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Research report

Maternal presence or absence alters nociceptive responding and cortical anandamide levels in juvenile female rats

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ABSTRACT

The influence of parental support on child pain experiences is well recognised. Accordingly, animal studies have revealed both short- and long-term effects of early life stress on nociceptive responding and neural substrates such as endocannabinoids. The endocannabinoid system plays an important role in mediating and modulating stress, social interaction, and nociception. This study examined the effects of maternal support or acute isolation on nociceptive responding of female rats to a range of stimuli during the juvenile pre-adolescent period and accompanying changes in the endocannabinoid system. The data revealed that juvenile female Sprague Dawley rats (PND21 − 24) isolated from the dam for 1 h prior to nociceptive testing exhibited increased latency to withdraw in the hot plate test and increased mechanical withdrawal threshold in the Von Frey test, compared to rats tested in the presence of the dam. Furthermore, isolated rats exhibited reduced latency to respond in the acetone drop test and enhanced nociceptive responding in the formalin test when compared to dam-paired counterparts. Anandamide, but not 2-AG, levels were reduced in the prefrontal cortex of dam-paired, but not isolated, juvenile rats following nociceptive testing. There was no change in the expression of CB_1, FAAH or MAGL; however, CB_2 receptor expression was reduced in both dam-paired and isolated rats following nociceptive testing. Taken together the data demonstrate that brief social isolation or the presence of the dam modulates nociceptive responding of juvenile rat pups in a modality specific manner, and suggest a possible role for the endocannabinoid system in the prefrontal cortex in sociobehavioural pain responses during early life.

1. Introduction

It is well recognised that parental presence during painful experiences can modulate a child's responses to pain from the neonatal period through to adolescence [1–3]. For the most part the effects reported are positive, with parental presence associated with reductions in pain experienced by the child, however, the effects depend on the nature, timing and stress status of the caregiving [4–7]. Similarly, preclinical animal studies have manipulated dam-pup interactions during the first 2 weeks of life to model early-life stress [8,9], an effect which results in an array of physiological changes including altered nociceptive responding (for reviews, see: 10,11]. For example, several studies have demonstrated that short term (up to 30 min) isolation of neonatal rodent pups from the dam and siblings result in thermal hypoalgesia [12–17], while longer-term isolation (4–6h) is associated with either no change [16] or thermal hyperalgesia [18]. High levels of maternal care to pups following an early-life pain experience has been shown to result in reduced nociceptive responding (hypoalgesia) to thermal noxious stimuli in adulthood [19]. Female rats experiencing social rejection during adolescence exhibit decreased thermal sensitivity in adulthood [20,21]. Furthermore, social reunion between male, but not female adult sibling mice, following separation at weaning, has been shown to

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; ADT, acetone drop test; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CNS, central nervous system; CPS, composite pain score; FAAH, fatty acid amide hydrolase; HPT, hot plate test; MAGL, monoacylglycerol lipase; PFC, prefrontal cortex; VFT, Von Frey test

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result in an increased heat nociceptive threshold [22–24]. While the effects of maternal support or early neonatal/post-weaning isolation on thermal nociceptive responding have been examined, there has been a lack of studies examining the effects on nociceptive responding to mechanical, cold or inflammatory stimuli or examining effects during the juvenile pre-weaning period.

The endocannabinoid system (ECS) is a retrograde neuromodulatory lipid signalling system known to play a key role in a host of physiological functions including stress, pain and social responding [21,25–27]. Comprised of the CB_1 and CB_2 receptors, the naturally-occurring endogenous ligands (endocannabinoids) – namely, anandamide and 2-arachadonyl glycerol (2-AG) - and the enzymes involved in the synthesis and degradation of the endocannabinoids, the ECS is uniquely positioned to modulate synaptic transmission and plasticity. Early-life stress has been shown to result in immediate and lasting alterations in the ECS [28–30]. Furthermore, several studies have revealed an important role for the ECS in mediating and modulating social behaviour during adolescence and its impact on nociceptive responding [20,31,32]. In addition to its role in executive and social functioning, the prefrontal cortex (PFC) is a key brain structure involved in sensory and affective pain processing [(for reviews see [33,34]]. Furthermore, several studies have demonstrated an important modulator role for the ECS in the PFC in mediating and modulating nociceptive responding in preclinical pain models [35–37]. However, there have been no studies examining whether the ECS in the PFC is altered during acute social withdrawal and pain during the juvenile period.

The present study examined the effects of maternal presence versus absence on nociceptive responding to thermal heat, cold, mechanical and inflammatory noxious stimuli in juvenile female rat pups. Despite calls to include females in preclinical research [38], pain studies continue to be predominantly conducted in males. There has been a lack of studies examining pain responses to brief social isolation and to our knowledge no study investigating such responses in female rodents. Female rats have been demonstrated to exhibit pronounced changes in pain sensitivity and endocannabinoid function as a result of prolonged isolation and peer rejection [20,21,31]. Thus a further aim of this study was to examine endocannabinoid levels and expression of receptors and enzymes of the ECS in the prefrontal cortex, and determine if maternal presence/absence-induced changes in nociceptive responding in female rats are associated with alterations in the ECS.

2. Materials and methods

2.1. Animals and experimental design

Male and female Sprague-Dawley (200–340 g; Charles River Laboratories, UK) rats were group housed under controlled conditions (temperature 20 − 24 °C, 40 − 50% relative humidity, and 12/12 h light cycle with lights on at 07:00) and allowed 7 days to acclimatise before mating. Food and water were available *ad libitum*. Ten pregnant females were singly housed and allowed to raise their own pups. Litters ranged in size from 7 to 12 pups (3–7 females per litter; no litters were exclusively male or female) and weaning took place between PND21 − 24 (PND21: 4 litters; PND23: 5 litters; PND24:1 litter). At the time of weaning, three female pups from each litter were assigned to one of three groups [1] Dam-paired nociceptive tested [2], Isolated nociceptive tested, and [3] Behaviourally-naïve (n = 10 per group). Male and unselected female pups were rehoused in same-sex groups and used in other experiments. Pups assigned to the dam-paired nociceptive tested group were kept in the original home cage with the dam (siblings removed 1 h prior to testing), and the dam was present during nociceptive testing. The pups in the isolated group were taken from the dam and siblings and singly housed in a new cage for 1 h prior to, and throughout, nociceptive testing (total period of isolation approx. 4 h). Nociceptive responding was carried out between 09:00 − 15:00 h and each animal was tested in the same sequential order: responding to noxious heat was assessed using the hot plate (HPT), followed by mechanical responding using the Von Frey (VFT), cold hypersensitivity using the acetone drop test (ADT) and inflammatory nociception using the formalin test. In order to ensure that all testing occurred between 09:00 − 15:00, and animal were isolated/weaned between PND21 − 24, the experiment was conducted over a five day period with pups from two litters tested in the three experimental groups per day (2 pups x 3 groups x 5 days = 30 animals). Animals were sacrificed immediately following nociceptive testing (group 1 and 2), or taken directly from the home cage (group 3) and sacrificed. The brain was removed and frozen at −80 °C. PFC dissection involved cutting a 1.5 mm coronal tissue section containing the PFC (Bregma 4.7 to 3.2 mm) from the frozen brain using a rat brain matrix. The olfactory bulbs were removed from the section and PFC was dissected from the right and left sides (Fig. 2; [39]) and randomly assigned for either the assessment of endocannabinoid levels or mRNA expression of enzymes and receptors. The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Health Products Regulatory Authority, in compliance with the European Communities Council directive 2010/63/EU and the ARRIVE guidelines.

2.2. Behavioural testing

2.2.1. The hotplate test (HPT)

The HPT apparatus (Harvard Apparatus, Kent, United Kingdom) was set to a test temperature of 55 ± 1 °C, in line with previous studies [40,41]. The latency to hind-paw lick was recorded with a maximum cut-off at 40 s, to prevent tissue damage. To accommodate the dam-paired pups, their home cage (open top cage containing the dam only) was placed at a maximum distance of 40 cm from the HPT apparatus while the pup was tested. The animal was returned to its home cage (containing the dam (dam-paired) or singly (isolated)) following testing and the test arena was cleaned and wiped down between uses, using 0.5 % acetic acid solution.

2.2.2. The Von Frey test (VFT) and acetone drop test (ADT)

Both the VFT and ADT were conducted sequentially in a six-compartment Perspex arena (11cm × 20cm × 15 cm) with wire mesh flooring, as previously described [42,43]. To accommodate the dam-pup group, the divider walls were substituted for Perspex partitions punched with holes of 0.5 cm diameter at 2 cm intervals, to enable the dam and pup to see and smell each other without physical contact during both habituation and testing. Pups in the isolated group were tested in the same arena, but with no animal in the second testing chamber. Five minutes following the HPT, pups (+/- dam) were habituated to the arena for 25 min, after which time von Frey filaments (Touch-Test®Sensory Evaluators; North Coast Medical Inc., Gilroy, CA, USA) of increasing forces (0.16 g – 180 g) were applied perpendicular to the plantar surface of the hind paw, for up to a maximum of 5 s or until flinching, licking or withdrawal of the paw occurred. Each filament was applied five times on each hind paw (alternating between right and left paws, for a total of ten withdrawals), and filament force was increased until a 100 % positive response (5 responses to 5 applications) was observed for two consecutive filaments. Results were expressed as the lowest filament to elicit a response and the percentage response frequency of paw withdrawals (number of withdrawals/10 × 100) and an EC50 value generated for each animal.

Immediately following VFT, the animals remained in the apparatus for ten minutes, before being assessed for cold allodynia with the ADT. In brief, a 0.2 mL droplet of acetone (Sigma-Aldrich, Dublin, Ireland) was applied to the plantar surface of each hind paw in turn, ensuring to avoid foot pads. Behaviours were scored for latency to first response (flinch, lick, paw shake, or withdrawal of paw) within the cut-off time of 60 s. If the animal did not respond within 60 s, the cut-off time was

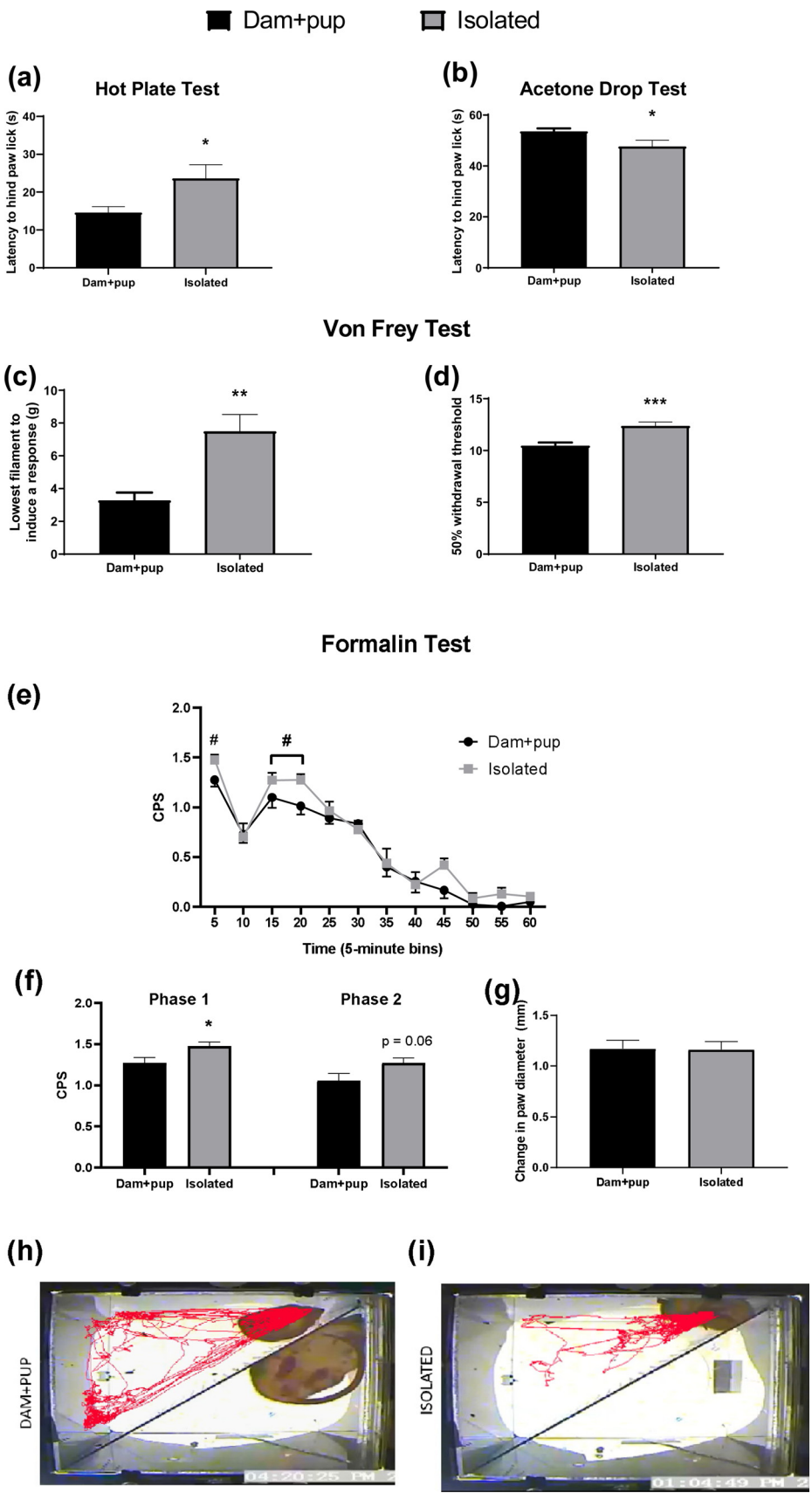

Fig. 1. The effect of acute isolation vs maternal support on nociceptive responding of juvenile female pups. (a) Latency to respond in the hot plate test. (b) Latency to hind paw lick in the acetone drop test. (c-d) Mechanical withdrawal thresholds in the von Frey test. CPS over the (e) 60 min formalin trial (5-minte bins) and (f) during the phase1 and 2 of the formalin response. (g) Change in paw diameter following formalin administration. (h-i) Representative images of the experimental setup during the formalin test. Data expressed as mean ± SEM (n = 8-10 per group). *** $p < .001$; ** $p < 0.01$; * $p < 0.05$ vs dam-paired pups. #represents phase 1 and 2 of the formalin test. CPS = composite pain score.

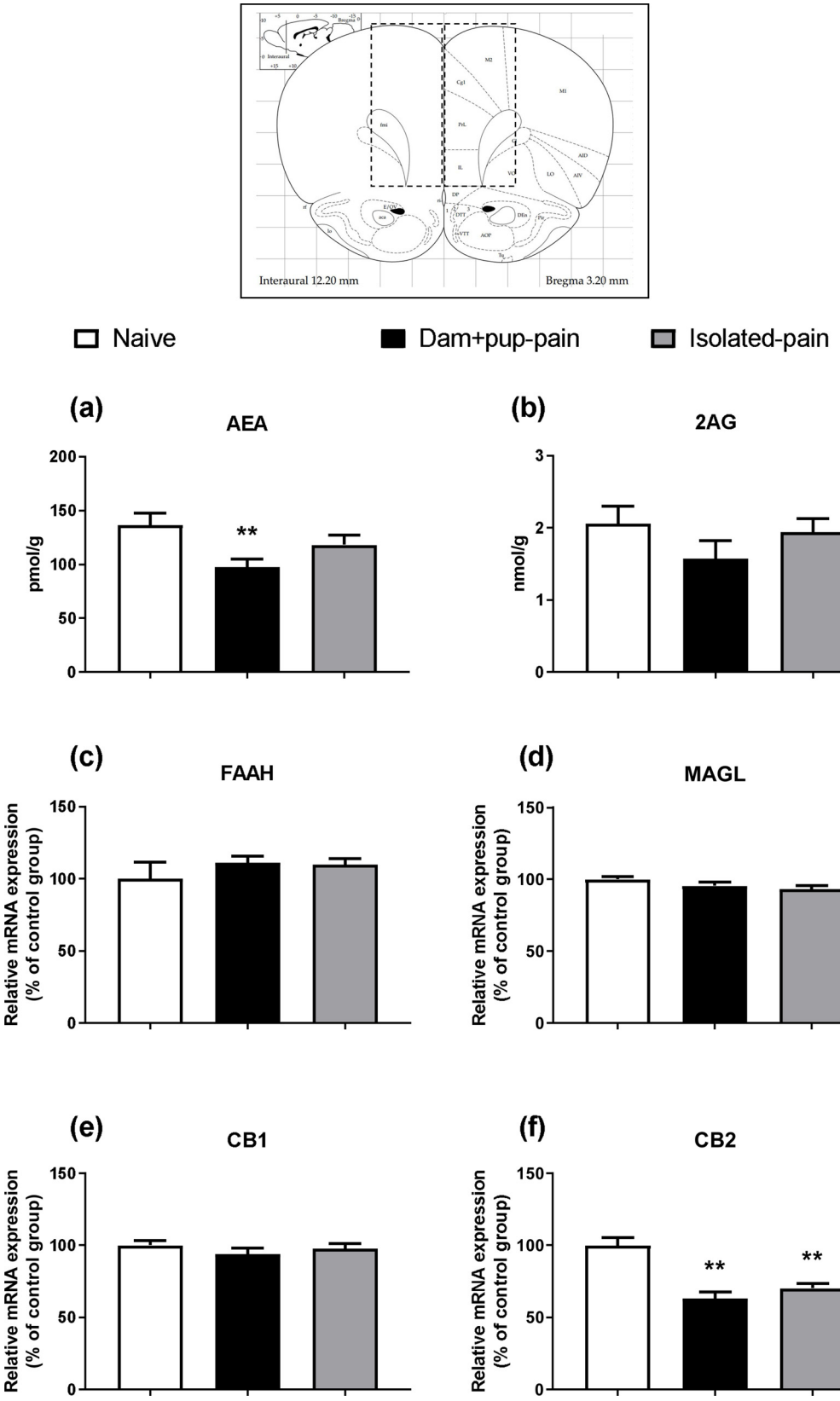

Fig. 2. The effect of intra-plantar formalin injection on endocannabinoid levels, enzyme and receptor expression in the PFC of juvenile female pups. Schematic of areas of PFC dissected for analysis. (a-b) AEA and 2-AG levels. (c-d) Expression of the catabolic enzymes FAAH and MAGL. (e-f) Expression of the cannabinoid receptors CB_1 and CB_2. Data expressed as mean ± SEM (N = 8-10). ** $p < 0.01$ vs behaviourally-naïve control group.

recorded as the latency. Three trials per paw were conducted, and the average of the 3 trials for each hind paw was calculated.

2.2.3. Formalin test

Immediately following the ADT, the pups were placed back into their home cage and transferred to a new procedure room where they were immediately placed into the formalin arena for a 10 min habituation period. The arena consisted of a black Perspex observation chamber (30 × 30 × 30 cm), divided in 2 equal parts by a Perspex panel (holes of 0.5 cm diameter punched at 2 cm intervals); this enabled the dam-pup pairs to see and smell each other, but not interact, during both the habituation and the formalin trial (Fig. 1). Light intensity was maintained at 35 lx and behaviour recorded by a video camera mounted beneath the observation chamber. Testing was carried out as previously described [41,44].

Distance moved, rearing and grooming during the 10 min habituation trial was recorded. Immediately following habituation, the pup was removed, gently restrained and the right hind paw diameter was measured using Vernier callipers, before administering an intra-plantar injection of 20 µl 1% formalin in sterile saline. The pup was then placed back into the arena for 60 min and behaviour was recorded onto video and analysed with the aid of EthoVision® XT11.5 video-tracking software (Noldus, Netherlands). Formalin-evoked nociceptive behaviour was scored over the entire 60 min trial and analysed in 5 min time bins using the weighted composite pain scoring technique (CPS-$WST_{0,1,2}$) [45]: time spent elevating the injected paw (C1), and licking, shaking, biting, or holding the injected paw (C2) were combined to obtain a composite pain score (CPS = C1 + 2(C2)/(duration of analysis period)). There are 3 distinct phases to the formalin test [46], phase 1 (0 − 5 min time period: which represents direct activation of peripheral nociceptors), the interphase (5 − 10 min period; hyperpolarization of nocicpeptors) and phase 2 (> 10 min period: which represents engagement of inflammatory and/or central nociceptive responses including sensitization). As such, CPS was also calculated for phase 1 (0 − 5 min) and phase 2 (10 − 20 min) of the formalin test. Distance moved, duration of rearing and grooming were also recorded. At the end of the test period, paw diameter were recorded again before sacrifice. Two pups from the isolated cohort received incorrect concentration of formalin into the hind paw and thus were excluded from formalin and post-mortem analysis (final group number for the formalin test are Dam-Pup group, n = 10; Isolated group, n = 8).

2.3. Quantification of endocannabinoid levels in the PFC using LC–MS/MS

Quantitation of endocannabinoids using mass spectrometry was carried out as described previously [44,47]. In brief, PFC samples were homogenized in 400 µl 100 % acetonitrile containing deuterated internal standards (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8), centrifuged at 14,000 *g* for 15 min at 4 °C, and then the supernatant was evaporated to dryness. Lyophilized samples were re-suspended in 40 µl 65 % acetonitrile and separated on a Zorbax C18 column by reversed-phase gradient elution (65%–100% acetonitrile with 0.1 % formic acid). Analyte detection was carried out in electrospray-positive ionization and multiple reaction monitoring mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantification of each analyte was performed by ratiometric analysis using Masshunter Quantitative Analysis Software (Agilent Technologies, Cheshire, UK) and expressed as nmol or pmol per gram of tissue.

2.4. Expression of endocannabinoid-related catabolic enzymes and receptors in the PFC

PFC tissue was placed into RA1 buffer and mRNA was extracted using the NucleoSpin RNA II total RNA isolation kit, in accordance with manufacturer instructions (Macherey-Nagel, Germany). mRNA was reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK) and Taqman gene expression assays (Applied Biosystems, UK) were used to quantify the gene of interest using real-time PCR performed using a StepOne Plus instrument (Applied Biosystems, UK), as previously described [44,47]. Assay IDs for the genes were CB_1 (Rn02758689), CB_2 (Rn03993699), FAAH (Rn00577086) and MAGL (Rn00593297), and VIC-labelled β-actin was used as an endogenous control to normalize gene expression data. PCR was performed using Taqman PCR Master Mix, and samples were run in duplicate. The cycling conditions were 90 °C for 10 min and 40 cycles of 90 °C for 15 min followed by 60 °C for 1 min. Relative gene expression was calculated using the ΔΔCT method and expressed as % change from behaviourally-naïve control group.

2.5. Statistical analysis

The SPSS 24.0 (IBM Corp, Armonk, NY) statistical program was used to analyse all data. Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene's test, respectively. Data from the two behavioural groups were analysed using unpaired t-tests, and CPS in the formalin test was assessed using repeated measures analysis of variance (ANOVA). Endocannabinoid levels and gene expression were assessed using one-way ANOVA with post-hoc Fisher LSD tests. If data were found to be non-parametric, three transformations were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed [48]. All data was parametric prior to or following these transformations. Statistical significance was set at $p \leq 0.05$. All graphs were constructed using GraphPad Prism and data are presented as group mean ± standard error of the mean (SEM).

3. Results

3.1. Isolated juvenile female rats exhibit thermal and mechanical hypoalgesia and cold allodynia when compared to pups tested in the presence of the dam

The effect of acute isolation versus social support (presence of dam) of juvenile rat pups on nociceptive responding to heat, cold and mechanical stimuli was examined. There was a significant difference on latency to respond in the hot plate test, with isolated juvenile pups demonstrating longer withdrawal latencies than dam-paired counterparts [$t(18) = 2.29$, $p = 0.034$] (Fig. 1a). During the acetone drop test, isolated juvenile pups demonstrated significantly reduced withdrawal latencies compared to dam-paired juveniles [$t(16) = 2.33$, $p = 0.033$] (Fig. 1b). Analysis of the sensitivity to mechanical stimuli during Von Frey testing revealed that isolated juvenile pups required heavier filaments to provoke a withdrawal response [$t(18) = 3.74$, $p = 0.002$] (Fig. 1c), and demonstrated a significantly increased paw withdrawal threshold compared to dam-paired juvenile pups [$t(18) = 4.15$, $p = 0.0006$] (Fig. 1d).

3.2. Nociceptive responding to an inflammatory stimulus is increased in isolated juvenile female rat pups compared to pups tested in the presence of the dam

Rats were exposed to a novel Perspex arena for 10 min; analysis of this habituation period revealed no significant difference between isolated and dam-paired juvenile pups in relation to distance moved, rearing or grooming behaviour (Table 1).

In order to examine the effect of isolation vs social support on inflammatory nociceptive responding, juvenile rats received an intraplantar formalin administration and behaviour was assessed over a 60 min period. Repeated measures ANOVA revealed a significant effect of

Table 1
Distance moved, rearing and grooming during habituation and formalin trial.

Habituation (10 min.)				
	Dam-pup	Isolated	Sig.	
Distance moved (cm)	1038 ± 60	868 ± 68	t(18) = 1.89; p = *0.07*	*ns*
Rearing (s)	95.45 ± 6.19	87.95 ± 14.93	t(18) = 0.46; p = *0.65*	*ns*
Grooming (s)	128.60 ± 11.11	117.23 ± 15.78	t(18) = 0.59; p = *0.56*	*ns*
Formalin trial (60 min.)				
	Dam-pup	Isolated	Sig.	
Distance moved (cm)	1523 ± 122	822 ± 73	t(16) = 4.62, p = *0.001*	***
Rearing (s)	69.17 ± 11.57	20.63 ± 7.69	t(16) = 3.30, p = *0.004*	**
Grooming (s)	60.60 ± 7.18	73.00 ± 13.50	t(16) = 0.86; p = *0.40*	*ns*

Data expressed as mean ± SEM (n = 8–10 per group); *** $p < 0.001$; ** $p < 0.01$; *ns* = not significant.

time [$F_{(11, 176)} = 83.61$, $p < 0.001$] on the composite pain score (CPS) per 5 min time bin over the course of the 60 min formalin trial (Fig. 1e). Further discrete analysis of the two main phases of the formalin test revealed that isolated pups exhibited a significant increase in CPS during the first phase of the formalin trial ($p = 0.035$), with a trend for a significant increase in responding in the second phase which just failed to reach statistical significance ($p = 0.06$) (Fig. 1f). There were no differences between dam-paired and isolated pups in relation to paw oedema at 60 min. post-formalin administration [t(16) = 0.063, $p = 0.950$] (Fig. 1g).

During the 60 min formalin trial, isolated juvenile pups moved significantly less [t(16) = 4.621, $p = 0.0003$] and reared less [t(16) = 3.30, $p = 0.004$] than dam-paired pups (Table 1). There was no significant difference between the groups in relation to the duration of grooming during the test [t(16) = 0.318, $p = 0.755$] (Table 1).

3.3. PFC anandamide levels are reduced in pups with a dam present during nociceptive testing

Endocannabinoid levels were assessed in the PFC following nociceptive testing and in a behaviourally-naïve control group. Analysis revealed a significant effect of nociceptive testing on levels of the endocannabinoid anandamide (AEA) in the PFC [$F_{(2,28)} = 5.176$, $p = 0.013$] (Fig. 2a). *Post-hoc* analysis revealed that levels of AEA were significantly lower in the PFC of dam-paired pups following nociceptive testing ($p = 0.010$), an effect not observed in isolated pups (Fig. 2a). There was no significant difference in 2-AG levels in the PFC between any of the groups [$F_{(2,28)} = 1.32$, $p = 0.284$] (Fig. 2b).

3.4. Nociceptive testing is associated with decreased in CB_2 receptor expression in the PFC

Fatty acid amide hydrolase (FAAH) is the enzyme primarily responsible for the catabolism of anandamide, while monoacylglycerol lipase (MAGL) preferentially metabolises 2-AG. There were no significant difference between the groups in mRNA expression of FAAH [$F_{(2,27)} = 0.01$, $p = 0.990$], MAGL [$F_{(2,27)} = 2.16$, $p = 0.137$] or the CB_1 receptor [$F_{(2,27)} = 0.730$, $p = 0.493$] in the PFC (Fig. 2c–e). Analysis revealed a significant effect group on the mRNA expression of CB_2 in the PFC [$F_{(2,27)} = 18.84$, $p = 0.0001$]. *Post-hoc* analysis revealed that levels of CB_2 were significantly lower in both isolated ($p = 0.0001$) and dam-paired pups ($p = 0.0001$), compared to the behaviourally-naïve control group (Fig. 2f).

4. Discussion

The data presented here demonstrate that nociceptive responding of juvenile female rat pups is altered depending on maternal presence or acute isolation. Specifically, isolated juvenile pups exhibit enhanced inflammatory and cold nociceptive responding, and reduced mechanical and heat nociceptive responding, when compared to pups tested in the presence of a dam. Conversely, the presence of the dam resulting in cold and inflammatory hypoalgesia, and mechanical and heat hyperalgesia in juvenile pups, when compared to pups tested in isolation. Taken together, the data indicating that maternal presence/isolation modulates nociceptive responding of juvenile female rat pups depending on the modality being examined.

To date, studies examining the effects of short term isolation and/or maternal support on nociceptive responding have been carried out in the early postnatal period and have only examined heat nociceptive thresholds. For example, 6 day old rats isolated from the dam for 30 min exhibit increased tail flick latencies [12] and 10–12 day old rat pups isolated for 5 − 8 min exhibit longer latencies in the hot plate test (i.e. hypoalgesia), [13–17]. Longer-term pup isolation (4–6 h) has been associated with either no change [16] or reduced [18] latency to respond in the hotplate test. Thus short, but not long term, isolation induces a decrease in nociceptive responding to noxious heat stimuli in the early neonatal period. The current data expand on this data demonstrating that juvenile pups (PND21 − 24) isolated from the dam and siblings for a 1 h period also exhibit an increase in nociceptive thresholds to a thermal noxious stimulus and expand this to include an increase in thresholds to mechanical noxious stimuli. Although this is the first study to examine effects in the juvenile period, it has been demonstrated that mice isolated for 1 h at 6–8 weeks of age exhibit increased mechanical thresholds and paw withdrawal latencies compared to non-isolated cage mates [49]. The precise mechanisms mediating isolation-induced thermal and mechanical hypoalgesia remain to be determined, however effects may be due to isolation stress-induced engagement of the descending pain pathway and resultant analgesia (stress-induced analgesia) [27,50]. Stress-induced analgesia is an important survival mechanism which results in reduced nociceptive responding to demanding acute internal and/or external stressors. However, stress-induced analgesia can be modality specific and effects are dependent on the type and intensity of the stressful event (psychological vs physical; conditioned vs unconditioned) [50,51]. Accordingly, while acute isolation results in inhibition of nociceptive responding to heat and mechanical noxious stimuli, our data demonstrated that rat pups tested in isolation exhibited enhanced responding to a cold innocuous stimulus (cold allodynia) compared to dam-paired counterparts. Thus, the effects of acute isolation on nociceptive responding of juvenile female pups depends on the modality under investigation, which may reflect the involvement of the different spinal and/or supraspinal mechanisms mediating the nociceptive responses to these varying heat, cold and mechanical stimuli.

During the formalin test, pups tested in isolation exhibited increased inflammatory nociceptive responding when compared to those tested in the presence of the dam. The increase in nociceptive responding occurred during the first phase, with a tendency for an increase during the second phase also noted. Furthermore, isolated rats exhibit reduced locomotor activity and rearing during the formalin test which may reflect indirect behavioural responses to pain (i.e. less movement due to greater pain). These data highlight effects of brief isolation on

nociceptive responding on both the initial somatosensory processing and the longer term supraspinal modulation of the inflammatory pain response. Although the mechanism mediating these effects remains to be determined, it is possible that the presence of the dam may act as a distractor from the inflammatory pain (distraction-induced analgesia). This is supported by the data demonstrating that rats tested in the presence of the dam exhibited greater distance moved and rearing during the formalin trial, primarily directed towards the partition separating the dam and pup (Fig. 1). Previous studies have demonstrated that the presence of a confined conspecific does not alter formalin-induced inflammatory nociceptive responding [52] or results in hyperalgesia to visceral inflammatory pain (acetic acid) [53]. However, the conspecific used in the aforementioned studies were unfamiliar to the test animal and testing was conducted using adult rodents. Furthermore, Langford et al. demonstrated that mild social threat produces hyperalgesia [53]. Thus, the presence of the dam may result in a distraction that promotes engagement of social circuits and descending pain pathways to modulate nociceptive responding.

It should be noted that isolated animals exhibit hypoalgesia the first two nociceptive tests conducted (hot plate and von frey tests) and hyperalgesia in the subsequent two tests (acetone and formalin tests). We cannot rule out that the heat and mechanical hypoalgesia was a transient effect of acute isolation or that sequential testing of the animals impacted on subsequent nociceptive responses. However regardless of the direction of change, the data confirm that acute isolation/maternal support alters nociceptive responding of female juvenile rat pups.

The ECS is a key modulator of stress- and distraction-induced analgesia [50,54] and plays an important role in the prefrontal cortex in mediating and modulating nociceptive responding [34,36,55]. The data herein demonstrate that AEA, but not 2-AG, levels were reduced in dam-paired, but not isolated, pups that underwent nociceptive testing. The decrease in AEA was not associated with a change in the expression of the metabolic enzyme FAAH, although activity of the enzyme may be altered. Recent data from our laboratory has demonstrated that increasing AEA tone within subregions of the prefrontal cortex modulates inflammatory nociceptive responding only under conditions of stress (attenuates fear-conditioned analgesia) [36]. Thus a decrease in AEA levels in the PFC may mediate stress- and/or distraction-induced analgesia. However, given the time at which the tissues were collected, it is possible and even likely that the decrease in AEA reflects an earlier increase in release, mobilisation and utilisation. Accordingly, Ford et al. demonstrated that distraction-induced analgesia was associated with an increase in AEA (and 2-AG) levels and could be attenuated by CB_1 antagonism [54]. Thus while additional studies are required to determine the precise mechanisms underlying the changes in nociceptive responding the presence or absence of the dam in juvenile rats, the data here suggest a possible role for AEA signalling in the PFC in mediating or modulating these effects. Interestingly both dam-paired and isolated juvenile rats that underwent nociceptive testing exhibited a decrease in CB_2 expression in the PFC. It is unknown if this effect is due to exposure to any one particular nociceptive test, removal from the homecage during testing, or an effect of cumulative nociceptive testing over a 3 h period. CB_2 receptors are expressed on glia and discrete populations of neurons within the brain, and increasing data suggests an important role for CB_2 receptors in the mediation and modulation of pain [26] Accordingly, recent data from our laboratory has demonstrated that enhancing 2-AG tone within the PFC attenuates fear-conditioned analgesia via CB_2 receptor activation [35]. However, the expression of CB_2 receptors are low under non-pathological conditions, which was confirmed in the current study (CB_1: average CT = 21 vs CB_2 average CT = 30). Future studies will be required determine if the decrease in CB_2 receptor expression following nociceptive testing translates into a change in protein expression, the location of such changes (neurons and/or glia, discrete areas of the PFC) and the physiological significance.

5. Conclusions

This study provides key insight into the effects of maternal presence vs acute isolation on nociceptive responding in juvenile female rat pups, effects dependent on the modality under investigation. The data also demonstrate that AEA levels are altered in a key brain region (PFC) involved in affect and nociception and thus provides further support for the ECS as a mediator and/or modulator of social support on nociceptive responding. This data contribute to our limited understanding of parent-child interactions during pain experiences, and may provide a framework for evaluating the role of the ECS in sociobehavioural responding during childhood painful experiences.

Author statement

GOS, BMcG, LC, and MR were involved in the experimental design and writing of the manuscript. GOS, RH, AT, DK and MR conducted and analysed the experiments. All authors read and approved the manuscript prior to submission.

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Declaration of Competing Interest

None.

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