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Author(s): Ford, Gemma K.; Moriarty, Orla; Okine, Bright N.; Tully, E.; Mulcahy, A.; Harhen, Brendan; Finn, David P.

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INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN ATTENTIONAL MODULATION OF NOCICEPTIVE BEHAVIOUR IN RATS

*G. K. Ford\textsuperscript{1,2,3}, *O. Moriarty\textsuperscript{1,2,3}, B. N. Okine\textsuperscript{1,2,3}, E. Tully\textsuperscript{1}, A. Mulcahy\textsuperscript{1}, B. Harhen\textsuperscript{2,3}, D. P. Finn\textsuperscript{1,2,3}

\textsuperscript{1} Pharmacology and Therapeutics, School of Medicine, \textsuperscript{2}NCBES Neuroscience Centre and \textsuperscript{3}Centre for Pain Research, National University of Ireland, Galway

* Equal contribution

Correspondence to: Professor David P. Finn,
Pharmacology and Therapeutics, School of Medicine,
National University of Ireland, Galway,
University Road,
Galway,
Ireland.
Tel. +353-91-495280. Fax. +353-91-495586.
Email: david.finn@nuigalway.ie.

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Running Title: Endocannabinoids in distraction-induced analgesia

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• Distraction-induced analgesia is a clinically useful form of pain modulation. However, the underlying mechanisms are poorly understood. We have recently developed a rat model for further investigation of these mechanisms.

• This article presents evidence for a role of the endocannabinoid system in the expression of distraction-induced antinociception in rats.
Abstract

**Background:** Distraction is used clinically to relieve and manage pain. It is hypothesised that pain demands attention and that exposure to another attention-demanding stimulus causes withdrawal of attention away from painful stimuli, thereby reducing perceived pain. We have recently developed a rat model that provides an opportunity to investigate the neurobiological mechanisms mediating distraction-induced analgesia, as these mechanisms are, at present, poorly understood. Given the well-described role of the endogenous cannabinoid (endocannabinoid; EC) system in the modulation of pain and attentional processing, the present study investigated its role in distraction-induced antinociception (DIA) in rats.

**Methods:** Animals received the CB1 receptor antagonist/inverse agonist, rimonabant or vehicle i.p., 30 mins prior to behavioural evaluation. Formalin-evoked nociceptive behaviour was measured in the presence or absence of a novel-object distractor. Liquid chromatography-tandem mass spectrometry was used to determine the levels of the endogenous cannabinoids anandamide and 2-arachidonoylglycerol (2-AG) in the ventral hippocampus (vHip).

**Results:** Exposure to a novel object distractor significantly reduced formalin-evoked nociceptive behaviour. The novel object-induced reduction in nociceptive behaviour was attenuated by rimonabant. Novel object exposure was also associated with increased tissue levels of anandamide and 2-AG in the vHip.

**Conclusions:** These data suggest that the reduction in formalin-evoked nociceptive behaviour which occurs as a result of exposure to a novel object may be mediated by engagement of the endocannabinoid system, in particular in the vHip. The results provide evidence that the EC system may be an important neural substrate subserving attentional modulation of pain.
1. Introduction

Pain is a multidimensional, subjective experience, and its perception requires supraspinal processing. Pain is modulated by cognitive factors, and distraction interventions are frequently used to relieve pain. Distraction results in withdrawal of attention from the noxious stimulus/input and reduced pain perception (Eccleston and Crombez, 1999; Wismeijer and Vingerhoets, 2005). Various distraction techniques, from simple mathematical tasks to sophisticated virtual-reality devices, have been used clinically to reduce pain (Hoffman et al., 2000; Maclaren and Cohen, 2007). Distraction has also been shown to reduce the perceived intensity of experimental pain in humans in laboratory settings (Bantick et al., 2002; Villemure and Bushnell, 2002).

The neural mechanisms underlying distraction-induced analgesia are poorly understood. Human brain imaging studies suggest involvement of brain regions associated with attention, affect, and endogenous analgesia (Petrovic and Ingvar, 2002; Valet et al., 2004). Evidence suggests a key role for the endogenous cannabinoid (endocannabinoid; EC) system in fear/stress-induced analgesia (Butler and Finn, 2009; Ford and Finn, 2008) and in the placebo response (Benedetti et al., 2011). The main constituents of the EC system are two endogenous ligands, \( N \)-arachidonoyl ethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) and two \( G_{i/o} \)-protein coupled receptors, cannabinoid\(_1\) (CB\(_1\)) and cannabinoid\(_2\) (CB\(_2\)) (Di Marzo, 2008). The EC system also regulates selective and sustained attention in humans and animals (Arguello and Jentsch, 2004; Solowij et al., 1995). In rodents, cannabinoid receptor agonists WIN55212-2 (Arguello and Jentsch, 2004; Pattij et al., 2007), AEA (Panlilio et al., 2009) and \( \Delta^9 \)-THC (Verrico et al., 2004) impaired attentional performance in operant reaction-time tasks, while the CB\(_1\) receptor antagonist/inverse agonist rimonabant enhanced attentional
responding (Pattij et al., 2007). Conditional CB₁ mutant mice also showed reduced attention towards an unfamiliar object (Lafenetre et al., 2009).

The hippocampus is activated by noxious stimuli in humans and rodents (Apkarian et al., 2005; Shih et al., 2008) and plays a role in cognitive modulation of pain; specifically, it is activated during exacerbation of pain by expectation (Ploghaus et al., 2001). The ventral hippocampus (vHip) is anatomically connected to the amygdala and prefrontal cortex (Ishikawa and Nakamura, 2006; Pitkanen et al., 2000), regions critically involved in attention/distraction and descending control of pain. Amygdalar and prefrontal cortical neurons project to the periaqueductal grey (Floyd et al., 2000; Rizvi et al., 1991), forming an integral part of the descending inhibitory pain pathway. Components of the EC system are highly expressed in the hippocampus (Felder et al., 1996; Freund et al., 2003). A recent study from our laboratory demonstrated that the vHip EC system plays an important role in expression of fear-conditioned analgesia in rats (Ford et al., 2011). We hypothesised that it may, therefore, be relevant to distraction-induced analgesia also.

We previously established and validated a rat model of distraction-induced analgesia whereby exposure to a non-aversive distractor suppressed formalin-evoked nociceptive behaviour (Ford et al., 2008). The present study aimed to investigate the effects of systemic administration of the CB₁ receptor antagonist/inverse agonist, rimonabant, on distraction-induced antinociception (DIA) in this rat model, and to determine whether DIA was associated with alterations in EC concentrations in the vHip.
2. Materials and Methods

2.1. Animals

Male Lister-hooded rats (260-300 g on the day of testing; Charles River, UK) were housed in groups of three in plastic-bottomed cages (45 cm x 25 cm x 20 cm) containing wood shavings as bedding. The animals were maintained at a constant temperature (21 ± 2° C) under standard lighting conditions (12 h:12 h light:dark cycle, lights on from 07:00 h to 19:00 h). Food and water were available ad libitum. The experimental protocol was carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee, National University of Ireland, Galway under license from the Irish Department of Health and Children and in compliance with the European Communities Council directive 86/609, and all efforts were made to minimise the number of animals used and their suffering.

2.2. Chemicals and Drug preparation

The CB1 receptor antagonist/inverse agonist rimonabant (SR141716A) [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-H-pyrazole-3-carboxamide, NIMH Chemical Synthesis Programme: Batch no. 10937-163-1] (1 mg/kg) was prepared fresh on the day of use, and dissolved in a vehicle of ethanol:cremaphor:saline (1:1:18) (Sigma, Ireland). The dose and time of administration was based on previous research demonstrating rimonabant-induced attenuation of fear-induced analgesia (Finn et al., 2004). The drug or vehicle was administered at a volume of 2 ml/kg via the intraperitoneal (i.p.) route. Formalin (Sigma-Aldrich, Ireland) was also prepared fresh on the day of use from 37% formaldehyde to give a final concentration of 2.5% in 0.9% saline, and 50µl was administered by intraplantar injection to the right hind paw. N-arachidonyl ethanolamide (AEA) and 2-arachidonylglycerol
(2AG) and their corresponding synthetic deuterated internal standards (N-arachidonyl ethanolamide-d8 (AEA-d8) and 2-arachidonylglycerol-d8 (2AG-d8)) were purchased from Cayman Europe (Tallinn, Estonia). Acetonitrile and formic acid were obtained from Lennox Laboratory Supplies (Dublin, Ireland). All solvents and chemicals were of HPLC grade or higher.

2.3. Apparatus

Habituation and testing took place in a specially constructed arena (30 cm x 30 cm x 40 cm) as described previously (Ford et al., 2008) and as shown in Figure 1. The arena floor was made of Perspex and the three remaining walls of each chamber were white, constructed from wood. The light intensity illuminating the arena was maintained at 200 lux. A bat detector (Batbox Duet, UK) was placed next to the arena to detect ultrasonic vocalization in the 22 kHz range, and a video camera was positioned under the arena. Live video images and audio signals were recorded onto DVD for subsequent analysis. Identical to that used previously (Ford et al., 2008), the novel object was an inverted plastic falcon tube filled with sand, attached to the base of the arena using Velcro®; the object could be explored freely but could not be displaced by the rat. To remove olfactory cues, the arena was cleaned and wiped down with 0.5% acetic acid solution pre- and post-habituation and testing of each rat; the novel object was also wiped down with acetic acid solution between trials.

2.4. Experimental Procedure

Subjects were randomly assigned to one of four groups (n = 9-11/group), and the sequence of habituation and testing was randomized throughout the experiment in order to minimize any confounding effects associated with the order of testing. Experiments were conducted between
08:30 h and 15:00 h. Subjects in all treatment groups were habituated to the arena for 10 minutes each day for seven days prior to experimentation. On the day of testing, all rats received an intraplantar injection of 50 µl formalin (2.5% in 0.9% saline) into the right hind paw under brief isoflurane anaesthesia, immediately followed by an i.p injection of rimonabant (1 mg/kg) or vehicle in an injection volume of 2 ml/kg. Rats were returned to their home cage for 30 minutes and were then placed back into the same arena to which they had been habituated for the previous days. The arena contained either no distracting stimulus (control) or the novel object (as described above) placed in the centre of the arena. Behaviour was then scored for a 30-minute period, 30-60 minutes post-formalin and post-i.p. drug injection, since our previous work has shown that this period of the second phase formalin response is subject to attentional modulation in this DIA paradigm (Ford et al., 2008). Immediately after the trial, rats were killed by decapitation. Brains were removed quickly, snap-frozen on dry ice, and stored at -80°C. The experimental groups were: no-object + formalin + vehicle (No Object-Vehicle; \( n = 10 \)), no-object + formalin + rimonabant (No Object-Rimonabant; \( n = 9 \)), object + formalin + vehicle (Object-Vehicle; \( n = 11 \)) and object + formalin + rimonabant (Object-Rimonabant; \( n = 10 \)).

2.5. Behavioural analysis

Behaviour during the 30-minute trial was analysed with the aid of Ethovision® behavioural tracking software (Noldus, Wageningen, The Netherlands). Formalin-evoked nociceptive behaviour was scored as time spent licking, biting or flinching the injected paw. Locomotor activity was assessed by tracking the distance moved by the rat. Directed attention towards the novel object and general, non-object directed behaviour (a composite duration of sniffing + rearing + grooming + walking) were also scored over the experimental period. Directed attention was defined and scored as the duration of time during which the rat’s head was directed towards the object and within a 2 cm annulus surrounding it, touching the object with
the nose or forepaws, or rearing against the object. Formalin-induced oedema was assessed as the difference between the post-mortem diameter of the right hind paw and the diameter before formalin administration measured using Vernier callipers. The number of faecal pellets was also recorded at the end of the 30-minute trial period.

2.6. Measurement of endocannabinoids from Palkovits punched tissue using liquid chromatography-tandem mass spectrometry (LC-MS-MS)

To determine the effect of exposure to the novel object distractor on endocannabinoid concentrations, frozen brain sections were cut (300 µm thickness) on a cryostat from Object-Vehicle and No Object-Vehicle group rats; the vHip was isolated from frozen sections using a cylindrical brain punch (Harvard Apparatus, internal diameter 2.0mm) and stored at -80°C prior to extraction for LC-MS-MS as described previously (Ford et al., 2011; Olango et al., 2012; Rea et al., 2013). The length of tissue punched was approximately 1.6 mm (start: bregma -4.3 mm) (Paxinos and Watson, 1998) and the average weight of the punches was 9.2 mg. During extraction, punched tissue was first homogenised in 100% acetonitrile containing deuterated internal standards added in fixed amounts to all samples (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8). Homogenates were centrifuged at 14,000 g for 15 minutes at 4°C and the supernatant was collected and evaporated to dryness in a centrifugal evaporator. Lyophilised samples were resuspended in 40 µl 65% acetonitrile, and 2µl was injected onto a Zorbax® C18 column (150 mm × 0.5 mm internal diameter) from a cooled autosampler maintained at 4°C. Mobile phases consisted of A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid), with a flow rate of 12 µl/min. Reversed-phase gradient elution began initially at 65% B and over 10 minutes was ramped up linearly to 100% B. At 10 minutes, the gradient was held at 100% B up to 20 minutes. At 20.1 minutes, the gradient returned to initial conditions for a further 10 minutes to re-equilibrate the column. The total run time was 30 minutes. Under these
conditions, AEA and 2-AG eluted at retention times of 9.1 minutes and 9.8 minutes respectively. Analyte detection was carried out in electrospray-positive ionisation mode on an Agilent 1100 HPLC system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Instrument conditions, in particular source parameters such as fragmentor voltage and collision energy, were optimised for each analyte by infusing standards separately. Quantitation of target endocannabinoids was achieved by positive-ion electrospray ionization and multiple-reaction monitoring (MRM) mode, allowing simultaneous detection of the protonated precursor and product molecular ions [M + H+] of the analytes of interest and the deuterated form of the internal standard. Precursor and product ion mass-to-charge (m/z) ratios for all analytes and their corresponding deuterated forms were as follows: AEA (m/z = 348.3-62.1); AEA-d8 (m/z = 356.3-63.1); 2-AG (m/z = 379.3-287.2); 2-AG-d8 (m/z = 387.3-294.2). Quantitation of each analyte was performed by determining the peak area response of each target analyte against its corresponding deuterated internal standard. This ratiometric analysis was performed using Masshunter Quantitative Analysis Software (Agilent Technologies Ltd, Cork, Ireland). The amount of analyte in unknown samples was calculated from the analyte/internal standard peak area response ratio using a 10-point calibration curve constructed from a range of concentrations of the non-deuterated form of each analyte and a fixed amount of deuterated internal standard. The values obtained from the Masshunter Quantitative Analysis Software are initially expressed in ng per mg of tissue by dividing by the weight of the punched tissue. To express values as nmol or pmols per mg of tissue, the corresponding values are then divided by the molar mass of each analyte expressed as ng/nmole or pg/pmole. Linearity (regression analysis determined R² values of 0.99 or greater for each analyte) was determined over a range of 18.75 ng to 71.5 fg for AEA and 187.5 ng – 715 fg for 2-AG. The limit of quantification was 1.32 pmol/g for AEA and 12.1 pmol/g for 2-AG.
2.7. Statistical analysis

All data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene tests, respectively. Behavioural data were analysed using either repeated-measures or two-way analyses of variance (ANOVA), with time as the within-subjects factor, and distractor (no object or object) and drug treatment (vehicle or rimonabant) as the between-subjects factors. Fisher’s least-significant-difference (LSD) post hoc tests were used to make pairwise comparisons where appropriate. All neurochemical data were analysed by Student’s unpaired two-tailed t-tests. Data were considered significant when $p<0.05$. Results are expressed as group means ± standard error of the mean (SEM). A Pearson correlation analysis was used to investigate the relationship between the duration of attention and the duration of nociceptive behaviour. Data were analysed using SPSS software for Windows and results were depicted graphically, where appropriate, with the aid of GraphPad Prism ® software.

3. Results

3.1 Effects of rimonabant on DIA
Intraplantar injection of formalin produced robust licking, biting and flinching of the injected paw. There was a significant time x drug interaction ($F_{(2.72)} = 3.32, p<0.05$) and the drug x object interaction approached the level of statistical significance ($F_{(1,36)} = 3.86, p=0.057$). As there was no effect of time, data at each time point were analysed by a one-way ANOVA followed by Fisher’s LSD post hoc tests. Rats exposed to the novel object distractor displayed significantly less formalin-evoked nociceptive behaviour in the first 10 minutes of the trial compared with rats not exposed to the object (Figure 2, Object-Vehicle vs No Object-Vehicle; *$p<0.05$), confirming the expression of DIA. A similar trend was observed throughout the trial, though this did not reach the level of statistical significance for the second and third time bins. The antinociceptive effect of novel object exposure in the first 10 minutes was blocked by the $CB_1$ receptor antagonist/inverse agonist rimonabant (Figure 2, Object-Vehicle vs Object-Rimonabant; #$p<0.05$). Rimonabant had no significant effect on formalin-evoked nociceptive behaviour in rats not exposed to the novel object (Figure 2, No Object-Vehicle vs No Object-Rimonabant $p>0.05$), though rimonabant was associated with a non-significant trend for a reduction in nociceptive behaviour in the third time bin ($p=0.06$).

3.2 Effects of rimonabant on attention directed at the novel object in the presence of formalin-evoked nociceptive tone

A significant negative correlation between duration of directed attention and formalin-evoked nociceptive behaviour was observed in formalin-treated rats exposed to novel object distractor (vehicle- and rimonabant-treated) during the first 10 minutes of the trial ($r^2 = 0.43$, **$p<0.01$; Figure 3A). However, there was no significant effect of rimonabant administration on attention directed towards the novel object in the first 10 minutes of the trial (Figure 3B).
3.3 Effects of rimonabant and novel object distractor on exploratory and fear-related behaviours

Non-object directed behaviour and locomotor activity were not significantly different between groups in the first 10 minutes of the trial (Figure 4A, 4B). Formalin injection resulted in an increase in paw diameter which was similar in all treatment groups (Figure 4C). There was a significant main effect of drug ($F_{(1,36)}=5.66$, $p<0.05$) on the number of faecal pellets produced over the 30 min trial, and the object x drug interaction was close to the level of statistical significance ($F_{(1,36)}=3.57$, $p=0.067$). Post hoc tests showed that the number of faecal pellets produced over the 30-minute trial was unaltered by drug treatment in rats not exposed to the distractor. However, in rats exposed to object, the number of defecations was reduced compared with animals not exposed to the object (No Object-Vehicle vs Object-Vehicle; *$p<0.05$), an effect that was attenuated by rimonabant administration (Object-Rimonabant vs. Object-Vehicle **$p<0.01$). No specific fear-related behaviours (freezing or 22 kHz ultrasonic vocalisations) were detected in rats from any of the experimental groups (data not shown), suggesting that rats did not express neophobic behaviours in the arena or during exposure to the novel object.

3.4 Effect of novel object exposure on EC levels in the vHip

Exposure of vehicle-treated rats to the novel object was associated with significant elevations in tissue levels of AEA (No Object-Vehicle vs. Object-Vehicle; $t_{(1,18)} = 4.01$; **$p<0.001$
Figure 5A) and 2-AG (No Object-Vehicle vs. Object-Vehicle; \( t_{(1, 19)} = 3.02 \); **\( p<0.01 \) Figure 5B) in the vHip. Thus, the expression of DIA was accompanied by elevations in EC levels in the vHip.

4. Discussion

Exposure to a novel object significantly reduced formalin-evoked nociceptive behaviour in rats in agreement with our previous findings (Ford et al., 2008). This expression of DIA was associated with increased AEA and 2-AG concentrations in the vHip. The CB\(_1\) receptor antagonist/inverse agonist rimonabant prevented DIA, suggesting that this is a CB\(_1\) receptor-mediated phenomenon. These data constitute the first evidence from an animal model for a neurochemical- and receptor-mediated basis for DIA, and suggest that the EC system may be an important neural substrate subserving attentional modulation of pain.

The results of the present study corroborate those of our earlier study demonstrating that exposure to a neutral, non-aversive novel object results in a robust reduction in formalin-evoked nociceptive behaviour (Ford et al., 2008). Consistent with the original description of the model (Ford et al., 2008), overt aversive behaviours (such as avoidance of the unfamiliar object, or fear-related behaviours) were not observed, suggesting that the reduction in nociceptive behaviour in these animals is not related to aversion (Ford et al., 2008). Indeed, the reduction in defecation in animals exposed to the novel object may be indicative of reduced emotionality in these rats, an effect that was attenuated by rimonabant. One minor difference between the studies is that Ford et al. (2008) observed significant effects on both formalin-evoked elevation and licking/biting/flinching of the injected hind paw (expressed as a
composite pain score) while in the present study we report effects on the latter pain-related behaviour only. Minor methodological differences between the two studies, such as the inclusion of i.p. injection in the present study, may account for this discrepancy. Overall, however, the DIA model originally described by Ford et al. (Ford et al., 2008) appears to be robust and reproducible.

Our results demonstrate that DIA was completely reversed by the CB₁ receptor antagonist/inverse agonist rimonabant during the first 10 minutes of the trial. We chose rimonabant because this compound, at the same dose as that used in the present study, has been shown to attenuate suppression of pain by an aversive stimulus (Finn et al., 2004). Taken together, these findings suggest that the endocannabinoid system mediates antinociception induced by non-aversive stimuli in addition to its well-described role in fear/stress-induced analgesia. The attenuation of DIA during the first 10 minutes of the trial only, reflects the fact that this was the period when a statistically significant object-induced reduction in formalin-evoked nociceptive behaviour was observed. Rimonabant had no effect on formalin-evoked nociception in rats not exposed to the novel object, indicating a specific effect on DIA rather than a non-specific effect on nociceptive behaviour per se. This finding suggests that the CB₁ receptor may not be directly involved in the tonic regulation of formalin-induced pain, which is supported by some previous studies (Beaulieu et al., 2000; Costa et al., 2005; Finn et al., 2004) but is in contrast with others (Calignano et al., 1998; Strangman et al., 1998). The discrepancies in the literature most likely relate to methodological differences in species or strain, dose, and time or route of drug administration. However, the study by Finn et al. (Finn et al., 2004) was the most similar in design to the present experiment, and also reported no effects of the same dose of rimonabant on formalin-evoked nociceptive behaviour in Lister-hooded rats. In the present study, a different dose or route of administration of rimonabant may
have had an effect on formalin-evoked nociceptive behaviour in rats not exposed to the object. However, such a result would then complicate and potentially confound interpretation of the effects of rimonabant in rats exposed to the novel object. For this reason, the dose of rimonabant administered in the present study was chosen to selectively block CB₁ receptors without having overt effects on formalin-evoked nociceptive behaviour per se. Neither rimonabant nor exposure to the novel object had any effect on non-object directed behaviour or locomotor activity in the present study, suggesting that the effects of object exposure on formalin-evoked nociceptive behaviour, and the effects of rimonabant thereon, are not due to overt, non-specific effects on behaviour, but instead likely represent specific effects on nociceptive processing.

Attention directed at the novel object was negatively correlated with the duration of this object-directed attention and the duration of nociceptive behaviour. Thus, rats exhibiting more attention directed at the novel object expressed less formalin-evoked nociceptive behaviour. These data support human studies demonstrating that cognitive distraction tasks reduce the perceived intensity and unpleasantness of experimental pain (Bantick et al., 2002; Bushnell et al., 2013; Valet et al., 2004; Villemure and Bushnell, 2002). Intriguingly, rimonabant did not have any significant effect on attention directed at the novel object, despite its effects in blocking DIA. Directed attention was defined as the duration of time during which the rat’s head was directed towards the object and within a 2 cm annulus surrounding it, touching the object with the nose or forepaws, or rearing against the object. Thus, it appears that rimonabant blocks DIA via a mechanism which is independent of any direct effects on (a) these measures of directed attention or (b) general locomotor activity. It is likely, therefore, that rimonabant blocks DIA not through an action in brain sites regulating the motor components of attention.
but rather at sites which subserve non-motor aspects of attention and/or modulation of pain-related behaviour.

Given the role of the vHip in endocannabinoid-mediated fear-induced analgesia (Ford et al., 2011) and its role in differential modulation of fear responding by endocannabinoids in the presence of persistent pain state (Rea et al., 2014), we investigated whether DIA was associated with alterations in levels of endocannabinoids in this region. Our results indicate that object exposure was associated with marked increases in the levels of AEA and 2-AG in the vHip. These elevations in levels of AEA and 2-AG in the vHip, coupled with the rimonabant-induced attenuation of DIA, support the hypothesis that endocannabinoids may act at CB1 receptors in the vHip to mediate DIA and provide a solid framework upon which to design future studies to test this hypothesis. Studies have demonstrated robust projections from the ventral hippocampus to the ventral medial prefrontal cortex (PFC), including the medial orbital area, the infralimbic area and the prelimbic areas, which subserve higher order functions including selective attention (Vertes 2006). Furthermore, the vHip input to the PFC converges with dopaminergic and serotonergic signalling pathways (Azmitia and Segal 1978; Gasbarri et al., 1994), which are known to exert a powerful modulatory influence over attentional behaviour (Robbins 2000). Lesions to the vHip have been shown to reduce response accuracy in the attention-demanding 5-choice serial reaction time task (Abela et al., 2013). A direct connection has also been demonstrated between the ventral CA1 region of the hippocampus and the amygdala (van Groen and Wyss 1990). The central nucleus of the amygdala is involved in control of attentional aspects of stimulus processing, and lesions to this region are associated with impaired visuospatial attention in a continuous-performance task (Holland et al., 2000). It is also possible that the vHip, being part of the cortico-limbic system, is capable of modulating the activity of brain regions classically associated with the descending inhibitory
pain pathway, including the PFC, amygdala, periaqueductal grey and rostroventromedial medulla, which mediate top-down endogenous modulation of pain. This hypothesis is supported by the presence of reciprocal connections between the vHip and the PFC and amygdala (Ishikawa and Nakamura, 2006; Pikkarainen et al., 1999; Pitkanen et al., 2000; Thierry et al., 2000). In human brain imaging studies, activity in the PFC (including the anterior cingulate cortex) is increased during DIA (Bantick et al., 2002; Petrovic et al., 2000; Valet et al., 2004). Moreover, vHip neurons contain cannabinoid, opioid and GABAergic receptors, and intrahippocampal microinjection of morphine produces antinociception mediated by the descending inhibitory pain pathway (Blasco-Ibanez et al., 1998; Favaroni Mendes and Menescal-de-Oliveira, 2008). Therefore, distraction-related alterations in EC signalling within the vHip could modulate nociceptive transmission through afferent projections to regions comprising the descending inhibitory pain pathway. Further studies are warranted to test this theory.

In conclusion, the present study has confirmed that exposure of rats to a novel object transiently reduces second-phase formalin-evoked nociceptive behaviour and represents a useful model of distraction-induced analgesia. Furthermore, we have demonstrated, for the first time, involvement of the EC system in DIA. The behavioural expression of DIA was attenuated by the CB1 antagonist/inverse agonist rimonabant and was accompanied by increases in the levels of AEA and 2-AG in the vHip. The results offer the first insight into the neurochemical and receptor mechanisms that mediate DIA and provide a framework for the design of future studies aimed at further elucidation of the molecular mechanisms and circuitry that subserve attentional modulation of pain.

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**Author Contributions**

GKF, OM, BNO, ET, AM, BH and DPF contributed substantially to study conception and design, and/or to acquisition, analysis and interpretation of data. GKF, OM, and DPF drafted the article or revised it critically for important intellectual content. DPF is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflict of interest to declare.

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Figure Legends

**Figure 1:** Distraction-induced analgesia model apparatus setup. Testing was carried out in a specially constructed arena of dimensions 30cm x 30cm x 40cm. The arena floor was made of Perspex and the walls were white and constructed from wood. The novel object was an inverted plastic falcon tube filled with sand, attached to the base of the arena. The light intensity at floor-level was 200 lux, and a video camera was positioned under the arena to record behaviour to DVD.

**Figure 2:** Time course of the effects of object exposure and rimonabant on formalin-evoked nociceptive behaviour. Nociceptive behaviour was significantly reduced in rats exposed to the novel object in the first 10 min of the trial (*p*<0.05 Object-Vehicle vs. No Object-Vehicle), indicating expression of distraction-induced antinociception. The effect was attenuated by administration of rimonabant (#*p*<0.05 Object-Rimonabant vs. Object-Vehicle). In the last 10 min of the trial, there was a trend for rimonabant to reduce nociceptive behaviour in rats not exposed to the novel object, but this failed to reach statistical significance (*p*=0.06). Data are expressed as mean ± SEM, *n*=9-11/group.

**Figure 3:** Directed attention in object-exposed rats for 0-10 min. (A) Pearson’s correlation analysis revealed a significant negative correlation between the duration of directed attention towards the novel object and the duration of formalin-evoked nociceptive behaviour in vehicle- and rimonabant-treated rats in the first 10 min of the trial (*r*² = 0.43, **p**<0.01, *n*= 21). (B) Rimonabant did not significantly alter the duration of attention in the first 10 min of the trial. Data are expressed as mean ± SEM, *n*= 10-11.
**Figure 4:** Effect of object exposure and rimonabant on general, non-object directed behaviour and other physiological parameters (hind-paw oedema and faecal pellets). Data are mean ± SEM, \(n=9-11\). (A) Duration of composite non-object directed behaviour (grooming + sniffing + rearing + walking) during the first 10 min of the trial. (B) Total distance moved (cm) during the first 10 min of the trial. (C) Change in paw diameter calculated at the end of the trial (post-formalin minus pre-formalin diameter). (D) Number of faecal pellets recorded at the end of the trial. The number of pellets was significantly lower in the vehicle-treated rats exposed to the novel object than in vehicle-treated rats not exposed to the object (*\(p<0.05\) Object-Vehicle vs. No Object-Vehicle). This reduction was prevented by administration of rimonabant in the object-exposed rats (##\(p<0.01\) Object-Rimonabant vs. Object-Vehicle).

**Figure 5:** The effect of novel object exposure, in the presence of nociceptive tone, on EC measurements in the ventral hippocampus (vHip). (A) AEA (B) 2-AG. Object exposure in vehicle-treated groups increased levels of AEA (***\(p=0.001\) and 2-AG (**\(p<0.01\) in the vHip. Data are presented as mean ± SEM, \(n=9-11\) group.
A

Graph showing the relationship between duration of nociception (s) and duration of attention (s). The coefficient of determination ($r^2$) is 0.43, and the p-value is $<0.01^*$.

B

Bar graph comparing the duration of directed attention (s) between Object-Vehicle and Object-Rimonabant groups. The Object-Vehicle group has a higher duration of attention compared to the Object-Rimonabant group.
**Figure A**

*Y-axis: Total Behaviour (s)*

- No Object: Vehicle (white bar), Rimonabant (black bar)
- Object: Vehicle (white bar), Rimonabant (black bar)

**Figure B**

*Y-axis: Distance Moved (cm)*

- No Object: Vehicle (white bar), Rimonabant (black bar)
- Object: Vehicle (white bar), Rimonabant (black bar)

**Figure C**

*Y-axis: Change in Paw Diameter (mm)*

- No Object: Vehicle (white bar), Rimonabant (black bar)
- Object: Vehicle (white bar), Rimonabant (black bar)

**Figure D**

*Y-axis: No. of Pellets*

- No Object: Vehicle (white bar), Rimonabant (black bar)
- Object: Vehicle (white bar), Rimonabant (black bar)

*Significance markers:*
- *: p < 0.05
- **: p < 0.01