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Central functional response to the novel peptide cannabinoid, hemopressin

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ABSTRACT

Hemopressin is the first peptide ligand to be described for the CB_1 cannabinoid receptor. Hemopressin acts as an inverse agonist *in vivo* and can cross the blood–brain barrier to both inhibit appetite and induce antinociception. Despite being highly effective, synthetic CB_1 inverse agonists are limited therapeutically due to unwanted, over dampening of central reward pathways. However, hemopressin appears to have its effect on appetite by affecting satiety rather than reward, suggesting an alternative mode of action which might avoid adverse side effects. Here, to resolve the neuronal circuitry mediating hemopressin's actions, we have combined blood-oxygen-level-dependent, pharmacological-challenge magnetic resonance imaging with c-Fos functional activity mapping to compare brain regions responsive to systemic administration of hemopressin and the synthetic CB_1 inverse agonist, AM251. Using these complementary methods, we demonstrate that hemopressin activates distinct neuronal substrates within the brain, focused mainly on the feeding-related circuits of the mediobasal hypothalamus and in nociceptive regions of the periaqueductal grey (PAG) and dorsal raphe (DR). In contrast to AM251, there is a distinct lack of activation of the brain reward centres, such as the ventral tegmental area, nucleus accumbens and orbitofrontal cortex, which normally form a functional activity signature for the central action of synthetic CB_1 receptor inverse agonists. Thus, hemopressin modulates the function of key feeding-related brain nuclei of the mediobasal hypothalamus, and descending pain pathways of the PAG and DR, and not higher limbic structures. Thus, hemopressin may offer behaviourally selective effects on nociception and appetite, without engaging reward pathways.

1. Introduction

To date, all known endogenous cannabinoids, such as 2-arachidonoylglycerol (2-AG) and anandamide, are eicosanoid fatty acid derivatives (Bisogno, 2008; Petrosino and Di Marzo, 2010). These endocannabinoids are released by postsynaptic neurons "on demand," following the Ca^{2+} influx produced in response to postsynaptic depolarization or activation of metabotropic receptors (Kano et al., 2009). When released into the synaptic cleft, endocannabinoids activate presynaptic cannabinoid 1 (CB_1) receptors, and impart an inhibitory action on further presynaptic transmission (Di Marzo et al., 2004; Wilson and Nicoll, 2002). Pharmacological manipulation, coupled with expression studies, has implied integral roles for CB_1 in a diverse range of physiological functions, including memory, cognition, reward, appetite and nociception (Pertwee, 2005; Valverde et al., 2005). Due to this diverse functionality, CB_1 receptors have immense therapeutic potential and are, in fact, established targets particularly in nociceptive and metabolic disorders (Di Marzo, 2011; Di Marzo et al., 2011; Walker and Hohmann, 2005).

The development of CB_1-based therapies, particularly as anti-obesity treatments, has been hindered following the withdrawal of rimonabant (Acomplia; SR141716) by the European Medicines

Abbreviations: 2-AG, 2-arachidonoylglycerol; AM251, N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AP, area postrema; BOLD, blood-oxygen-level-dependent; CB_1, cannabinoid 1 receptor; DR, dorsal raphe; GABA, γ-aminobutyric acid; Arc, hypothalamic arcuate nucleus; DMN, hypothalamic dorsomedial nucleus; VMN, hypothalamic ventromedial nucleus; i.p., intraperitoneal; i.v., intravenous; MABP, mean arterial blood pressure; Acb, nucleus accumbens; AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; NTS, nucleus of the solitary tract; OFC, orbitofrontal cortex; PAG, periaqueductal grey; phMRI, pharmacological-challenge magnetic resonance imaging; PACAP, pituitary adenylate cyclase-activating peptide; RVM, rostral ventromedial medulla; tcp, transcutaneous partial pressure; VP, ventral pallidum; VTA, ventral tegmental area.

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Agency. Rimonabant is a synthetic CB_1 inverse agonist, capable of producing weight-reducing effects over extended periods in rodents and humans (Di Marzo, 2008; Van Gaal et al., 2005). Rimonabant antagonises central CB_1 receptors to acutely reduce motivational appetite (Griebel et al., 2005; Thornton-Jones et al., 2005, 2007). This effect involves a corticostriatal–hypothalamic pathway thought to provide a link whereby reward/motivational circuits impinge on the hypothalamic control of feeding (Dodd et al., 2009; Kelley et al., 2005). In addition to affecting motivational appetite, rimonabant reduces body-fat mass via peripheral interaction with lipid mobilization pathways in adipose tissue and liver, and via energy expenditure and cellular glucose uptake (Di Marzo, 2008; Dodd et al., 2009; Kunos et al., 2009; Nogueiras et al., 2008). Despite being an effective anti-obesity treatment, the withdrawal of rimonabant was a consequence of undesirable central nervous system effects resulting in depression, anxiety or suicidality (Christensen et al., 2007). The discovery of selective CB_1 antagonists capable of maintaining therapeutic benefits whilst lacking these adverse side effects is highly coveted.

Hemopressin is a nine-amino acid peptide, derived from the alpha chain of haemoglobin, which was discovered originally using substrate capture on rat brain homogenates (Heimann et al., 2007; Rioli et al., 2003). Hemopressin acts *in vitro* to functionally antagonise CB_1 receptors, and demonstrates selective CB_1 inverse agonism with sub-nanomolar potency (Dodd et al., 2010b; Heimann et al., 2007). Like rimonabant, hemopressin inhibits food intake in both normal and obese rodent models, and hemopressin can block CB_1 agonist-induced hyperphagia *in vivo* (Dodd et al., 2010b). Interestingly, whereas rimonabant's anorectic effects are by inhibiting hedonic motivation, hemopressin appears to have a specific effect on satiety rather than on reward, and does not exhibit some of rimonabant's off-target behavioural side effects, at least in rodents (Dodd et al., 2009, 2010b; Kirkham, 2009). Hemopressin also induces robust non-opioid antinociception (Heimann et al., 2007), again devoid of non-selective behavioural effects, such as hypothermia, catalepsy and hypoactivity, seen previously with synthetic CB_1 ligands (Hama and Sagen, 2011b; Rahn and Hohmann, 2009). Taken together, these studies suggest that hemopressin may confer its anorectic and antinociceptive actions via more defined CB_1-mediated mechanisms. However, owing to its small size and high sequence homology with haemoglobin, hemopressin's expression, mode of action, and the neuronal targets underlying the peptide's behavioural effects are yet to be explored fully. Understanding the central circuits mediating hemopressin's action at a whole-brain level may be of high significance as it will offer a deeper insight into modes of CB_1 receptor antagonism.

By using the complementary techniques of c-Fos immunohistochemical activity mapping and blood-oxygen-level-dependent (BOLD), pharmacological-challenge magnetic resonance imaging (phMRI), we aim to describe the central circuitry modulated by hemopressin in rodents. Through comparison with the effects in $CB_1^{-/-}$ mice and to that of the rimonabant structural analogue, AM251, we hypothesise that hemopressin may act as a functional antagonist at CB_1 receptors capable of selectively modulating the activity of hypothalamic appetite centres in the brain.

2. Materials and methods

2.1. Animals

Experiments were carried out using adult male Sprague–Dawley rats, CD1 mice (Charles River Laboratories, Inc., Sandwich, UK) and $CB_1^{+/+}$ and $CB_1^{-/-}$ littermate mice (Marsicano et al., 2002). Animals were group housed in The University of Manchester animal unit in a constant environment of 21 ± 2 °C and 45 ± 10% humidity, on a 12:12 h light–dark cycle with the dark phase commencing at 20:00. Chow (Beekay International, Hull, UK) and tap water were available *ad libitum*. All procedures conformed with the requirements of the UK Animals (Scientific Procedures) Act, 1986 and local ethical review. The experiment involving the CB_1 knock-out mice was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the local Government of Rheinland-Pfalz, Germany.

2.2. c-Fos protein immunohistochemistry

Mice (30 ± 3 g; $n = 6$ per group) were housed singly five days prior to the experiment. During this acclimatisation period, mice were handled daily and food intake monitored. Mice were assigned randomly to receive intraperitoneal (i.p.) injections of either vehicle (0.9% NaCl, 10% DMSO, 20% β-cyclodextrin), 500 nmol/kg body weight hemopressin (Tocris Bioscience Ltd., Brighton, UK) or 5.4 μmol/kg (3 mg/kg body weight) AM251 (Tocris Bioscience Ltd., Brighton, UK). All injections were given in a volume of 4 ml/kg body weight. The doses of both hemopressin and AM251 are based on published effects to cause anorexia (Dodd et al., 2010a). Ninety minutes post injection, the mice were deeply anaesthetized with 5% isoflurane (Concord Pharmaceuticals Ltd., Essex, UK) in oxygen (1 l/min) and perfused transcardially with heparinised saline (10,000 units/l heparin in 0.9% NaCl) followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.3). The brains were post fixed overnight and then kept for two days in 30% sucrose in 0.1 M PB to cryoprotect the tissue, before freezing on dry ice. 30 μm sections (120 μm apart) were cut in the coronal plane throughout the entire rostrocaudal extent of the brain and incubated in 20% methanol, 0.2% Triton X-100, 1.5% hydrogen peroxide for 30 min to deactivate endogenous peroxidases. Sections were then incubated at room temperature for 1 h in the blocking buffer: 0.1 M PB, 0.3% Triton X-100, 1% normal sheep serum, and then overnight at 4 °C in rabbit anti-c-Fos antibody (Oncogene Science Inc., Bayer Healthcare, MA, USA) diluted to 1:8000 in blocking buffer. After washing, the sections were incubated sequentially at room temperature for 1 h in goat anti-rabbit IgG–biotin complex (Vector Laboratories, CA, USA) diluted 1:500 in blocking buffer, followed by avidin–biotin–peroxidase complex (GE Healthcare, UK) diluted 1:500 in PB and, finally, visualised with nickel-intensified diaminobenzidine (Vector Laboratories, UK).

For qualitative analysis, the distribution of c-Fos immunoreactivity was examined throughout the rostrocaudal extent of the brain by two independent investigators blind to the treatment groups. Attention was paid to areas previously identified as expressing c-Fos following induction by CB_1 receptor ligands (Allen et al., 2003; Dodd et al., 2010a), or by other psychoactive drugs (Dodd et al., 2010b; Gozzi et al., 2012; Stark et al., 2006; Sumner et al., 2004). Quantification of the number of c-Fos immunoreactive neurones was then performed in regions of interest, defined according to the Paxinos and Watson brain atlas (Paxinos and Watson, 1998). On this basis, the following regions of interest were quantified: nucleus accumbens core (AcbC); nucleus accumbens shell (AcbSh); ventral pallidum (VP); hypothalamic arcuate nucleus (Arc); hypothalamic ventromedial nucleus (VMN); hypothalamic dorsomedial nucleus (DMN); periaqueductal grey (PAG); area postrema (AP); nucleus of the solitary tract (NTS).

Brain regions were photographed using an Axiovision upright microscope (Zeiss, UK) and an Axiocam colour CCD camera. Results are presented as mean and standard error for the number of c-Fos-immunoreactive cells per section in each brain area. Drug challenge was compared with vehicle using the two-tailed, unpaired *t*-test and interactions of drug challenge and genotype were compared using a two-way ANOVA followed by Bonferroni's multiple-comparison *post hoc* test. All statistical tests were performed using the Prism statistical package (GraphPad Software Inc, San Diego, CA, USA).

2.3. BOLD phMRI

2.3.1. MRI acquisition

To measure BOLD activity using phMRI, eighteen rats (200 ± 25 g, $n = 6$ per group) were assigned randomly to receive i.p. injections of vehicle (0.9% NaCl, 10% DMSO, 20% β-cyclodextrin), 500 nmol/kg hemopressin, or 5.4 μmol/kg (3 mg/kg) AM251. Animal preparation followed a similar protocol to previous imaging experiments (Dodd et al., 2009, 2010a,b). Briefly, rats were anaesthetized with 2.5% isoflurane in 100% oxygen (2 l/min) to allow cannulation of a tail vein and subsequent anaesthetic maintenance by intravenous (i.v.) α-chloralose-HBC (Sigma–Aldrich Corp. Ltd., Poole, UK). A bolus of α-chloralose (60 mg/kg body weight; i.v.) was injected manually over a period of 5 min whilst the isoflurane and oxygen were turned off. Then α-chloralose was infused continuously at a rate of 30 mg/kg/hr i.v. by infusion pump for the remainder of the experiment. To confirm that neither drug challenge produces confounding actions on mean arterial blood pressure (MABP) and circulating blood gas levels, the effects of hemopressin and AM251 on MABP, and the transcutaneous partial pressure of blood CO_2 and O_2 were tested in additional anaesthetised rats (see Supplemental data).

After surgery, rats were secured into a temperature-controlled stereotaxic cradle using ear and bite bars. Rectal temperature (RS 51 K-type thermometer; RS Components Ltd, Northants, UK) and respiration rate (MR10 respiration monitor; Graseby Medical Ltd, Hertfordshire, UK) were continually monitored, while the rats were allowed to breathe spontaneously.

MRI data were acquired using a Bruker Avance III 7T pre-clinical system, with a 72 mm birdcage resonator for RF transmit and a Bruker curved "Rat Brain"