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Antihyperalgesic activity of a novel nonpeptide bradykinin B_1 receptor antagonist in transgenic mice expressing the human B_1 receptor

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1 We describe the properties of a novel nonpeptide kinin B_1 receptor antagonist, NVP-SAA164, and demonstrate its *in vivo* activity in models of inflammatory pain in transgenic mice expressing the human B_1 receptor.

2 NVP-SAA164 showed high affinity for the human B_1 receptor expressed in HEK293 cells (K_i 8 nM), and inhibited increases in intracellular calcium induced by desArg^{10}kallidin (desArg^{10}KD) (IC_{50} 33 nM). While a similar high affinity was observed in monkey fibroblasts (K_i 7.7 nM), NVP-SAA164 showed no affinity for the rat B_1 receptor expressed in Cos-7 cells.

3 In transgenic mice in which the native B_1 receptor was deleted and the gene encoding the human B_1 receptor was inserted (hB_1 knockin, hB_1-KI), hB_1 receptor mRNA was induced in tissues following LPS treatment. No mRNA encoding the mouse or human B_1 receptor was detected in mouse B_1 receptor knockout (mB_1-KO) mice following LPS treatment.

4 Freund's complete adjuvant-induced mechanical hyperalgesia was similar in wild-type and hB_1-KI mice, but was significantly reduced in mB_1-KO animals. Mechanical hyperalgesia induced by injection of the B_1 agonist desArg^{10}KD into the contralateral paw 24 h following FCA injection was similar in wild-type and hB_1-KI mice, but was absent in mB_1-KO animals.

5 Oral administration of NVP-SAA164 produced a dose-related reversal of FCA-induced mechanical hyperalgesia and desArg^{10}KD-induced hyperalgesia in hB_1-KI mice, but was inactive against inflammatory pain in wild-type mice.

6 These data demonstrate the use of transgenic technology to investigate the *in vivo* efficacy of species selective agents and show that NVP-SAA164 is a novel orally active B_1 receptor antagonist, providing further support for the utility of B_1 receptor antagonists in inflammatory pain conditions in man.

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Abbreviations: BSA, bovine serum albumin; BK, bradykinin; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; FCA, Freund's complete adjuvant; HBSS, Hank's balanced salt solution; HEK293, human embryonic kidney cell line; HEPES, *N*-[Hydroxyethyl]piperazine-*N*-2-ethanesulphonic acid; KD, kallidin; K_d, dissociation constant; K_i, inhibition constant; LPS, lipopolysaccharide; hB_1-KI, human B_1 receptor knockin; mB_1-KO, mouse B_1 receptor knockout; SDS, sodium dodecyl sulphate; RT–PCR, reverse transcriptase–polymerase chain reaction; TES, *N*-tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid; Tris, Tris[hydroxymethyl]aminomethane

Introduction

The kinins bradykinin (BK) and kallidin (KD) are formed from kininogen precursor proteins following the activation of plasma or tissue kallikrein enzymes by pathophysiological stimuli such as inflammation, tissue damage or anoxia. Considerable evidence suggests that kinins contribute to the pathophysiological processes accompanying both acute and chronic inflammation. The activity of kinins is terminated by several degradative enzymes: kininase I liberates the biologically active metabolites desArg^{9}BK and desArg^{10}KD, while kininase II and endopeptidases form inactive metabolites (Calixto *et al*., 2000).

Two distinct receptors for kinins have been defined, based initially on the pharmacological criteria (Farmer & Burch, 1992; Hall, 1992) and more recently confirmed by molecular cloning techniques (Hess *et al*., 1992; Menke *et al*., 1994; Pesquero *et al*., 1996). B_2 receptors have higher affinity for BK and KD, while B_1 receptors have higher affinity for the metabolites desArg^{9}BK and desArg^{10}KD. For some time, it

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was thought that the majority of actions of BK were mediated *via* B_2 receptors, which are constitutively expressed on a variety of cell types, including the central and peripheral nervous systems. In contrast, B_1 receptors are generally not present at high levels under normal conditions but are induced during conditions of inflammation, bacterial infection or tissue trauma (Marceau *et al*., 1998).

BK has long been established as one of the most algogenic substances known in man and animals, and is a potent activator of nociceptive neurones (Lang *et al*., 1990; Dray *et al*., 1992; Messlinger *et al*., 1994), as well as producing sensitisation of polymodal nociceptors (Koltzenburg *et al*., 1992; Rueff & Dray, 1993). These actions are mediated *via* B_2 receptors, and peptide and nonpeptide B_2 receptor antagonists have well-established analgesic actions in animal models of chronic pain (Dray & Perkins, 1993; Perkins & Kelly, 1993; 1994; Asano *et al*., 1997; Burgess *et al*., 2000). There is now compelling evidence that B_1 receptors also have an important role in persistent inflammatory pain, with peptide B_1 antagonists such as desArg^{10}HOE140 and [desArg^{9}Leu^8]BK inhibiting thermal and mechanical hyperalgesia in numerous models of joint or paw inflammation (Perkins & Kelly, 1993; Perkins *et al*., 1993; Davis & Perkins, 1994; Campos *et al*., 1995; Rupniak *et al*., 1997; Fox *et al*., 2003). Furthermore, while B_1 agonists do not normally affect nociceptive thresholds in normal animal, they have been shown to evoke hyperalgesia following inflammation, indicating the induction of B_1 receptors (Davis & Perkins, 1994; Perkins & Kelly, 1994; Fox *et al*., 2003).

Peptide antagonists for kinin receptors have been vital in elucidating the role of kinins and their receptors in chronic pain and other pathophysiological processes. However, their utility is limited by poor oral bioavailability and limited selectivity. While several nonpeptide antagonists have been described for B_2 receptors (Asano *et al*., 1997; De Campos *et al*., 1999; Burgess *et al*., 2000), examples of nonpeptide antagonists for B_1 receptors have only recently been reported (Gougat *et al*., 2004). We have recently described a novel nonpeptide B_1 receptor antagonist, NVP-SAA164, that is orally absorbed in rats and dogs and has high affinity for the human B_1 receptor (Ritchie *et al*., 2004). With the recent cloning of the B_1 receptor, it has become clear that there may be significant pharmacological differences between the rodent and human receptor (Jones *et al*., 1999), and characterisation of NVP-SAA164 showed that it exhibits marked selectivity for the human receptor, with no affinity for the rat B_1 receptor. In order to demonstrate *in vivo* efficacy in models of chronic pain, we have created a transgenic mouse in which the gene encoding the native mouse B_1 receptor has been deleted, and the gene for the human B_1 receptor inserted. Here, we describe the *in vitro* pharmacological characteristics of NVP-SAA164 (Figure 1), and the characterisation of a human B_1 receptor 'knockin' mouse, which was then used to demonstrate *in vivo* activity of NVP-SAA164 in models of inflammatory pain.

Methods

Radioligand binding studies

Human B_1 receptor binding Membranes were prepared from an HEK293 cell line expressing the human BK B_1 receptor. Cells were homogenised at 10,000 r.p.m. for 30 s and the suspension centrifuged for 30 min at 18,000 r.p.m., followed by washing twice in Tris-HCl (50 mM, pH 7.4) and recentrifugation. The final pellet was resuspended at a protein concentration of 2–8 mg ml^{-1} in Tris-HCl with an 8.8% glycerol solution and stored at −70°C. Binding assays were carried out in micronic polypropylene tubes in a final volume of 0.5 ml. Membranes (15 μg tube^{-1}) were incubated with [^{3}H]-desArg^{10}KD plus test compound or DMSO in a binding buffer (10 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.3 mM $NaHPO_4$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.6 mM glucose, pH 7.4) containing 1 mM 1–10 phenanthroline and 0.14 mg ml^{-1} bacitracin. Samples were incubated for 60 min at 4°C, and then filtered through GF/B filter plates presoaked in 0.3% polyethylenimine for 2–3 h at room temperature. Microscint-40 scintillation cocktail (50 μl) was added to each filter sample, which was counted in a Packard Topcount scintillation counter. The affinity constant (K_d), obtained by incubating the cells with a range of [^{3}H]-desArg^{10}KD concentrations, was estimated to be 0.36 nM. For displacement experiments, the cells were incubated with 1.5 nM [^{3}H]-desArg^{10}KD and various concentrations of test compound. Nonspecific binding was determined using 10 μM desArg^{10}KD.

Rat B_1 receptor binding Cos-7 cells were transiently transfected with rat B_1 receptor cDNA (150 μg ml^{-1}) using the Promega ProFection calcium phosphate transfection kit according to the manufacturer's instructions. For membrane preparation, cells were spun at 340 × *g* for 5 min at room temperature in 1 mM EDTA made up in calcium- and magnesium-free Hank's balanced salt solution (HBSS) containing 10 mM HEPES (pH 7.4). The pellet was homogenised in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 μg ml^{-1} bacitracin, 50 μg ml^{-1} chymotrypsin, 4 μg ml^{-1} leupeptin and 4 μg ml^{-1} trypsin inhibitor and the suspension centrifuged at 40,000 × *g* for 30 min, followed by rehomogenisation and centrifugation a further two times. The final pellet was resuspended in 50 mM Tris-HCl (pH 7.4) plus 10% glycerol and stored at −70°C. Membranes (50 μg well^{-1}) were incubated with [^{3}H]-desArg^{10}KD and various concentrations of test compound for 1 h at 4°C in a buffer containing 10 mM TES (pH 7.4), 1 mM EDTA, 0.1% BSA, 1 μM Plummer's inhibitor, 1 μM enalapril, 10 μM thiorphan in 96-deep-well plates in a volume of 500 μl. Nonspecific binding was determined with 3 μM desArg^{10}KD. Following incubation, the membranes were filtered and counted as above. The K_d was determined to be to 1.0 nM and displacement studies were carried out using a radioligand concentration of 1.5 nM. Nonspecific binding was determined using 5 μM desArg^{10}KD.

Monkey B_1 receptor binding Whole-cell radioligand binding was carried out using the Rhesus monkey fibroblast cell line DBS-FRhL-2 treated with IL-1β to induce B_1 receptor expression. Cells were plated onto 24-well plates at approximately 35,000 cells well^{-1} and grown overnight, and then treated with 100 U ml^{-1} IL-1β for 2 h prior to assay. Cells were then incubated for 1 h at 4°C in 500 μl binding buffer (10 mM HEPES in HBSS (pH 7.4) plus 1 mM phenanthroline and 0.14 mg ml^{-1} bacitracin) containing [^{3}H]-desArg^{10}KD and various concentrations of test compound. At the end of the incubation, the cells were washed three times with 50 mM Tris-HCl (pH 7.4) containing 300 mM sucrose, solubilised with

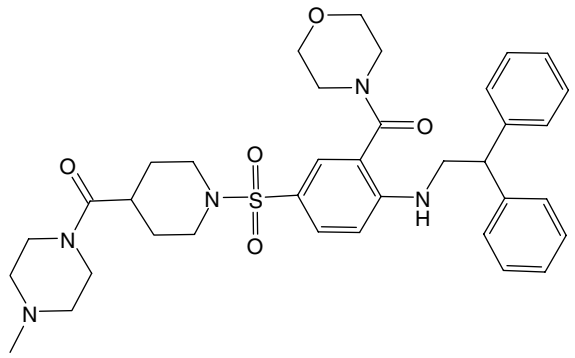

Figure 1 Chemical structure of NVP-SAA164.

0.2% SDS and the amount of radioactivity in the samples determined by liquid scintillation counting. The K_d was determined to be 2.3 nM, and displacement experiments were carried out using a radioligand concentration of 1.8 nM. Nonspecific binding was determined using 5 μM desArg^{10}KD.

For all studies, IC_{50} values were calculated in ORIGIN 4.1 using a logistic fit from at least four curves carried out in triplicate. K_i values were calculated from the IC_{50} values using the Cheng–Prussoff equation ($K_i = IC_{50}/(1 + ([RL]/K_d))$, where [RL] is the radioligand concentration.

Intracellular calcium measurements

Functional antagonist activity of NVP-SAA164 and desArg^{10}Hoe140 was determined by measuring inhibition of agonist-induced increases in intracellular calcium in transiently transfected Cos-7 cells as described previously (Jones *et al*., 1999). Antagonist activity was tested against either 30 nM desArg^{10}KD or 30 μM ATP, used here to activate endogenous purinoceptors as a test for selectivity.

Generation of mouse B_1 knockout–human B_1 knockin mice

Generation of targeting constructs and ES cell recombinants Elucidation of the structural organisation of the human B_1 gene has shown that the entire coding sequence for this gene resides in exon 3 (Yang & Polgar, 1996). We cloned the B_1 receptor genomic sequence from a bacterial artificial chromosome library derived from 129/SvJ mice (Genome Systems, St Louis MO, U.S.A.) and identified a 19 kb *Spe*I fragment containing the entire mouse B_1 gene, for generating the targeting constructs. We used a three loxP strategy and tried to preserve the murine intron 2 sequences to preserve any transcriptional regulation signals in this region. The translation start site to translation termination site in exon 3 was precisely replaced with a cassette containing the corresponding human B_1 receptor coding sequence flanked by loxP sites and an adjacent PGK-neo/Hsv-tk selection cassette with a third loxP site at the distal end. The human cDNA and the selection cassette was flanked by 3.7 kb of 5′ upstream sequence and 5.7 kb of downstream sequence (Figure 2a).

The construct was linearised with Xyz 1 and transfected into RW4 ES cells (Genome Systems, St Louis, MO, U.S.A.). G418-resistant clones were selected and identified by Southern blotting after *Eco*RV digestion and blotting to a 5′ probe (2.4 kb *Eco*RV–*Eco*R1 fragment). Four out of seven clones were correctly targeted by homologous recombination and this was confirmed by Southern blotting using a flanking 3′ probe (0.9 kb *Bam*H1–*Spe*1 fragment).

Cre transfection and identification of type I and type II deletion clones Recombination between loxP sites was carried out by transiently transfecting homologous recombinant clones with a Cre expression vector and selecting for deletion of the selection cassette with gancyclovir. Two possible homologous deletion events are possible between the three loxP sites (Figure 2a). Southern analysis was carried out in order to distinguish between the endogenous allele, homologous recombinant allele, deletion I (floxed allele in which the TK marker is recombined out and the human B_1 cDNA flanked by loxP sites is retained) and deletion II (knockout allele in which TK marker and the human B_1 cDNA are recombined out) clones. Analysis of nearly 200 gancyclovir-resistant clones resulted in the identification of two type I deletion clones (floxed) and five type II deletion clones, and these cells were used to generate mouse B_1 receptor knockout (mB_1-KO) mice and human B_1 receptor knockin (hB_1-KI) mice.

Generation of mice Two independent type I or type II clones were injected into blastocysts derived from C57Bl/6 animals and implanted into pseudopregnant CD-1 females to generate chimeras. The resulting male chimeras were backcrossed with C57Bl/6 females and the agouti progeny from these crosses were analysed by Southern blot to identify mB_1-kO/hB_1-KI heterozygous mice. Heterozygotes were backcrossed to a C57Bl/6 background for at least five generations and then intercrossed to generate homozygotes for the hB_1-KI mutation to be used for behavioural experiments. In all studies, comparisons were made with litter-mate wild-type animals. Genotyping was routinely carried out using Platinum taq (Invitrogen, Paisley, U.K.) PCR with PCR primers forward GCCATCATAACGCACGAAAAT, reverse CCAG CAGGACAGTTGGAAGAG, and 30 cycles of 95°C for 10 s, 60°C for 15 s followed by 68°C for 1 min. This produced products of 400 bp for the knockout, 1.1 kb for the wild-type and 1.5 kb for the knockin mice on DNA gels.

Measurement of B_1 receptor expression

B_1 receptor mRNA expression was examined in tissues from mB_1-KO and hB_1-KI mice by RT–PCR. Female mice were treated with oestradiol benzoate (0.5 mg kg^{-1}, i.p.) for uterus priming, followed 18 h later by an intravenous injection of bacterial lipopolysaccharide (LPS) (3 mg kg^{-1}) to induce B_1 receptor expression. Animals were killed 4 h later. Total RNA was prepared from the lung, heart, bladder, uterus, and spinal cord using Trizol (Invitrogen) with purification using an RNeasy Mini Kit (Qiagen).

cDNA was synthesised from the RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) and 1 μl of cDNA was used in triplicate for normalisation with a β-actin primer pair provided in the QuantumRNA β-actin Internal Standards Kit (Ambion) using a LightCycler Instrument and the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) according to the manufacturer's instructions. Normalised cDNA was used to analyse the expression of mouse and human B_1 receptor mRNA using the following TaqMan probes and primers: mouse B_1 – forward

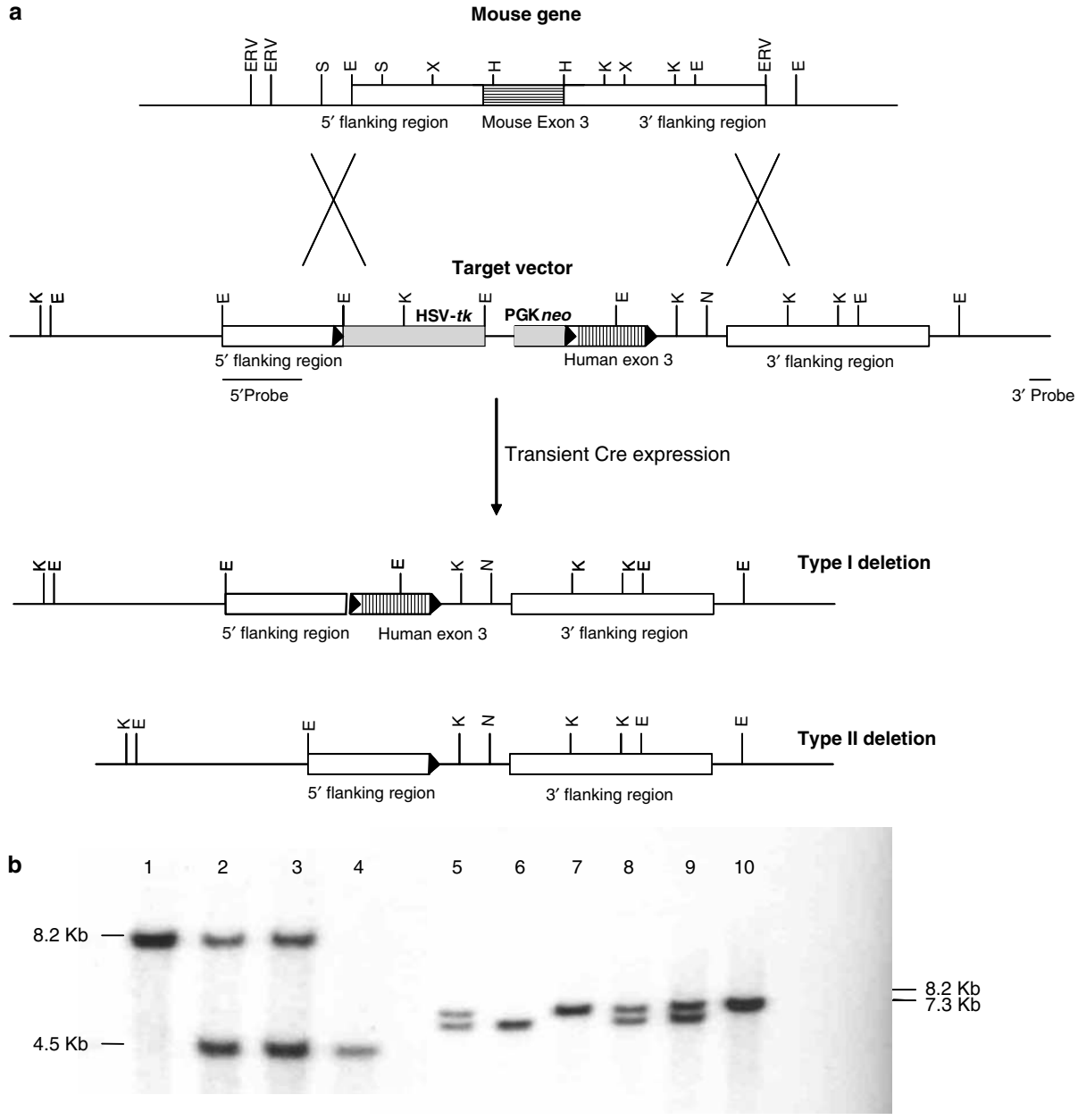

Figure 2 Generation of mB_1-KO/hB_1-KI mice. (a) A mouse gene targeting vector was generated by ligating the hB_1 receptor coding sequence flanked by selection markers, HSV-thymidine kinase (HSV-tk) gene and PG neomycin phosphotransferase gene (PG-neo) and loxP sites. The mouse coding sequence was replaced with this fragment such that the human coding sequence and selectable markers were flanked by 3.7 kb of 5′ mouse genomic sequence and 5.7 kb of 3′ mouse genomic sequence in pBluescript SKII. The construct was linearised and transfected into ES cells and homologous recombinants were selected and identified as described in Methods. Recombinant ES cell clones were transiently transfected with a Cre recombinase encoding vector and two types of recombinant deletion mutants were selected and isolated. Type I has an intact human coding region replacing the mB1 receptor encoding exon and Type II is a null allele. E = *Eco*R1, ERV = *Eco*RV, K = *Kpn*1, H = *Hind*III, N = *Not*1, S = *Sma*1, and X = *Xba*1. (b) Southern analysis of genomic DNA from wild-type, knockout and knockin mice. Mouse tail DNA was digested with *Eco*RI and hybridised with the 5′ probe. The predicted fragments of 8.2 kb band from wild-type allele, a 4.5 kb band from the knockin allele and a 7.3 kb band from the knockout allele were observed confirming that mice with all five possible genotypes were created (hB_1-KI heterozygote in lanes 2 and 3; hB_1-KI homozygote in lane 4; KO heterozygote in lanes 5, 8, and 9; KO homozygote in lane 6; and wild-type mice in lanes 1, 7, and 10). Mice 1, 7, and 10 have homozygous wild-type DNA. Mice 2 and 3 have heterozygous knockin alleles and mouse 4 has homozygous knockin alleles 5, 8 and 9 are heterozygous knockout and 6 is a homozygous knockout.

CTGGGATCTGCTGTGTCGG, mouse B_1 – reverse CCC AGGAGGCCAAAGAAAC, and the mouse B_1 – probe TGC TGCCAGGGTTCGTCATCACTG; human B1 – forward GC ATCCCCACATTCCTGC, human B1 – reverse GCAGGC GGTGATGTTCAGA, and the human B_1 – probe CGATC CATCCAAGCCGTCCCAG.

PCR was performed on all cDNA in triplicate in a 20 μl volume using the LightCycler-FastStart DNA Master

Hybridisation Probe Kit (Roche Diagnostics), with PCR primers at 0.5 μM and the TaqMan probe at 0.6 μM (in 3 mM $MgCl_2$) with thermal cycling at 95°C for 5 s and 60°C for 45 s for 60 cycles. Standard curves for mouse and human B_1 receptors were generated using the same PCR method on 0.1–100 pg of plasmid containing mouse B_1 receptor or human B_1 receptor fragment.

Behavioural studies

All animal studies were carried out according to U.K. Home Office Animal Procedures (1986) Act. All data shown are from male and female homozygote knockout and knockin mice, with comparisons to litter-mate wild-type mice.

Locomotor performance Changes in motor performance were assessed using the accelerating rotarod (Ugo Basile, Italy), with the speed increasing from 4 to 40 r.p.m. over 5 min. The time taken to fall off the rotarod was recorded as latency (s). At 24 h prior to drug testing, mice were given two training periods on the rotarod 1–2 h apart until they were able to remain on the rotarod for at least 60 s. In all experiments, a 300 s cutoff was employed.

Acute pain Responsiveness of mice to an acute noxious thermal stimulus was assessed using the tail-flick assay. Animals were placed inside a cotton pouch and the tail exposed to a focused beam of radiant heat using a tail-flick unit (Ugo Basile, Italy) with an infrared intensity of 14. Latency to tail withdrawal from the beam was measured, with 15 s cutoff to prevent tissue damage.

Inflammatory pain Mechanical hyperalgesia was examined in a model of persistent inflammatory pain, as previously described in the rat (Fox *et al*., 2003), by measuring paw withdrawal thresholds to an increasing pressure stimulus using an Analgesymeter (Ugo Basile). Initial experiments compared the time course of hyperalgesia induced by an intraplantar injection of 15 μl Freund's complete adjuvant (FCA) into one hindpaw in wild-type, hB_1-KI mice, and mB_1-KO. In experiments to assess the effect of test compounds on the mechanical hyperalgesia, paw withdrawal thresholds were measured prior to (naïve) and 24 h following FCA injection, and then up to 6 h following drug or vehicle administration.

Data are presented as mean $\pm$ s.e.m. withdrawal threshold, or percentage reversal of hyperalgesia calculated using the following formula:

$$\%\ \text{reversal} = \frac{\text{postdose} - \text{threshold} - \text{predose threshold}}{\text{naïve threshold} - \text{predose threshold}} \times 100$$

All experiments used six to eight animals per group and statistical analysis was carried out on withdrawal threshold readings using ANOVA with repeated measures followed by Tukey's HSD test.

Neuropathic pain Mechanical hyperalgesia was examined in a model of neuropathic pain induced by partial ligation of the sciatic nerve. Mice were anaesthetised, the left sciatic nerve exposed at mid-thigh level through a small incision and $\frac{1}{3}$ to $\frac{1}{2}$ of the nerve thickness tightly ligated within a 7.0 silk suture. The wound was closed with a single muscle suture and skin clips and dusted with Aureomycin antibiotic powder. Mechanical hyperalgesia was measured up to 3 weeks later as described above.

Drugs For *in vitro* experiments, all compounds were dissolved in DMSO and diluted in assay buffer to give final DMSO concentrations of 0.5–3%. For *in vivo* studies, NVP-SAA164 was administered orally in 0.5% methylcellulose containing 10 mg ml^{-1} malic acid and buffered to pH 4 with NaOH. Morphine and peptides were administered in 0.9% saline.

Results

B_1 receptor affinity of NVP-SAA164

NVP-SAA164 binds with high affinity to the human B_1 receptor, displacing [^{3}H]-desArg^{10}KD with a K_i value of 8 ± 1 nM compared to a K_i value of 63 ± 13 nM obtained for the peptide B_1 receptor antagonist desArg^{10}HOE140 (Figure 3a). NVP-SAA164 has a similar high affinity for B_1

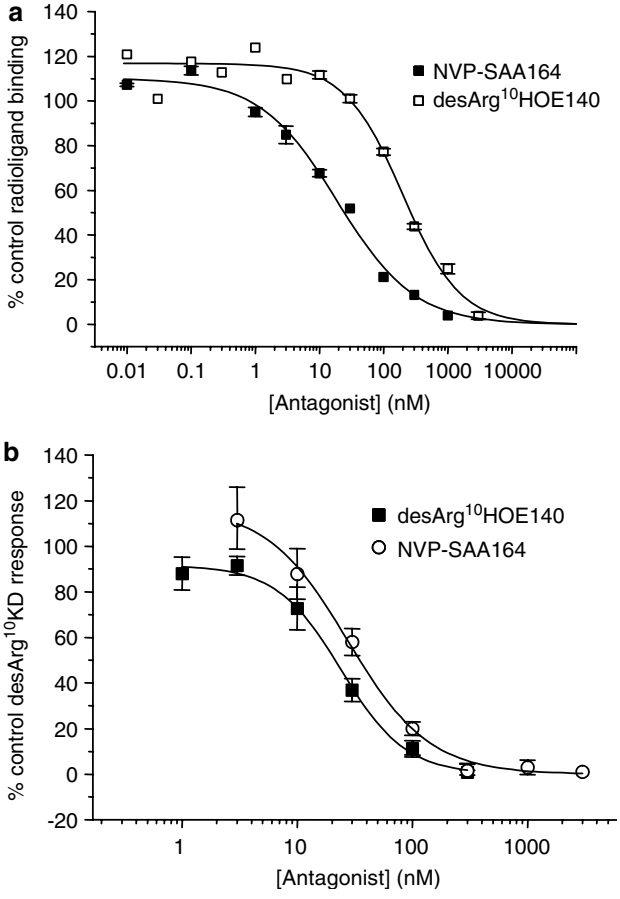

Figure 3 *In vitro* activity of NVP-SAA164 at the human B_1 receptor. (a) Inhibition of [^{3}H]-desArg^{10}KD binding in HEK293 cells expressing the human B_1 receptor. Data show mean $\pm$ s.e.m. from four displacement curves, each carried out in triplicate derived from at least two independent experiments. (b) Antagonist activity in Cos-7 cells expressing the human B_1 receptor. Data show means $\pm$ s.e.m. % inhibition of increases in intracellular calcium induced by 30 nM desArg^{10}KD from three to six experiments performed in quadruplicate.

receptors expressed in monkey fibroblasts (K_i 7.7±1.1 nM). However, it did not displace binding of [^{3}H]-desArg^{10}KD in membranes from Cos-7 cells expressing the rat B_1 receptor at concentrations up to 30 μM. In a receptor selectivity screen, NVP-SAA164 (10 μM) did not show significant affinity at 33 other G-protein-coupled receptors, including rat and human BK B_2 receptors, except for minor affinity for the human κ-opiate receptor (IC_{50} 6.6 μM). In addition, it showed no inhibitory activity for human COX-1 and COX-2 enzymes. NVP-SAA164 thus shows at least 1250-fold selectivity for the human B_1 receptor.

In functional studies using Cos-7 cells expressing the human B_1 receptor, NVP-SAA164 produced a concentration-dependent inhibition of increases in intracellular calcium evoked by the B_1 agonist desArg^{10}KD, with a calculated IC_{50} value of 33±6 nM compared to that of 23±7 nM obtained for desArg^{10}HOE140 (Figure 3b). At concentrations up to 3 μM, NVP-SAA164 did not itself produce increases in intracellular calcium, and did not affect responses evoked by 30 μM ATP.

B_1 receptor expression

Quantitative RT–PCR analysis showed that treatment of mice with bacterial LPS induced the expression of mRNA for the mouse B_1 receptor in the bladder, lung, heart, and uterus from wild-type animals (Figure 4). This expression was not observed in mB_1-KO or hB_1-KI animals (Figure 4). In contrast, human B_1 receptor expression was observed in tissues taken from hB_1-KI animals treated with LPS. In general, this induction of expression of the human receptor mirrored that of the mouse receptor observed in wild-type animals, although in the bladder the increase in expression of the human receptor, while significant, was not as great as that seen in the wild types. The reasons for this discrepancy in this one tissue are unclear but could be a sign of a more variable efficiency of gene regulation in the knockin animals.

Behavioural studies

hB_1-KI and mB_1-KO mice were fertile and showed no obvious signs of behavioural abnormalities. Locomotor coordination assessed by rotarod performance was similar in hB_1-KI mice (225±23 s) compared to wild-type littermates (256±13 s), and in mB_1-KO mice (267±17 s) compared to their wild-type littermates (231±21.6 s). In the tail-flick test for acute pain, there was no difference in withdrawal latencies between hB_1-KI (5.2±0.5 s) and wild-type littermates (4.6±0.4 s), or between mB_1-KO animals (5.1±0.5 s) and their respective wild-types (5.2±0.2 s).

Intraplantar injection of FCA in hB_1-KI mice produced a pronounced mechanical hyperalgesia that lasted for at least 7 days. This pattern of hyperalgesia was not different from that observed in wild-type mice (Figure 5a). Intraplantar injection of the B_1 agonist desArg^{10}KD into the contralateral paw 24 h, following FCA injection into the opposite paw evoked a contralateral mechanical hyperalgesia in hB_1-KI mice, that was again not different from that observed in wild-type mice (Figures 5b and c). Similar results were observed in wild-type and hB_1-KI mice using the B_1 agonist desArg^{9}BK (not shown). In contrast, in mB_1-KO animals, there was a moderate but significant reduction in the level of hyperalgesia induced by FCA injection (Figure 6a). In these animals, neither desArg^{9}BK nor desArg^{10}KD produced hyperalgesia following intraplantar injection, confirming deletion of the B_1 gene product (Figure 6b). In mB_1-KO mice, there was no difference in the time course or degree of mechanical hyperalgesia

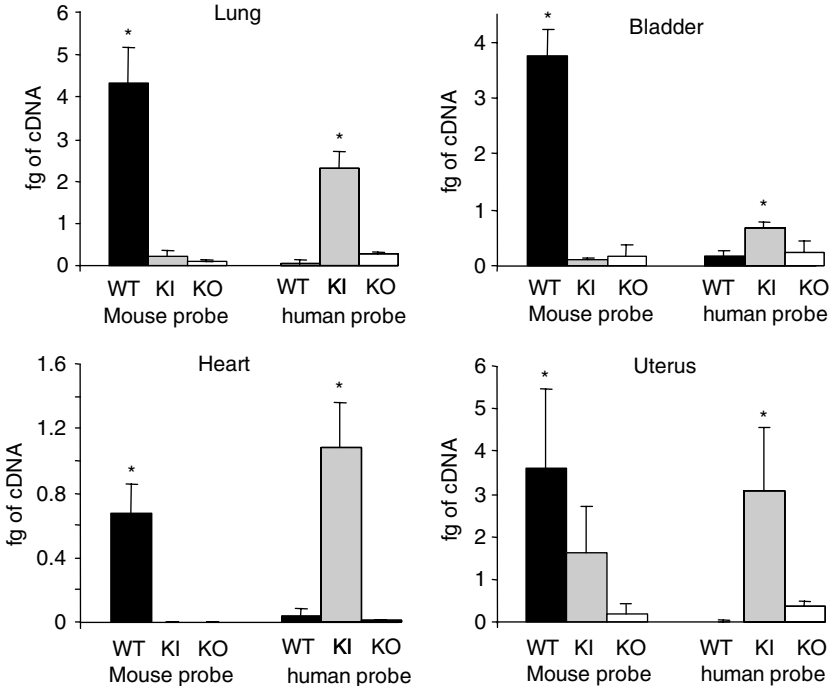

Figure 4 B_1 receptor expression in tissues from wild-type (WT), mouse B_1 receptor knockout (KO), and human B_1 receptor knockin (KI) mice. RNA levels were measured by RT–PCR from tissues obtained 4 h following treatment of mice with LPS, using probes directed against the mouse or human B_1 receptor. Each column represents mean±s.e.m mRNA level from three separate animals, with each determination performed in triplicate. *$P<0.05$ compared to KI and KO groups for mouse probe, and compared to WT and KO for human probe.

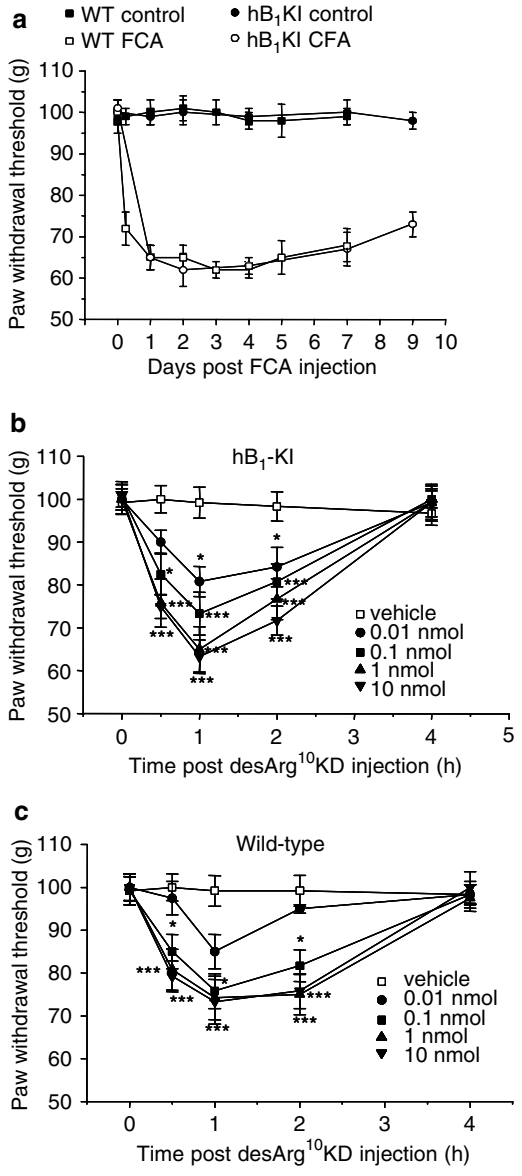

Figure 5 Inflammatory pain in hB_1-KI mice. (a) Time course of mechanical hyperalgesia induced by intraplantar injection of FCA in wild-type (WT) and hB_1-KI mice. (b) B_1 agonist-induced mechanical hyperalgesia in hB_1-KI mice. At 24 h following FCA injection into one hindpaw, intraplantar injection of desArg^{10}KD into the opposite paw produces mechanical hyperalgesia measured as a reduction in paw withdrawal threshold. (c) B_1 agonist-induced hyperalgesia in wild-type mice. In (a–c), data represent mean ± s.e.m. paw withdrawal threshold from six to 10 animals/group. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle-treated animals.

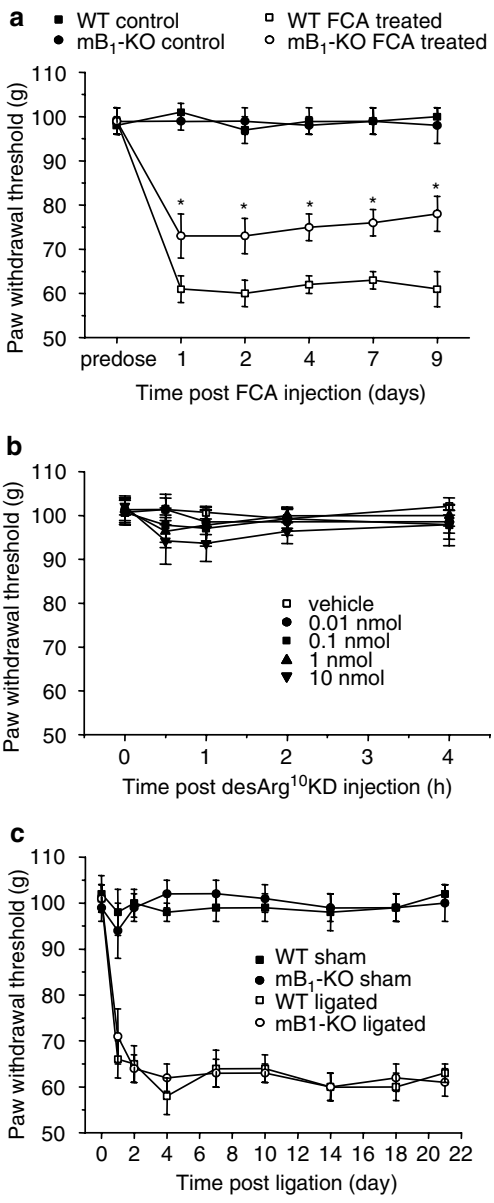

Figure 6 Inflammatory and neuropathic pain in B_1 receptor knockout mice. (a) Time course of mechanical hyperalgesia induced by intraplantar injection of FCA in wild-type (WT) and mB_1-KO. (b) B_1 agonist-induced mechanical hyperalgesia in mB_1-KO mice. At 24 h following FCA injection into one hindpaw, intraplantar injection of desArg^{10}KD into the opposite paw produces a reduction in paw withdrawal threshold. (c) Time course of mechanical hyperalgesia induced by partial sciatic nerve ligation in wild-type (WT) and mB_1-KO mice. In (a–c), data show mean ± s.e.m. from six to 10 animals/group. $^*P<0.05$ compared to WT FCA-treated animals.

induced by partial sciatic nerve ligation, a model of peripheral neuropathy (Figure 6c).

NVP-SAA164 did not affect FCA-induced mechanical hyperalgesia in wild-type mice (Figure 7a). However, in hB_1-KI mice, oral administration of NVP-SAA164 produced a dose-related reversal of inflammatory mechanical hyperalgesia, with a maximal 58% reversal achieved 1 h following oral administration and a calculated D_{50} value of 15.3 μmol kg^{-1} (10.6 mg kg^{-1}). In terms of potency and efficacy, the activity of NVP-SA164 in this model was similar to that observed with diclofenac, which produced a maximal reversal of 57% with a calculated D_{50} value of 12.3 mg kg^{-1} following oral administration (not shown). In addition, NVP-SAA164 significantly inhibited the hyperalgesia induced by intraplantar injection of desArg^{10}KD into the contralateral paw 24 h following ipsilateral injection of FCA (Figure 7c).

Discussion

The data presented here describe the generation of a transgenic mouse expressing the human B_1 receptor specifically designed

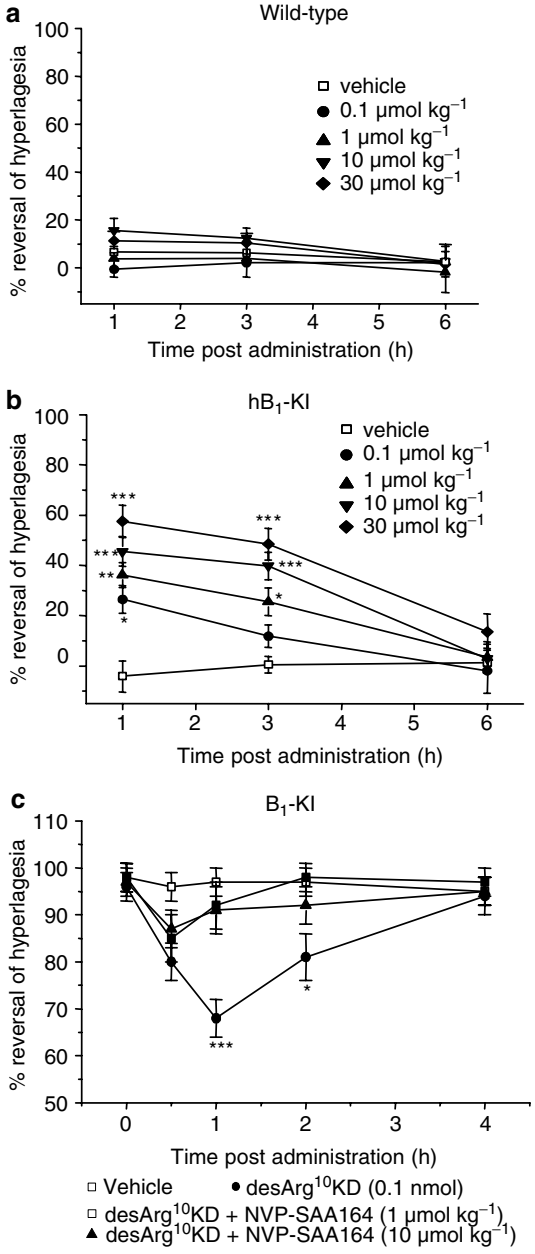

Figure 7 Activity of NVP-SAA164 in wild-type and hB_1-KI mice. (a) NVP-SAA164 is inactive in a model of inflammatory pain in wild-type mice. NVP-SAA164 was administered orally 24 h following intraplantar injection of FCA and mechanical hyperalgesia measured up to 6 h later. (b) NVP-SAA164 reverses FCA-induced mechanical hyperalgesia in hB_1-KI mice. NVP-SAA164 was administered orally 24 h following intraplantar injection of FCA. The graph shows combined data from three separate experiments with a total of six to 18 animals per group. (c) Inhibition of B_1 agonist-induced mechanical hyperalgesia. At 24 h following FCA injection into one hindpaw, desArg^{10}KD was injected into the opposite paw. NVP-SAA164 was administered orally 30 min prior to desArg^{10}KD injection. Data show mean ± s.e.m. from six animals per group. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to vehicle-treated animals.

to enable the testing of human receptor-specific B_1 compounds, and demonstrate the analgesic activity of a nonpeptide B_1 receptor antagonist in a model of inflammatory pain.

As described in the Introduction, there is now compelling evidence to indicate that B_1 receptors play a key role in persistent inflammatory pain (Marceau *et al*., 1998; Calixto *et al*., 2000). Thus, peptide B_1 receptor antagonists such as desArg^{10}HOE140 and [desArg^{9}Leu^8]BK have been shown to reverse mechanical or thermal hyperalgesia induced by a range of different stimuli, including FCA (Davis & Perkins, 1994; Fox *et al*., 2003), zymosan (Belichard *et al*., 2000), formalin (Correa & Calixto, 1993), UV irradiation (Perkins & Kelly, 1993), and cytokines such as IL-1β (Davis & Perkins, 1994; Perkins & Kelly, 1994). These findings imply the induction of B_1 receptors during the inflammatory insult. This is observed more directly through the use of the B_1 agonists desArg^{9}BK or desArg^{10}KD, which do not affect nociceptive thresholds in normal animals, but produce a marked hyperalgesia in the contralateral paw following ipsilateral paw inflammation (Perkins & Kelly, 1994; Fox *et al*., 2003). Until recently, it was generally accepted that the role of B_1 receptors in pain processing is indirect *via* the release of mediators from inflammatory cells and that, unlike B_2 receptors, they do not directly affect the activity of sensory neurons (Davis *et al*., 1996). However, recent findings suggest that they may also be expressed on the central and peripheral terminals of sensory neurones, directly mediating hyperalgesia in response to administered agonists (Levy & Zochodne, 2000; Pesquero *et al*., 2000; Fox *et al*., 2003).

NVP-SAA164 is a novel nonpeptide B_1 antagonist that shows good oral bioavailability in rats and dogs (Ritchie *et al*., 2004). As shown here, it has high affinity for the human and primate B_1 receptors, and acts as a functional antagonist at the human receptor producing a concentration-related inhibition of increases in intracellular calcium induced by the B_1 receptor agonist desArg^{10}KD. It is highly selective, with at least a 1200-fold selectivity over BK B_2 receptors or a range of other G-protein-coupled receptors and enzymes. However, NVP-SAA164 demonstrates a pronounced species selectivity and has no significant affinity for the rat B_1 receptor. Although not described here, additional data has shown that it also does not affect contractions of the isolated rabbit aorta evoked by B_1 agonists, the archetypal B_1 receptor preparation (Marceau *et al*., 1998). While some degree of species selectivity has been observed for agonists acting at the human and rodent B_1 receptors (Jones *et al*., 1999), the recently described nonpeptide antagonist SR240612 shows similar apparent affinities for human, rat, and rabbit B_1 receptors (Gougat *et al*., 2004).

In order to be able to assess the *in vivo* efficacy of NVP-SAA164, we generated a transgenic mouse in which the gene encoding the native B_1 receptor was deleted and that for the human B_1 receptor was inserted (hB_1-KI mouse). During this process, we additionally generated a mB_1-KO. The deletion of the mouse gene and insertion of the human gene was confirmed using Southern blot analysis, and also by examining expression of B_1 receptors in different tissues. B_1 receptors are not generally expressed in cell lines or tissues under normal conditions, but are induced following tissue injury or treatment with inflammatory stimuli such as cytokines or LPS (Marceau, 1995; Wohlfart *et al*., 1997; Haddad *et al*., 2000; Pesquero *et al*., 2000). In the present study, we confirmed the induction of B_1 receptor mRNA following LPS treatment in a number of tissues from wild-type animals, that could be detected by RT–PCR when using primers and probe specific

for the mouse but not human B_1 receptor. Conversely, B_1 receptor mRNA was detected by RT–PCR in tissues from hB_1-KI animals when using the human-specific primers and probe, but RNA encoding the mouse receptor could not be detected. As expected, B_1 receptor mRNA was not detected in tissues from LPS-treated mB_1-KO animals, confirming gene deletion. A recent elegant study (Hess *et al*., 2004) reports the generation of a transgenic rat line expressing the human B1 receptor. While this group similarly recognised the problems in testing human B1 receptor selective compounds, they took a rather different approach, in that they generated a rat line that constitutively expresses high levels of the human B1 receptor, in order to avoid having to induce expression with LPS. Moreover, this rat expresses high levels of B_1 receptors in the brain, which is not generally seen in wild-type animals, to exploit the potential role of neuronal B_1 receptors in pathophysiological processes. While they show an *ex vivo* binding model to demonstrate brain receptor occupancy, no behavioural data are given in this paper.

A key goal of the present study was to generate a mouse line that would express human B_1 receptors under similar conditions to wild-type mice, so that established animal models could be used, but would enable the *in vivo* testing of novel compounds that showed selectivity for the human B1 receptor. At the behavioural level, hB_1-KI animals were viable and showed no differences from wild-type animals in terms of locomotor coordination or responses to an acute noxious thermal stimulus. In addition, they showed a similar hyperalgesic profile in response to FCA injection, both in terms of degree of hyperalgesia and time course. Following FCA injection, administration of a B_1 agonist into the contralateral paw produced a dose-related mechanical hyperalgesia, as reported previously (Fox *et al*., 2003). Again, this profile was not different in hB_1-KI animals compared to wild-type animals, indicating that functional B_1 receptors are similarly induced in hB_1-KI animals. The antihyperalgesic activity of peptide B_1 antagonists in a model of inflammatory pain induced by FCA is well established, as reported by our group and others (Pesquero *et al*., 2000; Ferreira *et al*., 2001; Fox *et al*., 2003). These antagonists have similar affinities for the rodent and human B_1 receptor. In contrast, NVP-SAA164, which is selective for the human receptor, did not affect inflammatory hyperalgesia in wild-type mice. However, in hB_1-KI mice, it produced a dose-related reversal of mechanical hyperalgesia following oral administration. Moreover, NVP-SAA164 inhibited agonist-induced mechanical hyperalgesia following FCA treatment. These data confirm the role of B_1 receptors in persistent inflammatory pain, and demonstrate the *in vivo* efficacy of the nonpeptide human receptor selective B_1 antagonist NVP-SAA164.

Data obtained using the mB_1-KO mice provide additional evidence for a role of B_1 receptors in inflammatory pain. As with the hB_1-KI mice, the mB_1-KO animals were behaviourally normal and showed no differences in locomotor activity compared to wild-type mice, or responses to acute noxious stimuli. However, FCA-induced mechanical hyperalgesia was significantly reduced in mB_1-KO compared to wild-type mice, confirming findings previously reported by Ferreira *et al*. (2001). We additionally found no evidence for a role of B_1 receptors in neuropathic pain since there was no difference in the development of mechanical hyperalgesia following partial sciatic nerve ligation in mB_1-KO mice compared to wild-type animals. While B_1 receptors may be constitutively expressed on sensory neurons (Fox *et al*., 2003), these data suggest that they are not activated to a significant degree, presumably due to a lack of generated agonist, either under normal conditions to contribute to acute pain or during prolonged nerve injury with no ongoing inflammation. Our findings contrast with reports from other groups that have suggested a potential role for B_1 receptors in neuropathic pain models (Levy & Zochodne, 2000; Gabra & Sirois, 2003; Gougat *et al*., 2004), although there are clear methodological differences between the studies. Thus, these studies examined thermal hyperalgesia in models of neuropathic pain induced by chronic constriction of the sciatic nerve (Levy & Zochodne, 2000; Gougat *et al*., 2004) or streptozotocin-induced diabetes. Both of these models can have a significant inflammatory component to the neuropathy, which could partially account for the activity of the B_1 antagonists, although it should also be noted that effects of the antagonists in these studies was relatively modest.

There is accumulating evidence to indicate that the B_1 receptor induction observed in animal studies is also seen in man during conditions of inflammatory disease. Thus, B_1 receptor expression has been demonstrated in lung tissue from patients with interstitial lung disease (Nadar *et al*., 1996), gastric mucosa during active gastritis (Bhoola *et al*., 1997), and in lymphocytes from patients suffering from active multiple sclerosis (Prat *et al*., 1999). Most recently, B_1 receptor activation has been shown to produce signs of neurogenic inflammation in man following local UV irradiation of the skin, providing a direct translational model from animal studies (Eisenbarth *et al*., 2004). In the present study, we have described a novel nonpeptide B_1 receptor antagonist that is orally active in a model of persistent inflammatory pain in a transgenic mouse expressing the human B_1 receptor. Together with the data obtained from B_1 receptor knockout mice, these findings provide further support for the utility of B_1 antagonists in conditions of pain associated with inflammation in man.

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