatment with valproic acid or sodium butyrate attenuates novel object recognition deficits and hippocampal dendritic spine loss in a mouse model of autism. *Pharmacology Biochemistry and Behavior* 126, 43–49 (2014).

99. Alaee, E., Moazen, P., Pattij, T., Semnanian, S. & Azizi, H. Prenatal exposure to morphine impairs attention and impulsivity in adult rats. *Psychopharmacology* 238, 2729–2741 (2021).

100. Liu, J. *et al.* Sodium butyrate exerts protective effect against Parkinson's disease in mice via stimulation of glucagon like peptide-1. *J Neurol Sci* 381, 176–181 (2017).

101. Mahajan, S. *et al.* Tight Junction Regulation by Morphine and HIV-1 Tat Modulates Blood–Brain Barrier Permeability. *Journal of clinical immunology* 28, 528–41 (2008).

102. Sharma, U. *et al.* Prescription Opioids induce Gut Dysbiosis and Exacerbate Colitis in a Murine Model of Inflammatory Bowel Disease. *Journal of Crohn's and Colitis* 14, 801–817 (2020).

103. Dmytriv, T. R., Storey, K. B. & Lushchak, V. I. Intestinal barrier permeability: the influence of gut microbiota, nutrition, and exercise. *Front Physiol* 15, 1380713 (2024).

104. Sanchez, E. S., Bigbee, J. W., Fobbs, W., Robinson, S. E. & Sato-Bigbee, C. Opioid addiction and pregnancy: Perinatal exposure to buprenorphine affects myelination in the developing brain. *Glia* 56, 1017–1027 (2008).

105. Vestal-Laborde, A. A., Eschenroeder, A. C., Bigbee, J. W., Robinson, S. E. & Sato-Bigbee, C. The opioid system and brain development: effects of methadone on the oligodendrocyte lineage and the early stages of myelination. *Dev Neurosci* 36, 409–421 (2014).

106. Qi, J., Ma, L. & Guo, W. Recent advances in the regulation mechanism of *SOX10*. *Journal of Otology* 17, 247–252 (2022).

107. Hornig, J. *et al.* The transcription factors Sox10 and Myrf define an essential regulatory network module in differentiating oligodendrocytes. *PLoS Genet* 9, e1003907 (2013).

108. Barbian, M. E. *et al.* Butyrate Supplementation to Pregnant Mice Elicits Cytoprotection Against Colonic Injury in Offspring. *Pediatr Res* 92, 125–134 (2022).

109. Huang, Y. *et al.* Maternal butyrate supplementation induces insulin resistance associated with enhanced intramuscular fat deposition in the offspring. *Oncotarget* 8, 13073–13084 (2016).

110. Huang, Y., Gao, S., Jun, G., Zhao, R. & Yang, X. Supplementing the maternal diet of rats with butyrate enhances mitochondrial biogenesis in the skeletal muscles of weaned offspring. *Br J Nutr* 117, 12–20 (2017).

111. Maternal sodium butyrate supplement elevates the lipolysis in adipose tissue and leads to lipid accumulation in offspring liver of weaning-age rats - PubMed. https://pubmed.ncbi.nlm.nih.gov/27449927/.

112. Caldji, C. *et al.* Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. *Proc Natl Acad Sci U S A* 95, 5335–5340 (1998).

113. Zhang, Y., Chen, H., Cao, J., Gao, L. & Jing, Y. Maternal separation alters peripheral immune responses associated with IFN-γ and OT in mice. *Peptides* 182, 171318 (2024).

114. Banqueri, M., Méndez, M., Gómez-Lázaro, E. & Arias, J. L. Early life stress by repeated maternal separation induces long-term neuroinflammatory response in glial cells of male rats. *Stress* (2019).

115. Romanowicz, M. *et al.* The effects of parental opioid use on the parent–child relationship and children's developmental and behavioral outcomes: a systematic review of published reports. *Child Adolesc Psychiatry Ment Health* 13, 5 (2019).

116. Wallin, C. M., Bowen, S. E. & Brummelte, S. Opioid use during pregnancy can impair maternal behavior and the Maternal Brain Network: A literature review. *Neurotoxicology and Teratology* 86, 106976 (2021).

117. Taplin, S. & Mattick, R. P. Mothers in methadone treatment and their involvement with the child protection system: A replication and extension study. *Child Abuse & Neglect* 37, 500–510 (2013).

118. Schlagal, Caitlin. R. *et al.* Maternal Opioid Exposure Culminates in Perturbed Murine Neurodevelopment and Hyperactive Phenotype in Adolescence. *Neuroscience* 463, 272–287 (2021).

119. Smith, C. *et al. Prenatal Opioid Exposure Inhibits Microglial Sculpting of the Dopamine System during Adolescence*. (2021). doi:10.1101/2021.11.28.468091.

120. Smith, J. W., Seckl, J. R., Evans, A. T., Costall, B. & Smythe, J. W. Gestational stress induces post-partum depression-like behaviour and alters maternal care in rats. *Psychoneuroendocrinology* 29, 227–244 (2004).

121. Khayat, S., Fanaei, H. & Haji Bagheri, K. A. Social isolation during pregnancy disrupts maternal behavior and hippocampal neurochemistry in rats: A role for BDNF, corticosterone, and GABAARα1. *Comprehensive Psychoneuroendocrinology* 21, 100282 (2025).

122. McDougall, S. *et al.* Myelination of Axons Corresponds with Faster Transmission Speed in the Prefrontal Cortex of Developing Male Rats. *eNeuro* 5, ENEURO.0203-18.2018 (2018).

123. Korsten, S. G. P. J., Vromans, H., Garssen, J. & Willemsen, L. E. M. Butyrate Protects Barrier Integrity and Suppresses Immune Activation in a Caco-2/PBMC Co-Culture Model While HDAC Inhibition Mimics Butyrate in Restoring Cytokine-Induced Barrier Disruption. *Nutrients* 15, 2760 (2023).

124. Korsten, S. G. P. J., Vromans, H., Garssen, J. & Willemsen, L. E. M. Butyrate Protects Barrier Integrity and Suppresses Immune Activation in a Caco-2/PBMC Co-Culture Model While HDAC Inhibition Mimics Butyrate in Restoring Cytokine-Induced Barrier Disruption. *Nutrients* 15, 2760 (2023).

125. Chang, P. V., Hao, L., Offermanns, S. & Medzhitov, R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences* 111, 2247–2252 (2014).

126. Singh, N. *et al.* Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* 40, 128–139 (2014).

127. Liu, L. *et al.* Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. *Cellular Immunology* 277, 66–73 (2012).

128. Wachman, E. M. *et al.* Epigenetic Variation in the Mu-opioid Receptor Gene in Infants with Neonatal Abstinence Syndrome. *J Pediatr* 165, 472–478 (2014).

129. Page, G. P. *et al.* Prenatal opioid exposure and the early life epigenome: Results from ECHO. (2024).