0022-3565/07/3201-64–71$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 320:64–71, 2007
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Vol. 320, No. 1
106971/3160500
Printed in U.S.A.

Cannabinoid (CB_1) Receptor Activation Inhibits Trigeminovascular Neurons

Simon Akerman, Philip R. Holland, and Peter J. Goadsby

Headache Group, Institute of Neurology and the National Hospital for Neurology and Neurosurgery, Queen Square, London, United Kingdom

Received April 29, 2006; accepted October 2, 2006

ABSTRACT

Migraine is a common and disabling neurological disorder that involves activation or the perception of activation of the trigeminovascular system. Cannabinoid (CB) receptors are present in brain and have been suggested to be antinociceptive. Here we determined the effect of cannabinoid receptor activation on neurons with trigeminovascular nociceptive input in the rat. Neurons in the trigeminocervical complex (TCC) were studied using extracellular electrophysiological techniques. Responses to both dural electrical stimulation and cutaneous facial receptive field activation of the ophthalmic division of the trigeminal nerve and the effect of cannabinoid agonists and antagonists were studied. Nonselective CB receptor activation with *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl) (WIN55,212; 1 mg kg^{-1}) inhibited neuronal responses to A- (by 52%) and C-fiber (by 44%) afferents, an effect blocked by the CB_1 receptor antagonist SR141716 [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; 3 mg kg^{-1}] but not the CB_2 receptor antagonist AM630 (6-iodopravadoline; 3 mg kg^{-1}). Anandamide (10 mg kg^{-1}) was able to inhibit both A- and C-fiber-elicited TCC firing, only after transient receptor potential vanilloid 1 receptor inhibition. Activation of cannabinoid receptors had no effect on cutaneous receptive fields when recorded from TCC neurons. The data show that manipulation of CB_1 receptors can affect the responses of trigeminal neurons with A- and C-fiber inputs from the dura mater. This may be a direct effect on neurons in the TCC itself or an effect in discrete areas of the brain that innervate these neurons. The data suggest that CB receptors may have therapeutic potential in migraine, cluster headache, or other primary headaches, although the potential hazards of psychoactive side effects that accompany cannabinoid treatments may be complex to overcome.

Migraine, which is an episodic brain disorder that affects approximately 15% of the population (Steiner et al., 2003), can be highly disabling (Menken et al., 2000) and has been estimated to be the most costly neurological disorder in the European Community at more than €27 billion per year (Andlin-Sobocki et al., 2005). The pathophysiology of migraine is not fully understood, although it is thought to involve activation or the perception of activation of the trigeminal afferents that densely innervate dural structures (Goadsby et al., 2002). Stimulation of dural structures is painful in humans (Ray and Wolff, 1940), and in animal models, stimulation of dural sites, including the superior sagittal sinus and middle meningeal artery, results in neuronal activation in the trigeminovascular system. This activation is significantly inhibited by acute antimigraine drugs, and inhibition of neuronal firing is highly predictive of antimigraine potential (Lambert et al., 1992; Cumberbatch et al., 1998). Therefore, inhibition of trigeminal nerve activation is helpful in evaluating new targets for the treatment of migraine.

There are two cloned cannabinoid receptors; the CB_1 receptor is present on neurons in the brain and peripheral nervous system (Matsuda et al., 1990), and the CB_2 receptor is found predominantly in immune cells (Munro et al., 1993). These receptors are negatively coupled to $G_{i/o}$ proteins that inhibit adenylyl cyclase (Felder et al., 1993), block voltage-dependent Ca^{2+} channels (Twitchell et al., 1997), and activate voltage-dependent K^+ channels (Mackie et al., 1995). The commonly known effects of the cannabinoids, as highlighted in animal studies, are loss of concentration, catalepsy, hypothermia, depression of motor activity, and importantly, antinociception (Crawley et al., 1993; Smith et al., 1994).

Cannabinoids may have therapeutic use in pain, and cer-

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.106971.

ABBREVIATIONS: CB, cannabinoid; AM404, *N*-(4-hydroxyphenyl) arachidonylethanolamide; AM630, 6-iodo-pravadoline; MMA, middle meningeal artery; TCC, trigeminocervical complex; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; WIN55,212, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2, 3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl); DMSO, dimethyl sulfoxide; TRPV, transient receptor potential vanilloid.

tainly, they have been suggested to be of use in treating migraines in both 19th century Western and non-Western cultures (Russo, 1998). CB_1 receptors are widely distributed throughout the central nervous system, including the dorsal root ganglia and spinal dorsal horn (Hohmann and Herkenham, 1999; Farquhar-Smith et al., 2000). In vitro studies indicate that there are CB_1-immunoreactive cells in the spinal trigeminal tract and nucleus (Tsou et al., 1998), with a distribution suggesting input predominantly from the mandibular and maxillary divisions of the trigeminal nerve (Price et al., 2003, 2004). In vivo studies have shown that spinal application of anandamide, one of several endogenous cannabinoid agonist, inhibits nociceptive firing of both Aδ- and C-fiber mediated neurons (Harris et al., 2000; Kelly and Chapman, 2001). Furthermore, activation of the ophthalmic division of the trigeminal nerve, specifically corneal afferents, increased the number of Fos-positive neurons in the spinal trigeminal nucleus, and this response was significantly inhibited by CB_1-specific receptor activation (Bereiter et al., 2002).

The ophthalmic division of the trigeminal nerve also supplies inputs to the dura mater, and activation of these fibers causes vasodilation of dural blood vessels (Akerman et al., 2004b). We have shown that CB_1 receptor activation inhibits the dural vessel dilation caused by electrical stimulation at both pre- and postjunctional sites (Akerman et al., 2004b). In the present study, we wished to examine the effect of cannabinoid receptor activation centrally after stimulation of the ophthalmic division of the trigeminal nerve. We employed electrical stimulation of the dura mater adjacent to the middle meningeal artery and facial cutaneous stimulation to activate trigeminal afferents and recorded neuronal responses in the trigeminocervical complex. We studied the response of trigeminal neurons to cannabinoid receptor activation. The work has been presented in preliminary form at the XIth International Headache Congress in Kyoto, Japan (October 2005).

Materials and Methods

Surgical Preparation. All experiments were conducted under a project license issued by the UK Home Office under the Animals (Scientific Procedures) Act (1986). Thirty-three male Sprague-Dawley rats (310–375 g) were anesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.; Sigma-Aldrich, Dorset, Poole, UK) for induction of anesthesia and maintained with α-chloralose (10–20 mg kg^{-1} h^{-1}) made up with 2-hydroxy-β-cyclodextrin. During electrophysiological recording, the animals were paralyzed with pancuronium bromide (0.4 mg kg^{-1}; Faulding, Royal Leamington Spa, Warwickshire, UK) initially and maintained with 0.4 mg kg^{-1} every 45 min. The left femoral artery and vein were cannulated for blood pressure recording and intravenous infusion of anesthetic and test compounds, respectively. Temperature was maintained throughout using a homeothermic blanket system (Harvard Apparatus Inc., Holliston, MA). The rats were ventilated with oxygen-enriched air (2–2.5 ml; 60–80 strokes per min; Small Rodent Ventilator, model 683; Harvard Apparatus, Kent, UK). End-tidal CO_2 was monitored (Capstar-100; CWE Inc., Ardmore, PA) and kept between 3.5 and 4.5%, and blood pressure was monitored continually. Depth of anesthesia was judged by the absence of paw withdrawal and corneal blink reflex and by the lack of fluctuations in blood pressure during muscular paralysis.

Middle Meningeal Artery and TCC Exposure. The rats were placed in a stereotaxic frame, the skull was exposed, and a craniotomy of the left parietal bone was performed with a saline-cooled drill to provide access to the dural middle meningeal artery (MMA). The exposed dural area was covered in mineral oil. The muscles of the dorsal neck were separated, a partial C_1 laminectomy was carried out, and the dura mater was incised to expose the brainstem at the level of the caudal medulla. The electrode was slowly lowered into the brainstem at 5-μm increments with a hydraulic microstepper (David Kopf Instruments, Tujunga, CA).

Stimulation of MMA and Recording from TCC. A bipolar stimulating electrode (NE 200; Clarke Electromedical Instruments, Edenbridge, Kent, UK) was placed on the dura mater adjacent to or on either side of the MMA, and square-wave stimuli (0.6 Hz) of 1-ms duration (8–20 V) was applied (S88 stimulator; Grass Instruments, Quincy, MA) to activate trigeminal afferents. Extracellular recordings were made from neurons in the TCC, activated by MMA-stimulation, or cutaneous facial receptive fields, with tungsten microelectrodes (impedance 0.5 MΩ, tip diameter 0.5 μm; WPI, Stevenage, Herts, UK). The signal from the recording electrode attached to a high-impedance headstage preamplifier (NL100AK; Neurolog, Digitimer, Herts, UK) was fed via an AC preamplifier (Neurolog NL104, gain × 1000) through filters (Neurolog NL125; bandwidth typically 300 Hz to 10 kHz) and a 50-Hz noise eliminator (Humbug, Quest Scientific, North Vancouver, BC, Canada) to a second-stage amplifier (Neurolog NL106) providing variable gain (×20 to ×90). This signal (total gain ×20,000 to ×95,000) was fed to a gated amplitude discriminator (Neurolog NL201) and an analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and to a microprocessor-based personal computer (Dell Latitude; Dell, Berkshire, UK) where the signal was processed and stored. Filtered and amplified electrical signals from action potentials were fed to a loudspeaker via a power amplifier (Neurolog NL120) for audio monitoring and were displayed on analog and digital-storage oscilloscopes (Goldstar; LG Precision, Seoul, Korea; and Metrix Electronics, Chauvin Arnoux, Paris, France) to assist the isolation of single unit activity from adjacent cell activity and noise. Post and peristimulus time histograms of neural activity were displayed and analyzed using Spike2 version 5 (Cambridge Electronic Design).

Characterization of Neurons. Neurons were characterized for their cutaneous and deep receptive fields. The cutaneous receptive field, including cornea, was assessed in all three territories of the trigeminal innervation and identified as the recording electrode was advanced in the spinal cord. The receptive field was assessed for both non-noxious, with gentle brushing, and noxious inputs; the latter was assessed by pinching with forceps or applying heavy pressure that was painful when applied to humans. When a neuron sensitive to stimulation of the ophthalmic dermatome of the trigeminal nerve was identified, it was tested for convergent input from the dura mater (Bartsch et al., 2004). According to the cutaneous receptive field properties, neurons were classified as low-threshold mechanoreceptors that responded only to innocuous stimulation, nociceptive-specific receptors that responded to only noxious input, or wide-dynamic range that responded to both noxious and non-noxious stimuli (Hu et al., 1981).

Experimental Protocol. Trains of 20 stimuli were delivered at 5-min intervals to assess the baseline response to dural electrical stimulation. Responses were analyzed using poststimulus histograms with a sweep length of 100 ms and a bin width of 200 μs, which separated Aδ-fiber and C-fiber-activated firing (5–20 and 20–100 ms), respectively. Spontaneous activity (spikes per second, Hz) was recorded for 120 to 180 s preceding the dural stimulation using peristimulus histogram. Once it had been established that there was a TCC neuronal response to dural stimulation and a cutaneous receptive field input in the ophthalmic division of the trigeminal nerve, the responses were tested before and after drug intervention using the following experimental protocols: 1) three baseline collections of dural stimulation and a single receptive field characterization; 2) agonist drug intervention; 3) dural stimulation collections at 5-, 10-, 15-, 20-, 25-, 30-, and 45-min intervals, with a receptive field characterization after 15 min; 4) antagonist drug intervention

(where appropriate); 5) dural stimulation collection at 5 min followed immediately by a repeat of agonist drug intervention; and 6) same as 3.

In addition, there was a separate series of control experiments using the protocol above, with the solvents used to dissolve the test compounds saline, Tocrisolve (Tocris Bioscience, Bristol, UK), 45% w/v solution of 2-hydroxy-β-cyclodextrin, and a 1% solution of DMSO diluted in 1:1:8 solution of Tween 80/ethanol/0.9% NaCl.

Data Analysis. Data collected for A-fiber inputs represents the number of cells firing over a 10-ms time period in the region 5 to 20 ms poststimulation over the 20 collections. Likewise, data collected for C-fiber inputs represents the number of all cells firing over 80 ms (20–100 ms poststimulation) from the 20 collections. Spontaneous activity was measured in cell firings per second (in Hertz). Analysis of variance for repeated measures with Bonferroni's post hoc correction for multiple comparisons applied was used to measure the time cause of significant drug intervention, which included the three baselines. If Mauchly's test of sphericity was violated, we made appropriate corrections to degrees of freedom according to Greenhouse-Geisser (Field, 2000). Student's paired t test was used for post hoc analysis of the significance of individual time points, using the average of the three baselines for comparison (version 11.0; SPSS, Inc., Chicago, IL). Statistical significance was set at $P < 0.05$.

Drugs. The infusion of anesthetic and experimental drugs were all through the same femoral catheter intravenously in a volume of 0.2 to 0.3 ml; however, the line was always flushed with saline first, several min before administering the different compound. Anandamide (arachidonylethanolamide) and AM404 [*N*-(4-hydroxyphenyl)-arachidonylethanolamide] (both from Tocris Bioscience) came prepared in a soya oil/water (1:4) water-soluble emulsion (Tocrisolve), which was further diluted in water for injection. It has been shown previously that dosing of anandamide between 1 and 10 mg kg^{-1} is antinociceptive in the rat, and this effect can last up to 30 min (Adams et al., 1995); we chose to use 10 mg kg^{-1} because this was the most effective inhibitory dose in the neurogenic dural vasodilation model (Akerman et al., 2004a). SR141716 [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide], a gift of National Institute of Mental Health (Bethesda, MD), AM630 (6-iodo-pravadoline), and capsazepine (Tocris Bioscience) were initially dissolved in 0.05 ml of DMSO (Sigma-Aldrich) and made up to a 1% solution of DMSO with a 1:1:8 solution of Tween 80 (polyoxyethylene-sorbitan mono-oleate; Sigma-Aldrich), ethanol, and 0.9% NaCl. WIN55,212 [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-yl]-(1-naphthalenyl))methanone; Tocris Bioscience) was dissolved in a 45% w/v solution of 2-hydroxy-β-cyclodextrin (Sigma-Aldrich). The dose of WIN55,212 of 1 mg kg^{-1} was selected, taking into account its greater potency compared with anandamide, and data showed effects in neuropathic pain at a dose range of 0.5 to 2.5 mg kg^{-1} (LaBuda and Little, 2005).

Results

Recordings were made from 20 neurons for the control studies and 26 neurons for the active drug studies. These were responsive to dural stimulation, all classified as wide dynamic range with cutaneous receptive fields restricted to the first (ophthalmic) division of the trigeminal nerve, including the cornea. Neurons were found in laminae III-VI of the TCC at a depth between 380 and 1135 μm (Fig. 1). Units had a baseline firing latency after dural stimulation of 10 $\pm$ 0.8 and 63 $\pm$ 3 ms, respectively, for A- and C-fiber inputs. The baseline spontaneous firing rate was 23.3 $\pm$ 3 Hz.

Controls. Separate animals were used for the recordings for the control experiments, which were made from 20 neurons responsive to dural stimulation, all classified as wide dynamic range and with cutaneous receptive fields restricted to the first (ophthalmic) division of the trigeminal nerve, including the cornea. Intravenous injection of saline (A-fiber, $F_{2.37,9.47} = 1.11$; C-fiber, $F_{2.53,10.14} = 0.84$, $n = 5$), Tocrisolve (A-fiber, $F_{1.88,10.14} = 1.19$; C-fiber, $F_{2.78,11.12} = 0.94$, $n = 5$), 45% w/v solution of 2-hydroxy-β-cyclodextrin (A-fiber, $F_{1.98,7.91} = 0.97$; C-fiber, $F_{2.57,10.27} = 1.45$, $n = 5$), and a 1% solution of DMSO made up in a 1:1:8 solution of Tween 80/ethanol/0.9% NaCl (A-fiber, $F_{2.57,10.29} = 1.70$; C-fiber, $F_{2.64,10.56} = 0.88$, $n = 5$) did not significantly affect the Aδ- and C-fiber-mediated responses of trigeminal second-order neurons.

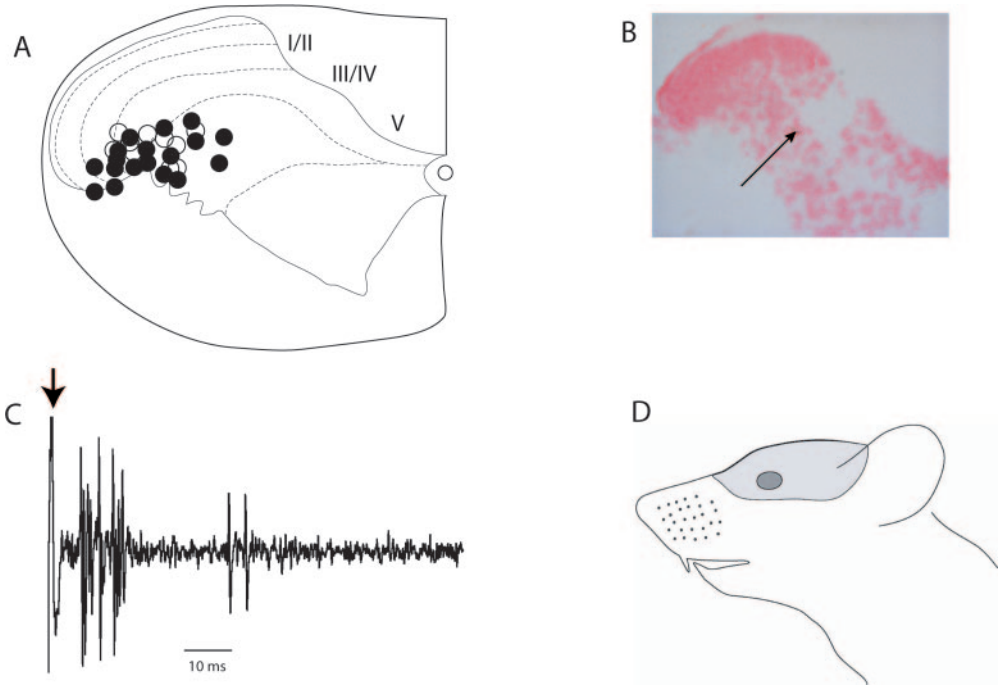

Fig. 1. The location of sites in the trigeminal nucleus caudalis from which recordings of nociceptive neurons, receiving convergent input from the dura mater and facial receptive field, were made. The locations were reconstructed from lesions (B, closed circles) or from microdrive readings (open circles). An original tracing from a typical unit responding to middle meningeal artery/dural stimulation (stimulus artifact arrow) is shown (C). All of the neurons studied were wide dynamic-type with receptive fields in the first (ophthalmic) division of the trigeminal nerve (D).

Activation of CB Receptor. WIN55,212 (1 mg kg^{-1}), a cannabinoid receptor agonist that activates both CB_1 and CB_2 receptors, was administered intravenously. Responses in the A-fiber input range were significantly inhibited over the 45-min course of the experiment ($F_{3.0,30.0} = 12.45$, $P < 0.001$, $n = 11$), with the maximal inhibition after 5 min of 52% ($t_{10} = 5.15$, $P < 0.05$, $n = 11$) that only returned to baseline after 45 min (Fig. 2A). Responses from C-fiber inputs were also inhibited ($F_{9,90} = 3.52$, $P < 0.05$, $n = 11$), with the maximal inhibition of 44% after 5 min ($t_{10} = 2.95$, $P < 0.05$, $n = 11$) that returned after 30 min (Fig. 2B). Spontaneous activity was also inhibited ($F_{3.0,29.8} = 6.47$, $P < 0.05$, $n = 11$), maximally by 58% after 5 min ($t_{10} = 3.85$, $P < 0.05$, $n = 11$). This returned after 30 min (Fig. 2C). There were no significant changes in responses to any of the three receptive fields tested, V_1 pinch ($t_6 = 0.64$, $P = 0.54$, $n = 7$), V_1 brush ($t_6 = 0.43$, $P = 0.68$, $n = 7$), and corneal brush ($t_6 = 0.73$, $P = 0.49$, $n = 7$).

Specific CB_1 and CB_2 Receptor Antagonists. When a specific CB_1 receptor antagonist, SR141716 (3 mg kg^{-1}), was given 5 min before treatment with the cannabinoid receptor agonist and the response of trigeminal neurons to dural stimulation was repeated over 45 min, there was no significant change in the responses to A-fiber input ($F_{3,15} = 1.03$, $P = 0.41$) or C-fiber input ($F_{2.1,11} = 1.1$, $P = 0.38$) or in spontaneous activity ($F_{2.9,14.7} = 0.78$, $P = 0.52$; Fig. 3, A–C). The response after administration of SR141617 alone compared with the control response was also not significant for A-fiber input ($t_5 = 0.71$, $P = 0.51$, $n = 6$), C-fiber input ($t_5 = 1.24$, $P = 0.27$, $n = 6$), or in spontaneous activity ($t_5 = 1.09$, $P = 0.33$, $n = 6$).

When a specific CB_2 receptor antagonist, AM630 (3 mg kg^{-1}), was given 5 min before treatment with the cannabinoid receptor agonist and the response of trigeminal neurons to dural stimulation was repeated over 45 min, there remained a significant inhibition in the responses to A-fiber input ($F_{2.1,8.5} = 8.17$, $P < 0.05$, $n = 5$) and C-fiber input ($F_{2.3,9.22} = 2.82$, $P < 0.05$, $n = 5$). Spontaneous activity was not significant ($F_{2.4,9.5} = 0.61$, $P = 0.52$, $n = 5$; Fig. 3, A–C). The response after administration of AM630 alone compared with the control response was not significant for A-fiber input ($t_4 = 1.5$, $P = 0.22$, $n = 5$), C-fiber input ($t_4 = -0.51$, $P = 0.64$, $n = 5$), or in spontaneous activity ($t_4 = 1.93$, $P = 0.13$, $n = 5$). Finally, there were no significant changes in responses in V_1 receptive field testing across the control, cannabinoid agonist pretreatment, and cannabinoid agonist and antagonist pretreatment trials ($n = 5$, data not shown).

Activation of Cannabinoid/Vanilloid Receptors with Anandamide. Anandamide (10 mg kg^{-1} i.v.) is one of several endogenous agonist of cannabinoid receptors and shows TRPV1 receptor agonist properties. It was administered, and the response of trigeminal neuronal firing to dural stimulation was examined. There was no significant change in the responses in the A-fiber range over the course of the experiment ($F_{2.6,18} = 1.75$, $P = 0.20$, Fig. 4A). There was a significant change in the response to C-fiber inputs in the TCC ($F_{3.4,24} = 5.19$, $P < 0.05$), with a significant inhibition of firing after 5 min ($t_7 = 3.37$, $P < 0.05$, $n = 8$). There was also a small steady increase in neurons with C-fiber inputs after 5 min that peaked at 40 min (Fig. 4B), but this was not significant. Spontaneous activity was also significantly reduced ($F_{3,21.3} = 3.54$, $P < 0.05$). There was a similar trend in

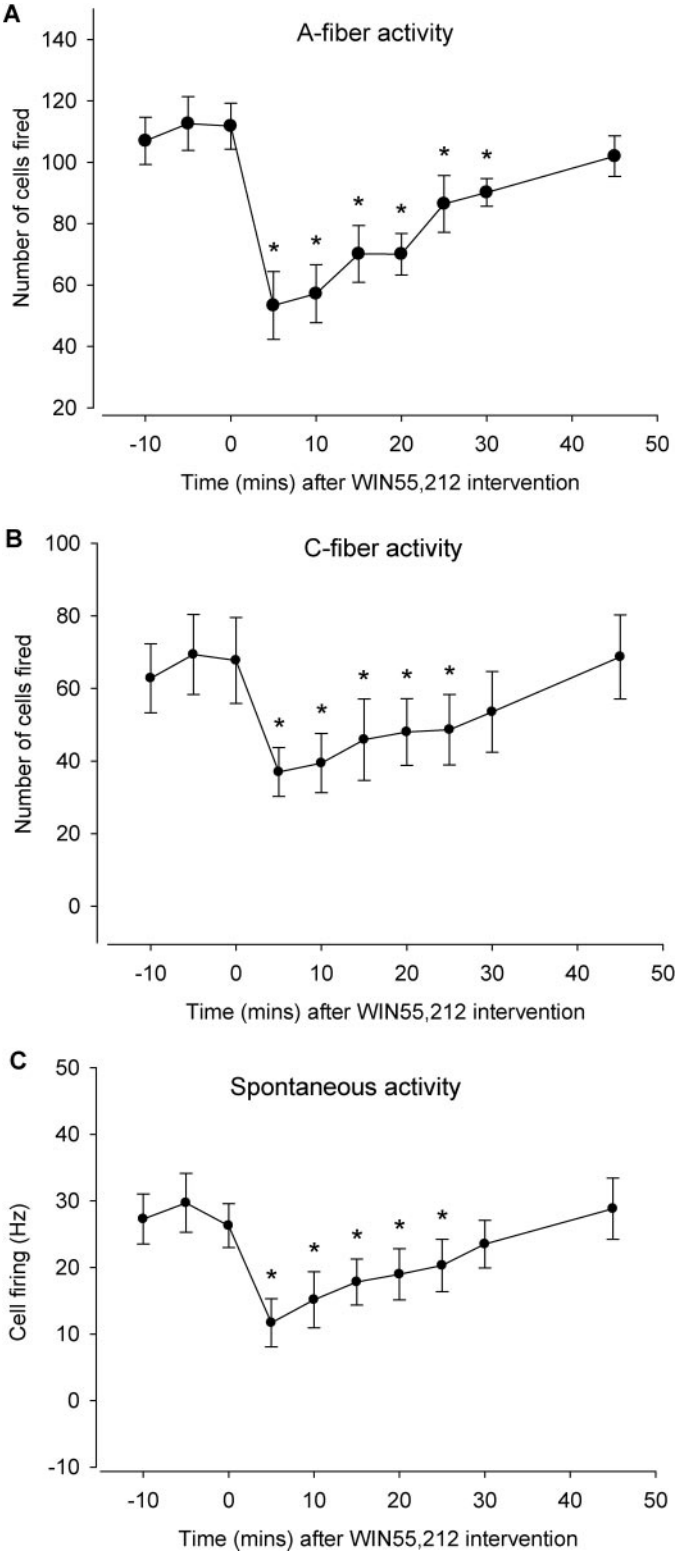

Fig. 2. Summary of changes in neural responses of second-order trigeminal neurons to injection of the cannabinoid receptor agonist, WIN55,212. After intravenous injection of WIN55,212, the excitability to electrical stimulation of the dura mater with respect to neurons with A-fiber (A) and C-fiber (B) inputs was significantly decreased. There was also a reduction in spontaneous activity (C) in the same region. Data are presented as mean ± S.E.M. *, $P < 0.05$ compared with an average of the three baselines, using Student's paired t test.

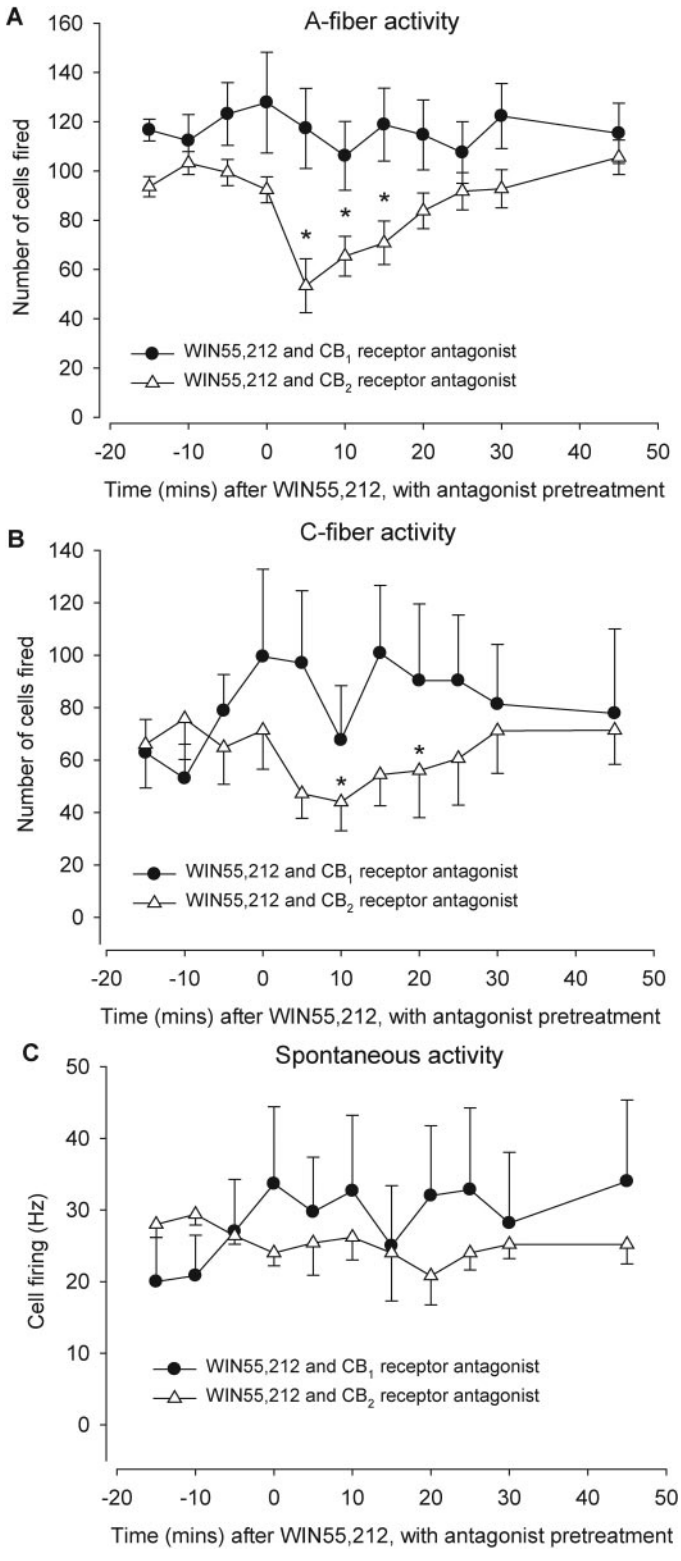

Fig. 3. Summary of changes in neural responses of second-order trigeminal neurons to pretreatment with both cannabinoid receptor agonist, WIN55,212 and either the specific CB_1 receptor antagonist SR141716 or the CB_2 receptor antagonist AM630. After intravenous injection of both SR141617 and WIN55,212, there was no significant change in neuronal firing with respect to neurons with A-fiber inputs (A) or C-fiber inputs (B) or in spontaneous activity (C). After intravenous injection of both AM630 and WIN55,212, neuronal firing with A-fiber (A) and C-fiber (B) inputs was significantly inhibited. Data are presented as mean ± S.E.M. ∗, $P < 0.05$ compared with an average of the three baselines, using Student's paired t test.

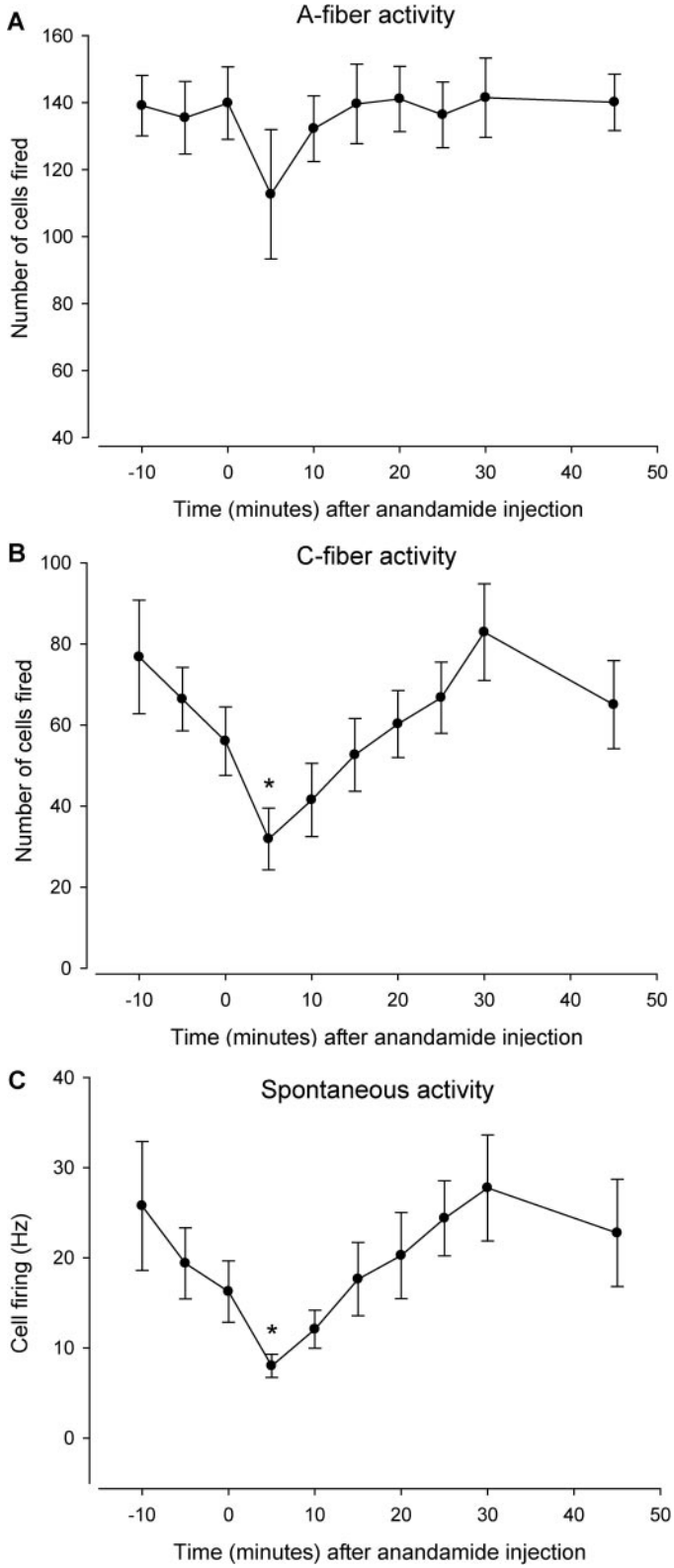

Fig. 4. Summary of changes in neural responses of second-order trigeminal neurons to treatment with the cannabinoid receptor agonist anandamide. After intravenous injection of anandamide (10 mg kg^{-1}), there was a significant decrease in the excitability to electrical stimulation of the dura mater of neurons with C-fiber input (B) and in spontaneous activity (C). There was no significant effect in responses from neurons with A-fiber input (A), although at the 5-min time point, there is a clear trend toward an effect. Data are presented as mean ± S.E.M. ∗, $P < 0.05$ compared with an average of the three baselines, using Student's paired t test.

spontaneous activity with a significant reduction in firing after 5 min ($t_7 = 2.60$, $P < 0.05$, $n = 8$, Fig. 4C) followed by a gradual nonsignificant increase in firing. There were no significant changes in the receptive field with anandamide, V_1 pinch ($t_7 = 0.82$, $P = 0.44$, $n = 8$), V_1 brush ($t_7 = -0.38$, $P = 0.72$, $n = 8$), and corneal brush ($t_7 = 0.74$, $P = 0.49$, $n = 8$).

TRPV1 Receptor Antagonist-Capsazepine. When the TRPV1-specific receptor antagonist capsazepine (3 mg kg^{-1}) was administered 5 min before anandamide, the response to neuronal firing was significantly reduced for neurons with A-fiber inputs from electrical stimulation of the dura mater ($F_{3,15} = 3.79$, $P < 0.05$). Specifically, significant reductions in firing at 5 ($t_5 = 3.41$, $P < 0.05$, $n = 6$), 10 ($t_5 = 4.50$, $P < 0.05$, $n = 6$), and 45 min ($t_5 = 2.82$, $P < 0.05$, $n = 6$) were observed (Fig. 5A). The response to C-fiber input was not significantly altered across the cohort ($F_{2.5,12.6} = 3.28$, $P = 0.063$). There was no significant effect of capsazepine on anandamide regarding spontaneous activity ($F_{1.4,6.9} = 0.74$, $P = 0.46$). The response after capsazepine alone after 5 min, just before the anandamide dose, compared with the control response was also not significant for A-fiber input ($t_5 = 1.13$, $P = 0.31$, $n = 6$), C-fiber input ($t_5 = 0.22$, $P = 0.84$, $n = 6$), or in spontaneous activity ($t_5 = 0.42$, $P = 0.69$, $n = 6$).

Injection of an Endocannabinoid Uptake Inhibitor. AM404 (3 mg kg^{-1}), the endocannabinoid uptake inhibitor, was administered intravenously, and dural stimulation was repeated over 45 min. There was no significant change in the response to A-fiber input ($F_{2,10} = 2.43$, $P = 0.14$, $n = 6$) or C-fiber input ($F_{2.2.10.6}$ 0.60, $P = 0.58$, $n = 6$) or in spontaneous activity ($F_{1.9,9.5} = 1.61$, $P = 0.25$, $n = 6$). There was no significant changes in responses to V_1 pinch ($t_4 = 1.0$, $P = 0.38$, $n = 5$), V_1 brush ($t_4 = 0.75$, $P = 0.50$, $n = 5$), and corneal brush ($t_4 = 0.65$, $P = 0.55$, $n = 5$).

Blood Pressure Effects. Anandamide (10 mg kg^{-1}) caused characteristic and significant triphasic blood pressure effect ($F_{2,10} = 22.08$, $P < 0.05$, $n = 6$), and phases II and III were not inhibited ($F_{2,6} = 18.52$, $P < 0.05$, $n = 4$) by capsazepine (3 mg kg^{-1}). WIN55,212 (1 mg kg^{-1}) caused a significant increase in blood pressure ($F_{2,20} = 3.81$, $P < 0.05$, $n = 11$), which was reversed by pretreatment with SR141716 (3 mg kg^{-1}, $F_{2,8} = 3.2$, $P = 0.1$, $n = 5$). AM404 (3 mg kg^{-1}) also caused significant triphasic blood pressure effects ($F_{2,8} = 60.63$, $P < 0.05$, $n = 6$), similar to that of anandamide. AM630 was unable to inhibit the blood pressure changes caused by WIN55,212 ($t_4 = 3.61$, $P < 0.05$, $n = 5$).

Discussion

In this study, we found that neurons in the TCC with input from the ophthalmic (first) division of the trigeminal nerve were inhibited by activation of the cannabinoid CB_1 receptor. Specifically, WIN55,212, a cannabinoid receptor agonist with activity at both CB_1 and CB_2 receptors, was able to inhibit significantly the activation of trigeminal neurons by electrical stimulation of the dura mater. WIN55,212 was able to inhibit neurons with A-fiber and C-fiber inputs, as well as spontaneous firing. There is evidence that WIN55,212 also acts at noncannabinoid receptors, evoking both calcitonin gene-related peptide release and trigeminal neuronal excitation in cell culture and inhibiting K^+-activated trigeminal neuronal firing (Price et al., 2004). It was demonstrated that

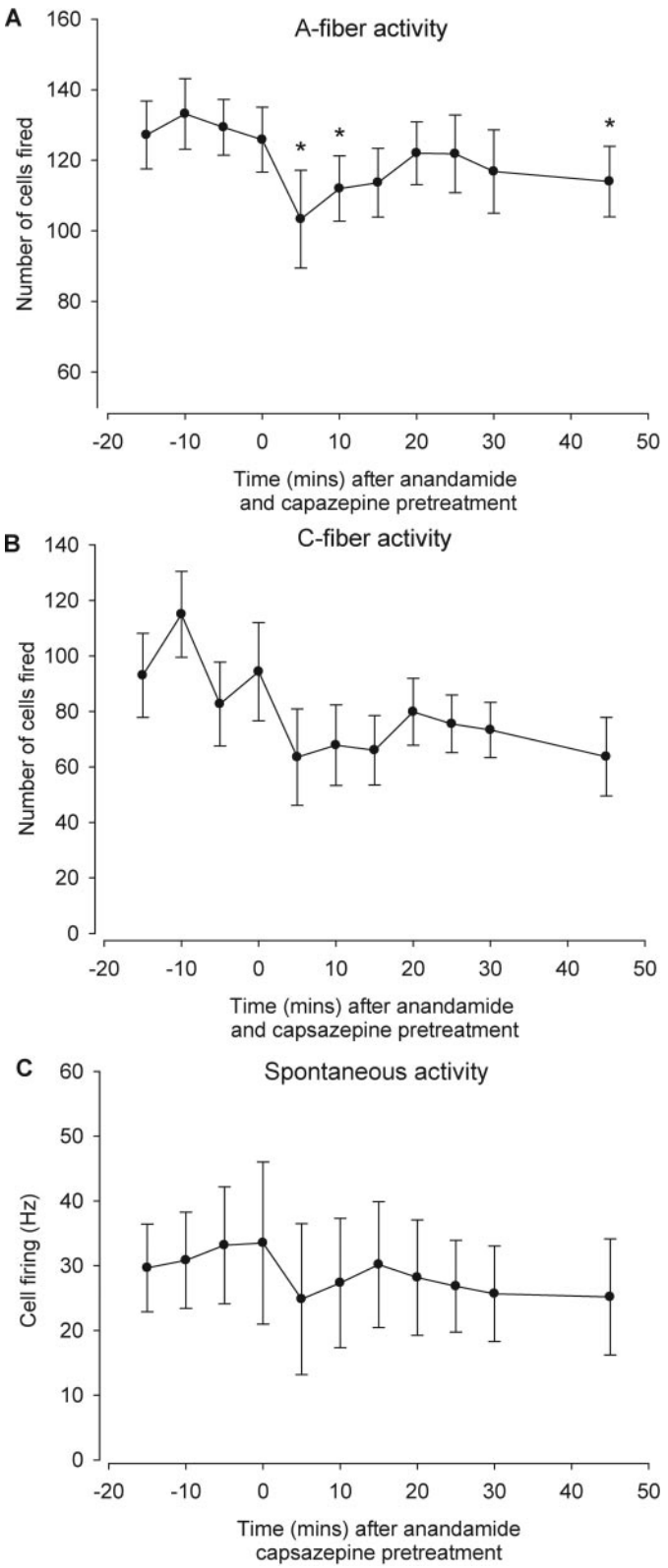

Fig. 5. Summary of changes in neural responses of second-order trigeminal neurons to pretreatment with both the cannabinoid receptor agonist anandamide and the specific TRPV1 receptor antagonist capsazepine. After intravenous injection of both anandamide and capsazepine, there was significant inhibition of firing compared with control responses after electrical stimulation of the dura mater in neurons with A-fiber (A) and C-fiber input (B). There was no significant effect on spontaneous activity (C). Data are presented as mean ± S.E.M. ∗, $P < 0.05$ compared with an average of the three baselines, using Student's paired t test.

inhibition of trigeminal firing was reversed by a specific CB_1 receptor antagonist, SR141716, and not by the CB_2 receptor antagonist AM630, indicating that the response observed is likely to be CB_1 receptor-mediated. These data offer a possible biological rationale to the historical observations that cannabinoid receptor activation has antimigraine effects (Russo, 1998).

Cannabinoid receptors have been found to be widespread in the brain and spinal cord of rats (Hohmann and Herkenham, 1999; Farquhar-Smith et al., 2000). In vitro studies have found CB_1 mRNA in the trigeminal ganglia with neurons that give rise to the mandibular and maxillary division of the trigeminal nerve (Price et al., 2003). CB_1 mRNA was predominantly found on large diameter myelinated fibers (Price et al., 2003). Another study demonstrates CB_1-like immunoreactivity in the deeper laminae of the trigeminal nucleus caudalis (Tsou et al., 1998), normally associated with inputs from the ophthalmic division of the trigeminal nerve and also predominantly associated with Aδ-fiber- and C-fiber-mediated activation (Millan, 1999). The physiologic effects observed compared with the mRNA data of Price et al. (2003, 2004) suggest differences between the trigeminal ganglion and cannabinoid influences on central trigeminal neurons. It is believed that the cardiovascular changes that take place after treatment with WIN55,212 are not directly responsible for the change in trigeminal neuronal firing, because similar and more dramatic changes occurred with both anandamide and AM404 and no significant alteration in firing was found.

The data presented suggest that CB_1 receptor activation is able to inhibit A- and C-fiber inputs to neurons that are activated by stimulation of the ophthalmic division of the trigeminal nerve and that have receptive field inputs from the ophthalmic dermatome. This is similar to neuronal responses at the L_4–L_5 level of the spinal cord, in response to innocuous and noxious stimuli (Harris et al., 2000; Kelly and Chapman, 2001). However, we found no changes in the neuronal firing of the cutaneous receptive field (V_1 pinch, brush and corneal brush) with cannabinoid receptor activation. Our data are consistent with other studies using in vivo techniques in both the TCC and other spinal regions. It was found in the cornea, which is densely innervated by Aδ- and C-fibers originating in the ophthalmic division of the trigeminal nerve (MacIver and Tanelian, 1993), that activation of corneal nociceptors by the application of mustard oil produced significant Fos-like immunoreactivity in the spinal trigeminal nucleus and in the subnucleus interpolaris/subnucleus caudalis (V_i/V_c) transition region of the trigeminal nucleus, and this response was only inhibited by topical application of specific CB_1 receptor agonists (Bereiter et al., 2002). In another study, recording of trigeminal nucleus caudalis neurons using electrical stimulation of characterized facial receptive field areas produced A-fiber- and C-fiber-mediated responses that were inhibited by bathing the brainstem area with WIN55,212, an effect partially reversed by application of a specific CB_1 receptor antagonist (Papanastassiou et al., 2004). It was also found that non-nociceptive neuronal firing was not inhibited by WIN55,212. This study did not discriminate between the divisions of the trigeminal nerve; however, they did demonstrate a role for neurons with Aδ- and C-fiber inputs in terms of modulation by WIN55,212.

Taking the literature together with the new data, it seems that in vivo cannabinoid receptor activation can modulate trigeminal neurons with ophthalmic division input from both A- and C-fibers. However, cannabinoid agonists do not act universally on neurons of the spinal trigeminal nucleus. Bereiter et al. (2002) and the data presented here are consistent with the notion that cannabinoid receptor activation does not affect neurons converging in the trigeminal nucleus caudalis/C_1 region of the trigeminal nucleus using cutaneous and corneal inputs. Whereas electrical stimulation of the ophthalmic division of the trigeminal nerve was influenced by cannabinoid receptor activation in the TCC region, the area was believed to be important for nociceptive inputs (Bereiter et al., 2002). However, mechanical stimulation of the ophthalmic division was influenced by cannabinoid activation in the V_i/V_c region, an area thought to be involved in the homeostasis and reflexive aspects of nociception (Bereiter et al., 2002), which is rostral to the TCC region. This may represent an important functional distinction between the two regions and thus an issue for further study. The trigeminovascular system also receives inputs from other higher brain centers, such as hypothalamus, thalamus, and periaqueductal gray (Bartsch et al., 2004; Shields and Goadsby, 2005); therefore, activation of cannabinoids in these sites may influence trigeminovascular neuronal firing.

Anandamide, an endogenous cannabinoid receptor agonist, was not able to affect trigeminal neurons with A-fiber inputs but did inhibit those with C-fiber inputs and spontaneous firing. When a TRPV1 receptor antagonist was used as a pretreatment to the anandamide response, trigeminal neuronal firing in response to A-fiber inputs was inhibited. Perhaps the dual action of anandamide acting on both CB_1 and TRPV1 receptors balances the effect on neurons with A-fiber inputs, and when TRPV1 receptors are inhibited, anandamide is able to inhibit neurons with A-fiber inputs, similar to WIN55,212. Likewise, C-fiber inputs were inhibited over a much greater time period with TRPV1 receptor antagonism. TRPV1 receptors are known to be present on trigeminal neurons with C-fiber inputs (Ichikawa and Sugimoto, 2001), perhaps highlighting the more prolonged C-fiber inhibition. The response to spontaneous activity was curious as there was inhibition with anandamide alone but not with both anandamide and TRPV1 receptor antagonist. The lack of an inhibitory response after TRPV1 intervention may be a reflection of interanimal variability (see Fig. 5C), as the means show a trend toward inhibition. Again, there was no significant effect on the receptive field with either combination of treatments, perhaps highlighting specialization in the TCC.

AM404, which is considered an endocannabinoid uptake inhibitor, although evidence exists that it is in fact a fatty acid amide hydrolase inhibitor (Glaser et al., 2003) (which is how it increases anandamide levels), was unable to alter responses to dural stimulation in any trigeminal neurons studied. This is less surprising given the responses of anandamide alone. It is possible that there is very little anandamide present tonically to alter the firing of trigeminal neurons. There is also evidence that AM404 may be a breakdown product in the brain of the nonsteroidal anti-inflammatory, paracetamol (acetaminophen) (Hogestatt et al., 2005). These data provide impetus to study paracetamol in the context of the cannabinoid system with regard pain relief and migraine.

The cannabinoids produce specific blood pressure effects. WIN55,212 caused a significant increase in blood pressure, which was inhibited by the specific CB_1 receptor antagonist. Anandamide has a characteristic triphasic effect that has been

observed previously (Akerman et al., 2004a). Phase I has been shown to be TRPV1-dependent (Pacher et al., 2004), and here it is shown that capsazepine is unable to inhibit phase II and III. It seems likely that the blood pressure increase caused by anandamide is mediated through the CB_1 receptor. AM404 also caused a triphasic blood pressure effect, similar to anandamide, which may reflect the ability of AM404 to prevent the breakdown and removal of anandamide from the synapse. The blood pressure data are consistent with biologically active levels of the agonists and antagonists in the studies as presented.

Studies of trigeminovascular neurons that receive input from the dura mater and large cranial vessels have proved useful in identifying targets for the development of antimigraine treatments (Goadsby, 2005). It has been suggested based on historical accounts that cannabinoid receptor activation may have antimigraine effects (Russo, 1998). Female migraineurs have increased degradation of anandamide in platelets compared with controls, consistent with lowered endocannabinoid tone (Cupini et al., 2006). Indeed cannabinoid receptor activation has been reported to alleviate headache in idiopathic intracranial hypertension (Evans and Ramadan, 2004). Whereas the evidence is far from overwhelming (we are not advocating for use of cannabinoid receptor agonists based on these data), the new data are completely consistent with the open label experience that has been reported.

In conclusion, activation of CB_1 receptors is able to inhibit trigeminal neurons with A-fiber and C-fiber input in the TCC in response to activation of the ophthalmic division of the trigeminal nerve. Anandamide was only able to inhibit neurons with A-fiber inputs after inhibition of the TRPV1 receptor, highlighting the dual agonist properties of anandamide in the brain. These results support an involvement of the cannabinoid CB_1 receptor and TRPV1 receptors in trigeminal neuronal firing, helping to further understand the pathophysiology of the trigeminovascular system and indicate potential directions for the development of new therapeutic agents, notwithstanding the potential difficulties of the psychoactive side effects accompanying cannabinoid treatments.

Acknowledgments

We thank Astrid Bergerot, Yolande Knight, Kevin Shields, James Storer, and Paul Hammond for both assistance and technical support during these experiments.

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Address correspondence to: Professor Peter James Goadsby, Institute of Neurology, Queen Square, London WC1N 3BG, UK. E-mail: peterg@ion.ucl.ac.uk