Pain 139 (2008) 225–236

PAIN

www.elsevier.com/locate/pain

The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain

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Received 25 September 2007; received in revised form 2 April 2008; accepted 7 April 2008

Abstract

It has been postulated that the G protein-coupled receptor, GPR55, is a third cannabinoid receptor. Given that the ligands at the CB_1 and CB_2 receptors are effective analgesic and anti-inflammatory agents, the role of GPR55 in hyperalgesia associated with inflammatory and neuropathic pain has been investigated. As there are no well-validated GPR55 tool compounds, a GPR55 knockout ($GPR55^{-/-}$) mouse line was generated and fully backcrossed onto the C57BL/6 strain. General phenotypic analysis of $GPR55^{-/-}$ mice revealed no obvious primary differences, compared with wild-type ($GPR55^{+/+}$) littermates. $GPR55^{-/-}$ mice were then tested in the models of adjuvant-induced inflammation and partial nerve ligation. Following intraplantar administration of Freund's complete adjuvant (FCA), inflammatory mechanical hyperalgesia was completely absent in $GPR55^{-/-}$ mice up to 14 days post-injection. Cytokine profiling experiments showed that at 14 days post-FCA injection there were increased levels of IL-4, IL-10, IFNγ and GM-CSF in paws from the FCA-injected $GPR55^{-/-}$ mice when compared with the FCA-injected $GPR55^{+/+}$ mice. This suggests that GPR55 signalling can influence the regulation of certain cytokines and this may contribute to the lack of inflammatory mechanical hyperalgesia in the $GPR55^{-/-}$ mice. In the model of neuropathic hypersensitivity, $GPR55^{-/-}$ mice also failed to develop mechanical hyperalgesia up to 28 days post-ligation. These data clearly suggest that the manipulation of GPR55 may have therapeutic potential in the treatment of both inflammatory and neuropathic pain.

Keywords: GPR55; Cannabinoid; Pain; Inflammation; Hyperalgesia; Cytokine

1. Introduction

Cannabinoids have numerous physiological effects resulting from interactions with specific receptors [29]. Cannabinoids include the bioactive constituents of the

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doi:10.1016/j.pain.2008.04.006

marijuana plant, *Cannabis sativa*, along with endogenous lipids, or endocannabinoids and synthetic compounds with cannabinoid-like activity. Two G protein-coupled cannabinoid receptors have been identified by molecular cloning; CB_1 [23] and CB_2 [25]. Studies using either selective CB_1 and CB_2 compounds or mice deficient in CB_1 and/or CB_2 have suggested that there are additional cannabinoid receptors present in neurones, the vasculature and immune cells, the so-called non-CB_1/CB_2 receptors [3,22].

The GPR55 receptor has recently been suggested to be an additional member of the cannabinoid receptor family [2] given patent literature indicating that several cannabinoid receptor ligands also bind to GPR55 [7,47]. Whilst GPR55 has low identity to CB_1 (13.5%) and CB2 (14.4%), lacks a CB_1 and CB_2 functional fingerprint [32] and is also activated by the potential endogenous ligand, lysophosphatidylinositol [28], three recent peer-reviewed publications [16,20,37] have described the actions of a range of cannabinoid compounds at the GPR55 receptor. Whilst there are currently discrepancies in the data, particularly in the pharmacology between GPR55 expressed recombinantly in yeast or mammalian hosts (described in [4]), such data are supportive of GPR55 being an additional cannabinoid receptor which selectively couples to $G_{\alpha 13}$ [2,37].

GPR55 has a concordant localisation to CB_1 and CB_2. CB_1 receptors are highly expressed in the brain and also to a lesser extent in several peripheral tissues (heart, reproductive and vascular tissues, immune cells, [30]). CB_2 receptors are expressed mainly by immune and haematopoietic cells [30] and recently they have also been observed in the CNS [26,42]. GPR55 mRNA has been shown in the CNS and peripheral tissues including immune tissues such as tonsil and spleen [38,47]. Given the therapeutic potential of CB_1 and CB_2 agonists as analgesic and anti-inflammatory agents [9], it would seem logical to postulate that the modulation of the GPR55 receptor may also produce analgesic activity. The aim of this study was to investigate the role of GPR55 in pain. Compounds with selective activity at some of the non-CB_1/CB_2 receptors are known (e.g. abnormal-cannabidiol, an agonist at the cardiovascular non-CB_1/CB_2 receptor [27]). However, the pharmacology of non-CB_1/CB_2 receptors is far from clear. In the absence of such tool compounds and in order to investigate the potential role of GPR55 in analgesia, a knockout mouse line was generated. This knockout mouse line has previously been used to investigate whether the cardiovascular non-CB_1/CB_2 receptor is GPR55 [16] and results from this study have suggested that it is not. In the present study, the GPR55 knockout mouse line has been tested in models of inflammatory (FCA-induced mechanical hyperalgesia) and neuropathic (partial ligation of the sciatic nerve) pain. In addition, given that CB_2 has a role in regulating immune function through cytokine release [31], these studies were extended to include cytokine profiling experiments to investigate whether GPR55 signalling is also involved in the regulation of immune function.

2. Methods

2.1. Generation and genotyping of $GPR55^{-/-}$ mice

Gene targeting was performed in E14.1 ES cells (129P2/OlaHsd). Two targeted clones were injected into C57BL/6-derived blastocysts. Male chimaeras were crossed with C57BL/6 females to produce N1F0 offspring. The animals carrying the GPR55 mutant allele from one injected clone were repeatedly bred, or backcrossed, onto the C57BL/6 genetic background for more than five generations. At each generation the C57BL/6 genetic composition was assessed using a panel of single nucleotide polymorphism (SNP) markers [24,45]. When heterozygous animals displayed greater than a 98% coverage with the C57BL/6 marker set, they were intermated to produce F1 animals homozygous for the GPR55 mutation ($GPR55^{-/-}$) and wild-type littermate controls ($GPR55^{+/+}$). PCR genotyping was performed using primers designed to amplify PCR products specific to either the GPR55 wild-type locus (GPR55: DW1: 5′-TCTTC CCCCTGGAGATCTTT-3′; DW2: 5′-CTGGGAGAAAG GAGACCACA-3′; 30 cycles of 94 °C (45 s), 58 °C (45 s), and 72 °C (45 s) generates an amplicon of 207 bp) or the targeted GPR55 locus (N-5′, neomycin gene specific 5′ primer: 5′-CCGGCCGCTTGGGTGGAGAGG-3′ and N-3′, neomycin gene specific 3′ primer: 5′-TCGGCAGGAGC AAGGTGA GATGACA-3′; 30 cycles of 94 °C (30 s), 68 °C (30 s), and 72 °C (30 s) generates an amplicon of 299 bp). All experiments were conducted according to the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and conformed to GlaxoSmithKline ethical standards.

2.2. Profiling GPR55, CB_1 and CB_2 gene expression

Brain tissues were taken from $GPR55^{-/-}$ and $GPR55^{+/+}$ mice ($n = 5$ per group) for the analysis of GPR55 and CB_1 gene expression. Spleen tissues from another cohort of $GPR55^{-/-}$ and $GPR55^{+/+}$ mice ($n = 4$ $GPR55^{-/-}$, $n = 6$ $GPR55^{+/+}$) were used for the analysis of CB_2 gene expression. All tissues were stored at −80 °C prior to extraction. RNA was isolated from brain tissues using RNeasy Lipid Tissue mini kit (Qiagen, Crawley, UK). RNA was isolated from spleen tissues by homogenising the tissue in Trizol and then using RNeasy maxi kit (Qiagen, Crawley, UK). Reverse transcription and quantitative PCR were performed using methods previously described [21]. The primer and probe sequences were designed using Primer3 software and checked for specificity using FASTA searches. Primers and probes were synthesised by Sigma-Proligo. The following primer and probe sequences were used:

Cyclophilin	
Forward	5′-TGTGCCAGGGTGGTGACTT
Reverse	5′-TCAAATTTCTCTCCGTAGATGGACCT
Probe	5′-ACACGCCATAATGGCACTGGCGG
GPR55	
Forward	5′-GATGAAGCAGATGGTGAAGACACTC
Reverse	5′-CCTGCCACAAGTGGAGTCCC
Probe	5′-AGGCACTCCACCAGAGTGCAGAAGGAC
CB1	
Forward	5′-GATGGCAAGGTGCAGGTGAC
Reverse	5′-ACCATGATCGCAAGCAGAGG
CB2	
Forward	5′-TGGGTATGTCCTCTGGAAAGC
Reverse	5′-TGAGAGCCAGTGCAGGGAAC
Probe	5′-GGACAGGCAGGTGCCTGGGATAGCTCG

2.3. Blood cell phenotyping in $GPR55^{-/-}$ and $GPR55^{+/+}$ mice

Blood from another cohort of male $GPR55^{-/-}$ and $GPR55^{+/+}$ mice ($n = 9/10$ per group) was collected into K^+EDTA tubes and incubated with APC-CD3, PE-CD45RA, FITC-CD4 and PerCP-CD8a (BD Biosciences; 1:100 dilution) for 30 min prior to fixing with FACSlyse (BD Biosciences, Cowley, Oxford, UK) and flow cytometric analysis. FL1 (PE-CD45RA fluorescence), FSC, SSC, FL2 (FITC-CD4 fluorescence), FL3 (PerCP-CD8a fluorescence) and FL4 (APC-CD3 fluorescence) parameters were collected for each sample and the total number of monocytes, neutrophils and T cells counted per 30 s determined.

2.4. Preliminary behavioural phenotypic analysis

A cohort of 10 male/female $GPR55^{-/-}$ and $GPR55^{+/+}$ mice were evaluated in a primary observational panel once the mice had been weaned. These tests included general observations (body weight, behaviour in cage, general appearance, posture/gait, age of eye opening, age of tooth eruption) followed by a modified SHIRPA protocol of primary observations [11] which included the observations of body position, transfer arousal, trunk curl, skin colour, palpebral closure, visual placing, contact righting reflex, aggression, pelvic elevation, vocalisation, tail elevation, bodyweight and touch escape, tremor, defecation, locomotor activity, pilorection, startle response, gait, positional passivity, limb grasping, other abnormal behaviour, grip strength, pinna reflex, corneal reflex, lacrimation, fear and irritability. In addition, cohorts of male and female $GPR55^{-/-}$ and $GPR55^{+/+}$ mice were tested in rotarod ($n = 10$ per group) and hot-plate tests ($n = 12$ per group) to determine if knocking out GPR55 caused any motor or thermal nociception deficits. For the rotarod test, Acceler.-ROTA-ROD for mice 7650 (Johnes & Roberts) apparatus was used and each mouse was tested on the rod which rotated between 4 and 40 rpm over the course of 5 min. For each animal three fall-off times were recorded and the best fall-off time for each animal was used to calculate mean ± SEM values for each group of mice. In the hot-plate test, mice were confined individually within a Perspex cylinder placed on a Harvard Analgesia Meter (Harvard Instruments) and were observed for signs of nociception, i.e. rapid fanning or licking of their paws, and the response latency recorded. Any mice failing to react within 40 s (60 s for 50 °C temperature) were removed from the test apparatus and assigned a latency of 40 s (60 s for 50 °C temperature). The same mice were tested on three separate occasions at 50 °C, 52.5 °C and 55 °C. Only one hot-plate temperature was tested per day and at least 24 h separated tests. Results were expressed as mean response latency for each group.

2.5. Pain phenotyping

Mice were housed in groups of 1–5 prior to use and maintained under a standard 12 h light:dark cycle with food and water available *ad libitum*. Four groups of animals were used throughout these studies; male and female homozygous $GPR55^{-/-}$ mice and male and female $GPR55^{+/+}$ mice. Estrous cycle was not noted in the female mice. All testing began when the mice were ≈10 weeks of age.

2.6. FCA-induced inflammatory hyperalgesia

Twelve male and female $GPR55^{-/-}$ and 12 male and female $GPR55^{+/+}$ littermates were employed for these studies. Mice were assessed for changes in mechanical hyperalgesia using the analgesymeter method [35]. This involved placing the paw under test between two platforms, the top most one consisting of a blunt pointed tip and the bottom one comprising a flat base plate. Once placed between these two platforms a linearly increasing mechanical force was applied to the paw. The end point of the test was reached when the mouse struggled to withdraw the paw. At this point the stimulation was stopped and the withdrawal threshold was recorded. Both ipsilateral and contralateral withdrawal thresholds were measured. Following the establishment of baseline latencies (day −1), all mice received 30 μl Freund's complete adjuvant (FCA, Sigma–Aldrich, Poole, UK) sub-plantar injected into the left hind paw. Male mice were re-tested for mechanical hyperalgesia on days 1, 5, 9 and 14 post-FCA. Female mice were re-tested 24 h post-FCA. Given that no sex differences were expected, female mice were sacrificed 1 day post-FCA and male mice at 14 days post-FCA to provide tissues for cytokine analysis representing both acute and chronic inflammatory time points.

2.7. Partial ligation of the sciatic nerve

A separate cohort of 15 male and 15 female $GPR55^{-/-}$ and 15 male and 15 female $GPR55^{+/+}$ littermates were used for this study with each sex being used in a separate study. All mice were tested for withdrawal responses to mechanical stimuli using the analgesymeter method [35] as described previously. Both ipsilateral and contralateral thresholds were measured. Following baseline testing, mice underwent surgery to partially ligate the sciatic nerve using a method based on that described by Seltzer et al. [39]. Mice were anaesthetised with isoflurane and ≈1 cm of their left sciatic nerve was exposed by blunt dissection through an incision at the level of the mid thigh. A suture (10/0 Virgin Silk:

Ethicon®) was then passed through the dorsal third of the nerve and tied tightly. The incision was sutured (7/0 Mersilk: Ethicon®) and the mice were allowed to recover for 3 days before testing commenced. On day 3 mice were tested for mechanical hyperalgesia in the manner previously described above. Testing was repeated at days 7, 10, 14, 17, 21 and 28 days post-ligation.

2.8. Cytokine analysis in $GPR55^{-/-}$ and $GPR55^{+/+}$ mice

Paw samples from both FCA studies ($n = 8$ $GPR55^{+/+}$ and $n = 9$ $GPR55^{-/-}$ mice) were homogenised in PBS containing 'complete' protease inhibitor cocktail (Boehringer Mannheim, Burgess Hill, UK) using a Mixer Mill MM300 (Qiagen, Crawley, UK). Homogenates were centrifuged and supernatants were harvested and analysed for cytokine content. A multi-cytokine bead array detection system capable of detecting mouse IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, Interferon γ (IFNγ), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and TNFα was used according to manufacturer's instructions (LINCO 10-plex, Upstate, Chandlers Ford, UK). Briefly, a monoclonal antibody directed against the desired analyte was covalently coupled to dyed 5.5 μm polystyrene beads (2.5×10^6 beads ml^{-1} $cytokine^{-1}$). The conjugated beads were exposed to 50 μl of sample or standard solutions containing a known amount of cytokine, in a 96-well filter plate and incubated overnight at 4 °C, protected from light. After a series of washes and vacuum filtration to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the analyte was added to the reaction. After incubation, the unbound antibody was removed; the reaction mixture was detected by the addition of streptavidin–phycoerythrin (streptavidin–PE), which binds to the biotinylated detection antibodies. Following a further series of washes and vacuum filtration, the beads were resuspended in 200 μl 5% BSA in PBS; the plate was stored at 4 °C in the dark until analysis. The reaction mixture was read using a Luminex Data Collector in a Luminex 100 flow cytometer (Luminex). Excel data files were generated containing the individual bead numbers and the associated median fluorescence intensities (MFI). Standard curves were plotted to calculate the relative amount of each cytokine in samples, using the aliquoted serial dilutions of a positive control solution for calibration. Unknown sample cytokine concentrations were calculated from the curve.

2.9. Data analysis

Taqman data were analysed using ANCOVA performed using Microsoft Excel with an add-in toolkit for the analysis of real-time PCR data (PRISM Training and Consultancy Ltd, Cambridge, UK). Cyclophilin expression in the brain and spleen was used as the covariate for normalisation. All other analysis was carried out using Statistica (Statsoft Inc., Tulsa, USA). Results for the hot-plate, FCA and partial nerve ligation studies were analysed using ANOVA. In all cases, pairwise comparisons, where appropriate, were made using Duncan's test. Results for the cytokine analysis were analysed using ANOVA followed by Fishers LSD test for post hoc comparisons.

3. Results

3.1. Generation of $GPR55^{-/-}$ mice and primary behavioural phenotypic analysis

To investigate the functional role of GPR55 a knockout mouse was generated (Fig. 1a). To generate the targeting vector 5′ and 3′ homology arms (4.1 kb and 3.6 kb, respectively) were isolated from a 129 SVj BAC library and inserted either side of the internal ribosyme entry site (IRES)-LacZ-polyA (pA) expression cassette and a positive selection cassette containing the neomycin phosphotransferase gene driven by the PGK promoter (Neo). Homologous recombination at the GPR55 genomic locus was confirmed by Southern blot analysis of NdeI digested genomic DNA using a 5′ external probe (black bar). This probe hybridised to a ~16 kb or an 11 kb fragment at the wild-type or targeted locus, respectively (Fig. 1b). Similarly, targeting at the 3′ end of the locus was confirmed by Southern blot analysis of NdeI digested genomic DNA using a 3′ external probe (blue bar) which hybridised to a ~16 kb or an 8 kb fragment at the wild-type or targeted locus, respectively (data not shown). The mutation retains the first 118 bp of the GPR55 open reading frame (ORF) encoding 39 amino acids, containing 20 of the 22 amino acids of the first transmembrane domain (Fig. 1c). In total, the deletion removes the sequences encoding 241 amino acids of the GPR55 ORF, from lysine 39 to lysine 281. The deleted amino acids encompass the second to sixth predicted transmembrane domains of this seven transmembrane domain receptor molecule. $GPR55^{-/-}$ mice were viable and born in normal Mendelian ratios. The $GPR55^{-/-}$ mice in both the general and the primary observational tests showed no significant differences to their $GPR55^{+/+}$ littermates (data not shown). This was also the case in the rotarod test (Fig. 2a). In the hot-plate test there were no differences in withdrawal latency between male $GPR55^{-/-}$ and $GPR55^{+/+}$ mice at any temperature (Fig. 2b). However, there was a significant reduction in withdrawal latency in female $GPR55^{-/-}$ mice at 50 °C when compared to $GPR55^{+/+}$ littermates (Fig. 2c).

3.2. GPR55, CB_1 and CB_2 gene expression

In order to confirm that the targeted deletion of GPR55 had been achieved, GPR55 gene expression levels were investigated using quantitative PCR. Given that the targeting strategy retains the first 118 bp of the GPR55 coding sequence, primers were designed to a region downstream of this N-terminal region. GPR55 mRNA was present in brain tissues from $GPR55^{+/+}$ mice and completely absent in brain tissues from $GPR55^{-/-}$ mice (Table 1). In order to determine if the targeted deletion of the GPR55 gene would cause

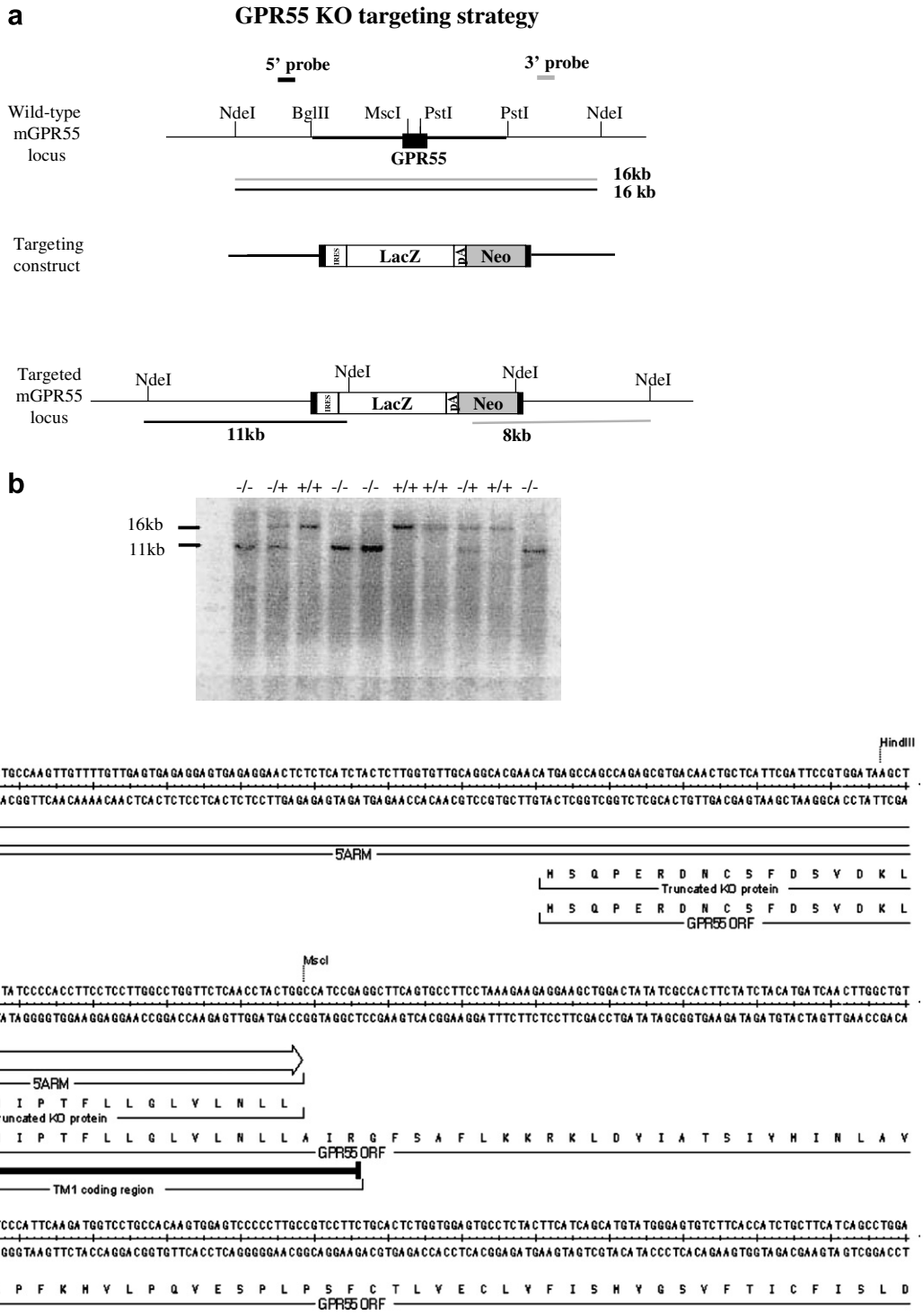

Fig. 1. Targeted deletion of the mouse GPR55 locus. (a) Targeting strategy showing the schematic representation of partial restriction maps of the wild-type GPR55 locus, targeting vector and targeted allele. The thickened lines indicate the 5′ and 3′ arms of DNA used to direct homologous recombination. The black and grey bars indicate the 5′ and 3′ external probes used to confirm the desired targeting event. Corresponding bars below the wild-type and mutant alleles indicate the predicted size of fragments hybridising to these probes after NdeI digestion. (b) Southern blot analysis of NdeI digested genomic DNA extracted from tail biopsies from F1 animals. Using the 5′ external probe GPR55$^{+/+}$ animals ($^{+/+}$) shows hybridisation to a 16 kb wild-type fragment whilst GPR55$^{-/-}$ animals ($^{-/-}$) show hybridisation to an 11 kb knockout fragment. Hybridisation to both fragments is seen in heterozygous ($^{+/-}$) animals. (c) Sequence detail of the GPR55 targeted mutation. The deletion begins at the Msc1 site located at the end of the 5′ arm of homology (arrowhead). As shown by the single letter amino acid code open reading frame (ORF) translation below the DNA sequence, the mutated GPR55 transcript encodes small 39 aa peptide containing 20/22 aa of the first transmembrane domain of the receptor (shown as a black bar).

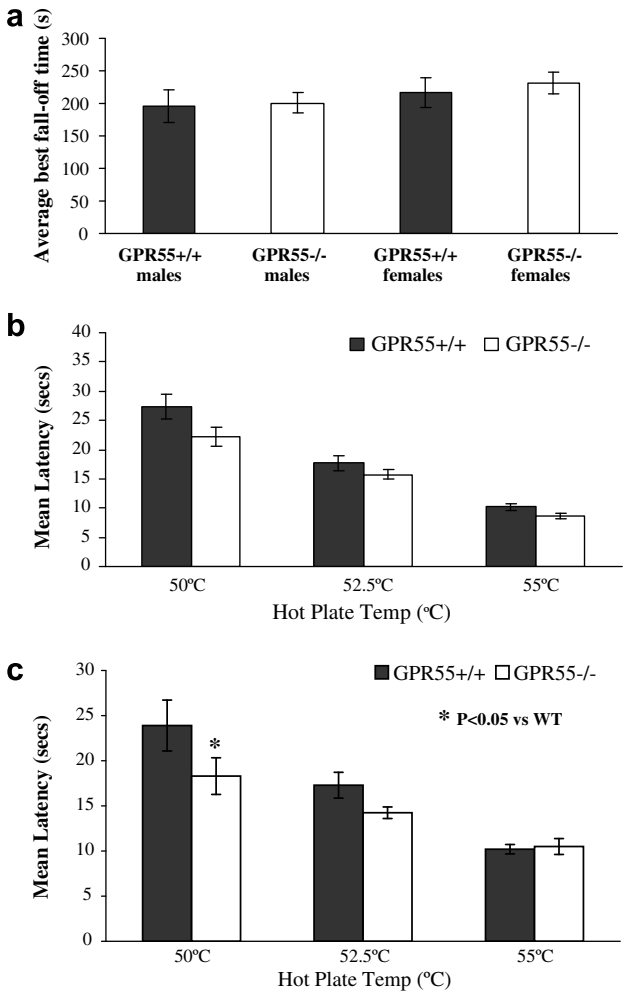

Fig. 2. Rotarod and hot-plate data for male and female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice. (a) Average rotarod best fall-off times (s) for groups of male and female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice were no different. (b) Mean hot-plate withdrawal latency (s) for male GPR55$^{-/-}$ and GPR55$^{+/+}$ mice ($n = 15$ per group) was no different at any temperature. (c) The mean hot-plate withdrawal latency (s) for female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice ($n = 15$ per group) was significantly reduced in GPR55$^{-/-}$ mice at 50 °C.

changes in the expression of other cannabinoid receptor genes, CB_1 and CB_2 gene expression levels were also investigated using quantitative PCR. There was no significant difference in CB_1 mRNA levels in brain tissue taken from GPR55$^{-/-}$ and GPR55$^{+/+}$ mice (Table 1).

Table 1
GPR55, CB_1 and CB_2 mRNA levels in brain or spleen samples from GPR55$^{+/+}$ and GPR55$^{-/-}$ mice

Gene	Tissue	GPR55$^{+/+}$	GPR55$^{-/-}$
GPR55	Brain (2 μg RNA/RT)	734 ± 37	0 ± 0
CB_1	Brain (2 μg RNA/RT)	11,270 ± 181	12,054 ± 926
CB_2	Spleen (1 μg RNA/RT)	1031 ± 79	903 ± 77

Data are expressed as mean copy numbers ± SEM. Brain RNA was isolated from $n = 5$ mice per group and spleen RNA was isolated from $n = 4$ GPR55$^{-/-}$ and $n = 6$ GPR55$^{+/+}$ mice.

Similarly, there were no significant differences in CB_2 mRNA levels in spleen tissues taken from GPR55$^{-/-}$ and GPR55$^{+/+}$ mice (Table 1).

3.3. Blood cell phenotyping

In order to determine whether there were any underlying differences in the blood cell populations within the GPR55$^{-/-}$ mice, FACS analysis was used to determine the relative proportions of monocytes, neutrophils, cytotoxic T cells and T helper cells counted in 30 s ± SEM in blood from groups of GPR55$^{-/-}$ and GPR55$^{+/+}$ mice. No significant differences were detected in the numbers of the different cell types counted between GPR55$^{-/-}$ and GPR55$^{+/+}$ mice (Table 2).

3.4. Inflammatory mechanical hyperalgesia

The established model of intraplantar FCA-induced inflammation was used to compare mechanical hyperalgesia in GPR55$^{+/+}$ and $^{-/-}$ mice. In this model there were no significant differences in baseline ipsilateral or contralateral withdrawal responses between male and female or GPR55$^{+/+}$ and $^{-/-}$ mice (Fig. 3). Following intraplantar FCA injection, the analysis of the ipsilateral withdrawal response revealed a significant effect of genotype (Fig. 3). At all post-FCA time points GPR55$^{+/+}$ mice developed a significant hyperalgesia ipsilaterally compared to baseline values (Fig. 3). Male GPR55$^{-/-}$ mice (Fig. 3a), however, failed to develop mechanical hyperalgesia up to 14 days post-FCA. Female GPR55$^{-/-}$mice (Fig. 3b) failed to develop mechanical hyperalgesia up to 1 day post-FCA. No significant differences were evident between GPR55$^{+/+}$ and $^{-/-}$ contralateral withdrawal thresholds at any time point (Fig. 3 inset panels).

3.5. Neuropathic hyperalgesia

The partial nerve ligation model [39] is an established pre-clinical model of neuropathic hyperalgesia. Prior to ligation, there were no significant differences in baseline ipsilateral or contralateral withdrawal responses between male and female or GPR55$^{+/+}$ and $^{-/-}$ mice

Table 2
Blood cell populations in GPR55$^{+/+}$ and GPR55$^{-/-}$ mice

Blood cell type	GPR55$^{+/+}$	GPR55$^{-/-}$
Monocytes	509 ± 69	502 ± 81
Neutrophils	960 ± 148	1226 ± 143
Cytotoxic T cells	551 ± 67	648 ± 39
T helper cells	700 ± 81	838 ± 57

Data are expressed as mean cell numbers ± SEM. Blood GPR55$^{-/-}$ mice was isolated from $n = 9$ mice and blood from GPR55$^{+/+}$ mice was isolated from $n = 10$ mice.

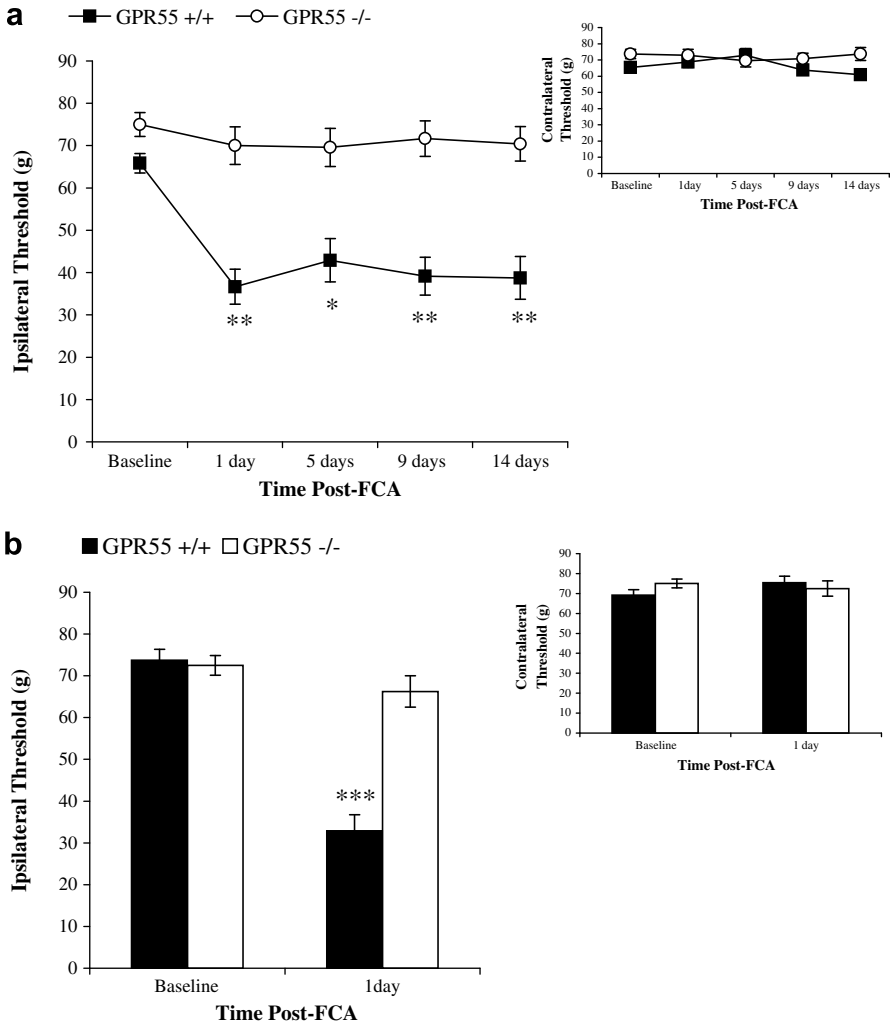

Fig. 3. Effect of FCA on paw pressure in male and female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice following intraplantar injection. (a) Male and (b) female GPR55$^{+/+}$ mice displayed mechanical hyperaglesia ipsilaterally post-FCA. This hyperalgesia persisted in the GPR55$^{+/+}$ mice for up to 14 days post-FCA in the male mice (a) and was present up to 1 day post-FCA in the female mice (b). No significant differences in contralateral withdrawal thresholds were observed at any time point in the GPR55$^{+/+}$ or $^{-/-}$ mice (inset panels). $^{*}P < 0.5$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs GPR55$^{-/-}$ thresholds.

(Fig. 4). Following partial nerve ligation a profound difference was evident in mechanical hyperalgesia between GPR55$^{-/-}$ and GPR55$^{+/+}$ mice (Fig. 4). At all post-ligation time points GPR55$^{+/+}$ mice showed a significant reduction in ipsilateral withdrawal thresholds when compared with baseline values. Male (Fig. 4a) and female (Fig. 4b) GPR55$^{-/-}$ mice, however, failed to develop mechanical hyperalgesia at any time point tested up to 28 days post-ligation. No significant differences were evident between GPR55$^{+/+}$ and $^{-/-}$ contralateral withdrawal thresholds at any time point (Fig. 4 inset panels).

3.6. Cytokine analysis

In order to investigate the mechanisms by which the deletion of the GPR55 gene might abolish FCA-induced mechanical hyperalgesia, local changes in cytokine levels were determined in paw tissue samples from GPR55$^{-/-}$ and GPR55$^{+/+}$ mice. Tissues were sampled at time points of 1 day post-FCA (for the female cohort, Table 3) or 14 days post-FCA (for the male cohort, Table 4). In the absence of FCA treatment, basal cytokine levels were not markedly different between GPR55$^{-/-}$ and GPR55$^{+/+}$ mice. The exception was IL-2, which was detected at 2-fold greater levels ($P < 0.05$) in male GPR55$^{-/-}$ paws compared to male GPR55$^{+/+}$ paws (Table 4), though notably this level was not greater than that in GPR55$^{+/+}$ female paws. As expected, at 1 day post-FCA, the majority of cytokines tested were significantly elevated compared to untreated paws (IL-1β, -4, -5, -6, -12, TNFα and GM-CSF; significance levels indicated in Table 3). No significant differences were observed in induced cytokine levels 1 day post-FCA between GPR55$^{-/-}$ and GPR55$^{+/+}$ paws. IL-10 levels appeared to be moderately elevated 1 day post-FCA in

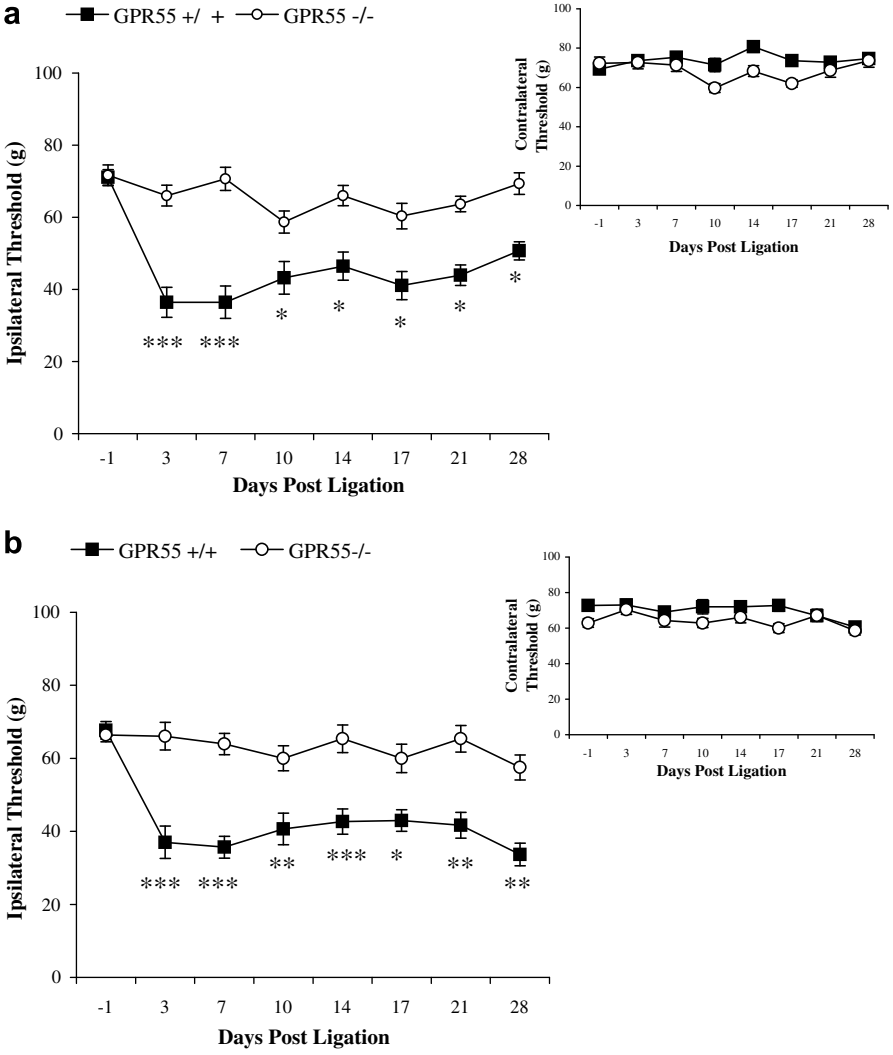

Fig. 4. Effect of partial nerve ligation on paw pressure in male and female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice. (a) Male and (b) female GPR55$^{+/+}$ mice (filled squares) developed mechanical hyperalgesia ipsilaterally 3 days post-ligation which was maintained for up to 28 days. In both cases, hyperalgesia completely failed to develop in the GPR55$^{-/-}$ mice (open circles) at all time points. No significant differences were observed contralaterally at any time point in the GPR55$^{+/+}$ or $^{-/-}$ mice (inset panels). $^{*}P < 0.5$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs GPR55$^{-/-}$ thresholds.

both GPR55$^{-/-}$ and GPR55$^{+/+}$ mice, though this reached statistical significance only in GPR55$^{-/-}$ tissues (Table 3). Thus, no overt differences were detected in the cytokine response to inflammatory insult that might underlie the absence of hypersensitivity observed in GPR55$^{-/-}$ mice at the 1 day post-FCA time point. Pro-

Table 3
Cytokine levels in paw samples from untreated and 1 day post-FCA female GPR55$^{-/-}$ and GPR55$^{+/+}$ mice

Cytokine (pg/ml)	Untreated GPR55$^{+/+}$	Untreated GPR55$^{-/-}$	1 Day post-FCA GPR55$^{+/+}$	1 Day post-FCA GPR55$^{-/-}$
IL-1β	0.92 ± 0.2	0.82 ± 0.07	**224.31 ± 35.9*****	**191.41 ± 45.56*****
IL-2	3.93 ± 1.23	2.3 ± 0.27	3.63 ± 0.51	3.77 ± 0.73
IL-4	0.11 ± 0.02	0.12 ± 0.01	**9.87 ± 2.34*****	**9.05 ± 1.74*****
IL-5	0.30 ± 0.08	0.51 ± 0.16	**5.09 ± 1.09*****	**4.18 ± 0.4*****
IL-6	3.31 ± 0.77	2.26 ± 0.23	**2659.25 ± 777.6*****	**2599.47 ± 653.2*****
IL-10	4.03 ± 0.85	4.40 ± 0.39	11.41 ± 3.76	**16.11 ± 3.09***
IL-12	2.91 ± 0.45	4.30 ± 0.88	**32.24 ± 7.24*****	**31.68 ± 6.03*****
TNFα	0.24 ± 0.06	0.29 ± 0.01	**53.75 ± 14.08*****	**78.42 ± 20.37*****
IFNγ	1.22 ± 0.32	1.40 ± 0.67	2.25 ± 0.41	4.02 ± 0.83
GM-CSF	6.58 ± 1.59	12.34 ± 5.19	**68.53 ± 2076****	**62.26 ± 17.79***

Cytokine concentrations ± SEM (pg/ml) from untreated mice are mean values from $n = 8$ mice per group and cytokine concentrations ± SEM (pg/ml) from FCA-injected mice are $n = 9$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs untreated values.

Table 4
Cytokine levels in paw samples from untreated and 14 day post-FCA male GPR55$^{-/-}$ and GPR55$^{+/+}$ mice

Cytokine (pg/ml)	Untreated GPR55$^{+/+}$	Untreated GPR55$^{-/-}$	14 Days post-FCA GPR55$^{+/+}$	14 Days post-FCA GPR55$^{-/-}$
IL-1β	1.26 ± 0.09	0.46 ± 0.09	50.69 ± 8.87	78.13 ± 22.65
IL-2	1.48 ± 0.52	**3.39 ± 0.75***	1.36 ± 0.25	1.78 ± 0.44
IL-4	0.16 ± 0.04	0.06 ± 0.02	3.71 ± 0.7	**11.42 ± 2.12*****
IL-5	0.33 ± 0.08	0.15 ± 0	0.27 ± 0.06	0.73 ± 0.25
IL-6	3.78 ± 0.52	1.81 ± 0.17	185.67 ± 64.24	201.27 ± 67.38
IL-10	8.04 ± 2.05	1.97 ± 0.15	10.73 ± 2.22	**32.21 ± 8.32*****
IL-12	4.99 ± 0.99	3.77 ± 0.94	5.37 ± 0.69	8.77 ± 3.07
TNFα	0.43 ± 0.09	0.27 ± 0.04	13.52 ± 1.92	28.57 ± 5.38
IFNγ	–	0.22	0.72 ± 0.52	**5.36 ± 2.75****
GM-CSF	13.79 ± 4.49	8.26 ± 1.28	23.68 ± 4.57	**112.72 ± 29.7*****

Cytokine concentrations ± SEM (pg/ml) from untreated and FCA-injected mice are mean values from $n = 8$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs GPR55$^{+/+}$ values.

filing of paw samples from the male GPR55$^{+/+}$ mice 14 days post-FCA similarly showed elevations in the majority of cytokines tested compared with untreated samples (IL-1β, -4, -6, -10, TNFα and GM-CSF; significance levels not shown, Table 4). Significant differences were also evident in the levels of some cytokines between GPR55$^{-/-}$ and GPR55$^{+/+}$ mice post-FCA. Levels of IL-4, -10, IFNγ and GM-CSF were all elevated in paw samples from the FCA-injected GPR55$^{-/-}$ mice, compared to GPR55$^{+/+}$ mice post-FCA (significance levels indicated in Table 4).

4. Discussion

These data show that the targeted deletion of GPR55 in mice abolished hyperalgesia to mechanical stimuli following inflammatory or neuropathic insults but without profound alteration of normal nociceptive thresholds. Following intraplantar FCA injection, there was no inflammatory mechanical hyperalgesia present in male GPR55$^{-/-}$ mice up to 14 days post-FCA. Following partial nerve ligation, there was no mechanical hyperalgesia present in male or female GPR55$^{-/-}$ mice at any time point up to 28 days post-ligation. This is the first report of a role for this receptor in pain.

To analyse the role of GPR55 in pain, GPR55$^{-/-}$ mice were generated. Quantitative PCR analysis confirmed the lack of GPR55 mRNA expression in the GPR55$^{-/-}$ mice, although it should be considered that there is likely to be a truncated N-terminal protein present in the GPR55$^{-/-}$ mice. Quantitative PCR showed no compensation in the levels of CB_1 or CB_2 mRNA in the GPR55$^{-/-}$ mice, nor were there any differences in the proportions of neutrophils, monocytes and T cells in the blood of the GPR55$^{-/-}$ mice. Targeted deletion of the GPR55 gene conferred no overt phenotypic changes as judged in a battery of general and primary observational tests. The rotarod data suggested no motor deficits in the GPR55$^{-/-}$ mice and whilst the hot-plate data did show a decreased withdrawal latency in female GPR55$^{-/-}$ mice at 50 °C, this was not a consistent effect as it was not observed at 52.5 or 55 °C. Similar to $CB_1{}^{-/-}$ [48] and $CB_2{}^{-/-}$ mice [5], the GPR55$^{-/-}$ mice appeared healthy with no gross morphological defect. GPR55$^{-/-}$ mice did not show an increased mortality rate at adulthood as was described for $CB_1{}^{-/-}$ mice [48].

GPR55$^{-/-}$ mice showed a lack of mechanical hyperalgesia following both intraplantar FCA injection and partial nerve ligation. This suggests that the targeted deletion of GPR55 results in the disruption of an event common to inflammatory and neuropathic pain behaviour. Given that a number of cannabinoid ligands activate GPR55 [4,37] and the recent finding that GPR55 activation can inhibit potassium current through M-type potassium channels [20], it is possible that GPR55 also has a pro-nociceptive role.

Previous work describing an involvement of cannabinoid receptors in pain has utilised both CB_1- and CB_2-selective compounds and knockout mice. For example, the CB_1/CB_2-selective agonists WIN-55212-2 and CP-55940 and the endocannabinoid anandamide are effective in models of acute and chronic inflammatory and neuropathic pain [9, for review]. Recently there has been greater focus on the use of CB_2-selective agonists in the animal models of neuropathic and inflammatory pain to avoid the centrally mediated side effects of cannabinoids such as hypothermia, catalepsy and loss of motor coordination. CB_2-selective agonists such as GW405833 and AM1241 have been shown to reverse carrageenan-induced oedema and thermal hypersensitivity [6,8,34], capsaicin- and incisional-allodynia [12,19] and hypersensitivity following intraplantar FCA, spinal nerve ligation [13] and partial nerve ligation [41]. In addition, the use of $CB_2{}^{-/-}$ mice in combination with CB_2-selective agonists has confirmed a role for CB_2 in the anti-hyperalgesic effects of GW405833 [46] and the anti-nociceptive effects of AM1241 [15]. Alternatively, recent work deleting CB_1 receptors peripherally suggests that peripherally

acting CB_1 agonists may be effective analgesics without any central side effects [1].

In terms of effectors of cannabinoid analgesia it has been suggested that the anti-nociceptive effects of CB_2 agonists are due to the peripheral release of beta-endorphins [14]. This was the case for AM1241 [14] but not GW405833 [46] and so an involvement of beta-endorphins is currently unclear. It has already been documented that it is the cascade of cytokines, rather than the inflammatory stimulus itself, which results in the release of the prostaglandins and sympathetic amines which actually cause pain in inflammatory and neuropathic hypernociception models ([43], for review). For this reason it was pertinent to measure the levels of a number of cytokines in $GPR55^{-/-}$ and $GPR55^{+/+}$ mice at a site local to the FCA injection (within the paw).

At day 1 post-FCA, there were elevated levels of the majority of cytokines measured in the paws of $GPR55^{-/-}$ and $GPR55^{+/+}$ mice. This suggested a typical acute inflammatory response local to the site of the FCA injection, where the levels of both pro-inflammatory (IL-1β, -6, -12, TNFα) and anti-inflammatory (IL-4) cytokines were raised. There were also increases in IL-5 and GM-CSF, two cytokines from the same family which are involved in B cell growth and immunoglobulin function and growth of white blood cells, respectively. The only differences in cytokine levels between paws taken from $GPR55^{-/-}$ and $GPR55^{+/+}$ mice at this time point were that the levels of the anti-inflammatory cytokine IL-10 were only significantly elevated from untreated controls in the $GPR55^{-/-}$ mice. Whilst the levels of IL-10 were still elevated in the $GPR55^{+/+}$ mice this did not reach statistical significance. In comparison with the behavioural data, the cytokine data at day 1 does not suggest a direct link between local inflammatory changes in the paw and pain behaviour given the similarity in cytokine levels following FCA in the $GPR55^{-/-}$ and $GPR55^{+/+}$ paws. At day 14, however, there were cytokine changes specifically in the paws of $GPR55^{-/-}$ mice that could explain the sustained lack of inflammatory mechanical hyperalgesia. At this later time point, levels of the majority of the cytokines were elevated compared to untreated controls; however, these levels were noticeably lower than the levels at day 1. Comparing cytokine levels between $GPR55^{-/-}$ and $GPR55^{+/+}$ mice showed differences mainly in the FCA-treated paws. There were increased levels of IL-4, -10, IFNγ and GM-CSF in the paws from FCA-injected $GPR55^{-/-}$ mice, compared to $GPR55^{+/+}$ paws. IL-4 and -10 are anti-inflammatory cytokines and there is increasing evidence that the cascade of pro-inflammatory cytokines involved in inflammation and pain is modulated by a parallel release of anti-inflammatory cytokines [10,17,18,33,40]. There is less known about the roles of IFNγ and GM-CSF in pain models. However, $IFN\gamma R^{-/-}$ mice do not develop signs of neuropathic pain following peripheral nerve injury [36] and there is increased responsiveness of rat dorsal horn neurones in vivo following prolonged intrathecal exposure to IFNγ [44].

The cytokine data at day 14 in this study clearly supports the previous findings of a role for IL-4 and IL-10 in inflammatory pain. It is possible that they are working together to reduce levels of the pro-inflammatory cytokines which could result in a subsequent reduction in the cytokine cascade responsible for pain. This would explain the lack of inflammatory mechanical hyperalgesia in the FCA model, though this does not appear to be the case at day 1. It should be considered that a reason for this may be that the tissues for the day 1 studies were from female mice and those for the day 14 studies were from male mice. Direct comparison of the mechanical hyperalgesia data at day 1 in the FCA model indicated no sex differences, as did the results from the partial nerve ligation study. In addition, there were no sex differences identified in the primary and general observations made on the $GPR55^{-/-}$ and $GPR55^{+/+}$ mice. However, without repeating the study in female mice up to day 14 it is unknown whether sex differences account for the discrepant results between the two time points. Alternatively, GPR55 signalling could also play a role in parallel/additional processes involved in the establishment of the inflammatory response at day 1, resulting in the lack of mechanical hyperalgesia. It may be that under the more chronic inflammatory conditions at day 14 the increase in levels of the anti-inflammatory cytokines was able to influence the downstream behavioural results.

Regardless of the differential cytokine profiling results at the different time points studied, the behavioural results suggest that manipulating GPR55 signalling will be of utility for treating inflammatory and neuropathic pain. Whilst gene deletion in mice provides important phenotypic information it is possible that there are compensatory changes in genes other than CB_1 and CB_2 which could influence the pain phenotype. Identification of selective GPR55 antagonists for testing in pre-clinical pain models is underway and these data in combination with the knockout data will be important in determining the role of GPR55 in experimental analgesia. In addition, selective GPR55 antagonists will be important in determining whether modulation of this receptor has any benefits over modulation of CB_2 or other pain targets currently being evaluated clinically.

Acknowledgements

Special thanks to LAS colleagues responsible for breeding of the $GPR55^{-/-}$ and $GPR55^{+/+}$ mice. The authors state no conflict of interest.

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