Gestational poly(I:C) attenuates, not exacerbates, the behavioral, cytokine and mTOR changes caused by isolation rearing in a rat 'dual-hit' model for neurodevelopmental disorders

Short title: Poly(I:C) attenuates responses to isolation rearing

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Abstract

Many psychiatric illnesses have a multifactorial etiology involving genetic and environmental risk factors that trigger persistent neurodevelopmental impairments. Several risk factors have been individually replicated in rodents, to understand disease mechanisms and evaluate novel treatments, particularly for poorly-managed negative and cognitive symptoms. However, the complex interplay between various factors remains unclear. Rodent dual-hit neurodevelopmental models offer vital opportunities to examine this and explore new strategies for early therapeutic intervention. This study combined gestational administration of polyinosinic:polycytidylic acid (poly(I:C); PIC, to mimic viral infection during pregnancy) with post-weaning isolation of resulting offspring (to mirror adolescent social adversity). After *in vitro* and *in vivo* studies required for laboratory-specific PIC characterization and optimization, we administered 10 mg/kg i.p. PIC potassium salt to time-mated Lister hooded dams on gestational day 15. This induced transient hypothermia, sickness behavior and weight loss in the dams, and led to locomotor hyperactivity, elevated striatal cytokine levels, and increased frontal cortical JNK phosphorylation in the offspring at adulthood. Remarkably, instead of exacerbating the well-characterized isolation syndrome, gestational PIC exposure actually protected against a spectrum of isolation-induced behavioral and brain regional changes. Thus isolation reared rats exhibited locomotor hyperactivity, impaired associative memory and reversal learning, elevated hippocampal and frontal cortical cytokine levels, and increased mammalian target of rapamycin (mTOR) activation in the frontal cortex – which were not evident in isolates previously exposed to gestational PIC. Brains from

adolescent littermates suggest little contribution of cytokines, mTOR or JNK to early development of the isolation syndrome, or resilience conferred by PIC. But notably hippocampal oxytocin, which can protect against stress, was higher in adolescent PIC-exposed isolates so might contribute to a more favorable outcome. These findings have implications for identifying individuals at risk for disorders like schizophrenia who may benefit from early therapeutic intervention, and justify preclinical assessment of whether adolescent oxytocin manipulations can modulate disease onset or progression.

Keywords

Maternal immune activation; poly(I:C); post-weaning isolation rearing; animal model; neurodevelopment; cognition; cytokine; mTOR; resilience; oxytocin

1. Introduction

Many psychiatric illnesses have a complex etiology involving genetic and early-life environmental risk factors spanning from gestation to adolescence. These lead to persistent neurodevelopmental impairments that represent a major cause of disability worldwide. For example schizophrenia, depression and bipolar disorder have a combined annual cost of over $542 billion in the USA alone (MacEwan et al., 2016), and poor management of negative and cognitive symptoms often prevents patient reintegration into society (Strassnig et al., 2018). Despite several compounds entering clinical trials for these domains very few have progressed beyond Phase III. High attrition is partly attributed to a need for improved preclinical models to elucidate disease neurobiology and enable predictive evaluation of novel therapeutics (Hutson et al., 2017). One recent approach to more refined modeling involves ‘dual-hit’ combinations of established neurodevelopmental manipulations that mirror delayed symptom onset and multiple neurotransmitter involvement. These

combinations may provide insight into how environmental interactions increase disease susceptibility and ultimately allow more effective targeting of personalized medicine to groups at high risk.

Social adversity is linked to schizophrenia, depression, anxiety disorders and substance abuse. For example parental separation or loss, abuse or bullying during childhood, frequent relocation during adolescence, and social disadvantage or exclusion extending into later life are all risk factors for individual disorders or their comorbidity (Cerdá et al., 2010; Stilo and Murray, 2010). Social deprivation can be replicated in rodents by post-weaning isolation of gregarious rat pups in individual cages. This well-established model is associated with lasting neuroanatomical, neurochemical and behavioral alterations that emerge post-puberty and have therefore traditionally been thought to resemble some of the core features of schizophrenia (for review see Jones et al., 2011). In addition we recently identified isolation-induced changes in both the gut microbiome and brain regional cytokines (Dunphy-Doherty et al., 2018; Shortall et al., 2018), both topical areas for mental health research (Bauer and Teixeira, 2019). However, sensitivity of isolation-induced changes to handling interference can limit opportunities to assess pharmacological reversal or prevention, changes in pre-pulse inhibition (PPI) of the acoustic startle response (a useful indicator of sensorimotor gating) are strain-dependent and do not occur in every cohort, and isolates often show increased aggression in social paradigms instead of reduced interaction more akin to negative symptoms (Jones et al., 2011).

Maternal immune activation (MIA) during pregnancy, by infection, allergy, stress or obesity, is associated with increased susceptibility of the offspring to several neurodevelopmental disorders, so may act as a disease primer. For example epidemiological and birth cohort

studies have linked schizophrenia and autistic spectrum disorders (ASD) with influenza, rubella, cytomegalovirus or herpes simplex viruses, as well as respiratory, urinary tract and parasitic infections (Brown, 2012; Knuesel et al., 2014). Although direct pathogen exposure can be replicated in rodents, for biosecurity reasons most studies use alternative challenges to induce MIA, such as the synthetic double-stranded RNA viral mimetic polyinosinic–polycytidylic acid (Poly(I:C); PIC), which activates toll-like receptor (TLR) 3 to stimulate cytokine release. Seminal studies demonstrated the potential for profound neurodevelopmental consequences of PIC exposure in mid-gestation, typically gestational day (GD) 15 in rats (for reviews see Macêdo et al., 2012; Meyer and Feldon, 2012). However, it is becoming increasingly clear that such models are variable, subject to PIC batch effects (Careaga et al., 2018; Kowash et al., 2019; Mueller et al., 2019), and at least in some laboratories do not adequately model the full range of cognitive or motivational impairments seen in patients (Bates et al., 2018; Gray et al., 2019).

It has been suggested that combining separate neurodevelopmental interventions, like MIA and isolation rearing, into a dual-hit model might overcome some of their individual limitations (Bates et al., 2018; Shortall et al., 2018). Crucially it appears that even subclinical MIA can increase vulnerability of the offspring to a second hit, thus increasing the likelihood of environmental stressors causing a schizophrenia- or ASD-like phenotype (Estes and McAllister, 2016). This study therefore preceded isolation rearing with gestational PIC (PIC-Iso), which we hypothesized would be associated with more robust changes than either intervention alone, according to the cumulative stress theory (McEwen, 1998). We have previously used a similar approach for neonatal phencyclidine (PCP) with isolation rearing (PCP-Iso), to produce a broader array of relevant cognitive changes (Gaskin et al., 2014) with apparent predictive validity for schizophrenia (Watson et al., 2016), although PPI deficits still

remained variable (Shortall et al., 2020). Our current goal was to examine whether combined exposure to two highly relevant risk factors might predict increased susceptibility to mental illness later in life, in which case we expected to achieve a translationally relevant dual-hit model with good construct validity (Venkatasubramanian and Debnath, 2013) that could be used to further investigate potential mechanisms of environment x environment interaction, and ultimately evaluate novel preventative or disease-modifying therapeutics.

To address requirements for laboratory-specific optimization (Roderick and Kentner, 2019; Murray et al., 2019; Gray et al., 2019) we confirmed expected responses to our batch of PIC *in vitro*, by assessing induction of the early-response immune and inflammatory regulator microRNA-155 (upregulated by TLR3 ligands like PIC: O'Connell et al., 2007; Swaminathan et al., 2012) in primary bone marrow-derived macrophages (Supplementary material 1). We also performed pilot studies to verify PIC-induced 'sickness behavior' *in vivo* (Supplementary material 1). Intravenous (i.v.) PIC administration is commonly used in MIA studies (e.g. Zhang et al., 2012; Wallace et al., 2014; Ballendine et al., 2015; Vernon et al., 2015), but a single intraperitoneal (i.p.) injection can also induce acute sickness responses in pregnant rats and lasting developmental changes in their offspring (Gilmore et al., 2005; Bronson et al., 2011; Richtand et al., 2012a; Richtand et al., 2012b; Vorhees et al., 2012; Murray et al., 2019). We therefore adopted the latter route as a potential refinement associated with reduced acute restraint stress. Resulting offspring of both sexes were initially assessed in adolescence for sensitivity to PIC, isolation and their combination. Remaining studies focused solely on males as they were more responsive to our neurodevelopmental interventions; an observation that may parallel the incidence of conditions like schizophrenia and ASD.

We used brain tissue obtained in adulthood to further understand changes that might have relevance to psychiatric illnesses (Abdolmaleky et al., 2014; Gururajan and van den Buuse, 2014; Morris and Pratt, 2014; Uhrig et al., 2016; McGuire et al., 2017) and contribute to the final behavioral phenotype. These included cytokine (Dunphy-Doherty et al., 2018) and oxytocin levels (Gilles and Polston, 2017), activation of mammalian target of rapamycin (mTOR; Meffre et al., 2012) and stress-related c-Jun N-terminal Kinase (JNK; Shortall et al., 2018) intracellular signaling pathways (measured using magnetic bead-based multiplex assays), as well as expression of serotonin (5-hydroxytryptamine, 5-HT; Burke et al., 2017) and its reuptake transporter SERT (via immunohistochemistry). Gestational PIC and/or isolation rearing have been individually reported to influence all these measures and we hypothesized that the alterations would be exacerbated by combined replication of two separate environmental risk factors. We followed this work by performing similar multiplex analysis of samples obtained from adolescent littermates in an attempt to understand early developmental mechanisms contributing to the isolation syndrome, and its modification by gestational PIC exposure.

2. Methods

2.1 Animals

This research involved a total of 250 animals, which were all maintained under controlled conditions (21 ± 2 °C, 55 ± 10% humidity, 12 h light-dark cycle; on at 07.00 h) and had unlimited access to food and water unless stated. The main gestational study involved 16 time-mated nulliparous female Lister hooded rats obtained (Charles River, UK) on GD 7-10 (with the day of vaginal plug detection designated as GD 1) and housed separately (n = 8 per treatment). Housing conditions influence PIC-induced MIA in mice (Mueller et al., 2018) and although parallel comparisons of different caging systems have yet to be performed in

rats, for consistency with maintenance of our chosen strain (Murray et al., 2019, published in abstract form when our experiments were designed and conducted) we housed females (and resulting litters until weaning) in individually ventilated cages (GR1800 Double-Decker; Tecniplast, UK) with sawdust bedding and standard environmental enrichment (cardboard play tube, wooden chew block and paper nest material). PIC administration and behavioral testing occurred in the light phase (10:00-11:00 and 09:00-17:00 h, respectively), using doses and an experimental design chosen to comply with the three R's of humane animal testing. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and ARRIVE guidelines (Kilkenny et al., 2010), with University of Nottingham Animal Welfare and Ethical Review Board approval. Group sizes were based on previous studies employing similar techniques (McLean et al., 2008; Woods et al., 2012; Gaskin et al., 2016; Watson et al., 2016; Kohli et al., 2019). Data were obtained by trained observers unaware of neurodevelopmental history. To support a recent initiative aimed at improving the rigor, reproducibility and transparency of MIA models (Kentner et al., 2019) we have included a completed reporting checklist (Supplementary material 2).

2.2 Gestational PIC administration

Dams (GD 14) were non-surgically injected with s.c. temperature sensing microchips into the nape of the neck (idENTHICHIP with Bio-Thermo®; Animalcare, UK) to allow subsequent physiological monitoring in freely-moving animals with minimal disturbance. On GD 15 body weight and temperature were recorded immediately prior to i.p. injection of 10 mg/kg PIC or vehicle (as described in Supplementary material 1). The gestational timing of PIC administration matched 70% of over 50 studies published at the time we started this research and corresponds to migration of cortical neurons in the rat, which occurs during the mid-point of the human second trimester (Sarkar et al., 2019). Hourly temperature monitoring and

rating of sickness behavior on a simple five-point scale (described in Supplementary material 1) continued for 6 h, with further recording of weight, temperature and sickness behavior on GD 16-18 (24, 48 and 72 h post-injection). Immediately after the final monitoring point on GD 18 dams, together with their existing environmental enrichment and a small amount of sawdust, were transferred to clean cages with additional fresh sawdust bedding and generous amounts of nesting material (to occupy approximately half the total cage volume). To minimize stress throughout the remainder of gestation, and to avoid disruption of mother-pup interactions throughout the pre-weaning period (Wallace et al., 2014) dams and litters were then left undisturbed until counting and sexing of pups on postnatal day (PND) 20.

2.3 Retrospective analyses of PIC fragment size and potential LPS contamination

Recognized batch variations in PIC immunogenicity, even from the same supplier (Harvey and Boksa, 2012), can be attributed to differences in molecular weight composition (Zhou et al., 2013; Mian et al., 2013) and variable contamination with bacterial endotoxin (which induces MIA through TLR4). Emerging awareness of the profound implications for MIA models emphasizes the importance of characterizing each PIC batch (Careaga et al., 2018; Kowash et al., 2019; Mueller et al., 2019), and we therefore conducted retrospective analyses of both molecular weight composition and potential endotoxin contamination.

Endotoxin content was determined using a ToxinSensor^{TM} chromogenic limulus amebocyte lysate (LAL) endotoxin assay kit (L00350; GenScript, USA). Molecular weight was quantified by multi-angle light scattering (MALS) as described by Kowash et al. (2019). In summary an input of 50 μg PIC was loaded onto a Superose 6 10/300 GL column (GE Healthcare Life Sciences, UK) flushed with PBS Dulbecco A (Thermo Fisher Scientific) at a rate of 0.75 ml/min. MALS data were analyzed using 1 ml fractions from 7 to 17 ml of

column flow-through to determine both the mean molecular weight and molecular weight distribution.

2.4 Post-weaning housing of gestational vehicle- and PIC-exposed litters

On PND 22 pups were weaned into single-sex treatment-matched groups (3-4 per cage) or isolation (1 per cage), resulting in four neurodevelopmental conditions per sex; vehicle group-housed (Veh-Gr), PIC group-housed (PIC-Gr), vehicle isolation (Veh-Iso) and PIC isolation (PIC-Iso). Post-weaning cages (Gr 32 x 51 cm, Iso 25 x 42 cm) contained sawdust bedding but no environmental enrichment, and were of the conventional open-top style with grid lids to ensure singly housed rats maintained olfactory and auditory as well as visual contact with conspecifics, according to our established protocol (Jones et al., 2011). Handling was restricted to a single weekly cage change and body weight measurement until the start of behavioral testing. To minimize the chances of any litter effect (Spencer and Meyer, 2017) pups from each Veh- and PIC-treated dam were assigned to both Gr and Iso housing conditions, and further subdivided to ensure equivalent contribution to different aged test groups. This randomized allocation of littermates to different experimental subgroups avoided artificial sample size inflation by pseudo replication.

2.5 Behavioral evaluation of offspring

Locomotor hyperactivity in a novel environment is one of the earliest features of the isolation syndrome to emerge. Adolescent male and female rats underwent this single behavioral evaluation two weeks post-weaning to establish the most suitable sex for remaining studies, and reduce cumulative lifetime adversity by avoiding the opposite sex undergoing long-term maintenance and additional test procedures. This cohort of adolescent rats were then immediately killed to collect tissue samples at this developmental time point. Starting four

weeks post-weaning separate male littermates, which appeared more sensitive to isolation alone or combined with PIC, underwent a battery of tasks selected for translational relevance to the positive, negative and multiple cognitive symptoms of schizophrenia, some of which overlap with symptoms of depression, attention deficit hyperactivity disorder (ADHD) and ASD (Young et al., 2009; Fabricius et al., 2011; Millan et al., 2012). These include assessment of locomotor activity, non-spatial novel object discrimination (NOD), object-based novel location discrimination (NLD), social interaction and concomitant ultrasonic vocalizations (USVs), PPI, fear-motivated contextual freezing responses (CFR) and a bowl-digging attentional set-shifting task (ASST). These are all described in detail elsewhere (McLean et al., 2008; ElBeltagy et al., 2010; Shortall et al., 2018; Kohli et al., 2019) and were assessed in order from least to most aversive at approximately one week intervals (Fig. 1). In cases where the number of animals and duration required to assess each individual necessitated testing to be split over multiple days care was taken to ensure a mix of neurodevelopmental conditions across days. Animals underwent balanced allocation to one of two possible final tasks, CFR or ASST, so that each individual only experienced one task involving fear conditioning or requiring food restriction, to limit cumulative lifetime severity.

2.5.1 Locomotor activity

Spontaneous activity (in the form of ambulation, rearing and fine movement) in a novel arena was assessed on either PND 36 (adolescent rats of both sexes) or PND 51-57 (males only). Rats spent 1 h in individual photobeam activity chambers (39 x 23.5 x 24.5 cm; San Diego Instruments, USA), where a single ambulation count was recorded for every two consecutive adjacent lower beam breaks, a single rearing count for every upper beam break, and a single fine movement count for each repeated lower beam break (Shortall et al., 2018).

2.5.2 NOD

Non-spatial visual recognition memory was assessed the following day in the same arena (PND 52-58). Rats received 3 min habituation, then two consecutive 3 min object exploration trials separated by either a 1 or 2 h inter-trial interval (ITI). In the familiarization trial rats encountered two identical bottles. For the choice trial one was randomly replaced with a novel object (striped bottle). Object exploration (sniffing, licking, chewing or having moving vibrissae while directing the nose towards and ≤ 1 cm) was timed and used to calculate the choice trial discrimination ratio (exploration of novel/total choice trial object exploration; Shortall et al., 2018).

2.5.3 NLD

A variant of the NOD procedure was used to assess spatial visual recognition memory. The day before testing rats were habituated to different individual test arenas (39 x 23.5 x 24.5 cm) with spatial cues (black and white striped/spotted patterns) on opposite walls. On test days (PND 59-65) object exploration trials were separated by a 1 min ITI and in the choice trial one of the identical objects was randomly moved to a novel location (ElBeltagy et al., 2010).

2.5.4 Social interaction and USVs

Social interaction between unfamiliar conspecifics was assessed to provide an indicator of social engagement or withdrawal. All rats were individually housed the night before testing to facilitate behavioral interaction. On PND 66-70 pairs of neurodevelopmental condition- and weight-matched (<33 g difference) rats from different litters were differentially colored by light application of pink or blue hairspray to the back (Superdrug, UK; 30 min prior to the test) to facilitate video tracking (Ethovision XT v8.5, Noldus). Each pair was placed in an

unfamiliar circular arena (75 cm diameter, 45 cm walls), under dim light (40 lx) for 10 min. Movement was automatically tracked and total social interaction as well as individual components (anogenital sniffing, body sniffing, crawling under and over, following, lying side by side, boxing and biting, pinning) exhibited by each member of the pair were scored from video-recordings. Because the behavior of each rat is dependent on that of its partner, mean values were calculated for each pair (Kohli et al., 2019).

As an additional index of interaction and communication concomitant USVs were recorded using an electret microphone (Emkay, Avisoft Bioacoustics, Germany) connected to an ultrasound detection unit (Ultrasound Gate customized model 112, Avisoft Bioacoustics) to capture both 22 kHz alarm and 50 kHz prosocial USVs. The resulting signal was digitalized and saved as .wav files. Temporal and frequency characteristic of each call were extracted using Avisoft analysis software (SAS-LabPro, v4.38, Avisoft Bioacoustics), verified by eye, and categorized into flat (peak frequency change ≤5 kHz), step (at least one short element >5 kHz higher or lower than the fundamental call) or trill (at least two common zig-zag patterns appearing as a series of inverted-U shapes) subtypes (Kohli et al., 2019).

2.5.5 PPI

Sensorimotor gating was assessed (PND 74-78) in SR-Lab startle response chambers (San Diego Instruments, USA) where rats received 5 min acclimatization to background white noise (62 dB), ten 120 dB startle trials, a pseudorandom mix of 50 trials with or without a preceding sub-threshold 72, 76, 80 or 84 dB pre-pulse, then five final startle trials (all separated by 10-20 s). Individual whole-body startle responses were recorded for 100 ms after startle pulse onset and used to calculate area under the curve (AUC). For each trial type the mean percentage PPI was calculated from mean AUC (after conditional elimination of

values greater than two standard deviations from the mean, attributed to movement during startle delivery), using the equation % PPI = ((pulse alone AUC – pre-pulse AUC)/pulse alone AUC) x 100 (Shortall et al., 2018).

2.5.6 CFR

Fear-motivated associative memory was assessed over a two day period starting on PND 79-83. Rats were placed into a two-compartment shuttle box with a light and dark side separated by an automated door and linked to shuttle box control and shocker units (Panlab SLab, Spain). After 30 s in the light side the door opened and the latency for the animal to transfer to the dark side was recorded using a floor sensor which also triggered door closure. After 30 s in the dark side a 5 s conditioned stimulus (light and 3 kHz 89 dB tone) was delivered, paired with an unconditioned stimulus (1 s x 0.4 mA footshock delivered through the grid floor) during the last second. Rats received a total of three stimuli pairings separated by 55 s and the duration of freezing behavior (immobility, except for respiration, in a hunched posture with inactive vibrissae) following each pairing was recorded separately using stopwatches. Twenty-four hours later rats returned to the dark side for a total of 600 s, without any further footshocks. Freezing to the context was measured during the first 300 s, then the conditioned stimuli were presented for 5 s and freezing to cue was measured for the remainder of the trial (Shortall et al., 2018).

2.5.7 ASST

Reasoning and problem solving were assessed using a bowl-digging ASST. The relatively labor-intensive nature of this task required a staggered start to the eight-day protocol (PND 79-125), at which point group-housed rats were transferred to pair housing (with an existing cage mate) to enable food restriction (12 g/rat/day). On the 1st day of restriction rats received

10 loops of honey flavored breakfast cereal in the home cage (Cheerios, broken into thirds; Nestlé, UK) and on three consecutive days between 2nd and 6th of food restriction they were habituated (15 min twice daily) to the test arena. The arena (black Perspex 70 x 50 x 50 cm) was modeled on that of McLean et al. (2008) and consisted of a start area (with access to water and separated from the rest of the apparatus by a lifting divider), open central area, and choice area (where two 11 cm diameter terracotta digging bowls were inserted through holes to expose only 3 cm of the rim above floor level). During habituation sessions rats were released from the start area and allowed uninterrupted access to both bowls, which contained unscented sawdust and were generously baited with honey loops at increasing depths to encourage vigorous digging. On the 7th day of food restriction all rats were trained (as described by McLean et al., 2008) to discriminate a rewarded from unrewarded digging medium (pink 'Carefresh' recycled paper pulp versus white paper flake rodent bedding) and rewarded from unrewarded odor (satsuma versus vanilla Bodyshop aromatic oils anointed around the rim and allowed to dry). 50% of each neurodevelopmental condition were trained on media first, and only 50% of these started with media as the relevant dimension on test day. In all cases a correct response (i.e. first dig in the correct bowl) was rewarded by location and consumption of one reward. For the first four 'discovery' trials on each discrimination rats were allowed to follow an incorrect response with a rewarded dig in the correct bowl. Training continued until rats achieved six consecutive correct trials, and the number of trials and errors to reach criterion were recorded. Two rats which failed to achieve this within 30 trials (1 Veh-Gr for media and 1 Veh-Iso for odor) were excluded from further testing. On the 8th (final) day of food restriction (PND 87-133) rats underwent a standard series of seven discriminations (simple, compound, reversal 1, intradimensional shift, reversal 2, extradimensional shift, and reversal 3; Birrell and Brown, 2000). These used previously unencountered exemplars that were rotated across discriminations to ensure any slight

differences in cue salience would not preferentially skew results for any particular discrimination. The order of rotation was replicated for one rat from each neurodevelopmental condition. As the number of examplars prevented full counterbalancing of all possible odor/media combinations and correct initial stimuli they were presented in constant sets, where the first member of each pair represents the initial correct response when that dimension was relevant (smooth fish tank gravel versus chinchilla bathing sand with lavender versus strawberry, mineral- versus cellulose-based cat litter with green tea and lemon versus pomegranate and raspberry, plastic versus wax beads with exotic versus jasmine and white frangipani, and wood chip versus compost reptile bedding with aloe and soft linen versus sandalwood and ginger). To minimize any likelihood of individuals becoming fatigued or satiated at different stages of the test day all rats received a standardized 1 h rest break in the home cage after completing reversal 1.

2.6 Tissue collection

Adolescent rats were killed immediately after locomotor activity assessment on PND 36 (2 weeks post-weaning) and adult rats from the CFR subgroup on PND 87-91 (9-10 weeks post-weaning) by concussion and immediate decapitation. The frontal cortex, hippocampus and striatum were dissected on a refrigerated table (4 ˚C) then frozen in liquid nitrogen and stored at -80 °C (for multiplex analysis). Adult rats from the ASST subgroup were killed immediately after completing the task (PND 87-133), via the same method. Intact brain hemispheres were immersed in 4% paraformaldehyde then 30% sucrose (each overnight, 4 °C) then frozen in isopentane on dry ice and stored at -80 °C (for immunohistochemistry).

2.7 Immunohistochemical analysis of frontal cortical 5-HT and SERT in adulthood

Serial coronal sections (60 μm) were obtained throughout the frontal cortex using a freezing microtome (Anglia Scientific, UK) and stored in anti-freeze (30% glycerol, 30% ethylene glycol in PBS) at -20 °C until free-floating immunohistochemistry. Ten evenly spaced sections per rat were washed (4 x 5 min in phosphate buffered saline; PBS) then incubated in 2% normal donkey or goat serum in buffer 1 (0.5% BSA, 0.3% Triton X-100 in PBS; 1 h), followed by goat polyclonal primary antibody against 5-HT (Abcam, UK: 1:500), rabbit polyclonal primary antibody against the 5-HT transporter (SERT; Immunostar, USA: 1:5000), or buffer 1 alone for negative control (overnight, 4 °C). Sections were washed (3 x 5 min in buffer 2; 0.15% BSA, 0.1% Triton X-100 in PBS), incubated in Alexa Fluor 594 donkey anti-goat or 568 goat anti-rabbit secondary antibody (Thermo Fisher Scientific: 1:500 in buffer 2; 1 h in the dark), washed (2 x 5 min buffer 2, 2 x 5 min PBS) then mounted on gelatin-subbed slides and air-dried. Slides were rinsed with PBS, counterstained with DAPI nuclear stain (Sigma-Aldrich: 1:2 000 in H_2O; 30 s), rinsed with H_2O and cover slipped with DABCO (Sigma-Aldrich: 0.2% in 90% glycerol in PBS) then stored at 4 °C. Sections were viewed on a Nikon EFD-3 fluorescence microscope and images obtained using an Insight QE camera and SPOT Imaging software (Diagnostic Instruments Inc., USA). The intensity of 5-HT and SERT immunoreactivity in consistently oriented and consistently placed x10 snapshot images throughout the orbitofrontal cortex (OFC) and prelimbic cortex (PrL), delineated using boundaries defined in Paxinos and Watson (1998; Supplementary material 5, Supplementary Fig. 6A-B), was quantified using the Analyze > Measure tool in Fiji (Schindelin et al., 2012) and normalized by manual subtraction of background staining. Data from OFC regions of interest from each individual were averaged to ensure representation of rostral medial/ventral and dorsolateral (Bregma +4.20-4.70) as well as caudal ventral/lateral subdivisions (Bregma +3.20-3.70). PRL images from the same individuals were also

averaged to ensure representation of Bregma +3.20-4.20. Technical issues during sectioning prevented inclusion of one Veh-Gr, PIC-Gr and Veh-Iso individual.

2.8 Multiplex analysis of cytokine and oxytocin levels, as well as mTOR and JNK activation in adulthood and adolescence

Brain regional tissue samples were homogenized (4 °C) by sonication in a 1:100 mix of RIPA buffer and protease inhibitor cocktail (both Sigma-Aldrich); 1 ml per 120 mg of tissue, by sonication (~15 s) then vertical disc rotation (1 h). Supernatants resulting from centrifugation (850 x g, 5 min) were subject to Lowry assay then adjusted to a common protein concentration (10.5 mg/ml) before determination of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-18 and TNFα), total and phosphorylated (Ser2448) mTOR, total and phosphorylated (Thr183/Tyr185) JNK, and oxytocin against standards and quality controls using multiplex kits (MILLIPLEX® RECYTMAG-65K, 48-652MAG, 48-622MAG and RMNPMAG-83K; Merck Millipore, UK) and a MAGPIX® system with xPONENT 3.1 software, as previously described (Hind et al., 2016; Dunphy-Doherty et al., 2018).

2.9 Statistical analysis

All analyses were performed using GraphPad Prism (v7) or IBM SPSS (v24). Normality and homogeneity of variance were confirmed prior to use of parametric analyses. Body weight and temperature data from dams were analyzed by two-way repeated measures ANOVA (with time as a within- and gestational treatment as a between-subjects factor). Due to the absence of a non-parametric equivalent this test was also applied to the sickness behavior time course. Total sickness scores across the post-injection monitoring period were analyzed by Mann-Whitney U test.

All data from offspring were analyzed by ANOVA with a minimum of two between-subject factors; gestational treatment and housing. For simplicity main effects of these factors are reported within the results section as effects of PIC or isolation, respectively. Sex was included as a third between-subjects factor during adolescence, since males and females were both studied at this developmental stage. For behavioral tests where performance of each rat was assessed over time (e.g. locomotor activity), or where behaviors scored in any one trial were mutually exclusive (e.g. exploration of novel versus familiar objects, or engagement in different components of the social interaction repertoire) a task-specific variable was included as a final within-subjects repeated measure. For cytokine analysis (which involved multiple inflammatory mediators in multiple brain regions) both cytokine and region were included as within-subjects repeated measures and ANOVAs were followed by Benjamini-Hochberg corrections for false discovery rate. In all cases post-hoc within- and between-subjects comparisons used Sidak's and Tukey's tests, respectively.

Additional Pearson's *r* correlation analyses were performed between summary measures for each behavioral test and brain regional tissue changes (specifically cytokine levels, mTOR activation, JNK activation or oxytocin levels that were significantly influenced by PIC, isolation or their combination). Similar inter-correlations were performed between tissue changes in the same brain region at the same developmental change. Cytokine correlations were limited to the single most pronounced change in that brain region and all correlations were subjected to Benjamini-Hochberg corrections for false discovery rate. $P<0.05$ was regarded as statistically significant and data are presented as the mean ± SEM.

3. Results

3.1 Confirmation of maternal PIC-induced sickness behavior

Full-scale gestational PIC administration was associated with hypothermia, sickness behavior and reduced weight gain. There was a PIC x time interaction for body temperature ($F_{(9,126)}$ = 4.840, P<0.001) which was lower in PIC than Veh-treated dams 2 h post-injection (Fig. 2A), a PIC effect ($F_{(1,14)}$ = 14.530, P = 0.002) and PIC x time interaction for sickness score ($F_{(5,70)}$ = 5.093, P = 0.001) which was elevated 3-4 h after PIC (Fig. 2B) and 367 ± 53% of Veh for the total 6 h post-injection period (P = 0.003; data not shown), plus a PIC effect for weight gain ($F_{(1,14)}$ = 8.494, P = 0.011) which was reduced 24 h after PIC (Fig. 2C). The PIC effect on body weight was equivalent to a 0.3 ± 0.5% decrease from GD 15 to GD 16, compared to a 2.0 ± 0.4% increase in Veh-treated dams.

A total of 215 pups were born to the 16 dams, with no observable malformations nor any effect of PIC on number of pups per litter (Veh 13.4 ± 0.9, PIC 13.5 ± 0.8), male:female ratio (1.1 ± 0.2 in each case) or pup weight at weaning (Veh males 38.4 ± 0.6 g, PIC males 38.2 ± 0.8 g, Veh females 36.7 ± 0.9 g, PIC females 34.1 ± 1.4 g).

3.2 Retrospective analyses of PIC fragment size and potential LPS contamination

The endotoxin concentration in our PIC batch was 15.65 EU/ml, while levels in our vehicle control were below the detection limit. The mean molecular weight of our PIC was 117.1 kDa and the majority (<98%) was distributed in the <260 kDa range. However a very small proportion (1.6%) of higher molecular weight species were in the >800 kDa range (Fig. 2D).

3.3 Evaluation of adolescent offspring for sex differences in susceptibility to PIC, isolation and their combination

3.3.1 Locomotor activity

Assessment of locomotor activity in adolescent offspring, two weeks post-weaning, revealed highly significant main effects of sex and isolation on ambulation, fine movement and rears. These were accompanied by a time x sex x isolation interaction for time course of ambulation ($F_{(11,528)} = 1.945$, $P = 0.032$), sex x isolation interaction for total ambulation ($F_{(1,48)} = 4.380$, $P = 0.042$) and sex x PIC x isolation interaction for total fine movement ($F_{(1,48)} = 6.396$, $P = 0.015$). When sexes were analyzed separately the isolation effect was more marked in males. Male Veh-Iso demonstrated higher counts than Veh-Gr for each of the three activity indicators at multiple time points (Fig. 3A, C, E; line graphs) and in total (Fig. 3A, C, E; bar graphs), whereas female Veh-Iso were only more active than Veh-Gr in terms of rears (Fig. 3F). The PIC effect was also more pronounced in males, where total fine movement was elevated in PIC-Gr (Fig. 3C; bar graph). Although the PIC-Iso combination elevated fine movement in both sexes (Fig. 3C-D) additional short-term increases in ambulation and rears were only evident in males (Fig. 3A, E; line graphs).

3.3.2 Brain regional cytokine levels

Assessment of cytokine levels in brain regional tissue samples collected immediately after locomotor activity testing revealed expected main effects of cytokine ($F_{(7,259)} = 1480.440$, $P<0.001$), brain region ($F_{(2,74)} = 12.641$, $P<0.001$) and sex ($F_{(1,37)} = 11.969$, $P = 0.001$), together with interactions between these factors ($F_{(14,518)} = 33.149$, $P<0.001$). Of note there was also a PIC x cytokine x sex interaction ($F_{(7,259)} = 2.611$, $P = 0.013$). When sexes were analyzed separately there was a PIC x cytokine interaction in males ($F_{(7,133)} = 3.030$, $P<0.001$), plus additional subtle changes which are described in detail in Section 3.5.3 (alongside other multiplex data from adolescent males). There was no PIC x cytokine interaction in females (Supplementary material 3).

Based on these sex differences in locomotor and cytokine susceptibility to PIC, Iso and their combination we restricted more extensive adulthood evaluation to males. This decision minimized cumulative lifetime severity experienced by female cohorts, who avoided additional time in isolation and did not undergo cognitive tests involving footshock or food restriction.

3.4 Behavioral changes in adult male offspring

Body weight of free-feeding males across the first eight weeks post-weaning showed the expected time effect ($F_{(8,224)} = 6086.990$, $P<0.001$), but no PIC or isolation effects (data not shown).

3.4.1 Locomotor activity

Locomotor testing of male offspring in early adulthood revealed main effects of time plus PIC and/or isolation on ambulation, fine movement and rears. These were accompanied by PIC x isolation interactions for total rears ($F_{(1,28)} = 8.285$, $P = 0.008$) and total fine movement ($F_{(1,28)} = 4.162$, $P = 0.050$), which were both higher in the Veh-Iso single model than Veh-Gr controls. But interestingly, neither rearing nor fine movement were elevated above Veh-Gr levels in the combined PIC-Iso model (Fig. 4A).

Locomotion during the social interaction test showed a similar pattern. There was a housing effect on distance moved ($F_{(1,28)} = 7.937$, $P = 0.009$), which was higher in Veh-Iso (82 ± 2 m; $P = 0.029$) but not PIC-Iso (73 ± 3 m) than Veh-Gr (67 ± 2 m). Mean velocity (treatment x housing $F_{(1,28)} = 10.76$, $P = 0.003$) was also higher in Veh-Iso (14.3 ± 0.3 cm/s; $P = 0.007$) than PIC-Iso (12.3 ± 0.5 cm/s).

3.4.2 NOD and NLD

There were no spatial preferences between identical objects during the familiarization trial, nor any between-group differences in total object exploration in either trial (data not shown). The main effects of object during choice trials of both the non-spatial NOD (1 h ITI: $F_{(1,28)}$ = 16.731, P<0.001; 2 h ITI: $F_{(1,28)}$ = 14.170, P = 0.001) and spatial NLD (1 min ITI: $F_{(1,28)}$ = 27.861, P<0.001) were unaffected by PIC or isolation (Supplementary material 4; Supplementary Fig. 4A-C).

3.4.3 Social interaction and USVs

Total durations of social interaction were equivalent across groups (Veh-Gr 156 ± 17 s, PIC-Gr 158 ± 13 s, Veh-Iso 157 ± 12 s, PIC-Iso 136 ± 14 s), but examination of components of the social repertoire revealed considerable between-group differences in the nature of the interaction. Aggressive behaviors (boxing and biting, pinning) showed subtype x PIC ($F_{(1,28)}$ = 8.086, P = 0.008), subtype x isolation ($F_{(1,28)}$ = 19.335, P<0.001) and subtype x PIC x isolation interactions ($F_{(1,28)}$ = 4.904, P = 0.035). Veh-Iso pairs engaged in more boxing and biting than Veh-Gr pairs but this increase was absent in PIC-Iso (Fig. 4B). Prosocial behaviors (anogenital sniffing, body sniffing, crawling under and over, following, lying side by side) also showed a subtype x isolation interaction ($F_{(4,112)}$ = 28.768, P<0.001) but were not modified by PIC. Thus Veh-Iso and PIC-Iso both performed less anogenital sniffing and more body sniffing than Veh-Gr and PIC-Gr, such that in this case there were no differences in either behavior between Veh-Iso and PIC-Iso (Fig. 4B).

The number of accompanying prosocial USVs showed a PIC x isolation interaction ($F_{(1,28)}$ = 6.613, P = 0.016, being higher in Veh-Iso (588 ± 168) but not PIC-Iso (327 ± 98) or PIC-Gr (394 ± 95) than Veh-Gr (90 ± 39). Segregation of calls into three main subtypes revealed the

elevation in Veh-Iso was restricted to step-type frequency-modulated calls (which were also elevated in PIC-Gr), while frequency-modulated trills and non-frequency-modulated flat calls were unaffected by PIC or isolation (Fig. 4B). Aversion-related 22 kHz calls were rare (only detected in 2 PIC-Gr pairs, 3 Veh-Iso pairs and 1 PIC-Iso pair) and their numbers were not significantly influenced by PIC or isolation (data not shown).

3.4.4 PPI

PPI testing showed the expected inhibition of startle with increasing pre-pulse volume ($F_{(2,56)}$ = 58.572, P<0.001), but neither startle reactivity (data not shown) nor inhibition were influenced by PIC or isolation (Supplementary material 4; Supplementary Fig. 4D).

3.4.5 CFR

Acquisition of the conditioned-unconditioned stimulus association, demonstrated by increased freezing with increased light, tone and footshock presentation ($F_{(2,56)}$ = 17.615, P<0.001), was unaffected by PIC or isolation (Supplementary material 4; Supplementary Fig. 4E). Retention of this association 24 h later, demonstrated by freezing on re-exposure to the apparatus in the absence of footshocks, showed a PIC x isolation interaction ($F_{(1,28)}$ = 5.634, P = 0.025). Light and tone stimuli still evoked freezing ($F_{(1,28)}$ = 14.013, P = 0.001), but its duration was reduced in Veh-Iso compared to Veh-Gr, and this deficit was absent in PIC-Iso (Fig. 4C).

3.4.6 ASST

There was no impact of discrimination type (i.e. media or odor) on number of trials or errors to criterion during simple discrimination training, nor any impact of PIC or isolation on acquisition of these simple discriminations (Supplementary material 4; Supplementary Fig.

4F). During complex discrimination testing there were main effects of discrimination type on trials ($F_{(6,156)} = 2.168$, $P = 0.049$) and errors ($F_{(6,156)} = 2.813$, $P = 0.013$) to criterion, which were both higher for reversal 1 than the immediately preceding compound discrimination (trials $P = 0.002$, errors $P = 0.001$), and the extradimensional than intradimensional shift (trials $P = 0.038$, errors $P = 0.037$). There was a PIC x isolation interaction for trials to criterion ($F_{(1,26)} = 4.884$, $P = 0.036$), which were higher in Veh-Iso (but not PIC-Iso) than Veh-Gr specifically during reversal 1 (Fig. 4D).

3.4 Brain regional tissue changes in male offspring

We observed multiple changes to adult brain regional cytokine levels and mTOR activation in the PIC and/or Iso single models that were not present upon combination in the PIC-Iso group (which matches the pattern of adulthood behavioral alterations described above), whereas JNK activation in the adult frontal cortex remained elevated in the combined as well as both single models (which does not parallel the behavioral deficits). Very few changes to individual cytokines were evident in adolescence, but hippocampal oxytocin was altered at this earlier developmental time point. These findings are described in detail below.

3.5.1 5-HT and SERT immunoreactivity in adult males, after ASST

Patterns of 5-HT and SERT immunoreactivity were consistent with the reported serotonergic innervation of frontal cortical regions (Linley et al., 2013) by projections from the dorsal raphe nucleus, which are characterized by fine axons with small varicosities (Kosofsky and Molliver, 1987). There was a main effect of isolation on 5-HT immunoreactivity in the OFC ($F_{(1,25)} = 6.638$, $P = 0.016$), which tended to decrease in isolates (Supplementary material 5; Supplementary Fig. 5A, Supplementary Fig. 6C). There was a PIC x isolation interaction for SERT immunoreactivity in the OFC ($F_{(1,25)} = 5.618$, $P = 0.026$) and PrL ($F_{(1,25)} = 4.311$, $P =$

0.048), which both tended to be higher in Veh-Iso than PIC-Iso (Supplementary material 5; Supplementary Fig. 5B, Supplementary Fig. 6D).

3.5.2 Cytokines, mTOR, JNK and oxytocin in adult males, after CFR

There were PIC x isolation interactions for multiple cytokines in multiple brain regions. These include five of the selected eight in the frontal cortex (IL-1β $F_{(1,28)} = 7.870$, $P = 0.043$; IL-2 $F_{(1,27)} = 6.831$, $P = 0.044$; IL-6 $F_{(1,27)} = 12.580$, $P = 0.011$; IL-10 $F_{(1,28)} = 14.430$, $P = 0.008$; IL-12 $F_{(1,27)} = 7.245$, $P = 0.048$), plus hippocampal IL-10 ($F_{(1,27)} = 6.651$, $P = 0.042$) and three in the striatum (IL-2 $F_{(1,26)} = 12.630$, $P = 0.009$; IL-4 $F_{(1,27)} = 6.966$, $P = 0.047$; IL-12 $F_{(1,26)} = 21.160$, $P = 0.002$). There were also isolation effects in the frontal cortex (IL-2 $F_{(1,27)} = 16.380$, $P = 0.010$; IL-6 $F_{(1,27)} = 11.060$, $P = 0.023$; IL-12 $F_{(1,27)} = 10.290$, $P = 0.027$; TNFα $F_{(1,27)} = 9.933$, $P = 0.023$) and striatum (TNFα $F_{(1,28)} = 8.518$, $P = 0.033$). PIC-Gr exhibited significantly higher striatal IL-2, IL-4, IL-12 and TNFα than Veh-Gr, while Veh-Iso showed higher frontal cortical IL-1β, IL-2, IL-6, IL-10, IL-12 and TNFα than Veh-Gr. However, these alterations in PIC-Gr and Veh-Iso were all either absent or significantly attenuated in PIC-Iso (Fig. 5A).

Total mTOR expression in the frontal cortex (isolation $F_{(1,28)} = 6.651$, $P = 0.016$; PIC x isolation $F_{(1,28)} = 15.830$, $P<0.001$) was higher in PIC-Gr (598 ± 58; $P = 0.026$) and Veh-Iso (718 ± 39; $P<0.001$), but not PIC-Iso (524 ± 48), than Veh-Gr (373 ± 61). Frontal cortical p-mTOR expression (PIC $F_{(1,28)} = 5.870$, $P = 0.022$; isolation $F_{(1,28)} = 15.160$, $P<0.001$; PIC x isolation $F_{(1,28)} = 40.400$, $P<0.001$) was also higher in both single PIC-Gr (11 ± 2; $P = 0.045$) and Veh-Iso (22 ± 2; $P<0.001$) models than Veh-Gr (3 ± 1), but lower in PIC-Iso (6 ± 2; $P<0.001$) than Veh-Iso. The ratio of p-mTOR to total mTOR (PIC $F_{(1,28)} = 6.761$, $P = 0.015$; isolation $F_{(1,28)} = 13.03$, $P = 0.001$; PIC x isolation $F_{(1,28)} = 36.580$, $P<0.001$) was

consequently also higher in Veh-Iso than Veh-Gr, and lower in PIC-Iso than Veh-Iso (Fig. 6A). Hippocampal p-mTOR (PIC x isolation $F_{(1,27)} = 8.698$, $P = 0.007$) and the hippocampal p-mTOR to total mTOR ratio (PIC $F_{(1,27)} = 6.109$, $P = 0.020$; PIC x isolation $F_{(1,27)} = 10.080$, $P = 0.004$) were also both lower in PIC-Iso than Veh-Iso (Fig. 6A).

There were no between-group differences total JNK expression in any brain region. In a pattern that contrasts with behavioral and other signaling observations, frontal cortical p-JNK expression (PIC $F_{(1,23)} = 4.675$, $P = 0.041$; PIC x isolation $F_{(1,23)} = 7.557$, $P = 0.011$) was higher in both single PIC-Gr ($P = 0.017$) and Veh-Iso models ($P = 0.016$), as well as the dual-hit PIC-Iso combination ($P = 0.037$), than Veh-Gr (data not shown). The ratio of p-JNK to total JNK (PIC $F_{(1,23)} = 4.879$, $P = 0.037$; isolation $F_{(1,23)} = 7.228$, $P = 0.013$) was consequently also higher in PIC-Gr, Veh-Iso and PIC-Iso than Veh-Gr (Fig. 7A).

There were no between-group differences in hippocampal or striatal oxytocin levels (Fig. 8A). Frontal cortical data are unavailable as insufficient sample volumes remaining after cytokine, mTOR and JNK analyses prevented analysis of several individuals.

3.5.3 Cytokines, mTOR, JNK and oxytocin in adolescent males, after locomotor activity

None of the frontal cortical cytokine alterations seen in adult males were evident in this sex during adolescence. There were a number of other subtle changes including a PIC x isolation interaction for frontal cortical IL-18 and hippocampal IL-1β, IL-4, IL-6, IL-10, IL-12 and TNFα, plus striatal TNFα, but these did not persist after correcting for false discovery rate (Fig. 5B). The pattern in adolescence therefore contrasts with the adulthood PIC-Gr elevation and its normalization in PIC-Iso (Fig. 5A).

No changes in mTOR (Fig. 6B) or JNK (Fig. 7B) activation were detected in adolescence, but interestingly hippocampal oxytocin (PIC x isolation $F_{(1,25)} = 9.558$, $P = 0.005$), which was unaltered in adulthood, was higher in adolescent PIC-Iso than Veh-Iso (Fig. 8B). Striatal oxytocin (PIC x isolation $F_{(1,22)} = 4.382$, $P = 0.048$) showed a similar trend. (Fig. 8B).

The patterns of behavioral and brain regional alterations in adult and adolescent male rats are summarized in Fig. 9.

3.6 Correlations between behavioral and brain regional tissue changes

In adult males there were positive associations between frontal cortical cytokines (represented by IL-6) and total prosocial USVs during social interaction testing ($P = 0.041$; Supplementary material 6; Supplementary Fig. 7A), frontal cortical mTOR activity ($P = 0.003$; Supplementary material 6; Supplementary Fig. 7B) and frontal cortical JNK activity ($P = 0.046$; Supplementary material 6; Supplementary Fig. 7C). There were also associations between frontal cortical mTOR activity and a number of behavioral indicators. These include positive correlations with total rearing during locomotor activity testing ($P = 0.028$; Supplementary material 6; Supplementary Fig. 7D), aggressive behavior ($P = 0.023$; Supplementary material 6; Supplementary Fig. 7E) and total prosocial USVs during social interaction testing ($P = 0.008$; Supplementary material 6; Supplementary Fig. 7F), plus a negative correlation with post-cue freezing during assessment of fear-motivated associative memory ($P = 0.010$; Supplementary material 6; Supplementary Fig. 7G). Scatter plots suggest these correlations are driven at least in part by clustering of Veh-Iso away from Veh-Gr. Separate analyses applied to each of the four treatment-housing combinations in turn did not reveal any within-group associations for any of the correlations that reached statistical

significance at the overall adult frontal cortical level. There were no overall correlations between frontal cortical JNK and behavior, nor any hippocampal or striatal correlations.

In adolescence there were fewer behavioral measures and fewer significant cytokine or signaling changes. Fewer correlation analyses were therefore performed at this developmental stage and no significant associations were detected.

4. Discussion

The use of rodent dual-hit neurodevelopmental manipulations is becoming increasingly common in an attempt to create a more comprehensive spectrum of changes with face, construct and predictive validity for psychiatric illnesses triggered by early-life environmental risk factors. Others have employed peripubertal PIC administration after social isolation of rats (Lukasz et al., 2013) to replicate environmental risk factor interactions in adolescence. However, to our knowledge no prior study has encapsulated our particular combination of MIA and adolescent social adversity by combining gestational PIC with post-weaning isolation rearing. Remarkably, instead of exacerbating the isolation syndrome, gestational PIC exposure actually protected against a spectrum of changes in behavior, brain regional cytokine levels and mammalian target of rapamycin (mTOR) activation in the adult offspring. The adolescent period may represent an important window for mechanistic insight into disease risk, and our preliminary data suggest the impact of early-life adversity on central oxytocin levels and oxytocinergic signaling should be a high priority for further investigation.

Because the effects of PIC appear to vary between batches and/or laboratories we were careful to perform pilot studies before moving to gestational administration. Our batch of

PIC induced expected upregulation of the early-response immune and inflammatory regulator microRNA-155 in primary macrophages *in vitro* and also induced hunched posture, increased respiratory rate and piloerection when administered i.v. or i.p. to non-pregnant Lister hooded females, with the apparently higher cumulative sickness score for the 3 h 30 min following i.p. administration taken as a predictor of more pronounced MIA via this route (Supplementary material 1). MicroRNA-155 is upregulated by TLR3 ligands including PIC but also TLR4 ligands including LPS (O'Connell et al., 2007; Swaminathan et al., 2012). It is therefore noteworthy that the endotoxin content of our PIC was approximately 3- to 5-fold lower than all other batches tested to date (Kowash et al., 2019). The mean molecular weight was also lower than most batches of the same product (and InvivoGen low molecular weight PIC), although it did contain a very small percentage of higher molecular weight species not detected in other batches (Kowash et al., 2019). These could potentially contribute to greater TLR3 activation and more potent cytokine induction, and also bind to retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) receptors for double-stranded RNA (Zhou et al., 2013). Crucially though our dams did not show mortality, abortion or reduced litter size, which are all reported upon intentional administration of predominantly high molecular weight PIC (Kowash et al., 2019; Mueller et al., 2019). However they did show transient hypothermia, sickness behavior and short-term weight loss, which appears to predict the level of change in resulting offspring (Vorhees et al., 2015).

Some groups successfully use gestational PIC-exposed (Lins et al., 2019) or isolation-reared females (Harte et al., 2007; McLean et al., 2010; Hermes et al., 2011) and indeed our evaluation of adolescent offspring did reveal some alterations in this sex. However, on the whole males were more sensitive to both neurodevelopmental interventions. This parallels the incidence of conditions like schizophrenia and ASD (Aleman et al., 2003; Werling and

Geschwind, 2013), as well as the apparent greater magnitude or earlier onset of behavioral, structural and functional responses in males exposed to PIC (Piontkewitz et al., 2011; Zhang et al., 2012; Patrich et al., 2016; De Felice et al., 2018; Drazanova et al., 2018; Lins et al., 2018; Gray et al., 2019; Haida et al., 2019; Lins et al., 2019) or reared in isolation (Weiss et al., 2001; Weiss et al., 2004; Fabricius et al., 2010; Pisu et al., 2016; Chmelova et al., 2019).

Behavioral alterations in adult males were broadly consistent with the literature, and more marked after isolation rearing than gestational PIC. For example, gestational PIC generally has little impact on spontaneous locomotion without a pharmacological challenge (Bronson et al., 2011; Richtand et al., 2012b; Vorhees et al., 2012; Vorhees et al., 2015), whereas we observed some degree of hyperactivity (attributed to sensitization of the mesolimbic dopaminergic pathway; Fabricius et al., 2011) in 96% of 25 isolation-reared cohorts over the last 14 years. Likewise isolation routinely increases aggression (Wongwitdecha and Marsden, 1996), reduces anogenital sniffing (Möller et al., 2011) and elevates frequency-modulated step-type 50 kHz USVs (Willey and Spear, 2013) during social interaction. Although these calls have traditionally been assumed to reflect a positive emotional state they are also emitted during aggression and submission (Wöhr et al., 2008) and can be dissociated from changes in prosocial behavior. For example, oxytocin-induced increases in anogenital and body sniffing can occur without alteration USV alterations (Kohli et al., 2019), whereas PIC-exposed offspring lacking marked social behavior changes (Gray et al., 2019) emitted more step-type 50 kHz USVs here. Cognitive deficits appear more variable, and indeed our desire for a more robust phenotype stimulated combination of these models. NOD was impaired in 72% of our 25 previous isolate cohorts, CFR in 67% of the 12 that underwent this task, and PPI in just 55% of 18. Thus current deficits in CFR and reversal learning (Powell et al., 2015), together with increased locomotion and aggression, appear reasonably consistent

with the typical scope of the isolation syndrome. Subtle opposing changes in 5-HT and SERT expression in the orbitofrontal cortex suggest the potential for a localized serotonergic deficit (Bickerdike et al., 1993) in a region where 5-HT depletion impairs reversal learning by increasing perseveration (Clarke et al., 2007), and isolation-induced mTOR activation is also linked to cognitive impairment (Meffre et al., 2012). Although gestational PIC may impair NOD (Vernon et al., 2015; Osborne et al., 2017; Gray et al., 2019) and reversal learning (Wallace et al., 2014; Lins et al., 2018) after i.v. administration, it does not always influence PPI (Vorhees et al., 2012; Lins et al., 2018; Gray et al., 2019), CFR (Vorhees et al., 2015) or frontal cortical 5-HT (Hadar et al., 2015). Nevertheless sickness responses in our dams together with locomotor, USV and brain regional changes in their offspring do support modest consequences of our MIA.

Numerous studies have seen additive effects of neurodevelopmental combinations in rats, like gestational PIC followed by cannabinoid exposure in adolescence (Dalton et al., 2012) or neonatal PIC followed by peripubertal stress (Monte et al., 2017). Additional examples of additive effects include (but are not limited to) neonatal MK-801 or PCP plus isolation rearing (Lim et al., 2012; Gaskin et al., 2014) and maternal separation with peripubertal stress (Novak et al., 2013), isolation rearing (Vargas et al., 2016) or early adulthood corticosterone administration (Hill et al., 2014). While other studies find no interaction (e.g. between gestational PIC and juvenile stress or isolation rearing and subchronic MK-801; Ashby et al., 2010; Yee et al., 2011) an increasing number actually report exposure to the first ‘hit’ confers resilience to a second, as observed here. For example, some effects of isolation are reduced by prior maternal separation (Ellenbroek and Cools, 2002), neonatal AMPA/kainite agonism (Marriott et al., 2016) or high fat diet (Arcego et al., 2016), and more recently gestational PIC has been found to blunt responses to an immune challenge in adolescence (Clark et al., 2019).

Accumulating evidence suggests isolation rearing induces plasma (Möller et al., 2013; Ko and Liu, 2015) and brain regional cytokine alterations (Shortall et al., 2018) and upregulates viral response genes (Murphy et al., 2010; Lukasz et al., 2013) despite the lack of any direct immune challenge, so in this regard the overlap between our findings and those of Clark et al. (2019) is perhaps not surprising.

Resilience in animal studies parallels suggestions that some level of cumulative lifetime adversity in humans is actually better than a relative lack of negativity (Seery et al., 2013). Disappointingly though there appears no clear distinction between the nature and timing of combinations that increase risk versus those that promote resilience, and additional work with three-hit models is still required to incorporate genetic x multiple environmental interactions (Daskalakis et al., 2013). Even without this added complexity many so-called two-hit models actually include additional and differing environmental contributors that can hinder comparisons between studies. We refined our PIC protocol to minimize gestational stress but it is likely that transport of time-mated dams (common in PIC studies; Zhang et al., 2012; Osborne et al., 2017; Lins et al., 2018) induced some hypothalamic-pituitary-adrenal (HPA) axis activation, which is itself a risk for anxiety, depression, ADHD and schizophrenia (Monk et al., 2019). Avoidance of gestational transport by commercial breeders performing PIC administration (Vernon et al., 2015) introduces an alternative transport stress in adolescent offspring, and even if animals are bred in-house (Bronson et al., 2011; Richtand et al., 2012a; Wallace et al., 2014; Vorhees et al., 2015; Murray et al., 2019) maternal stressors can still include general anesthesia for i.v. PIC administration (Zhang et al., 2012; Wallace et al., 2014; De Felice et al., 2018) and/or restraint for post-injection blood sampling (Murray et al., 2019). We acknowledge the value of plasma cytokine and corticosterone determination but less invasive monitoring of maternal body weight, sickness behavior and temperature change

can also indicate the intensity of MIA (Vorhees et al., 2015; Kentner et al., 2019). Since levels of maternal stress and the intensity of MIA determine the likelihood and extent of neurodevelopmental change in the offspring (Meyer, 2019) it seems entirely likely that the same factors could also influence susceptibility or resilience of the offspring to subsequent adversity. It should therefore be noted that the extent of MIA associated with the current resilience appears consistent with modeling of a mild self-limiting illness not requiring medical intervention and affecting less than 2% of the total gestational period (i.e. less than one day in rats, corresponding to less than one week in humans). It remains to be seen whether more severe MIA might instead be associated with increased susceptibility of the offspring to subsequent adversity, as per our original hypothesis.

Variations in the level of maternal care, including pup licking and grooming, can shape social and cognitive behavior of the offspring in adulthood (Liu et al., 2000; Starr-Phillips and Beery, 2014) as well as their reactivity to isolation rearing (Daskalakis et al., 2012). It is therefore important to emphasize that the profound consequences of post-weaning isolation rearing observed here cannot simply be attributed to confounding differences in maternal care, since pups from each of the Veh-treated dams were randomly assigned to ensure an equal contribution to both Veh-Gr and Veh-Iso subgroups. It should also be noted that the load on maternal care and lactation is unlikely to explain differences between our Veh- and PIC-exposed litters, because although we did not artificially standardize litter sizes PIC did not influence pup numbers. The current maintenance of natural litters is consistent with 43% of the approximately 50 rat PIC studies published when we commenced this research (e.g. Mattei et al., 2014; Wallace et al., 2014; Zavitsanou et al., 2014) and was chosen to prevent any maternal stress associated with number adjustment from disrupting care of remaining offspring. We acknowledge that an equal maternal load does not guarantee equivalent

maternal care, since gestational PIC can reduce pup licking and grooming in mice (Schwendener et al., 2009) and may contribute towards some deficits in their offspring (Meyer et al., 2008; Richetto et al., 2013). However consistent with 95% of rat PIC studies published before the start of our work we intentionally did not assess maternal care, since the required interference with nesting material and associated disruption of mother-pup interactions would constitute an additional environmental 'hit'. But given that low maternal care normally increases stress susceptibility in rats (Henningsen et al., 2012) any PIC-induced decrease in care would actually be expected to exacerbate, not protect against responses to subsequent post-weaning isolation. The current findings therefore remain equally remarkable regardless of whether the unexpected resilience is attributed to PIC-induced MIA during gestation, PIC-induced disruption of postnatal maternal care, or a combination of both.

We analyzed samples from adult rats in an attempt to understand brain regional changes underlying their final behavioral phenotype, and from adolescent littermates to investigate early developmental contributors to the isolation syndrome and resilience conferred by gestational PIC exposure. Although JNK signaling is important during neurodevelopment and disrupted in a variety of psychiatric illnesses (including ASD and schizophrenia; Coffey, 2014) the similar elevation of JNK phosphorylation in the adult frontal cortex after separate versus combined neurodevelopmental interventions, and the lack of any formal correlation between JNK phosphorylation and behavioral performance both suggest increased activation of this signaling cascade is probably unrelated to the particular isolation-induced behavioral changes in this study or resilience exhibited by PIC-Iso. In contrast, adulthood changes in brain regional cytokine levels and mTOR activation both closely mirrored patterns of behavioral change, being evident in one or both single-hit models and absent upon their combination. Formal correlations support a positive association of frontal cortical IL-6 with

prosocial USVs and mTOR signaling, which is especially interesting because recent findings suggest IL-6-induced changes in synaptic plasticity within the medial prefrontal cortex can lead to stress susceptibility in some individuals and resilience in others (Esquivel-Rendón et al., 2019). Correlation analyses also support positive associations of frontal cortical mTOR activation with locomotion, social interaction and concomitant USVs, and a negative association of frontal cortical mTOR activation with retention of conditioned fear memory. It is widely recognized that neural circuits involving the medial prefrontal cortex and amygdala are central to social behavior (Ko, 2017) and fear memory (Marek et al., 2013), and studies in wild-type or transgenic animals and models for depression or ASD have linked elevated mTOR signaling within the frontal cortex to increased locomotor activity, decreased sociability (Jiménez-Sánchez et al., 2016; Qin et al., 2016; Niu et al., 2018) and alterations in fear memory reconsolidation and extinction (Iafrati et al., 2014; Zubedat and Akirav, 2017). But although isolation-induced increases in frontal cortical mTOR activation have already been associated with visual recognition and social memory deficits (Meffre et al., 2012) we are the first to suggest increased frontal cortical mTOR activation may also be relevant to disturbances in social interaction and fear memory retention in this particular model. Since gestational PIC exposure has recently been shown to downregulate mTOR expression in the frontal cortex of adult mice (Amodeo et al., 2019), mTOR signaling would theoretically appear to represent a plausible point of interaction between gestational PIC and isolation rearing. However, current adolescent analyses reveal that changes in cytokine levels and mTOR signaling actually emerge later than two weeks post-weaning (when locomotor hyperactivity is already established), so in fact may not contribute to early development of the entire isolation syndrome, or its absence in gestational PIC-exposed animals. Despite known signaling links (Katholnig et al., 2013) mTOR activation also appears an unlikely trigger for some of the current cytokine elevations, since neither the tendency towards an

isolation-induced IL-18 elevation in the adolescent frontal cortex or the multiple robust PIC-induced increases in the adult striatum were accompanied by any change in mTOR phosphorylation in these regions.

Preliminary findings may point to the oxytocinergic system as an alternative explanation for the observed resilience, since hippocampal oxytocin levels during adolescence were higher in the 'protected' PIC-Iso offspring that 'susceptible' Veh-Iso counterparts. Chronic stress and isolation rearing both decrease plasma oxytocin (Harvey et al., 2019; Scarola et al., 2019) mirroring findings in schizophrenia (Jobst et al., 2014) and ASD (Zhang et al., 2016). Although the number of oxytocin-positive cells in the adult hypothalamus is unaffected by isolation (Gilles and Polston, 2017; Tanaka et al., 2010) lasting perturbation of central oxytocinergic systems is demonstrated by reduced oxytocin receptor expression in projection regions including the nucleus accumbens (Oliveira et al., 2019), where oxytocin coordinates the rewarding properties of social interaction (Dölen et al., 2013). Hippocampal oxytocin levels in PIC-exposed or isolation-reared rats have not previously been reported but oxytocin-expressing fibers and oxytocin receptors are abundant throughout this region, where oxytocin modulates inhibitory and excitatory activity, neurogenesis and social memory (Cilz et al., 2019). Since increased hippocampal oxytocin can protect against stress (Lee et al., 2015) it certainly appears plausible that the current elevation of hippocampal oxytocin in adolescent PIC-Iso could confer resilience to isolation. Oxytocin regulates the HPA axis and immune system (for reviews see Engelmann et al., 2004; Wang et al., 2015) and inhibits cytokine secretion (Garrido-Urbani et al., 2018), so it is interesting that adolescent PIC-Iso lacked the tendency towards elevated frontal cortical IL-18 seen in Veh-Iso, but disappointing we did not have sufficient frontal cortical tissue after other multiplex analyses to reliably measure oxytocin levels in this brain region. Future studies beyond the scope of the current

manuscript should focus on temporal changes in brain regional oxytocin levels and oxytocin receptor expression as well as activity of the HPA axis throughout the isolation period, and ultimately seek to elucidate the mechanisms via which environmental risk factors modulate central oxytocin signaling. Intracerebroventricular or intrahippocampal administration of exogenous oxytocin can attenuate stress-induced corticoterone release (Windle et al., 1997; Cohen et al., 2010) and microglial activation, and reverse neurodevelopmental consequences of maternal separation (Amini-Khoei et al., 2017) or gestational protein deficiency (Mairesse et al., 2019). Our future studies will therefore also examine whether oxytocin administration during adolescence can prevent the onset of isolation-induced neurodevelopmental deficits, thus offering hope for modulating disease progression in individuals at high risk of developing adult-onset mental illnesses triggered by early-life events and exposures.

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Author contributions

KCFF and MVK conceived the study. Work was designed and performed by NZ and AMP (bone marrow-derived macrophages), J-YG, MVK and KCFF (behavioral studies), SES and MVK (immunohistochemistry), J-YG, SEO'S and MVK (multiplex assays) and HGP (retrospective PIC analyses). J-YG and MVK performed statistical analysis and MVK wrote the first draft of the manuscript. All authors contributed to and have approved the final version. We acknowledge Ian Topham and Mark Trussell for assistance with PIC pilot

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Figure Legends

Fig. 1. Summary of the experimental protocol for the main gestational study. Sixteen time-mated females received Veh or PIC (10 mg/kg i.p.) on gestational day 15. Offspring were housed in same-sex treatment-matched groups of 3-4 (Gr) or isolation (Iso) from weaning on postnatal day (PND) 22 and adolescents of both sexes were assessed for locomotor sensitivity to PIC, isolation and their combination (one rat from each litter per sex-treatment-housing combination to give a final n = 8 unrelated individuals per group). This informed progression of separate males from the same litters (n = 16 per treatment-housing combination) to an adulthood battery of tests, including locomotor activity (LMA), novel object discrimination (NOD) and its spatial variant novel location discrimination (NLD), social interaction (SI) with concomitant recording of ultrasonic vocalizations (USV), and pre-pulse inhibition of acoustic startle (PPI). To limit cumulative lifetime severity rats underwent balanced allocation to one of two possible final tasks, a conditioned freezing response (CFR) test of fear-motivated associative memory or a food-motivated bowl-digging attentional set-shifting task (ASST). Brain tissue was collected after the final test for immunohistochemical (IHC) examination of serotonergic markers, or multiplex analysis of cytokine and oxytocin levels plus activation of mTOR and JNK intracellular signaling pathways. This experimental design resulted in one rat from each litter per treatment-housing combination for the final behavioral tasks and all brain regional measures. For other behavioral tests mean values were derived from the two littermates in each treatment-housing combination to maintain all group sizes at a consistent n = 8 unrelated individuals per treatment-housing combination.

Fig. 2. Confirmation of maternal PIC-induced sickness response, together with molecular weight distribution of the PIC batch from which all data were obtained. Mean ± SEM (**A**)

body temperature, (**B**) sickness score and (**C**) weight change in time-mated female Lister hooded rats that received a single i.p. injection of vehicle (Veh; saline, 1 ml/kg) or PIC (10 mg/kg) on gestational day 15 (n = 8 per treatment). Temperature was recorded via s.c. microchips implanted into the nape of the neck on gestational day 14. (**D**) PIC weight distribution was quantified by multi-angle light scattering (MALS) and each data point represents a 1 ml flow-through fraction from the column. **P*<0.05/***P*<0.01/****P*<0.001 versus Veh at the same time point (two-way repeated measures ANOVA with Sidak's post-hoc test).

Fig. 3. Sex differences in susceptibility of adolescent offspring to locomotor hyperactivity following gestational PIC exposure and/or post-weaning isolation rearing. Mean ± SEM time course (line graphs) and total (bar graphs) number of infra-red beam breaks for (**A-B**) ambulation (**C-D**) fine movement and (**E-F**) rears in (**A, C, E**) male and (**B, D, F**) female offspring during 1 h in a novel arena on PND 36 (n = 8 of each sex per treatment-housing combination). $^{\sim}P$<0.05 PIC-Gr versus Veh-Gr; **P*<0.05/***P*<0.01/****P*<0.001 Veh-Iso versus Veh-Gr; $^{\#}P$<0.05/$^{\#\#}P$<0.01 PIC-Iso versus Veh-Gr; $^{\dagger}P$<0.05/$^{\dagger\dagger}P$<0.01 PIC-Iso versus PIC-Gr; $^{\ddagger}P$<0.05 PIC-Iso versus Veh-Iso (two or three-way repeated measures ANOVA with Tukey's post-hoc test).

Fig. 4. Gestational PIC exposure prevents locomotor, social and cognitive consequences of post-weaning isolation rearing in adult male offspring. Mean ± SEM (**A**) number of infra-red beam breaks for total ambulation, fine movement and rears across a 1 h locomotor activity monitoring period in a novel arena on PND 51-57, (**B**) duration of social interaction sub-components (left y-axis) and number of accompanying 50 kHz USVs (right y-axis) between weight-, treatment- and housing-matched unfamiliar pairs during a 10 min social interaction

test on PND 66-70, (**C**) duration of conditioned freezing responses on PND 80-84, in the 5 min before and after 5 s tone and light cues previously (-24 h) paired with mild electric footshocks, and (**D**) number of trials required to achieve the criterion of six consecutive correct in seven different phases of a bowl-digging attentional set-shifting task on PND 87-133 (SD = simple discrimination, CD = compound discrimination, Rev 1 = reversal 1, IDS = intradimensional shift, Rev 2 = reversal 2, EDS = extradimensional shift, Rev 3 = reversal 3). In all cases data are n = 8 per treatment-housing combination, either individual values from 8 non-littermates per treatment-housing combination or mean values from each of the 8 litters per treatment-housing combination. $^{\sim\sim}P<0.01$ PIC-Gr versus Veh-Gr; $^{*}P<0.05/^{**}P<0.01/^{***}P<0.001$ Veh-Iso versus Veh-Gr; $^{\#}P<0.05/^{\#\#}P<0.01/^{\#\#\#}P<0.001$ PIC-Iso versus Veh-Gr; $^{\ddagger}P<0.05/^{\ddagger\ddagger}P<0.01$ PIC-Iso versus Veh-Iso (two or three-way repeated measures ANOVA with Tukey's post-hoc test).

Fig. 5. Brain regional cytokine changes produced by gestational PIC exposure or post-weaning isolation rearing are more pronounced in adulthood than adolescence, and absent following their combination. Mean ± SEM cytokine levels in brain regional tissue samples obtained from (**A**) adult male offspring on PND 87-91 or (**B**) adolescent male offspring on PND 36 (n = 8 per treatment-housing combination at each developmental stage). Absolute levels (pg/mg total protein) varied by cytokine and brain region, so to aid clarity of presentation data are presented as a percentage of expression in Veh-Gr controls. Absolute cytokine levels in Veh-Gr controls are shown in Supplementary material 3; Supplementary Table 1, and statistical analyses were performed on raw data not percentages. $^{\sim}P<0.05/^{\sim\sim}P<0.01/^{\sim\sim\sim}P<0.001$ PIC-Gr versus Veh-Gr; $^{*}P<0.05/^{**}P<0.01/^{***}P<0.001$ Veh-Iso versus Veh-Gr; $^{\dagger}P<0.05/^{\dagger\dagger}P<0.01$ PIC-Iso versus PIC-Gr; $^{\ddagger}P<0.05/^{\ddagger\ddagger}P<0.01$ PIC-Iso versus Veh-Iso (four- or five-way repeated measures ANOVA followed by Benjamini-

Hochberg correction for false discovery rate and Tukey's post-hoc test). Corresponding symbols in grey indicate significance prior to false discovery rate correction.

Fig. 6. Elevations in frontal cortical mTOR activation produced by post-weaning isolation rearing are only evident in adulthood, and absent when combined with gestational PIC exposure. Mean ± SEM expression of phosphorylated (Ser2448) mTOR (p-mTOR) as a proportion of total mTOR expression, in brain regional tissue samples obtained from (**A**) adult male offspring on PND 87-91 or (**B**) adolescent male offspring on PND 36 (n = 8 per treatment-housing combination at each developmental stage). $^{***}P<0.001$ Veh-Iso versus Veh-Gr; $^{\ddagger\ddagger}P<0.01$/$^{\ddagger\ddagger\ddagger}P<0.001$ PIC-Iso versus Veh-Iso (two-way ANOVA with Tukey's post-hoc test).

Fig. 7. Elevations in frontal cortical JNK activation produced by gestational PIC exposure or post-weaning isolation rearing are only evident in adulthood, and not further modified by their combination. Mean ± SEM expression of phosphorylated (Thr183/Tyr185) JNK (p-JNK) as a proportion of total JNK expression, in brain regional tissue samples obtained from (**A**) adult male offspring on PND 87-91 or (**B**) adolescent male offspring on PND 36 (n = 8 per treatment-housing combination at each developmental stage). $^{\sim}P<0.05$ PIC-Gr versus Veh-Gr; $^{*}P<0.05$ Veh-Iso versus Veh-Gr; $^{\#\#}P<0.01$ PIC-Iso versus Veh-Gr (two-way ANOVA with Tukey's post-hoc test).

Fig. 8. Hippocampal oxytocin levels are higher in adolescent male rats after combined gestational PIC exposure and post-weaning isolation rearing than after isolation rearing as a sole intervention. Mean ± SEM oxytocin levels in brain regional tissue samples obtained from (**A**) adult male offspring on PND 87-91 or (**B**) adolescent male offspring on PND 36 (n

= 8 per treatment-housing combination at each developmental stage). ‡$P<0.05$ PIC-Iso versus Veh-Iso (two-way ANOVA with Tukey's post-hoc test).

Fig. 9. Summary of behavioral, cytokine and signaling alterations in male rats exposed to gestational PIC and/or post-weaning isolation rearing. ⬆/⬇/- = increase/decrease/no change compared to Veh-Gr control; (↑)/(↓) restricted increase/decrease (of a single cytokine in a single brain region, before false discovery rate correction) compared to Veh-Gr control; Nd = not determined. In the case of social interaction there were simultaneous increases to some and decreases to other subcomponents. Full details of all these changes appear elsewhere (adults: sections 3.4.1-6, 3.5.1-2 and Fig. 4A-8A; adolescents: sections 3.3.1-2, 3.5.3, Fig. 3 and Fig. 4B-8B).

Figure 1

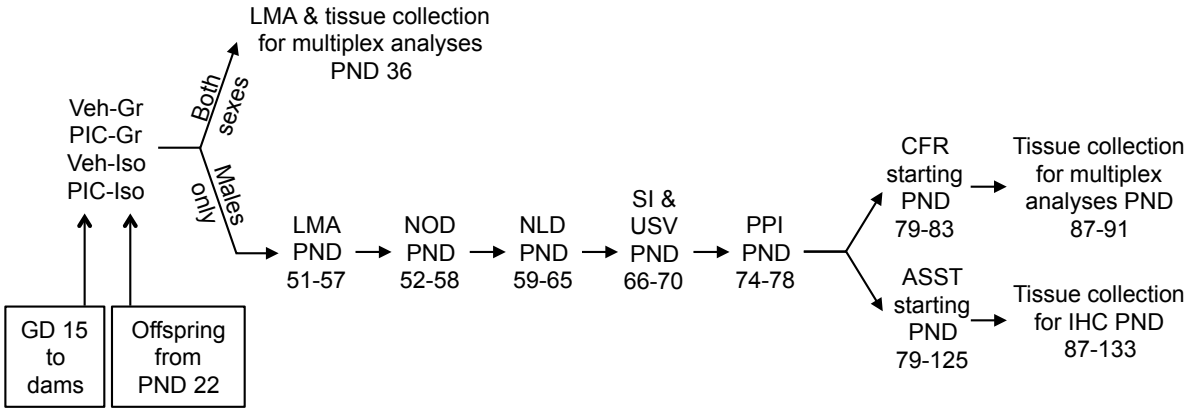

LMA & tissue collection
for multiplex analyses
PND 36
Both sexes
Males only
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso
GD 15
to
dams
Offspring
from
PND 22
LMA
PND
51-57
NOD
PND
52-58
NLD
PND
59-65
SI &
USV
PND
66-70
PPI
PND
74-78
CFR
starting
PND
79-83
Tissue collection
for multiplex
analyses PND
87-91
ASST
starting
PND
79-125
Tissue collection
for IHC PND
87-133

Figure 2

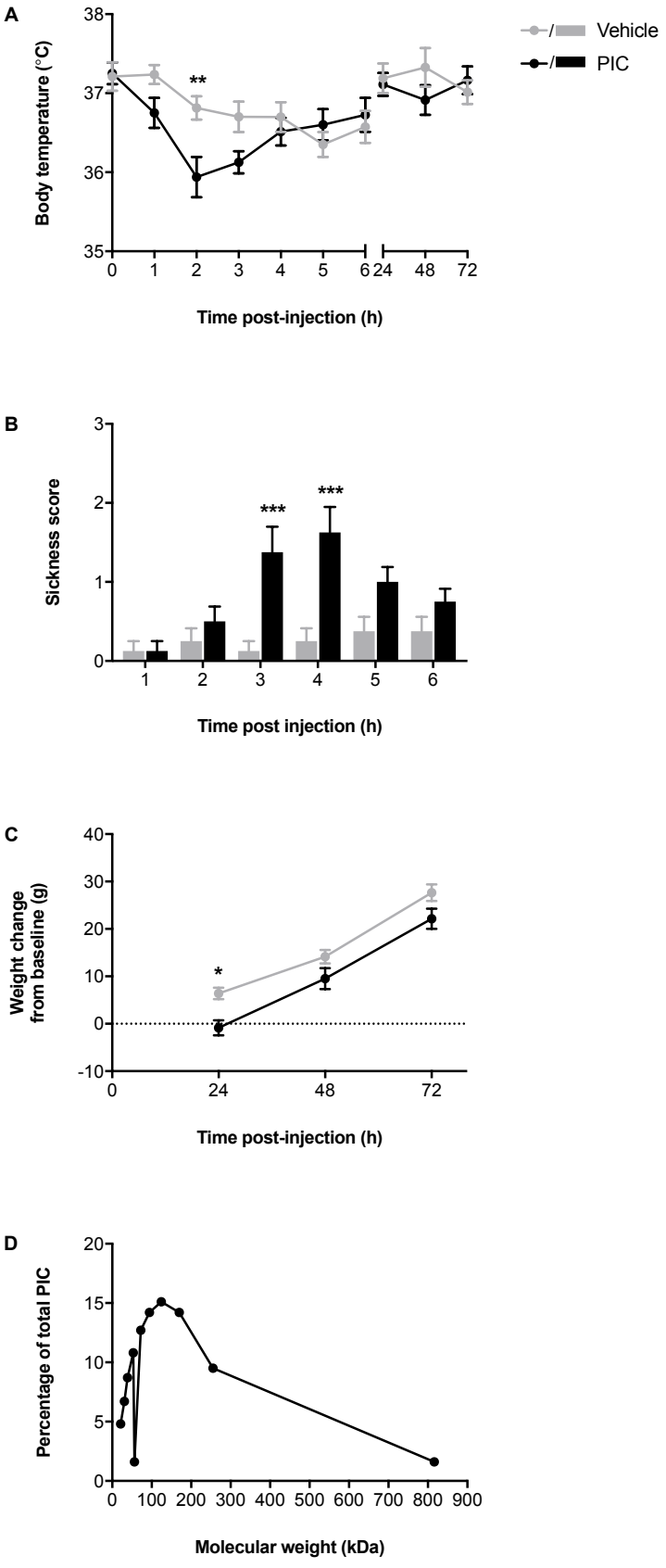

A
Body temperature (°C)
38
37
36
35
**
0 1 2 3 4 5 6 24 48 72
Time post-injection (h)
Vehicle
PIC
B
Sickness score
3
2
1
0
***
***
1 2 3 4 5 6
Time post injection (h)
C
Weight change from baseline (g)
40
30
20
10
0
-10
*
0 24 48 72
Time post-injection (h)
D
Percentage of total PIC
20
15
10
5
0
0 100 200 300 400 500 600 700 800 900
Molecular weight (kDa)

Figure 3

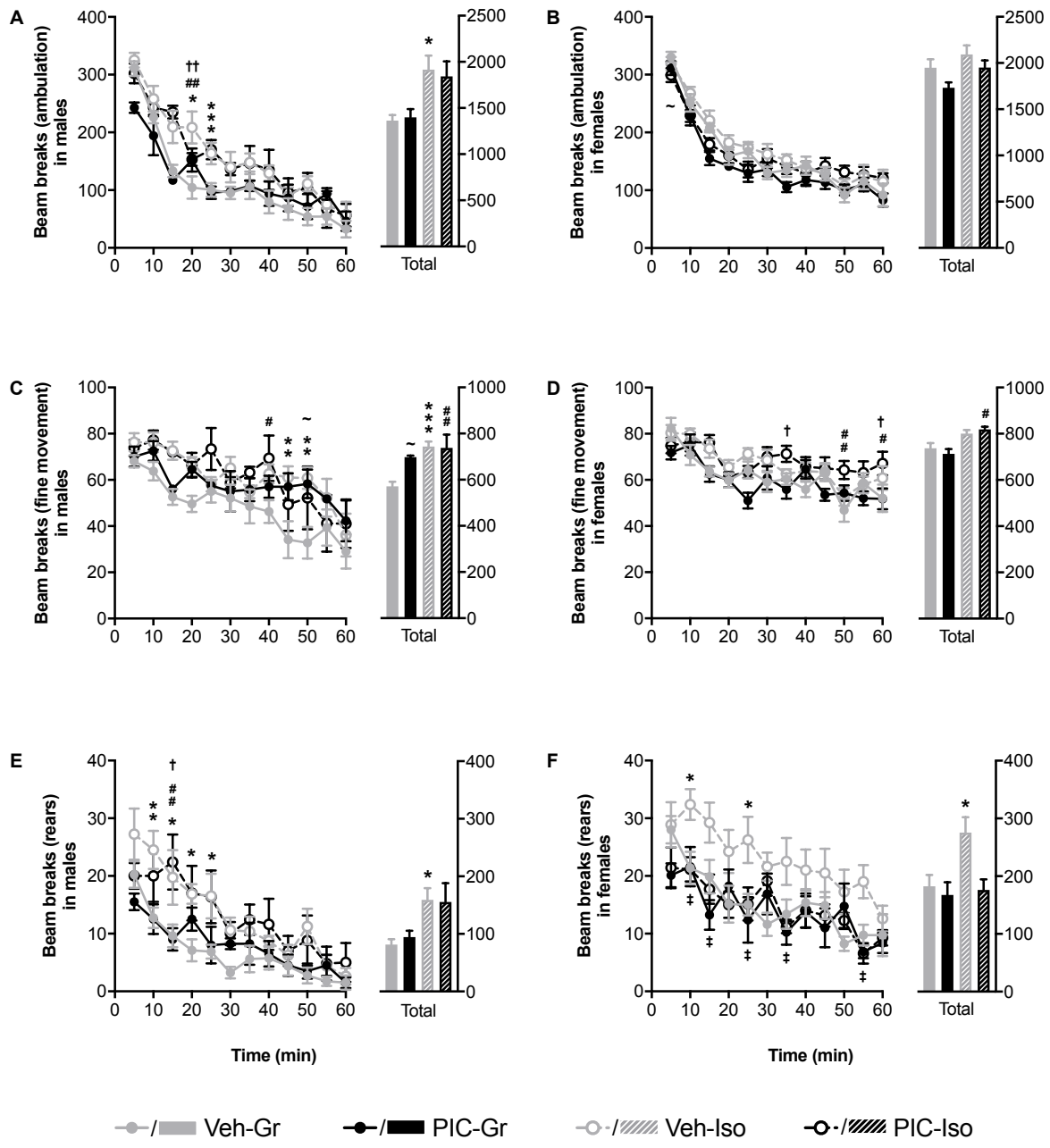

A
B
C
D
E
F
Beam breaks (ambulation) in males
Beam breaks (ambulation) in females
Beam breaks (fine movement) in males
Beam breaks (fine movement) in females
Beam breaks (rears) in males
Beam breaks (rears) in females
Total
Time (min)
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso

Figure 4

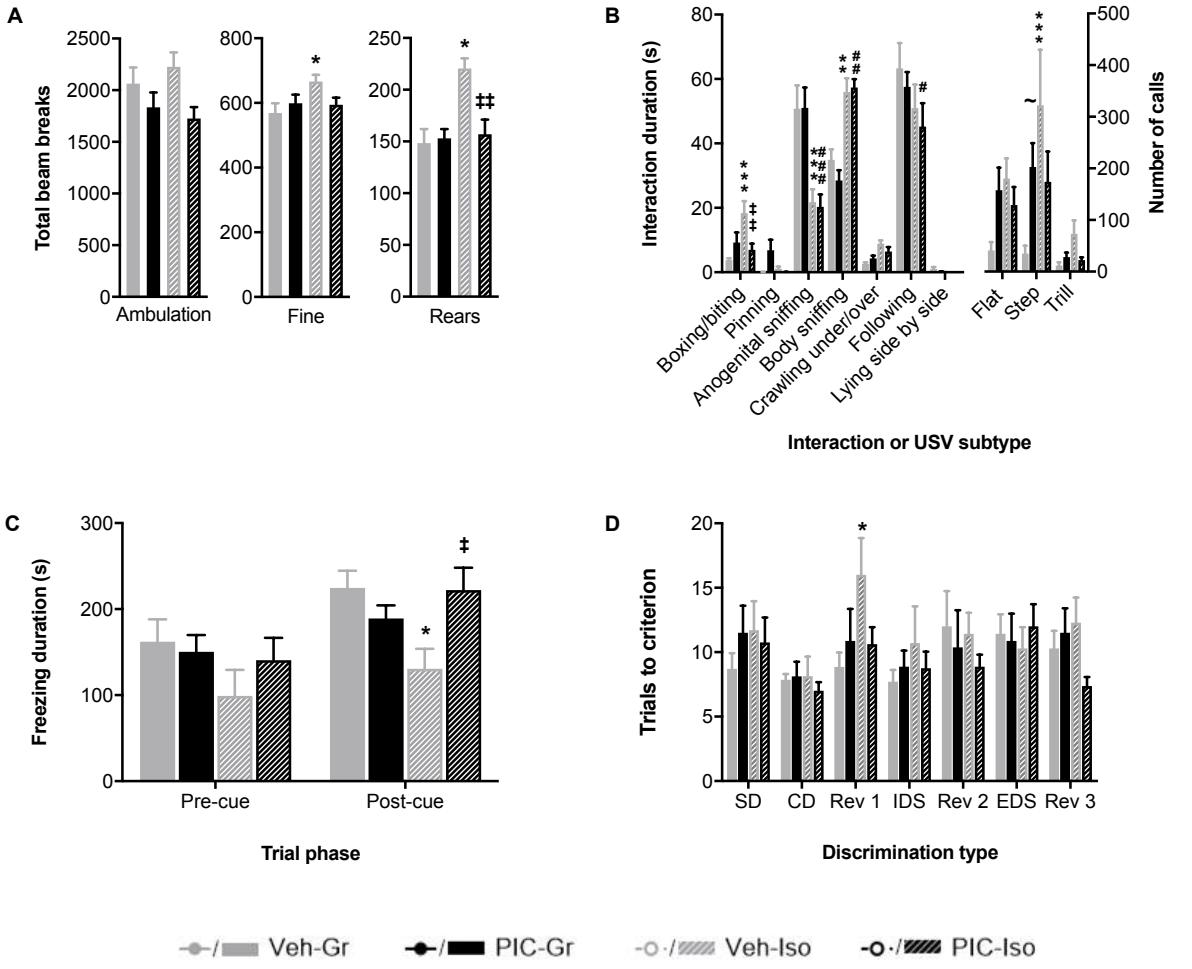

A
Total beam breaks
2500
2000
1500
1000
500
0
Ambulation
800
600
400
200
0
Fine
250
200
150
100
50
0
Rears
B
Interaction duration (s)
80
60
40
20
0
Number of calls
500
400
300
200
100
0
Boxing/biting
Pinning
Anogenital sniffing
Body sniffing
Crawling under/over
Following
Lying side by side
Flat
Step
Trill
Interaction or USV subtype
C
Freezing duration (s)
300
200
100
0
Pre-cue
Post-cue
Trial phase
D
Trials to criterion
20
15
10
5
0
SD
CD
Rev 1
IDS
Rev 2
EDS
Rev 3
Discrimination type
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso

Figure 5

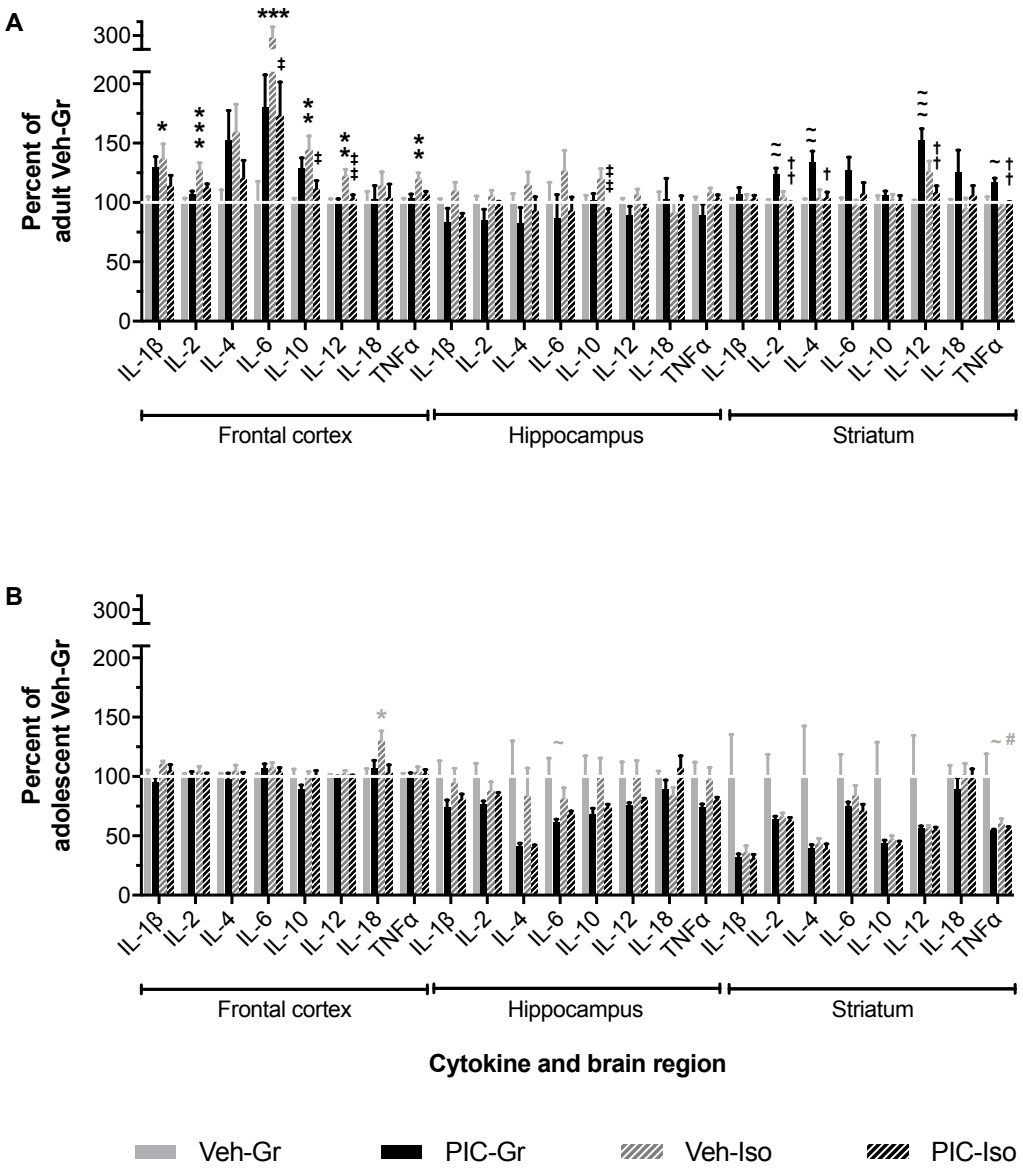

A
Percent of adult Veh-Gr
300
200
150
100
50
0
IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα
Frontal cortex
Hippocampus
Striatum
B
Percent of adolescent Veh-Gr
300
200
150
100
50
0
IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα IL-1β IL-2 IL-4 IL-6 IL-10 IL-12 IL-18 TNFα
Frontal cortex
Hippocampus
Striatum
Cytokine and brain region
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso

Figure 6

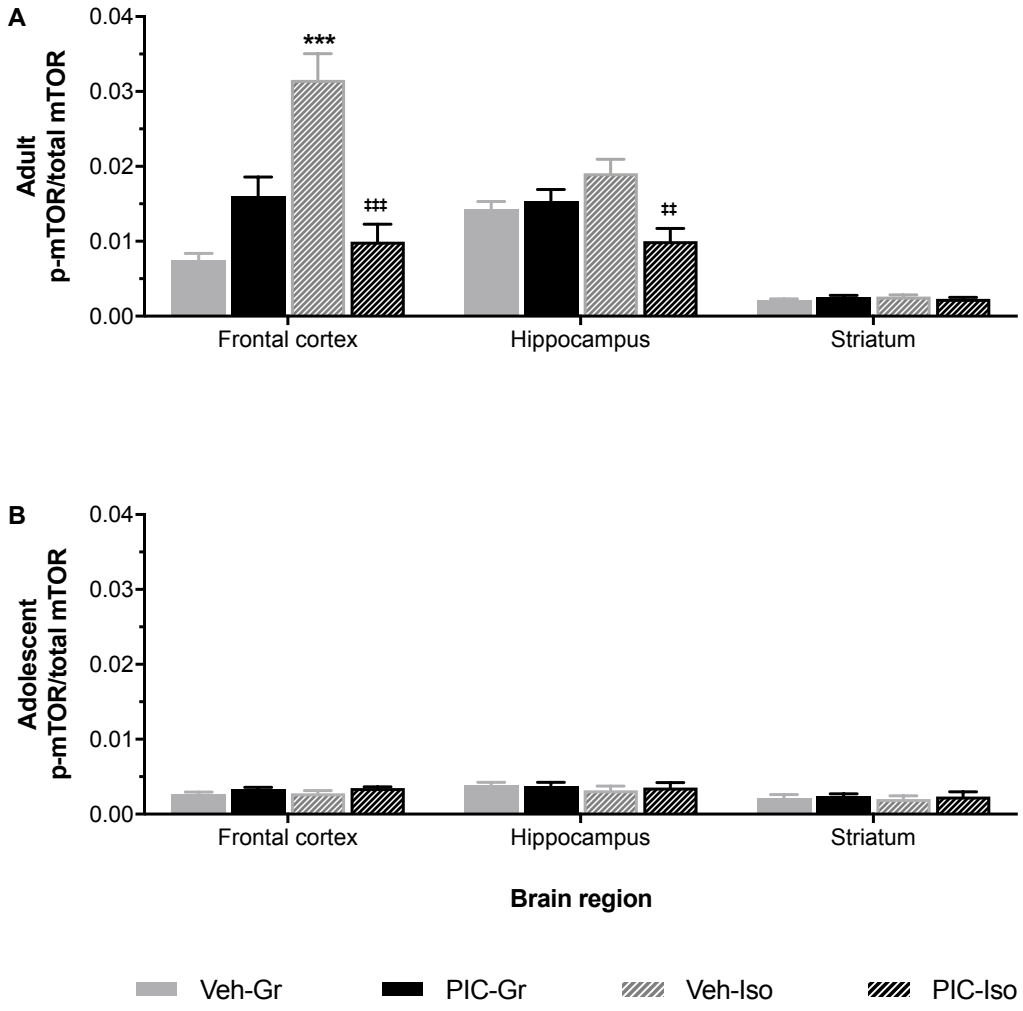

A
Adult
p-mTOR/total mTOR
0.04
0.03
0.02
0.01
0.00
***
##
#
Frontal cortex
Hippocampus
Striatum
B
Adolescent
p-mTOR/total mTOR
0.04
0.03
0.02
0.01
0.00
Frontal cortex
Hippocampus
Striatum
Brain region
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso

Figure 7

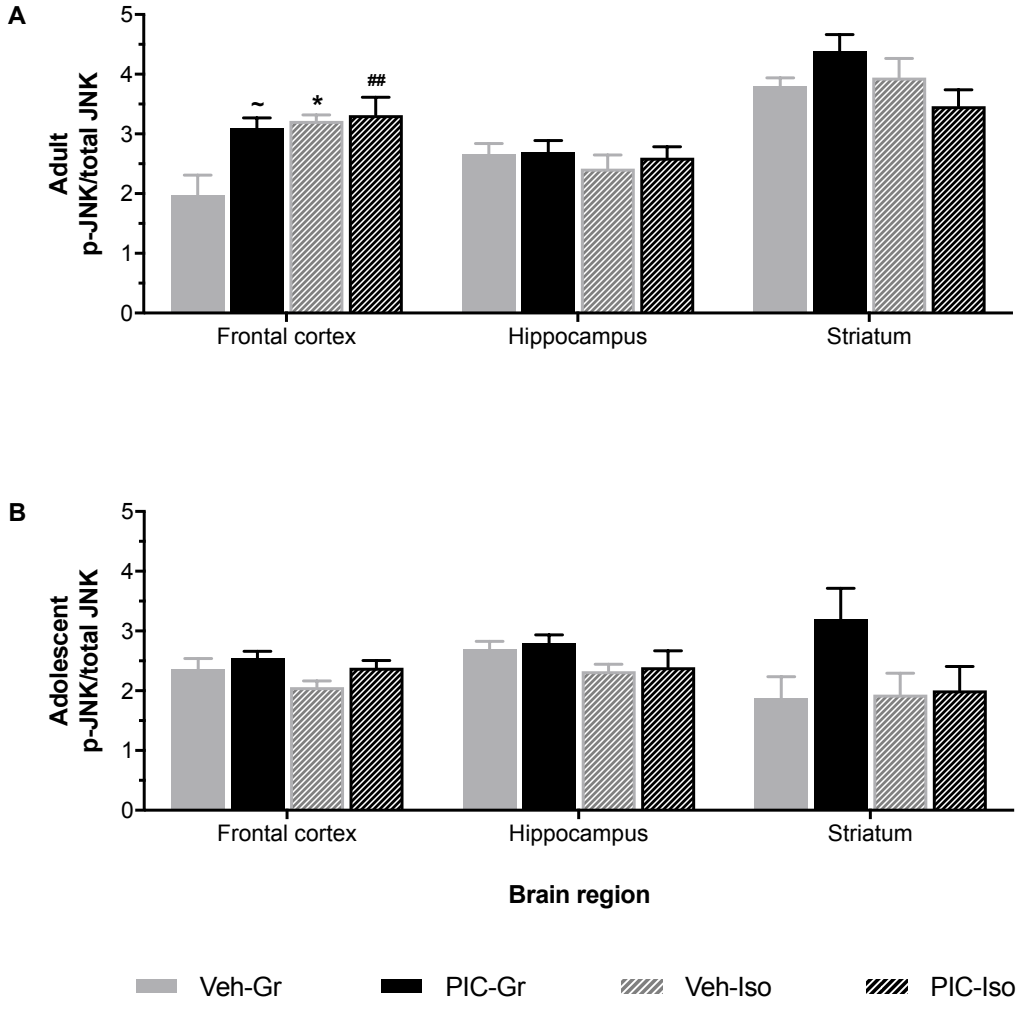

A
Adult
p-JNK/total JNK
5
4
3
2
1
0
~
*
##
Frontal cortex
Hippocampus
Striatum
B
Adolescent
p-JNK/total JNK
5
4
3
2
1
0
Frontal cortex
Hippocampus
Striatum
Brain region
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso

Figure 8

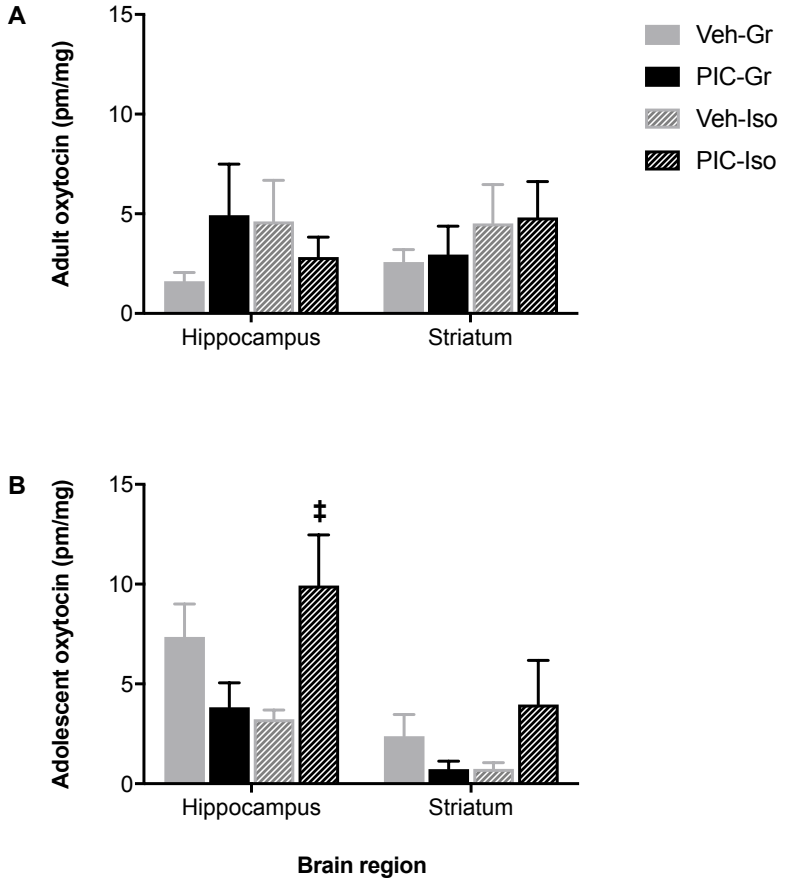

A
Adult oxytocin (pm/mg)
15
10
5
0
Hippocampus
Striatum
Veh-Gr
PIC-Gr
Veh-Iso
PIC-Iso
B
Adolescent oxytocin (pm/mg)
15
10
5
0
‡
Hippocampus
Striatum
Brain region

Figure 9

	Adult males			Adolescent males		
	PIC-Gr	Veh-Iso	PIC-Iso	PIC-Gr	Veh-Iso	PIC-Iso
Locomotor activity	↑	↑	-	↑	↑	↑
Visual memory	-	-	-	Nd		
Social interaction & concomitant USVs	- ↑	↑/↓ ↑	↑/↓ -			
Sensorimotor gating	-	-	-			
Associative memory	-	↓	-			
Reversal learning	-	↓	-			
5-HT markers	-	↓	-			
Cytokine levels	↑	↑	-	(↓)	(↑)	(↓)
mTOR activation	-	↑	-	-	-	-
JNK activation	↑	↑	↑	-	-	-
Oxytocin levels	-	-	-	-	-	>Veh-Iso

Supplementary material 1

Methods

Confirmation of expected PIC-induced inflammatory response in vitro

At the time we commenced this research, of approximately 20 independent groups using PIC for MIA in rats one used a high molecular weight product from InvivoGen, one an unspecified product from Amersham Biosciences, and the remainder PIC from Sigma-Aldrich. Of the latter ten used an unspecified product, four the sodium salt and five the potassium salt. The potassium salt was also the most common for PIC-induced MIA in mice (65% of over 80 studies) and was chosen here for its popularity and the availability of preliminary data from i.p. administration to non-pregnant Lister hooded rats (Murray et al., 2015). Before using our batch of PIC potassium salt (P9582 batch 075M4076V; Sigma-Aldrich, UK) *in vivo* we assessed its ability to induce microRNA-155 expression by primary macrophages *in vitro*, since this TLR-mediated upregulation is a well-characterized component of the macrophage inflammatory response to immune challenges (O'Connell et al., 2007; Swaminathan et al., 2012).

Primary bone marrow-derived macrophages for *in vitro* studies were isolated from three female 129/SvJ mice (9-10 weeks of age; Bio-Support Unit, University of Nottingham), which were killed by cervical dislocation. Bone marrow-derived macrophages were obtained using an established protocol (Piccinini and Midwood, 2012). Bone marrow was flushed from tibias and femurs, and erythrocytes lysed with Red Blood Cell Lysis Buffer (Sigma-Aldrich). The resulting cells were cultured in Lonza Dulbecco's Modified Eagle Medium (Scientific Laboratory Supplies, UK) supplemented with 20% Gibco Fetal Calf Serum, 1% Antibiotic-Antimycotic solution, and 50 μM Invitrogen β-mercaptoethanol (all from Thermo

Fisher Scientific, UK) containing 100 ng/ml recombinant murine M-CSF (PeproTech, UK). After 7 days adherent cells were washed, plated, and stimulated with 20 μg/ml of the PIC product detailed above for 24 h (Sun et al., 2012). Total RNA, including small RNA, was extracted from bone marrow-derived macrophages (1×10^6) using Invitrogen TRIzol (Thermo Fisher Scientific). For mature microRNA-155 detection RNA was reverse transcribed using the Applied Biosciences TaqMan microRNA reverse transcription kit, including microRNA-specific primers, followed by real-time PCR with individual microRNA TaqMan assays for the commonly used endogenous reference RNA RNU6B and microRNA-155 (Thermo Fisher Scientific). Quantitative real-time PCR was performed using a Corbett Rotor-Gene-Q machine. Changes in expression were calculated by the change-in-threshold ($\Delta\Delta C_T$) method with RNU6B as endogenous control, and normalized to data obtained in non-stimulated macrophages from the same mice (Piccinini and Midwood, 2012). Data were analyzed by Student's unpaired *t*-test.

Pilot PIC administration in vivo and assessment of acute sickness behavior

Pilot examination of PIC-induced sickness behavior in Lister hooded rats with the current PIC batch, routes of administration and dose levels used 16 non-pregnant females (100-170 g; Charles River, UK) housed in groups of four (n = 4 per condition). The majority of PIC studies use Sprague-Dawley or Wistar rats, but Lister hooded rats are the strain of choice for our established isolation paradigm (King et al., 2009; Schubert et al., 2009; Meffre et al., 2012; Watson et al., 2012; McIntosh et al., 2013; Napolitano et al., 2014; Dunphy-Doherty et al., 2018; Shortall et al., 2018). We wished to retain the benefit of our accumulated knowledge on the isolation syndrome in Lister hooded rats, which are more active during the light phase and more highly motivated to engage with planned behavioral procedures like the attentional set-shifting task (ASST; Clemens et al., 2014; Tait et al., 2018). We sought to

further this knowledge with the novel measurements outlined in the main body of the manuscript, and to compare the PIC-Iso approach with our PCP-Iso dual-hit model (Gaskin et al., 2014; Gaskin et al., 2016; Watson et al., 2016; King et al., 2018).

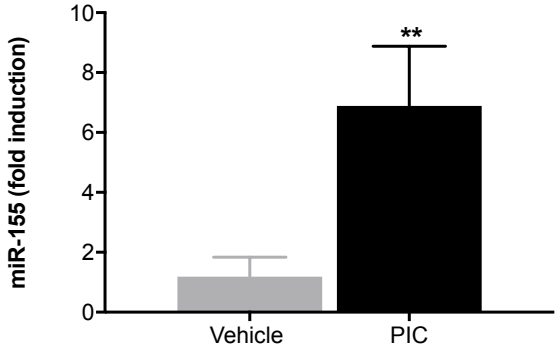
PIC (as above) was dissolved in sterile water for i.p. (10 mg/kg; Murray et al., 2015) or i.v. (4 mg/kg; Wallace et al., 2014) administration at the most commonly reported doses from studies involving Lister hooded rats. Gentle warming in a heat box (10-15 min) preceded i.v. tail vein injections, which were performed under mild physical constraint without anesthesia (Dalton et al., 2012). Doses were based on the pure PIC concentration being 10% of the potassium salt, and control animals received 1 ml/kg sterile saline. Rats were observed for a 30 s snapshot every 10 min to capture simple home cage behaviors indicative of sickness using a simple rating scale. This was based on the absence (0) or presence of hunched posture (1), increased respiratory rate (1), mild (1) or marked (2) piloerection, and shivering (1), giving a maximal possible score of 5 at any one observation point. Behavioral observation continued until 3 h 30 min post-injection, when the procedure terminated. Total sickness scores across the post-injection monitoring period were analyzed by Mann-Whitney U test. The timecourse of sickness behavior was analyzed by two-way repeated measures ANOVA (with time as a within- and treatment as a between-subjects factor), due to the absence of a non-parametric equivalent. These analyses were applied separately to data from i.p. and i.v. groups.

Results

Confirmation of expected PIC-induced inflammatory response in vitro

Stimulation of bone marrow-derived macrophages with PIC for 24 h resulted in a significant (P = 0.009) 7-fold upregulation of microRNA-155 (Supplementary Fig. 1). This is higher

than the reported 2.4- to 3.5-fold elevations after 14-16 h incubation in 10 μg/ml InvivoGen PIC (Swaminathan et al., 2012) or identical 24 h incubation in 20 μg/ml of an alternative Sigma-Aldrich PIC batch (Sun et al., 2012), confirming this batch of PIC was biologically active and suitable for progression to *in vivo* use.

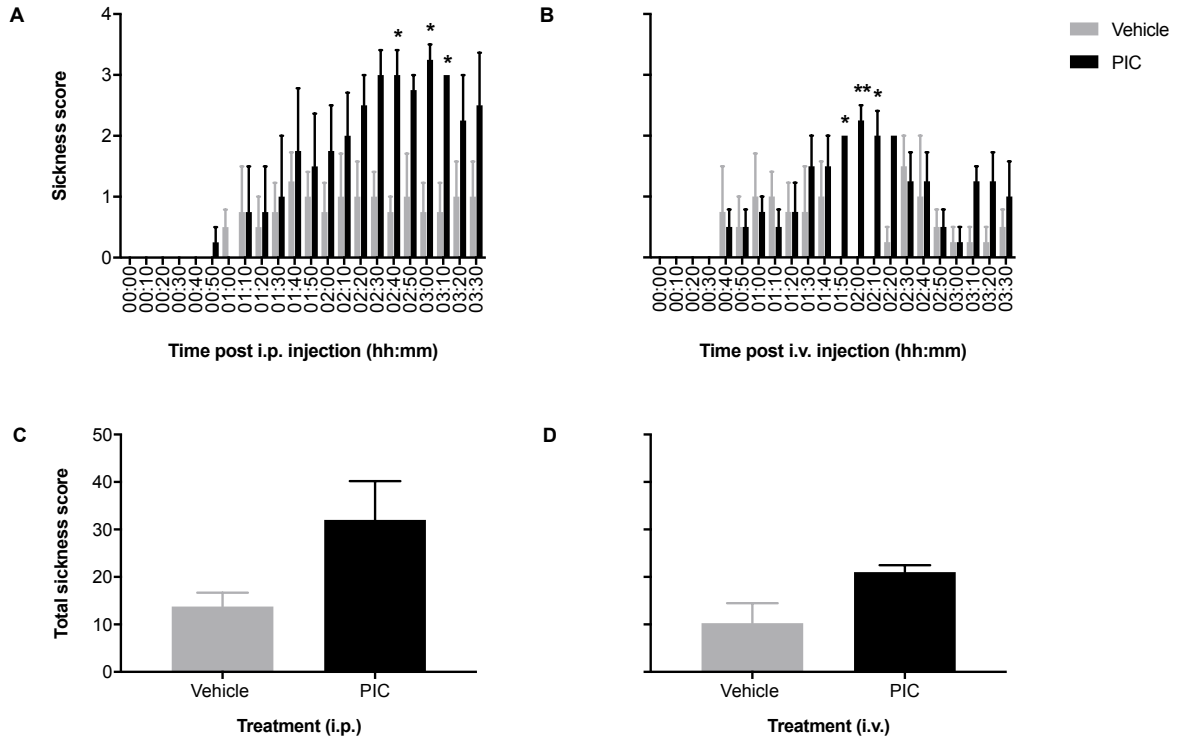


Supplementary Fig. 1. Confirmation of PIC-induced pro-inflammatory activity *in vitro*. Mean ± SEM microRNA-155 expression in primary bone marrow-derived macrophages treated for 24 h with 20 μg/ml PIC, expressed as change of non-stimulated control cells from the same animals (n = 3). $^{**}P<0.01$ versus control (unpaired *t*-test).

Pilot examination of PIC-induced sickness behavior in non-pregnant females

Small-scale comparison of PIC-induced sickness behavior following the most commonly used i.p. versus i.v. doses revealed hunched posture, increased respiratory rate, and/or shivering in each case. This resulted in significant PIC x time interactions and transient post-hoc elevations in sickness score from 2 h 40 min to 3 h 10 min after i.p. ($F_{(21,126)} = 2.139$, $P = 0.005$; Supplementary Fig. 2A) and 1 h 50 min to 2 h 10 min after i.v. ($F_{(21,126)} = 2.461$, $P = 0.001$; Supplementary Fig. 2B) administration. Low statistical power prevented PIC-induced increases in total sickness score over the entire 3 h 30 min post-injection period reaching significance, but the i.p. route appeared to produce more robust elevation (233 ± 59% of Veh;

Supplementary Fig. 2C) than the i.v. route (205 ± 14% of Veh; Supplementary Fig. 2D). We therefore selected i.p. injection of 10 mg/kg PIC for progression to the main gestational study, based on our desire to achieve refinement by producing the most effective MIA with the minimal level of acute restraint stress.



Supplementary Fig. 2. Pilot demonstration of greater PIC-induced sickness behavior after administration of the most commonly used i.p. than i.v. dose to non-pregnant female Lister hooded rats. Mean ± SEM (**A-B**) time course and (**C-D**) total cumulative sickness score for the 3 h 30 min after (**A, C**) i.p. administration of vehicle (Veh; saline, 1 ml/kg) or 10 mg/kg PIC, or (**B, D**) i.v. administration of Veh or 4 mg/kg PIC (n = 4). **P*<0.05/***P*<0.01 versus Veh at the same time point (two-way repeated measures ANOVA with Sidak's post-hoc test).

Although this preliminary evaluation of PIC-induced sickness behavior *in vivo* intentionally employed smaller group sizes than required for robust detection of statistical significance, we still saw indications of hunched posture, increased respiratory rate and piloerection in non-pregnant Lister hooded females. Their timescale matched previous behavioral observations (Yee et al., 2011; Clark et al., 2019) and the timeframe of typical plasma cytokine elevations after i.p. (Murray et al., 2019) and i.v. administration (Dalton et al., 2012) of the same dose levels. The apparently higher cumulative sickness score for the 3 h 30 min following i.p. administration was taken as a predictor of more pronounced MIA via this route. At first glance PIC-induced hyperthermia in males (Yamato et al., 2014; Bastos-Peereira et al., 2015) and non-pregnant females (Murray et al., 2019) seems contradictory to the hypothermia subsequently observed in our dams. However the anatomical location of the temperature reading may be an important contributor, because despite a generally close agreement between core and s.c. temperatures (Cilia et al., 1998; Kort et al., 1998) PIC can simultaneously elevate core and decrease skin surface temperature (Bastos-Pereira et al., 2015), and the latter could conceivably influence our s.c. readings. Pregnancy is an additional consideration since PIC doses that induce hyperthermia in non-pregnant females can decrease (Clark et al., 2019) or fail to influence (Murray et al., 2019) rectal temperature in dams, whilst still reducing body weight, elevating plasma IL-6 and producing changes in the offspring.

Supplementary material 2

(Note: a higher resolution copy is available online https://doi.org/10.1016/j.bbi.2020.05.076 or from the corresponding author)

Maternal Immune Activation Model Reporting Guidelines Checklist

ARRIVE Reporting Guideline & Recommendation	Arrive Item	MIA Model Specific Reporting Recommendation *Please complete this chart for each point outlined below. If not applicable, write N/A*
Study design ➢Overview of immune activation issues For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimize the effects of subjective bias when allocating animals to treatment (e.g. randomization procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	6	MIA Specific Reporting: a. General need for improved reporting in MIA model methods + reporting pilot data o Details on pilot data:
Experimental procedures ➢Compounds ➢Validation measures For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	7	Provide details of: a. Compounds – source, vehicle, preparation/storage, administration route, volume administered, whether anesthetics were used at time of immune challenge. o Name of compound: Polyinosinic–polycytidylic acid potassium salt o Catalogue number: Sigma-Aldrich P9582 o Lot number: 075M4076V o Vehicle control used: Poly (I:C) dissolved in sterile water for injection (due to o Route of administration: i.p. and i.v. in pilot study, then i.p. for MIA o Volume administered: 1ml/kg o Storage conditions: Powder at -20C, dosing solution prepared on morning of o Anesthetic (type, dose, duration) used: N/A b. Housing variables at injection - temperature of room at injection time, cage change at time of injection or not o Light cycle of animal housing room: 12h light-dark; on at 07:00 o Time of day of injection: 10:00-11:00 o Room temperature at injection time: 21 +/- 2C o Did a cage change occur at time of injection: NO

Kentner AC, Bilbo AD, Brown AS, Hsiao EY, McAllister AK, Meyer U, Pearce BD, Pletnikov MV, Yolken RH, Bauman MD. (2018). Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. Neuropsychopharmacology, https://doi.org/10.1038/s41386-018-0185-7.

		c. Validation of immune activation – behavior, physiological indices and/or cytokine data, including pilot dosing data ○ Method used to verify immune activation: Sickness behavior and temperature change during pilot studies (Supplementary material 1), and the same measures plus body weight change during main MIA study (manuscript main text) d. Validation of gestational timing – vaginal plug, estrous cycle, weight gain ○ Method of validating gestational timing: Time-mating by Charles River UK with validation of timing by vaginal plug. For the purpose of this manuscript the day of plug detection was designated as GD 1. Additional comments: N/A
Experimental animals ➢Species/strain/vendor a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	8	Provide details of: a. Species – considerations for appropriate species (mouse, rat, non human primate, other) ○ Species: Rat b. Strain – variability in strain can influence model ○ Strain: Lister hooded c. Maternal/Offspring Physiological Variables at time of immune challenge – age, body weight ○ Maternal Age at challenge: Unknown, but Charles River growth chart sugges ○ Maternal Body weight: 280-345g ○ Offspring Age at challenge: GD 15 ○ Offspring Sex: Both males and females tested ○ Offspring Body weight: First recorded at weaning minimise impact on mother-pup interad d. Vendor – even within the same strain, vendor can influence endpoints ○ Vendor: Charles River ○ Location of Vendor: UK ○ Room/area where animals originated from: A56

		Additional Comments: N/A
Housing and husbandry ➢Cage, ventilation, bedding, enrichment Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding program, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	9	Provide details of: a. Caging systems o *At breeding* Material of cage: Unknown Cage dimensions: Unknown o *After parturition* Material of cage: Details in main text (Section 2.1) Cage dimensions: o *At weaning* Material of cage: Details in main text (Section 2.4) Cage dimensions: b. Animal Holding room o Temperature in room: 21 +/- 2C o Humidity in room: 55 +/- 10% o Ventilation system: Individually ventilated cages o Specific pathogen free [SPF]: NO o Are males & females housed in the same or separate rooms: Housed in same room after weaning c. Bedding exchanges/bedding type o Type of cage bedding used: Sawdust plus paper nesting material o Frequency of cage changes per week *during gestation:* Once weekly *during neonatal period:* Once between partultion and weaning *following weaning:* Once weekly d. Breeding - bred on site or timed pregnant, how many different sires (are the same fathers breeding with both experimental and control dams) Breeding location: Charles River UK

- Gestational age at shipping: GD 6-9 on dispatch, GD 7-10 on arrival
- Biological age of dams (if not listed in Section 8c): See section 8c of this
- Number of Dams bred: 16
- How many times have dams been mated previously: 0
- How many times did the dams mate and not become pregnant: N/A
- Are the dams primiparous or multiparous? All dams are primiparous
- What was the frequency of maternal handling during the gestational/neonatal period (e.g. cage cleanings, weighing, blood collection manipulations): Daily weighing 3d prior and 3d post Poly (I:C)
- Biological age of sires: Unknown
- Number of sires bred: 16
- How many times have sires been mated previously: Unknown
- How many times did the sires mate successfully (e.g. mating resulted in pregnancy, full term birth): All 16 females delivered litters at full term
- If bred previously, what was the interval between mating times:
- Are sires matched to experimental and control dams: NO
- Describe the mating design (1:1, 1:2 etc): 1:1

e. Social enrichment – number of cage companions
- Number of cage companions prior to breeding: Unknown
- Gestational age when dam separated for parturition: Unknown, but
- Number of cage companions at weaning: Group housed = 3-4 per cage,

f. Physical enrichment – describe enrichment devices, and when enrichment is in the cage (removed when pups born? Or present throughout study), does the enrichment type change? How frequently?
- Describe what type of enrichment devices (and how many) are included in cage/housing room:

Shelf (1)
Cardboard tube (1)
Wooden chew block (1)
Paper nesting material (enough to fill 3/4 of lower cage level)

		○ Does enrichment type/access change across study? YES ○ If so, when does enrichment type/access change (e.g. enrichment removed prior to parturition and replaced in late neonatal period): No enrichment beyond weaning age Additional Comments: Animals were not SPF but sentinel animals from the same housing areas were free from all tested viruses, bacteria and parasites during routine organizational screening (full details of screening panel available on request)
Sample size ➢Litter versus offspring a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	10	Provide details of: a. Maternal N vs offspring N ○ What is the total number of dams/litters included in the study: 16 ○ What is the total number of offspring per litter included the study: 8, but becasue littemates were divided between male/female, group/isolation b. Litter size and sex distribution ○ What size was each litter maintained at: See below ○ What age did culling take place at: See below ○ How many males and females were maintained in each litter: See below c. Cross fostering ○ Did cross fostering occur: NO ○ If so, at what age did cross fostering occur: N/A Additional Comments: 215 pups were born to the 16 dams, of these only 128 were used in this study. Litters were maintained at natural sizes and male:female ratios until weaning

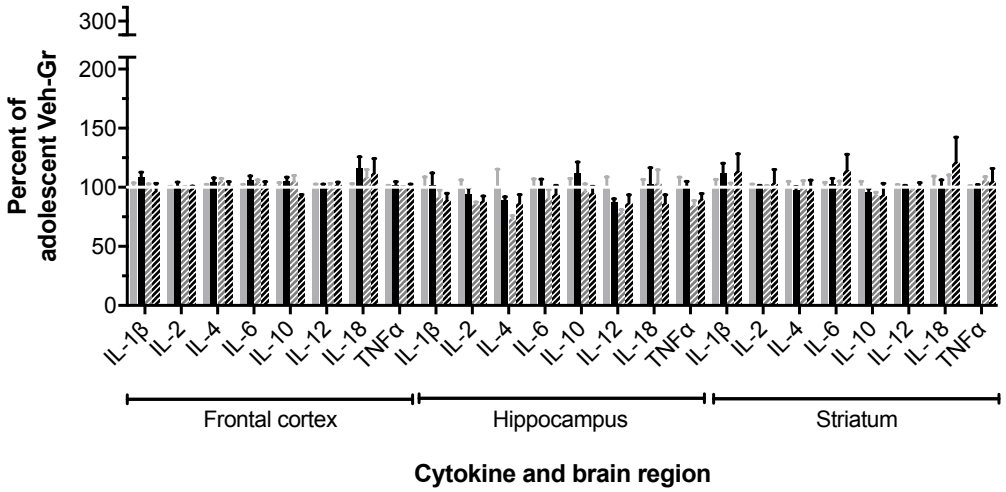
Allocating animals to experimental groups a. Give full details of how animals were allocated to experimental groups, including randomization or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	11	a. How many offspring per litter were used in each measure: 1, or if more then data from b. Randomization/Matching procedures o What procedures were used to assign animals to groups: Lots were drawn to assign animals to the various treatment/housing groups and test ages. This was done in a way that ensured balanced litter representation across groups c. Sex as a biological variable (behavioral and physiological outcomes) o Were both males and females evaluated in each behavioral and physiological outcome: YES Additional Comments: Males and females were both evaluated in the first behavioral test, 2 weeks post-weaning and males and females were also included in brain regional cytokine analyses at this age. Thereafter only males were used (as justified in the main text; Sections 2.5, 3.3.1 and 3.3.2)
Experimental outcomes ➢Behavioral testing ➢Physiological endpoints Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioral changes).	12	a. Maternal behavior and pup interactions o If maternal care was evaluated, were there differences following immunogen challenge (if so, please briefly describe): Not evaluated - mothers and pups were left undisturbed to minimize stress and associated disruption of care. Provision of extensive nesting material prevented non-disruptive observation b. Age(s) of offspring at behavioral testing/physiological evaluation endpoints: Detailed in main text (Sections 2.5.1-2.5.7 plus 2.6, and summarized in Fig. 1) c. Order of testing (e.g. behavioral test order) o Were animals evaluated in a counter-balanced order in terms of: *presentation of tests to each animal:* NO *order of experimental/control groups run through each test:* YES o What was the inter-test interval if a single animal underwent a battery of tests: See below (more space available)

		Additional Comments: The inter-test interval was typically ~1 week, but varied for tasks where the number of animals and duration to assess each required testing to be split over multiple days. In these cases care was taken to ensure a mix of neurodevelopmental conditions across test days and inter-test intervals: Locomotor activity to novel object discrimination = 1d (as activity recorded during NOD arena habituation); novel object discrimination to novel location discrimination = 7d; novel location discrimination to social interaction = 4-7d; social interaction to pre-pulse inhibition = 7-8d; pre-pulse inhibition to conditioned freezing response = 6d; OR pre-pulse inhibition to start of attentional set shifting task = 5-53d
Statistical methods a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	13	a. **Unit of analysis for each data set** ○ **Is the unit (n) of each analysis based on number of litters, or number of animals used per group:** Number of litters. Randomized allocation of littermates to different experimental subgroups avoided artificial inflation of sample size by pseudoreplication and in cases (adult male LMA, NLD, PPI) where more than one littermate underwent the same test at the same age and thus contributed data to the same experimental subgroup data from the littermates were averaged to provide one value per litter per experimental subgroup before statistical analysis
Other Disclosures		**Please make note of any other extraneous variables that you would like to report (e.g. fire alarms, construction, temporary relocations, other variables that you think we should be considering in our studies etc.):** N/A

The recommended use of this reporting form is to fill it out and include it as supplemental material for each of your laboratory's research publications. If there are difficulties utilizing/adapting this fillable form, please contact one of the corresponding authors to request a copy. The authors give permission for this table to be edited for use in reporting on other animal models (e.g. postnatal immune challenge models, early life stress models) as appropriate.

Kentner AC, Bilbo AD, Brown AS, Hsiao EY, McAllister AK, Meyer U, Pearce BD, Pletnikov MV, Yolken RH, Bauman MD. (2018). Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. Neuropsychopharmacology, https://doi.org/10.1038/s41386-018-0185-7.

Supplementary material 3

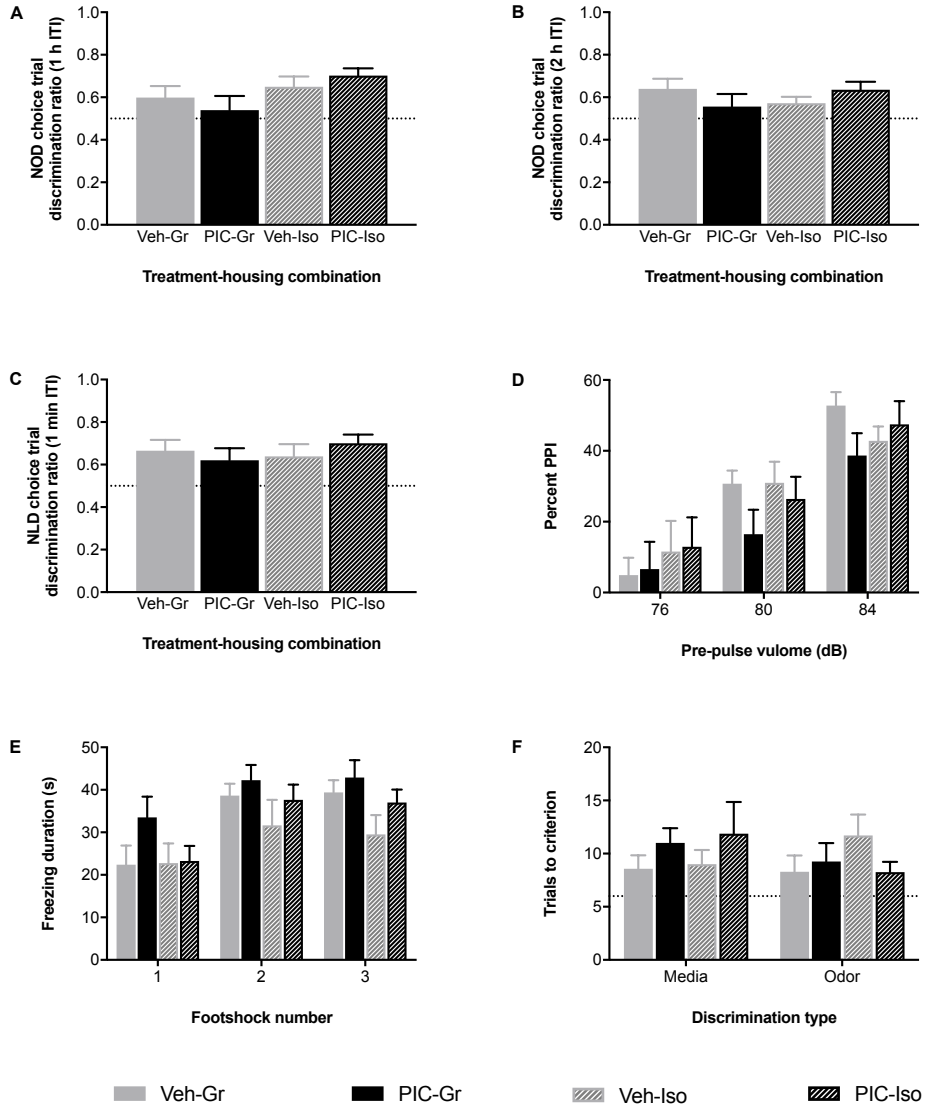


Supplementary Fig. 3. Mean ± SEM cytokine levels in brain regional tissue samples obtained from adolescent female offspring (PND 36; n = 8 per treatment-housing combination) are not influenced by gestational PIC exposure or post-weaning isolation rearing. Absolute levels (pg/mg total protein) varied by cytokine and brain region, so to aid clarity of presentation data are presented as a percentage of expression in Veh-Gr controls. Absolute levels in Veh-Gr controls are shown below in Supplementary Table 1, and statistical analyses were performed on raw data not percentages. The y-axis scale matches that used in Fig. 5 to permit direct comparison of data from males and females.

	Cytokine levels (pg/mg total protein) in Veh-Gr controls								
	Adult males			Adolescent males			Adolescent females		
	Frontal cortex	Hippocampus	Striatum	Frontal cortex	Hippocampus	Striatum	Frontal cortex	Hippocampus	Striatum
IL-1β	6.9 ± 0.33	11 ± 0.34	11 ± 0.38	12 ± 0.63	14 ± 1.9	46 ± 16	11 ± 0.42	18 ± 1.5	11 ± 0.75
IL-2	7.1 ± 0.27	9.2 ± 0.50	7.5 ± 0.18	8.5 ± 0.21	11 ± 1.2	26 ± 4.8	8.4 ± 0.073	12 ± 0.76	7.2 ± 0.20
IL-4	6.6 ± 0.71	12 ± 0.89	9.9 ± 0.31	11 ± 0.29	24 ± 7.2	75 ± 32	11 ± 0.28	31 ± 4.7	10 ± 0.50
IL-6	22 ± 3.8	88 ± 15	120 ± 4.7	90 ± 2.0	160 ± 24	270 ± 50	96 ± 2.5	170 ± 12	110 ± 4.6
IL-10	5.9 ± 0.21	8.3 ± 0.50	8.0 ± 0.44	10 ± 0.65	14 ± 2.4	36 ± 10	10 ± 0.40	16 ± 1.2	10 ± 0.50
IL-12	2.2 ± 0.066	2.6 ± 0.090	1.5 ± 0.033	2.0 ± 0.032	2.7 ± 0.34	5.8 ± 2.0	2.0 ± 0.056	3.5 ± 0.31	1.9 ± 0.041
IL-18	25 ± 2.3	29 ± 2.5	33 ± 0.90	44 ± 2.9	41 ± 1.9	53 ± 4.9	48 ± 1.5	41 ± 2.8	29 ± 2.7
TNFα	0.17 ± 0.0059	0.23 ± 0.011	0.22 ± 0.011	0.24 ± 0.0066	0.34 ± 0.041	0.67 ± 0.13	0.25 ± 0.0040	0.43 ± 0.038	0.23 ± 0.0031

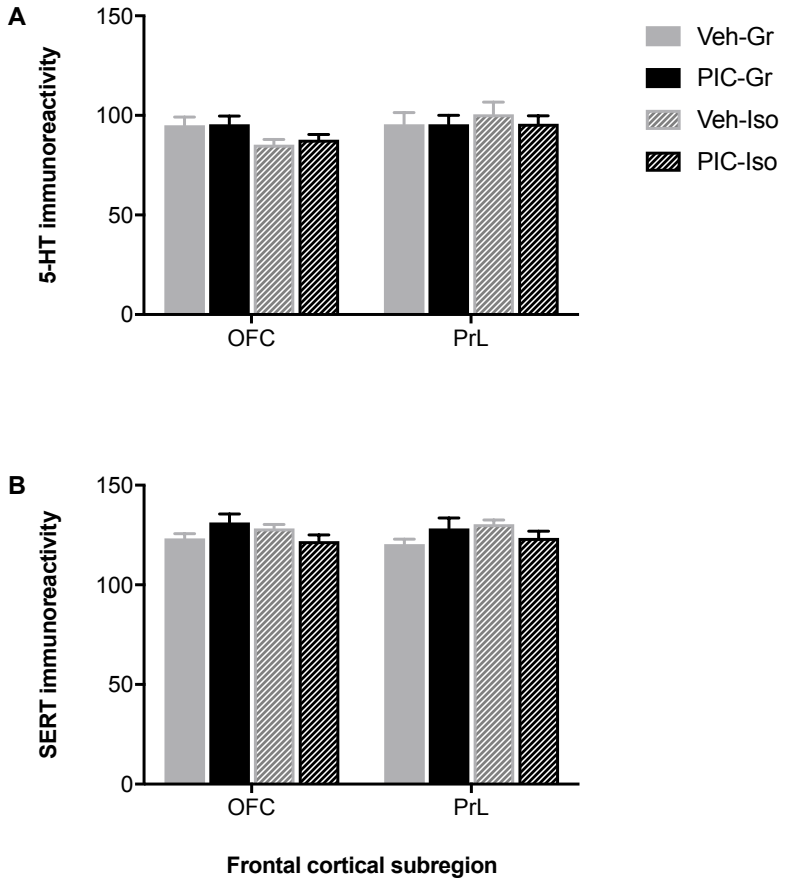
Supplementary Table 1. Absolute cytokine levels (mean ± SEM, to 2 significant figures) in brain regional tissue samples obtained from adult male (PND 87-91; n = 8) and adolescent male or female Veh-Gr controls (PND 36; n = 8 of each sex). Because levels vary by cytokine, brain region and age the above values were used to derive percentage differences in PIC-Gr, Veh-Iso and PIC-Iso for clarity of presentation in Fig. 5 (males) and Supplementary Fig. 3 (females).

Supplementary Material 4



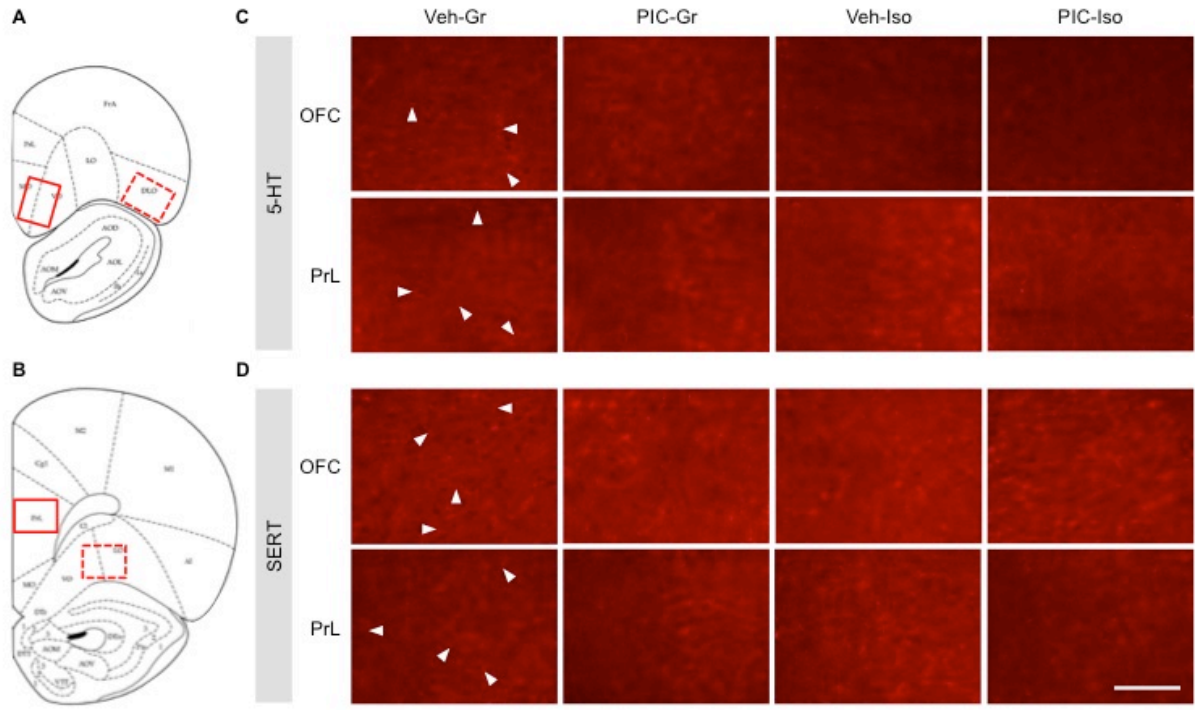
Supplementary Fig. 4. Summary of cognitive measures unaffected by gestational PIC exposure or post-weaning isolation rearing in adult male offspring. Mean ± SEM choice trial discrimination ratio in the novel object discrimination (NOD) task following an inter-trial interval (ITI) of (**A**) 1 h or (**B**) 2 h on PND 52-58, or (**C**) the novel location discrimination (NLD) task following a 1 min ITI on PND 59-65, (**D**) percent pre-pulse inhibition (PPI) with increasing pre-pulse volume on PND 74-78, (**E**) freezing duration with increasing footshock number during acquisition of a conditioned freezing response (CFR) on PND 79-83 and (**F**) trials to criterion during the simple discrimination training phase of the attentional set shifting task (ASST) on PND 86-132. In all cases data are n = 8 per treatment-housing combination, either individual values from 8 non-littermates per treatment-housing combination or mean values from each of the 8 litters per treatment-housing combination.

Supplementary Material 5



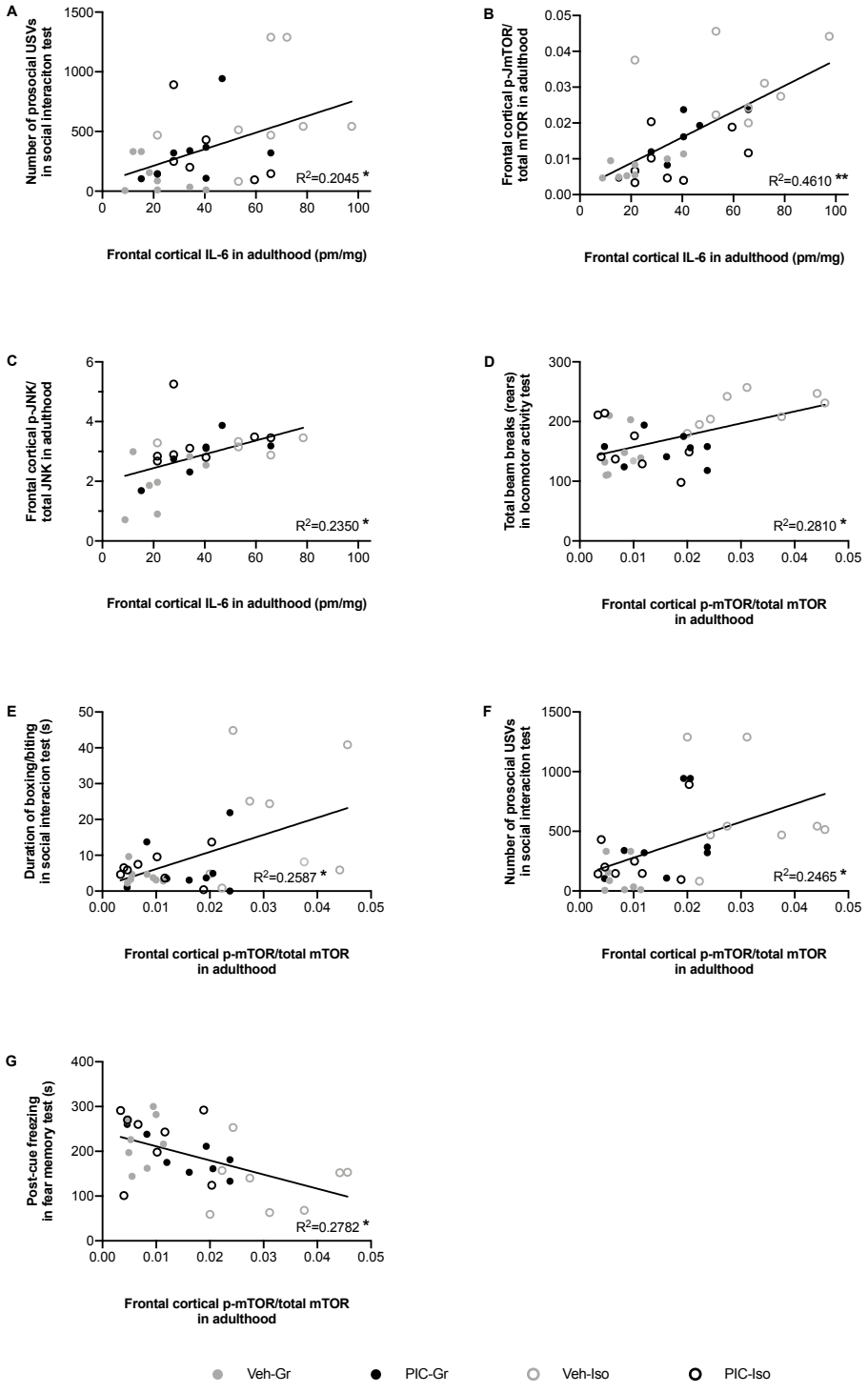
Supplementary Fig. 5. Serotonergic markers in the frontal cortex after gestational PIC exposure and/or post-weaning isolation rearing. Mean ± SEM intensity of (**A**) 5-HT or (**B**) 5-HT reuptake transporter (SERT) immunoreactivity in the orbitofrontal (OFC) or prelimbic (PrL) subregions of tissue obtained from adult male offspring on PND 87-133 (n = 7-8 per treatment-housing combination). Technical issues during sectioning prevented inclusion of one Veh-Gr, PIC-Gr and Veh-Iso individual. Regions of interest and representative images are shown in Supplementary Fig. 6.

Supplementary Fig. 6 (below). Images (x 10 magnification) were collected from orbitofrontal cortical (OFC) regions of interest in (**A**) rostral (Bregma +4.20-4.70) medial/ventral (MO/VO; solid red border), rostral dorsolateral (DLO; dashed red border) and (**B**) caudal (Bregma +3.20-3.70) ventrolateral (VO/LO; dashed red border) subdivisions, as well as prelimbic (PrL; solid red border) regions of interest (Bregma +3.20-4.20) delineated using Paxinos and Watson (1998). Representative (**C**) 5-HT and (**D**) 5-HT reuptake transporter (SERT) immunoreactivity in parts of the MO/VO and PrL regions of interest, with fine varicose axons marked by arrowheads and scale bar equivalent to 100 μm.



(Note: a higher resolution copy is available online https://doi.org/10.1016/j.bbi.2020.05.076)

Supplementary Material 6



Supplementary Fig. 7. Correlations between adulthood frontal cortical expression (PND 87-91) of cytokines or signaling intermediates (significantly influenced by gestational PIC exposure, post-weaning isolation rearing or their combination) and (**D**) rearing during locomotor activity testing on PND 51-57, (**E**) aggressive behavior and (**A**, **F**) prosocial ultrasonic vocalizations (USVs) during social interaction testing on PND 66-70, (**G**) post-cue

conditioned freezing responses during assessment of fear-motivated associative memory on PND 80-84, or (**B**, **C**) expression of other signaling intermediates in the same brain region. Each data point represents one of the eight male rats in each treatment-housing combination and lines represent best fit. **P*<0.05; ***P*<0.01 (Pearson's *r* followed by Benjamini-Hochberg false discovery rate correction).