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RESEARCH PAPER

Endocannabinoid modulation of hyperaemia evoked by physiologically relevant stimuli in the rat primary somatosensory cortex

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Background and purpose: *In vitro* studies demonstrate that cannabinoid CB_1 receptors subserve activity-dependent suppression of inhibition in the neocortex. To examine this mechanism *in vivo*, we assessed the effects of local changes in CB_1 receptor activity on somatosensory cortex neuronal activation by whisker movement in rats.
Experimental approach: Laser Doppler flowmetry and c-Fos immunohistochemistry were used to measure changes in local blood flow and neuronal activation, respectively. All drugs were applied directly to the cranium above the whisker barrel fields of the primary somatosensory cortex.
Key results: The CB_1 receptor agonist WIN55212-2 potentiated the hyperaemia induced by whisker movement and this potentiation was occluded by bicuculline. The CB_1 receptor antagonists, rimonabant and AM251, inhibited hyperaemic responses to whisker movement; indicating that activation of endogenous CB_1 receptors increased during whisker movement. Whisker movement-induced expression of c-Fos protein in neurons of the whisker barrel cortex was inhibited by rimonabant. Movement of the whiskers increased the 2-arachidonoylglycerol content in the contralateral, compared to the ipsilateral, sensory cortex.
Conclusions and implications: These results support the hypothesis that endocannabinoid signalling is recruited during physiologically relevant activation of the sensory cortex. These data support the hypothesis that the primary effect of CB_1 receptor activation within the activated whisker barrel cortex is to inhibit GABA release, resulting in disinhibition of neuronal activation. These studies provide physiological data involving endocannabinoid signalling in activity-dependent regulation of neuronal activation and provide a mechanistic basis for the effects of cannabis use on sensory processing in humans.
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Keywords: CB_1 cannabinoid receptor; rimonabant; hyperaemic response; laser Doppler flowmetry; whisker barrel cortex; endocannabinoid; 2-arachidonoylglycerol; anandamide

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, *N*-arachidonylethanolamine; AUC, area under the curve; BIC, bicuculline; CB_1 receptor, cannabinoid receptor type 1; DSI, depolarization-induced suppression of inhibition; Fos-LI, c Fos like immunoreactivity; LDF, laser Doppler flowmetry; PN, pyramidal neuron

Introduction

Integration and processing of sensory information by the somatosensory cortex is highly dependent upon regulation of pyramidal neuron (PN) firing by networks of interneurons that release the inhibitory neurotransmitter, GABA. The inhibitory interneurons segregate into subclasses that selectively innervate morphological domains of the PN resulting in modulation of distinct aspects of PN activity (Kawaguchi

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and Kubota, 1997). In particular, GABAergic somatic inputs provide tonic inhibition that can regulate both action potential timing by blocking the spread of depolarization towards the hillock (Soltesz *et al*., 1995) and back-propagation of the action potential into the dendrites, which is important for synaptic integration and plasticity (Larkum *et al*., 1999).

Cannabinoid CB_1 receptors (nomenclature follows Alexander *et al*., 2009) are expressed at moderate density (relative to other brain regions) in the primary sensory cortex of rat (Bodor *et al*., 2005; Deshmukh *et al*., 2007; Hill *et al*., 2007); primate (Eggan and Lewis, 2007) and human (Glass *et al*., 1997) brain. Within the sensory cortex, the highest levels of CB_1 receptors are found in layers 2/3, 5 and 6 (Bodor *et al*., 2005; Deshmukh *et al*., 2007). In the adult brain, immunohistochemical data demonstrate prominent CB_1 receptor expression in GABAergic axons (Bodor *et al*., 2005); however, functional data indicate that glutamatergic afferents, likely arising from neurons in layer 4 (Bender *et al*., 2006; Domenici *et al*., 2006), are CB_1 receptor positive as well.

Several types of endocannabinoid-mediated synaptic plasticity have been identified in the sensory cortex of neonatal rodents. Interneuron-perisomatic PN synapses throughout the neocortex are regulated by endocannabinoid/CB_1 receptor-mediated depolarization-induced suppression of inhibition (DSI) (Trettel and Levine, 2002; 2003; Fortin *et al*., 2004; Trettel *et al*., 2004; Bodor *et al*., 2005). DSI is a form of short-term plasticity in which depolarization of principal neurons evokes the release of a retrograde signal that inhibits GABA release through an action at the presynaptic terminal (Pitler and Alger, 1992). Since perisomatic GABAergic interneurons exert a tonic inhibition of PN depolarization, endocannabinoid-mediated DSI results in disinhibition and an increased probability that the PN will fire an action potential (Fortin *et al*., 2004). Interestingly, slice studies suggest that endocannabinoid-mediated DSI in the neocortex is recruited by very moderate levels of PN activity; as few as three action potentials in a 20 Hz train were sufficient to induce DSI (Fortin *et al*., 2004). Endocannabinoid-mediated signalling has also been implicated in long-term depression at glutamatergic afferents to layer 2/3 from layer 4 (Bender *et al*., 2006), Consistent with these data, a recent *in vivo* electrophysiological study suggests that blockade of CB_1 receptors increases visually-evoked potentials in layer 2/3, and that endocannabinoid signalling is required for the sensory deprivation-induced ocular dominance shifts that take place in rodents post natally (Liu *et al*., 2008).

Recent experiments have demonstrated a critical role for endocannabinoid signalling in the development and postnatal plasticity of the rat whisker-barrel (Li *et al*., 2009), a well-defined region of the cortex that is the primary target of sensory afferents from facial whiskers. Despite these data, the role of endocannabinoid signalling in the modulation of whisker-evoked neuronal activity in the mature barrel cortex has not been examined. Increases in excitatory neurotransmission in the whisker barrel cortex produce increased blood flow within the active region to match local metabolic demand (Raichle, 1998). As a result, laser Doppler flowmetry (LDF) can be used to assess the hyperaemic response and, therefore, excitatory neurotransmission, in real time. Our model, based upon the anatomical and electrophysiological data outlined previously, predicts that increased CB_1 receptor activity within the sensory cortex will result in increased PN activation as a result of disinhibition of tonic GABAergic inputs. Our data support this model as increased CB_1 receptor signalling potentiates, while decreased CB_1 receptor signalling reduces, the hyperaemic response to whisker movement. Taken together, our results suggest that inhibitory control of PN activation in the superficial layers of the somatosensory cortex is regulated by activity-dependent, endocannabinoid/CB_1 receptor signalling.

Methods

Measurement of functional hyperaemic response

All animal care and experimental procedures were in accordance with the National Institute of Health Guide for the Use and Care of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Male Sprague-Dawley rats (250–350 g, Harlan, Madison, WI) were used in these experiments. Rats were anaesthetized with ketamine/xylazine (100/7 $mg \cdot kg^{-1}$, intramuscular; supplemented as required). The left femoral artery was cannulated with heparinized polyethylene tubing (PE50) for blood pressure recording. The animals breathed room air with body temperature maintained at 37°C using a controlled heating pad. Local blood flow in capillaries or small arterioles in the upper 1 mm of the sensory cortex was measured by LDF. Briefly, a laser Doppler (LD) probe (BLF21 Transonic System, Ithaca, NY) was positioned over an area devoid of large, visible vessels in an open cranial window (with intact dura) in the right whisker barrel cortex (approximately 2 mm caudal and 5 mm lateral to bregma). The probe was adjusted until a LD response was elicited following contralateral whisker movement. A positive LD response represents an increase in blood flow and is an arbitrary, tissue perfusion unit. Whiskers were fitted through a comb connected to a solenoid-driven actuator with lateral movement of approximately 5 mm at 10 Hz. LD signals, whisker stimulator timing pulses and arterial blood pressure were recorded using Windaq Acquisition (DATAQ Instruments, Akron, OH).

Control LD responses were measured at the beginning of the experiments. Test responses were obtained at 15 min intervals (at 15, 30 and 45 min) after application of a test compound or its vehicle. Drugs were injected slowly just underneath the dura over the whisker barrel fields using a syringe in a volume of 400 μL to ensure that the drug solution covered the entire cranial window. At the end of the incubation period, excess solution was removed using cotton swabs. Drugs were left undisturbed on the dura for 15 min.

Each LD response includes a 10 s baseline period, 13 s of movement and a 17 s post-movement period. Whiskers were stimulated 8–15 times, and the mean of 8 individual LD responses was calculated. The mean LD response was expressed as percent change from baseline and the area under curve during whisker movement (i.e. percent change from baseline × unit time) represents the magnitude of hyperaemic

response. Arterial blood pressure was obtained by averaging blood pressure values during LD responses at each time point.

Distribution of rimonabant (SR1411716A) after subdural application

The distribution of rimonabant after subdural application was examined using [^{3}H]SR141716A. Male Sprague-Dawley rats were anaesthetized and open cranial windows prepared as described above. Whiskers were stimulated as in the LDF studies, but the corresponding changes in blood flow were not measured. Rimonabant (1 μM) containing [^{3}H]SR141716A (4–8 μCi) was injected under the dura and excess solution was removed after 15 min. The brain was removed after an additional 15 min, the time at which rimonabant displayed maximal effects on LDF. Each hemisphere (with the one not exposed to rimonabant acting as the control) was separately sliced in the horizontal plane, into six 1 mm sections using a brain matrix. Each slice was weighed and solubilized in 1 mL NaOH (1 M). The resultant solution was neutralized with acetic acid then 10 mL scintillation fluid was added (Econo Safe; Research Products International, Mt Prospect, IL). Radioactive content was measured using liquid scintillation counting (Beckman LS 6500).

c-Fos expression studies

Rats were anaesthetized and open cranial windows were prepared, as described previously. Subdural drug administration and whisker movement were performed as described earlier. At the end of the movement period, the rats were deeply anaesthetized using isoflurane and transcardially perfused with 200 mL 0.9% phosphate-buffered saline followed by 200 mL 4% formaldehyde. Brains were removed, placed in 30% sucrose in 0.1 M phosphate buffer for 48 h and sectioned (40 μm, coronal) using a cryostat. Fos-like immunoreactive (Fos-LI) neurons were detected using a rabbit anti-c-Fos antibody (Oncogene, Cambridge, MA; 1:25 000 dilution) and the immunohistochemical protocol described previously (Patel and Hillard, 2003). Reaction product for Fos was visualized using biotin-conjugated secondary antibodies (1:500 dilution; Jackson Immunoresearch, West Grove, PA) followed by processing with the ABC kit (Vector Laboratories, Burlingame, CA) with Ni/Co heavy metal intensification to yield a black reaction product.

For quantitative analysis, bright-field photomicrographs from matched coronal sections were obtained using a Nikon Eclipse E600 microscope (Melville, NY, USA) and SPOT advanced imaging software (Sterling Heights, MI, USA). All sections from a given experiment were obtained during the same microscopy session at which the light level and camera exposure times were kept constant. This was performed to minimize variability in the automated cell counting procedure. Photomicrographs were opened in Image J (available online from the NIH) for automated cell counting. Images were converted to 8 bit mono, the somatosensory cortex was outlined with the freehand draw tool, and the number of Fos-LI nuclei was determined within the specified region using the threshold and particle analysis functions of Image J. The regions quantified were of the same size and were chosen without knowledge of the *in vivo* drug treatment. Particles that met both optical density and size requirements were counted as Fos-LI nuclei by the Image J software.

Endocannabinoid measurement

Male Sprague-Dawley rats were anaesthetized as described previously. A bilateral craniotomy was performed creating two 5 mm × 5 mm cranial windows. The coronal and lambdoidal sutures were the ventro-dorsal boundaries and the sagittal suture was the medial boundary. A subdural injection of approximately 275 μL of toludine blue dye in physiological salt solution (PSS, composition described later) was performed bilaterally to identify the area of the whisker barrel cortex. Injections were made to avoid visible blood vessels. The animal's left whiskers were stimulated as described in the cerebral blood flow experiments for one hour. Brains were removed from the animals immediately after killing and frozen using liquid nitrogen followed by storage at –80°C. Within four weeks, the whisker barrel cortices were dissected using the dye as a landmark. Lipids were extracted from the tissues and *N*-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) contents were determined using isotope dilution, liquid chromatography/mass spectroscopy, exactly as described previously (Patel *et al*., 2005).

Data analysis and statistics

Data are reported as mean ± SEM Using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA), area under curve of mean LD responses, basal cerebral blood flow and arterial blood pressure were analysed by repeated measures ANOVA, followed by Dunnett's *post hoc* tests. The numbers of Fos-LI cells in different treatment groups were compared, where appropriate, by Student's paired *t*-test or one-way ANOVA followed by Dunnett's *post hoc* tests. $P < 0.05$ was taken as statistically significant.

Materials

Rimonabant (also known as SR141716A), SR144528 (NIDA Drug Supply Program, Research Triangle Park, NC), AM251 (Tocris Cookson, Ellisville, MO), WIN55212-2, and WIN55212-3 (Sigma-Aldrich Chemicals, St Louis, MO) were dissolved in 100% ethanol and diluted in Tocrisolve™ (Tocris Cookson) and physiological salt solution to achieve the desired concentrations for subdural injections. (-)-Bicuculline methiodide and GABA (Sigma-Aldrich Chemicals) were dissolved in deionized water. [^{3}H]SR141716A (Amersham Biosciences, Piscataway, NJ) was supplied in 100% ethanol. Composition of PSS was (mM): NaCl 119, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, glucose 5.5, EDTA 0.03.

Results

Measurement of the hyperaemic responses to whisker movement

Mechanical deflection of whiskers resulted in an increase in cerebral blood flow in the contralateral whisker barrel cortex. Figure 1 shows an example of a train of hyperaemic responses.

It can be seen that local cerebral blood flow increases with a slight delay following whisker movement and gradually returns to baseline value. Addition of Tocrisolve vehicle alone to the cranial window did not affect the hyperaemic response to whisker movement for at least 45 min following its application [area under the curve (AUC) prior to vehicle administration, 113 ± 26 s; AUC 15 min after vehicle, 118 ± 30 s; AUC 30 min after vehicle, 134 ± 24 s; AUC 45 min after vehicle, 130 ± 16 s; $n = 7$].

Effects of WIN55212-2 on the hyperaemic responses

Subdural application of the CB_1 receptor agonist WIN55212-2 (applied in Tocrisolve vehicle at a solution concentration of 1 μM), significantly enhanced the hyperaemic response induced by whisker movement (Figure 2A,B). The effect of WIN55212-2 on the hyperaemic response (the area under the curve of the time-change from baseline relationship; Figure 2A) was significantly affected by the time elapsed following drug administration (repeated measures ANOVA $F_{(3,18)} = 3.85$, $P = 0.026$). *Post hoc* analysis revealed a significant effect of Win 55212-2 occurred 30 min after drug administration (Figure 2B). When the concentration of Win 55212-2 in the applied solution was 100 nM, a hyperaemia was seen that did not reach statistical significance (AUC prior to WIN55212-2 administration: 138 ± 22 s; AUC 15 min after, 194 ± 32 s; AUC 30 min after, 196 ± 48 s; AUC 45 min after, 163 ± 43 s; $n = 5$; $F_{(3,16)} = 0.54$, $P = 0.66$, NS). Application of 1 μM WIN55212-3, an enantiomer of WIN55212-2 with very low affinity for both CB_1 and CB_2 receptors (Felder *et al.*, 1992; Slipetz *et al.*, 1995), did not affect the hyperaemic response over the time period examined (Figure 2C,D).

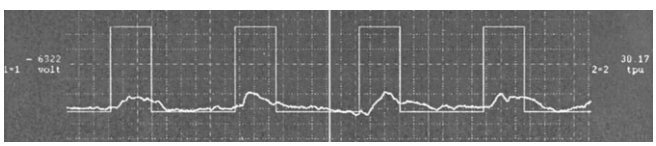

Figure 1 An original trace showing hyperaemic responses to 4 episodes of whisker stimulation in the same rat. Local blood flow is given as tissue perfusion unit (tpu) as determined by laser-Doppler flowmetry. Upward deflection of square pulses indicates whisker movement; this lasted for approximately 13 s.

Role of GABA in the effects of WIN55212-2 on hyperaemic responses

The effect of WIN55212-2 to enhance the hyperaemic response is consistent with previous findings that eCB/CB_1 receptor mediated signalling within the sensory cortex increases excitatory neurotransmission via inhibition of GABA release (Fortin *et al.*, 2004). This model predicts that increased GABA concentrations will reduce the hyperaemic response and oppose the effects of WIN55212-2; and that the hyperaemic effect of WIN55212-2 will be mimicked and occluded by inhibition of $GABA_A$ receptors.

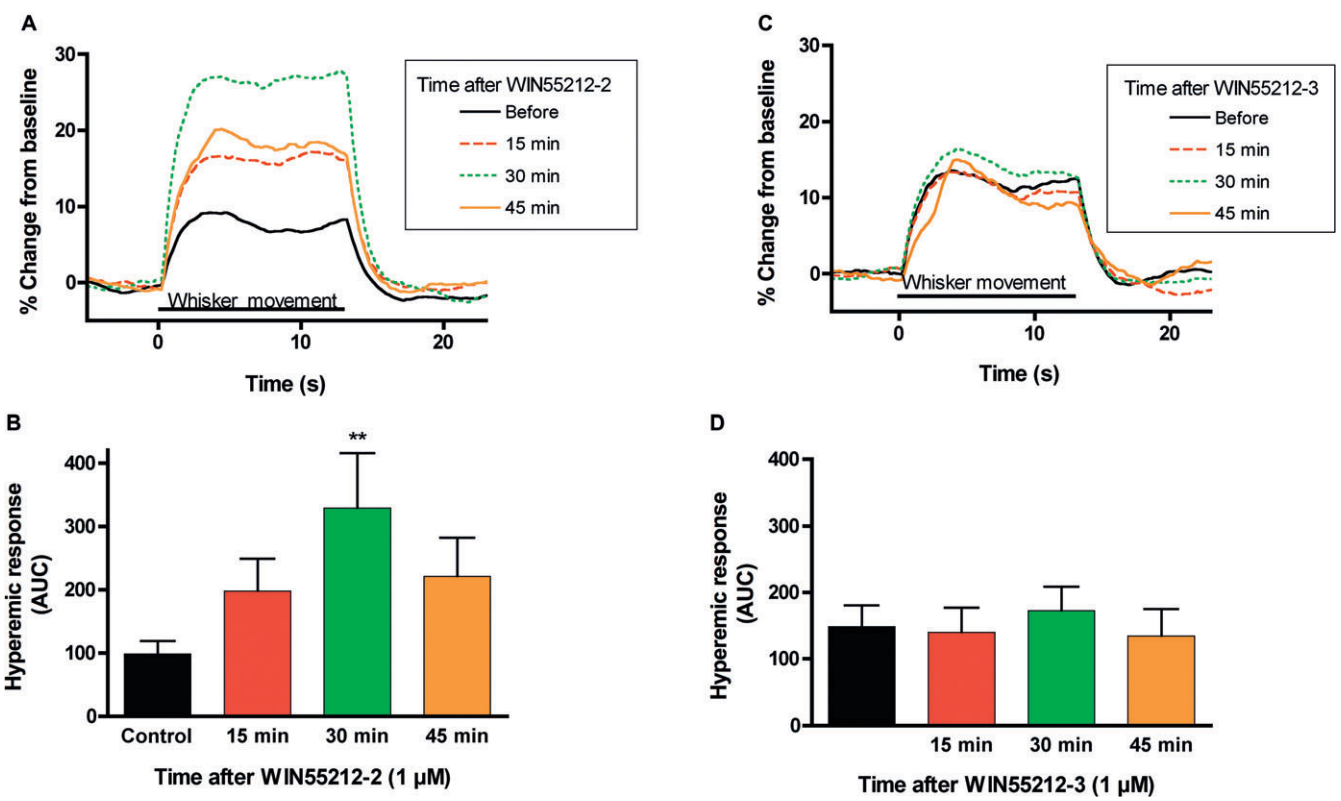

Figure 2 WIN55212-2, but not WIN55212-3, increases the hyperaemic response to whisker movement. Shown are the laser-Doppler tracings averaged from all subjects (A,C) and the corresponding magnitude of functional hyperaemic responses (B,D) before, and at regular time intervals, after 15 min exposure of the whisker-barrel cortex to 1 μM WIN55212-2 ($n = 7$) or 1 μM WIN55212-3 ($n = 5$). $^{**}P < 0.01$ from control, repeated measures ANOVA followed by Dunnett's *post hoc* tests. Vertical lines represent SEM.

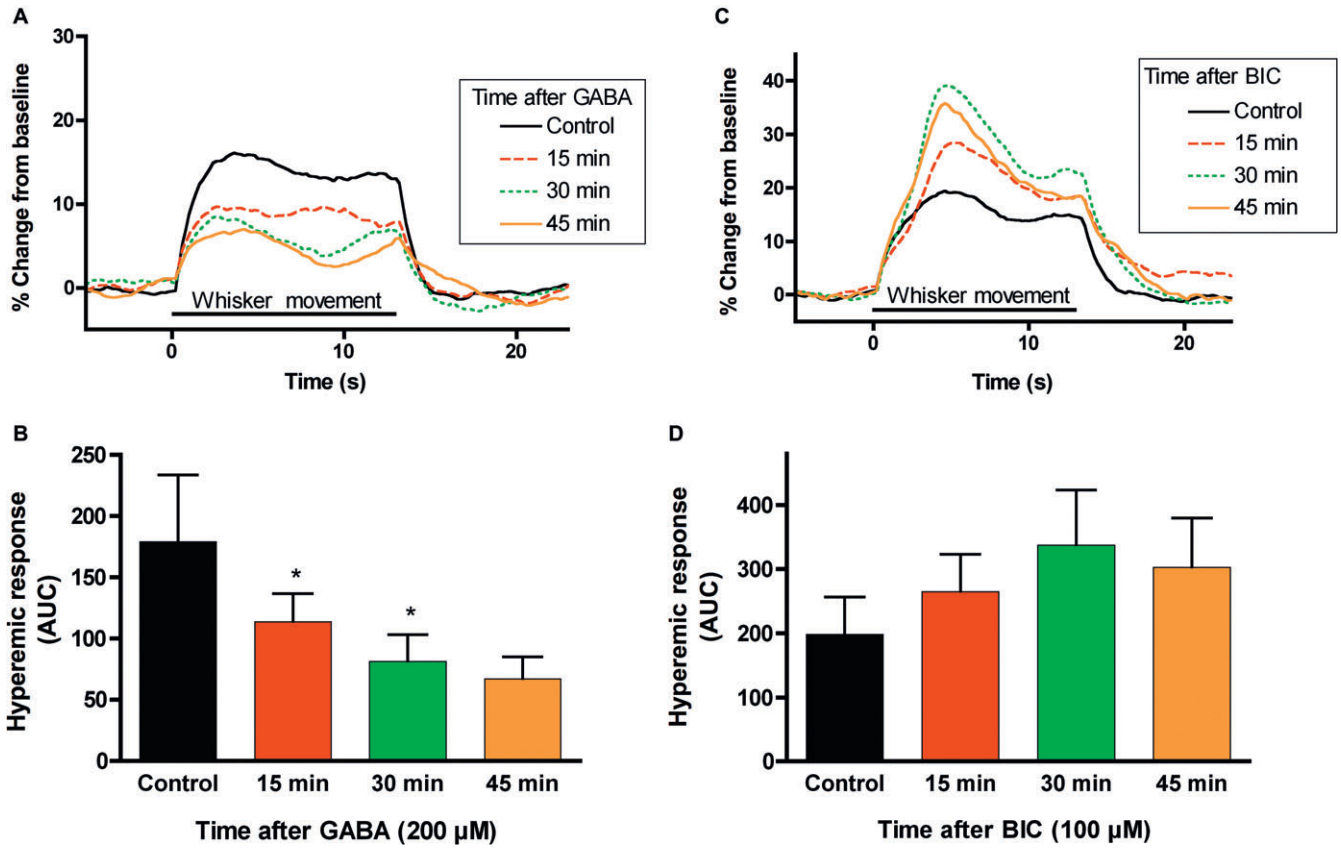

Figure 3 GABA inhibited, while bicuculline (BIC) tended to enhance, the hyperaemic response to whisker movement. Shown are the laser-Doppler tracings averaged from all subjects (A,C) the corresponding magnitude of functional hyperaemic responses (B,D) before, and at regular time intervals, after 15 min exposure of the whisker-barrel cortex to 200 µM GABA ($n = 5$) or 100 µM BIC ($n = 5$). *$P < 0.05$ from control, repeated measures ANOVA followed by Dunnett's *post hoc* tests. Vertical lines represent SEM.

Subdural application of GABA at a concentration of 100 µM produced a time-dependent inhibition of the hyperaemic response to whisker movement (Figure 3A,B). Repeated measures ANOVA indicated that the effect of GABA reached statistical significance ($F_{(4,16)} = 3.2$; $P = 0.04$); Dunnett's *t*-tests revealed significance at 15 and 30 min after drug administration. Subdural application of 100 µM bicuculline, an antagonist of $GABA_A$ receptors, tended to increase the hyperaemic response but the effect did not reach statistical significance as determined by repeated measures ANOVA ($F_{(3,12)} = 1.0$, $P = 0.43$; Figure 3C,D). At a higher concentration (500 µM), bicuculline increased the hyperaemic response at 15 and 30 min following drug administration; however, the kinetics of the change were very different from control in that the blood flow increase was considerably slower in onset and, in some cases, did not to return to baseline at the offset of whisker movement (data not shown).

We examined co-administration of either GABA or BIC together with WIN55212-2 to determine whether either of these drugs would affect the hyperaemic response to WIN55212-2. The data shown in Figure 4 are the results obtained at 30 min after drug administration. These data were analysed by two-way ANOVA, using WIN55212-2 treatment as one factor and control, GABA or bicuculline treatment group as the second factor. This analysis revealed that there were significant effects of WIN55212-2 treatment ($F_{(1,28)} = 5.7$, $P = 0.02$) and a highly significant effect of treatment group ($F_{(2,28)} = 12.5$, $P = 0.0001$). *Post hoc* tests indicate that WIN55212-2 significantly increased the hyperaemic response compared to vehicle in the control treatment group but not in the presence of either GABA or BIC. These data support the hypothesis that

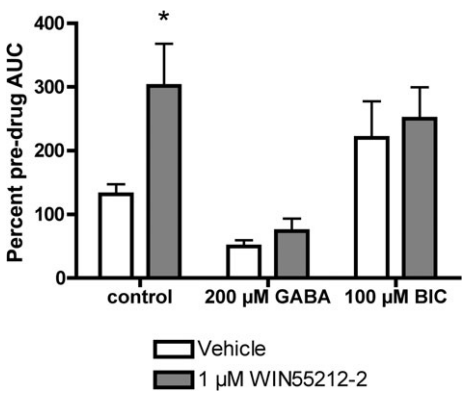

Figure 4 The presence of GABA and bicuculline (BIC) inhibit the effects of WIN55212-2 on functional hyperaemic responses. Shown are the mean ± SEM for the hyperaemic responses obtained before and 30 min after exposure to 1 µM WIN55212-2 alone ($n = 5$), or WIN55212-2 plus 200 µM GABA ($n = 7$) or 100 µM BIC ($n = 7$). *$P < 0.05$ from vehicle, two-way ANOVA followed by Bonferroni *post hoc* tests.

the mechanism of WIN55212-2-induced hyperaemia involves inhibition of GABAergic signalling.

Studies of the contribution of endogenous CB_1 receptor signalling to the hyperaemic response

The effects of CB receptor antagonists were determined to explore the hypothesis that CB_1 receptor signalling is involved in the regulation of the hyperaemic response and to examine the question of recruitment of endogenous cannabinoid signalling. The CB_1 receptor antagonist rimonabant, applied to the cranium at a solution concentration of 1 μM, produced a highly significant reduction in the hyperaemic response ($F_{(3,15)}$ = 8.5, P = 0.0015). *Post hoc* tests revealed significant effects at all times investigated, with maximal effect at 30 min after subdural application (Figure 5A,B). In a separate set of experiments, [^{3}H]SR141716A was used to estimate the distribution of rimonabant after local, subdural application. Radioactivity was found to concentrate in the surface of the hemisphere where the opened cranial window was made (SR hemisphere; Figure 5C), indicating that, in spite of its lipophilicity, rimonabant did not diffuse far from its site of application. These data support our assumption that the cannabinoid drugs affect the functional hyperaemic response locally.

A second CB_1 receptor antagonist, AM251 (1 μM), also significantly attenuated the hyperaemic response ($F_{(3,18)}$ = 4.0, P = 0.034; Figure 6), with significant reductions occurring at 30 min after administration. In contrast, the CB_2 receptor antagonist SR144528 (1 μM) had no significant effect (control, 145 ± 32 s; 15 min, 163 ± 63 s; 30 min, 153 ± 58 s; 45 min, 89 ± 23 s; n = 4).

Effects on basal cerebral blood flow and peripheral haemodynamics

The effects of each of the drug treatments on blood flow in the unstimulated state (basal blood flow) were determined immediately prior to the hyperaemic response measurements. This time point was at least 15 min after subdural drug application. Basal cerebral blood flow was significantly reduced after incubation with the cannabinoid receptor agonist WIN55212-2 at both 100 nM and 1 μM (Table 1). Neither WIN55212-3 nor the CB receptor antagonists (rimonabant, AM251 and SR144528) had significant effects on basal cerebral blood flow. Interestingly, although neither GABA nor bicuculline alone affected basal blood flow; the presence of either abolished the effect of WIN55212-2 on basal blood flow (Table 1). None of the agents used in this study significantly altered systemic arterial blood pressure (Table 1).

Effects of WIN55212-2 and rimonabant on c-Fos expression

To further examine the role of changes in neuronal activity in cannabinoid-induced modulation of hyperaemic responses, we examined the effects of whisker movement on c-Fos protein expression in the somatosensory cortex. c-Fos expression is increased by somatodendritic depolarization and is commonly used as a marker for neuronal excitation (Morgan *et al*., 1987; Morgan and Curran, 1988; Patel and Hillard, 2003). Vehicle, WIN55212-2 (1 μM) or rimonabant (1 μM) was administered subdurally, followed by whisker movement on one side, following the same pattern as used in the LDF studies. Two hours later, both cortices were removed and

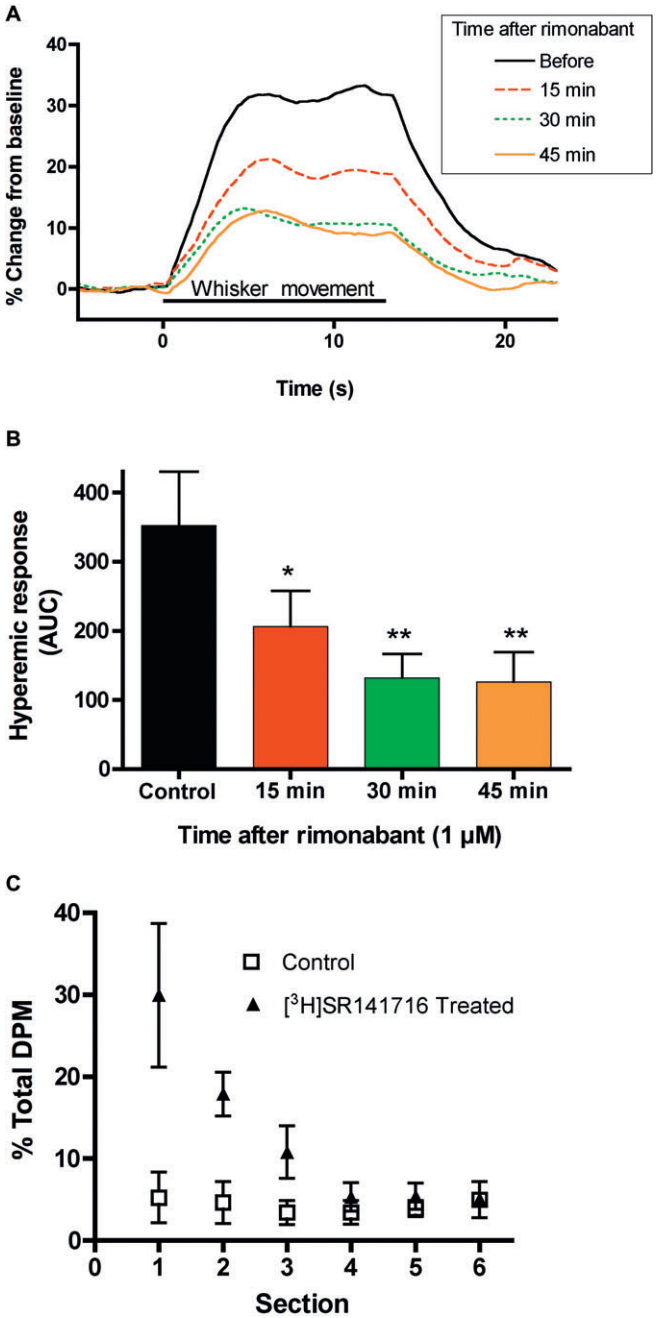

Figure 5 Rimonabant (SR141716A) inhibited the hyperaemic response to whisker movement through a local effect. Shown are the average laser-Doppler tracings from all subjects (A) and the corresponding magnitude of functional hyperaemic responses (B) before, and at regular time intervals, after 15 min exposure of the whisker-barrel cortex to 1 μM rimonabant (n = 5). *P < 0.05, **P < 0.01 from control, repeated measures ANOVA followed by Dunnett's *post hoc* tests. (C) Distribution of radioactivity after subdural application of [^{3}H]-SR141716A in the whisker-barrel cortex. One hemisphere was exposed to [^{3}H]SR141716A ([^{3}H SR])whereas the other hemisphere of the same brain was used as the control (n = 2). Each hemisphere was separately sliced into 1 mm sections from top (section 1) to bottom (section 6).

processed for c-Fos immunohistochemistry. Representative photomicrographs of a region of primary sensory cortex of rats from each treatment group are shown in Figure 7A–C. A high density of Fos-LI positive nuclei can be seen in cell layers close to the brain surface (bottom of the images) and in the inner-most layers. A higher magnification image of layer 2/3 from a rat treated with WIN55212-2 is shown in Figure 7D. This image shows expression of Fos occurs in neurons with pyramidal shape (an example is indicated by an arrow), consistent with Fos expression in principal neurons of the sensory cortex.

Two-way ANOVA demonstrated significant effects of both hemisphere ($F_{(1,30)}$ = 54, P < 0.0001) and drug administration ($F_{(2,30)}$ = 10.7, P = 0.0003). Whisker movement produced a significant increase in the number of cells that were Fos-LI in the sensory cortex contralateral to the side of the stimulated whiskers, consistent with neuronal activation in the whisker barrel cortex. The presence of rimonabant (1 μM) markedly reduced Fos expression induced by whisker movement, whereas WIN55212-2 (1 μM) had no significant effect on the number of Fos-LI positive cells, but appeared to increase the intensity of Fos staining in individual cells compared to vehicle (Figure 7A,B).

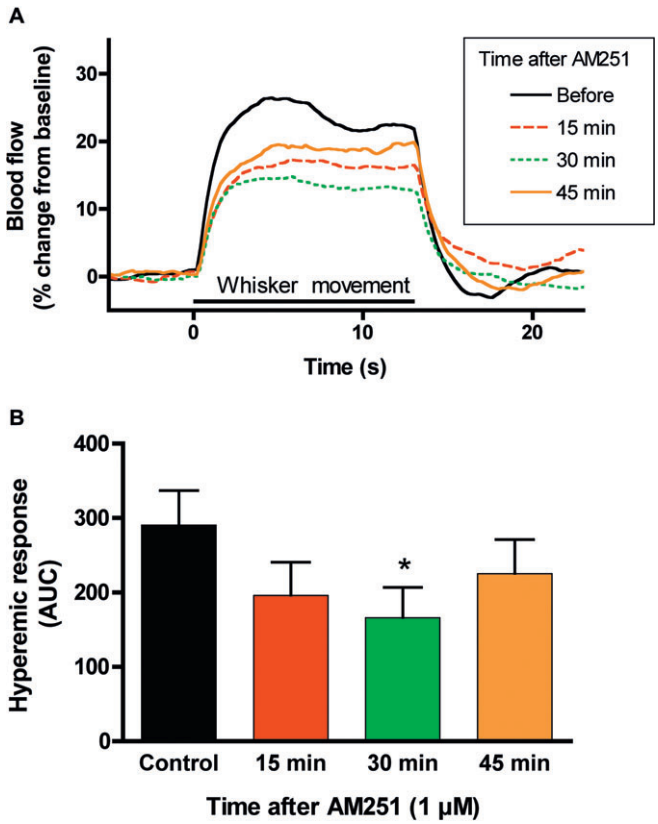

Figure 6 AM251 inhibits the hyperaemic response to whisker movement. (A) The average laser-Doppler tracings and (B) corresponding magnitude of functional hyperaemic responses before, and at regular time intervals, after 15 min exposure of the whisker-barrel cortex to 1 μM AM251 (n = 7). *P < 0.05 from control, repeated measures ANOVA followed by Dunnett's *post hoc* tests. Vertical lines represent SEM.

Effect of whisker movement on endocannabinoid content in the sensory cortex

The effect of rimonabant to reduce the LDF and c-Fos responses to whisker movement without an effect on the basal LDF response suggests that endocannabinoid signalling is mobilized by whisker movement. To test this hypothesis, whiskers on one side were moved for an extended period (30 min), followed by rapid removal of both sensory cortices. Lipids were extracted and AEA and 2-AG were quantified using isotope dilution, liquid chromatography-mass spectrometry (Figure 8). The tissue content of 2-AG was significantly greater in the sensory cortex contralateral to the moved whiskers, compared to the ipsilateral cortex (paired t-test, t = 4.9, P = 0.017, n = 4). The tissue contents of AEA were not significantly different (paired t-test, t = 0.21, P = 0.13, n = 4).

Table 1 Basal blood flow and arterial blood pressure before and after drug treatments

	Basal blood flow (tpu)				*Arterial blood pressure (mm Hg)*			
	Time after exposure to drug				*Time after exposure to drug*			
Drug	Control	15 min	30 min	45 min	Control	15 min	30 min	45 min
Vehicle†	17 ± 1	14 ± 3	15 ± 3	14 ± 2	96 ± 2	92 ± 4	92 ± 3	95 ± 4
WIN55212-2 (100 nM)	14 ± 2	9 ± 1**	8 ± 1**	8 ± 1**	99 ± 4	98 ± 3	98 ± 3	97 ± 2
WIN55212-2 (1 μM)	18 ± 2	12 ± 3	11 ± 2*	13 ± 3	95 ± 2	93 ± 1	94 ± 1	92 ± 1
WIN55212-3 (1 μM)	18 ± 4	13 ± 3	14 ± 4	16 ± 5	97 ± 1	97 ± 1	97 ± 1	96 ± 1
Rimonabant (1 μM)	20 ± 3	21 ± 2	27 ± 6	27 ± 7	96 ± 4	92 ± 3	91 ± 5	106 ± 8
AM251 (1 μM)	10 ± 1	9 ± 2	9 ± 1	10 ± 1	99 ± 2	97 ± 1	99 ± 1	99 ± 2
SR144528 (1 μM)	11 ± 1	11 ± 1	11 ± 1	12 ± 1	98 ± 2	99 ± 1	97 ± 1	98 ± 2
GABA (200 μM) + vehicle§	11 ± 2	9 ± 1	10 ± 1	11 ± 1	93 ± 3	94 ± 2	93 ± 1	92 ± 2
GABA (200 μM) + WIN55212-2 (1 μM)	11 ± 1	9 ± 1	9 ± 1	10 ± 1	95 ± 1	96 ± 1	95 ± 2	96 ± 2
BIC (100 μM) + vehicle§	18 ± 3	24 ± 4	15 ± 2	13 ± 1	97 ± 4	96 ± 5	91 ± 4	93 ± 4
BIC (100 μM) + WIN55212-2 (1 μM)	18 ± 2	23 ± 3	18 ± 4	26 ± 7	102 ± 5	104 ± 3	100 ± 3	98 ± 3

Data are given as mean ± SEM, n = 4–7.
*P < 0.05, **P < 0.01 from control using repeated measures ANOVA followed by Dunnett's *post hoc* tests.
†Vehicle for SR141716, AM251, SR144528.
§Vehicle for WIN55212-2.
BIC, bicuculline; tpu, tissue perfusion unit.

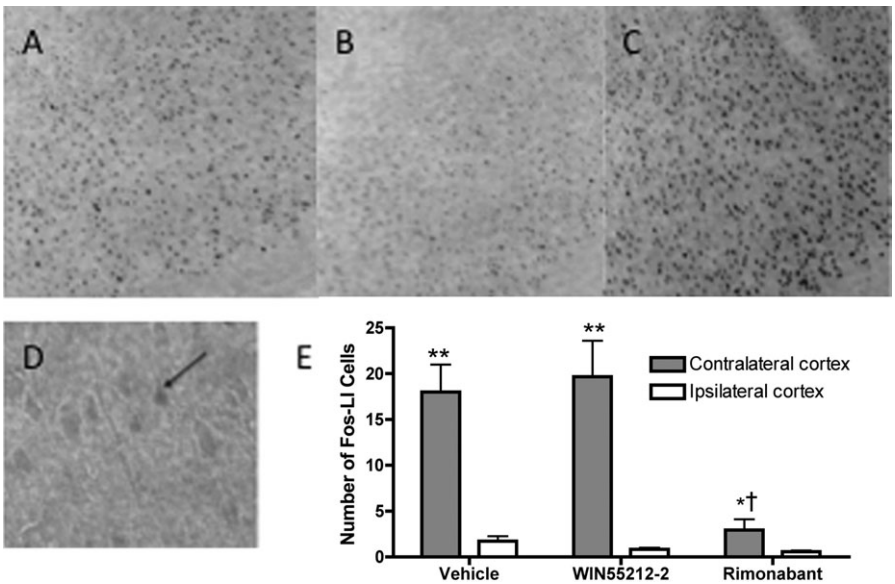

Figure 7 Whisker movement increases c-Fos-LI in the whisker-barrel cortex contralateral to the whiskers that were moved. Shown in A-C are representative images of sections of sensory cortex contralateral to the stimulated whiskers stained for c-Fos (10× magnification). The bottom of the images corresponds to the cortical surface. (A) Vehicle treated; (B) rimonabant (1 μM); and (C) WIN55212-2 (1 μM). In (D), a representative c-Fos-LI positive neuron is shown at the arrow (40× magnification). Grouped data from 6 rats are shown in E. *$P < 0.05$, **$P < 0.01$ from ipsilateral cortex of the same animals; †$P < 0.01$ from vehicle; one-way ANOVA followed by Dunnett's *post hoc* tests. Vertical lines represent SEM.

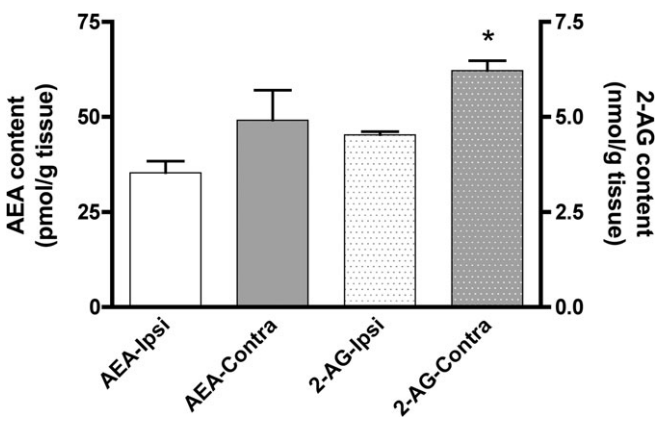

Figure 8 Whisker movement increases 2-AG, but not AEA, content in the contralateral whisker-barrel cortex, compared to the ipsilateral cortex ($n = 4$). *$P < 0.05$ from ipsilateral cortex from the same animals; Student's paired *t*-tests.

Discussion and conclusions

This study demonstrates that both exogenous and endogenous activation of CB_1 receptors modulate functional hyperaemic responses during activation of the rat primary somatosensory cortex in anaesthetized rats. CB_1 receptor antagonists attenuated, while a CB_1 receptor agonist, WIN55212-2, potentiated the increases in blood flow to the sensory cortex resulting from mechanical simulation of whiskers. WIN55212-2 potentiation was occluded by the $GABA_A$ antagonist BIC and did not occur in the presence of GABA, consistent with CB_1 receptor-mediated inhibition of GABA release. Furthermore, the CB_1 receptor antagonist, rimonabant, significantly reduced c-Fos activation in the sensory cortex induced by whisker movement. Taken together, these data are consistent with the hypothesis that activation of CB_1 receptors increases neuronal activity, which results in increased metabolic drive for regional blood flow. Furthermore, our data are consistent with CB_1 receptor-mediated inhibition of GABA release, thus providing an *in vivo* example of activity-dependent, endocannabinoid inhibition of GABA release. These findings are important as they provide evidence that endocannabinoid modulation of synaptic transmission occurs *in vivo* and that endocannabinoid signalling can be evoked by physiologically relevant increases in neuronal activity in adult rodents.

In considering the mechanisms of action, it is important to note that the route of administration we have used here avoided systemic effects of these agents. Arterial blood pressure remained unchanged when the hyperaemic response was significantly increased or decreased by modulators of the cannabinoid system. The distribution of radiolabelled rimonabant ([^{3}H]SR141716A) after subdural application was also consistent with a local site of action.

Exogenous, local CB_1 receptor activation using WIN55212-2 placed into the cranial window enhanced the whisker-stimulated hyperaemic response. In support of the involvement of CB_1 receptors, WIN55212-3, an enantiomer of WIN55212-2 with no affinity for CB receptors (Felder *et al.*, 1992), had no significant effect while application of two CB_1 receptor antagonists, rimonabant and AM251, profoundly inhibited the hyperaemic response. The CB_2 receptor antagonist, SR144528, had no significant effect. Although both rimonabant and AM251 are inverse agonists of CB_1 receptors (MacLennan *et al.*, 1998; New and Wong, 2003), their effects are more likely due to blockade of endocannabinoid

-mediated activation of the receptor. We examined this issue directly by measuring the tissue content of two of the best studied endocannabinoids: AEA and 2-AG. In this experiment, whiskers were moved for a long period to time to ensure a strong and sustained neuronal activation in the sensory cortex. We found that this protocol results in a significant increase in the tissue content of 2-AG but not AEA. These data are consistent with abundant data suggesting that activity- and CB_1 receptor-dependent changes in synaptic activity are mediated by 2-AG mobilization [(Pan *et al.*, 2009), for example].

To further examine the role of neuronal activity in cannabinoid-induced modulation of hyperaemic responses, we utilized Fos expression as an index of neuronal excitation (Morgan *et al.*, 1987; Morgan and Curran, 1988; Patel and Hillard, 2003). As expected, whisker movement greatly increased the density of Fos-LI cells in whisker barrel cortex. The finding that rimonabant caused a marked reduction of Fos expression is consistent with its profound inhibition of hyperaemic responses and supports a neuronal site of action for endocannabinoids in the regulation of local blood flow. Interestingly, WIN55212-2 treatment potentiated hyperaemic responses, but did not increase the number of neurons positive for Fos expression compared to those in the vehicle treated whisker barrel cortex. As we measured the *number* of Fos-LI positive cells; it is possible that WIN55212-2 treatment increased the excitatory drive on PN, increasing the *rate* of action potential firing by individual neurons, without changing the absolute number of PN that were active.

Taken together, these data are consistent with a mechanism by which whisker movement recruits endocannabinoid/CB_1 receptor signalling which modulates neurotransmission in the sensory cortex, which in turn affects the metabolic drive for blood flow. Cortical glutamate-mediated transmission is a major determinant of neuronal energy consumption and hence local blood flow (Bonvento *et al.*, 2002). There is strong evidence that endocannabinoids are synthesized and released from glutamatergic neurons in response to increased metabotropic glutamate receptor activation and depolarization (Freund *et al.*, 2003). The endocannabinoids released act locally as retrograde messengers, inhibiting transmitter release via presynaptic CB_1 receptors on both glutamatergic and GABAergic terminals (e.g. Kreitzer and Regehr, 2001; Fortin and Levine, 2007). If the predominant effect of CB_1 receptor activation is to reduce glutamate release, CB_1 receptor agonists would be expected to reduce the hyperaemic response. However, the opposite was observed in this study, suggesting that CB_1 receptor activation reduces GABA input onto glutamatergic neurons and thereby enhances neuronal activity and increases local blood flow. Anatomical studies reveal that a high density of CB_1 receptors are expressed in GABAergic interneurons in cortical areas (Katona *et al.*, 1999; Marsicano and Lutz, 1999; Hajos *et al.*, 2000; Bodor *et al.*, 2005). Activation of presynaptic CB_1 receptors has been shown to inhibit GABA release and reduce $GABA_A$ receptor-mediated, inhibitory postsynaptic currents in brain slices *ex vivo* (Hajos *et al.*, 2000; Hoffman and Lupica, 2000; D'Amico *et al.*, 2004). Importantly, GABAergic neurons appear to respond more vigorously to whisker stimulation than their glutamatergic counterparts and they exert a strong tonic inhibition on cortical excitability (Simons, 1978; Connors and Gutnick, 1990; McCasland and Hibbard, 1997). In support of this hypothesis, the $GABA_A$ receptor antagonist bicuculline attenuated the potentiation of hyperaemic responses induced by WIN55212-2. Interestingly, application of bicuculline alone also tended to potentiate the hyperaemic response, supporting the notion that whisker movement activates GABAergic signalling. We also found that direct application of GABA significantly reduced the hyperaemic response, as would be expected. Importantly, the presence of GABA blocked the WIN55212-2 potentiation of hyperaemia. Taken together, these data support the hypothesis that endocannabinoids are produced in the sensory cortex during whisker movement and activate CB_1 receptors on GABAergic terminals, resulting in reduced GABA release. This results in local disinhibition of glutamatergic transmission and an enhanced hyperaemic response. It is also possible that endocannabinoid -mediated modulation of other neurotransmitter systems, such as 5-HT (Nakazi *et al.*, 2000), could contribute to the alterations in hyperaemic response observed in the present study. The precise synaptic mechanisms underlying endocannabinoid modulation of sensory-evoked cortical activity in the mature cortex remains to be determined.

In contrast to results from the current study, we have previously reported that intravenous administration of rimonabant (SR141716; 1 $mg \cdot kg^{-1}$) significantly potentiated the hyperaemic response to whisker movement (Patel *et al.*, 2002). The CB_1 receptor is widely expressed by neurons in the brain (Herkenham *et al.*, 1991) but is also found in astrocytes (Sanchez *et al.*, 1998; Rodriguez *et al.*, 2001); cerebral arteries (Gebremedhin *et al.*, 1999; Rademacher *et al.*, 2005) and cerebral endothelial cells (Maccarrone *et al.*, 2006). All of these cell types play a role in the regulation of cerebral blood flow. Activation of CB_1 receptors in vascular smooth muscle cells dilates cerebral arteries and increases cerebral blood flow *in vitro* and *in vivo* (Gebremedhin *et al.*, 1999; Wagner *et al.*, 2001).

WIN55212-2 significantly reduced basal blood flow at the same concentrations that increased the hyperaemic response to whisker movement. The effect of WIN55212-2 to reduce basal blood flow was not shared by WIN55212-3. In light of previous studies showing that CB_1 receptors are expressed by cells of the microvasculature of the brain (Maccarrone *et al.*, 2006) and that CB_1 receptor agonists affect neurovascular coupling in nucleus accumbens (Cheer *et al.*, 2006), these data suggest that non-neuronal CB_1 receptor activation could contribute to the mechanism of action of WIN55212-2 to alter functional hyperaemia. Since the direction of change is not the same (i.e. WIN55212-2 reduced basal but increased whisker-stimulated blood flow) and in light of the Fos data discussed above, we conclude that direct effects on the vasculature play a minor role in the mechanism of action of WIN55212-2. It is interesting to note that WIN55212-2 had no effect on basal blood flow when either GABA or bicuculline were also added. It is possible that the effect of WIN55212-2 on basal blood flow is also the result of inhibition of neuronal GABA release; thus the effect is reversed by GABA and occluded by bicuculline. A lack of effect of BIC argues against this possibility; however, further studies are needed to clarify

the contribution of vascular CB_1 receptors to the response observed.

In conclusion, the results of this study are consistent with the hypothesis that activation of the CB_1 receptors by endocannabinoids positively modulates the recruitment of blood flow during neuronal stimulation in the rat somatosensory cortex. We hypothesize that increases in synaptic activity elevates synaptic endocannabinoid content, most likely as 2-AG, which acts via presynaptic CB_1 receptors on GABAergic interneurons to inhibit GABA release. These studies add to our understanding of the role of endocannabinoid signalling in normal function of brain circuitry, as they suggest that a physiologically relevant stimulus recruits endocannabinoid signalling and that this contributes to appropriate neuronal activation. It is possible that the mechanism identified in these studies is responsible for the effects of Δ^9-tetrahydrocannabinol to disrupt sensory processing in cats (Wilkison *et al*., 1982; Pontzer *et al*., 1986; Pontzer and Wilkison, 1987) and humans (Edwards *et al*., 2009). Furthermore, it is possible that this mechanism could have relevance to the connection between cannabis use and increased susceptibility for the development of schizophrenia (D'Souza, 2007), as impairment of sensory gating is thought to mirror the abnormal information processing that occurs in schizophrenia (Freedman *et al*., 2003).

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Statement of conflicts of interest

None.

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