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Endocannabinoids in the Brainstem Modulate Dural Trigeminovascular Nociceptive Traffic via CB_1 and "Triptan" Receptors: Implications in Migraine

Simon Akerman,* Philip R. Holland,* Michele P. Lasalandra, and Peter J. Goadsby
Headache Group, Department of Neurology, University of California, San Francisco, San Francisco, California 94158

Activation and sensitization of trigeminovascular nociceptive pathways is believed to contribute to the neural substrate of the severe and throbbing nature of pain in migraine. Endocannabinoids, as well as being physiologically analgesic, are known to inhibit dural trigeminovascular nociceptive responses. They are also involved in the descending modulation of cutaneous-evoked C-fiber spinal nociceptive responses from the brainstem. The purpose of this study was to determine whether endocannabinoids are involved in the descending modulation of dural and/or cutaneous facial trigeminovascular nociceptive responses, from the brainstem ventrolateral periaqueductal gray (vlPAG). CB_1 receptor activation in the vlPAG attenuated dural-evoked Aδ-fiber neurons (maximally by 19%) and basal spontaneous activity (maximally by 33%) in the rat trigeminocervical complex, but there was no effect on cutaneous facial receptive field responses. This inhibitory vlPAG-mediated modulation was inhibited by specific CB_1 receptor antagonism, given via the vlPAG, and with a 5-$HT_{1B/1D}$ receptor antagonist, given either locally in the vlPAG or systemically. These findings demonstrate for the first time that brainstem endocannabinoids provide descending modulation of both basal trigeminovascular neuronal tone and Aδ-fiber dural-nociceptive responses, which differs from the way the brainstem modulates spinal nociceptive transmission. Furthermore, our data demonstrate a novel interaction between serotonergic and endocannabinoid systems in the processing of somatosensory nociceptive information, suggesting that some of the therapeutic action of triptans may be via endocannabinoid containing neurons in the vlPAG.

Introduction

The endocannabinoid system is known for being physiologically analgesic. It is involved in the modulation of pain at the spinal level (Kelly and Chapman, 2001) and contributes to the descending modulation of pain transmission via brainstem nuclei, such as the ventrolateral periaqueductal gray (vlPAG) and rostral ventromedial medulla (RVM; de Novellis et al., 2005; Maione et al., 2006). The PAG-RVM pathway is believed to provide descending control of only noxious somatosensory spinal events (Waters and Lumb, 1997), as innocuous inputs appear to be unaffected. Furthermore, it is thought that PAG activation preferentially inhibits noxious cutaneous C-fiber responses at the spinal level (Waters and Lumb, 2008), more than any other fiber types.

Nociceptive activation and sensitization of specifically trigeminovascular neurons is thought to contribute to the neural substrate of the severe and throbbing nature of pain in migraine (Goadsby et al., 2002; Bernstein and Burstein, 2012). Inhibiting dural-evoked nociceptive activation of neurons in the trigeminocervical complex has proven to be predictive of therapeutic anti-migraine efficacy (Bergerot et al., 2006), including the acute migraine treatment, triptans; 5-$HT_{1B/1D}$ receptor agonists (Goadsby and Hoskin, 1996). Similar to responses at the spinal level, endocannabinoids, via CB_1 receptor activation, also inhibit trigeminovascular nociceptive processing with specifically dural inputs (Akerman et al., 2004, 2007). Furthermore it is known that descending projections from the vlPAG also modulate dural nociceptive trigeminovascular processing, including Aδ-fiber and C-fiber responses, as well as basal trigeminal neuronal tone (Knight and Goadsby, 2001; Knight et al., 2002, 2003). The role that brainstem endocannabinoids have in modulating trigeminovascular nociceptive responses is not known; therefore, the aim of this study was to determine whether vlPAG endocannabinoids provide descending modulation of noxious and innocuous inputs to trigeminovascular neurons. Using direct application of endocannabinoids into the vlPAG, we tested the hypothesis that CB_1 receptor activation in the vlPAG would modulate dural nociceptive trigeminovascular transmission in the trigeminocervical complex (TCC).

5-$HT_{1B/1D}$ receptor activation, using naratriptan, in the vlPAG, also provides descending modulatory inhibition of dural nociceptive Aδ-fiber and C-fiber neuronal responses and basal trigeminal neuronal tone in the trigeminal nucleus caudalis, but not cutaneous responses (Bartsch et al., 2004). These data imply that one of the therapeutic actions of triptans may be via the

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*S.A. and P.R.H. contributed equally to this work.

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Correspondence should be addressed to Dr. Simon Akerman, Department of Neurology, University of California, San Francisco, 675 Nelson Rising Lane, San Francisco, CA 94158. E-mail: akermans@neurology.ucsf.edu.

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vlPAG, and changes in activation here may play a crucial role in modulating trigeminovascular nociceptive responses believed to be involved in migraine pathophysiology. Endocannabinoids are known already to interact with serotonergic neurons in the brainstem dorsal raphe to modulate pain mechanisms (Palazzo et al., 2006; Haj-Dahmane and Shen, 2009). Whether there is also an interaction of these transmitter systems in the vlPAG in the modulation of trigeminovascular nociceptive processing is not known. We therefore tested the hypothesis that any effects of endocannabinoids in the vlPAG will be modulated by 5-$HT_{1B/1D}$ receptor activity, given the known effects of triptans in the vlPAG already. These studies may help us further understand the therapeutic mechanism of action of triptans in migraine, and the role endocannabinoid mechanisms may have in this process.

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) and under license of the University of California San Francisco Institutional Animal Care And Use Committee, and conforming to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and adhered to the guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain (Zimmermann, 1983).

The surgical preparation and recording setup has been reported in detail previously (Bartsch et al., 2004; Akerman et al., 2007). Briefly, 33 male Sprague Dawley rats (300–380 g) were anesthetized with sodium pentobarbitone (Sigma-Aldrich, 60 mg/kg^{-1}, i.p) for induction and anesthesia maintained with a propofol solution (PropoFlo, 25–30 mg/kg^{-1} h^{-1}, i.v. infusion). During electrophysiological recording the animals were paralyzed with pancuronium bromide (Pavulon; Organon), 0.4 mg initially, and maintained with 0.2 mg every 30 min. The femoral artery and vein were cannulated for on-line blood pressure recording and intravenous infusion of anesthetic and test compounds, respectively. The rats were mounted in a stereotaxic frame, core temperature maintained throughout using a homeothermic blanket system, ventilated with oxygen-enriched air, 2–2.5 ml, 85–100 strokes per minute, and end-tidal CO_2 was monitored and kept between 3.5 and 4.5%. This allows one to monitor for changes to respiration and blood pressure due to long-term anesthetic maintenance. A sufficient depth of anesthesia was judged by the absence of paw withdrawal and corneal blink reflex and during muscular paralysis by lack of fluctuations of blood pressure.

Middle meningeal artery, TCC, and vlPAG site exposure. To gain access to the dura mater and middle meningeal artery (MMA), the skull was exposed and a craniotomy of the parietal bone performed with saline-cooled drilling and the area covered in mineral oil. For access to the TCC, the muscles of the dorsal neck were separated, and a partial cervical (C1) laminectomy performed and the dura mater incised to expose the brainstem at the level of the caudal medulla. An electrode was slowly lowered into the brainstem at 5 μm increments with a hydraulic microstepper (Exfo; Burleigh). Finally an area of bone directly above the coordinates of the vlPAG was thinned and removed and the dura mater pierced to allow entry of a micropipette into the vlPAG. After the completion of surgery the animals were left to stabilize for at least 1 h before electrophysiological recording.

Stimulation of MMA and recording from TCC. Extracellular recordings were made from neurons in the TCC, activated by dural stimulation, with cutaneous facial receptive fields, using tungsten microelectrodes (WPI; impedance 0.5–1.0 MΩ, tip diameter 0.5 μm). The signal from the recording electrode was filtered and amplified and fed to an analog-to-digital converter (Power 1401; Cambridge Electronic Design) and then to a microprocessor-based personal computer (Dell Latitude) where the signal was processed and stored. Additionally it was fed to a loudspeaker for audio monitoring and displayed on analog and digital storage oscilloscopes to assist isolation of action potentials from adjacent cell activity and noise. Poststimulus and peristimulus time histograms of neural activity were displayed and analyzed using Spike2 v5 (Cambridge Electronic Design). Dural nociceptive neurons in the TCC were identified by applying square-wave electrical stimuli (0.6 Hz) of 0.1–0.5 ms duration, 8–20 V to the dura mater, to activate trigeminal afferents, via a bipolar stimulating electrode placed on the dura mater adjacent to or either side of the MMA. These stimulation parameters were able to activate both trigeminal Aδ-fibers (with approximate latencies between 3 and 20 ms) and C-fibers (with latencies >20 ms and up to 80 ms) that innervate the dura mater. This is based on the distance of the trigeminal ganglion from the dural stimulation site, plus the distance from the trigeminal ganglion to the recording site in the TCC (~30–40 mm; Burstein et al., 1998) and the approximate conduction velocities of Aδ-fiber (2.0–30.0 m/s) and C-fiber (0.5–2.0 ms) (Millan, 1999, 2002) primary afferents.

Characterization of neurons. Neurons were characterized for their cutaneous and deep receptive fields. The cutaneous receptive field, including the cornea tested separately, 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 using a cotton tip applicator or fine paint brush (cornea), and noxious inputs, with pinching with forceps that was painful when applied to humans. When a neuron sensitive to stimulation of the ophthalmic (V1) dermatome of the trigeminal nerve was identified it was tested for convergent input from the dura mater. According to the cutaneous receptive field properties, neurons were classified as low-threshold mechanoreceptors that responded only to innocuous stimulation, nociceptive specific that responded to only noxious input, or wide-dynamic range (WDR) that responded to both noxious and non-noxious stimuli (Hu et al., 1981).

vlPAG microinjection. A multibarreled glass capillary unit (MicroData Instruments) with tip diameter no more than 80 μm was used for microinjection of drugs into the vlPAG (Knight et al., 2003; Bartsch et al., 2004). The stereotaxic coordinates for the multibarrel pipette position in the vlPAG used were (Paxinos and Watson, 1998): 1.36 mm rostral, 4.2 mm dorsal from the interaural point and 0.5–0.7 mm from midline, ipsilateral to stimulation and recording sites. A summary of the experimental setup and the injection sites in the vlPAG can be found in Figure 1*A* and *B*. Drugs were injected slowly over 30 s with a volume up to 100 nl.

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 1 ms that separated Aδ-fiber-activated (3–20 ms) and C-fiber (20–80 ms)-activated firing. Spontaneous activity (spikes per second, Hz) was recorded for 120–180 s preceding the dural stimulation using peristimulus histogram. Once it had been established, using at least three stable baselines, that there was a TCC neuronal response to dural stimulation and cutaneous and deep receptive field inputs from the ophthalmic division of the trigeminal nerve, these responses were tested after pharmacological intervention. First, in 25/33 animals, a functional connection between a recorded trigeminal neuron and the vlPAG was demonstrated using bicuculline injection. Inhibition of nociceptive neuronal responses in the TCC, after bicuculline injection in the vlPAG, had been demonstrated previously to produce significant inhibition of responses (Knight et al., 2003), and there is no response when microinjected outside of the PAG (Bartsch et al., 2004). This also helps determine that we were in the correct region of the vlPAG. In some of the animals where no functional connection was demonstrated we injected WIN55,212 to determine whether there was a functional role of cannabinoid activation outside the PAG, and also to serve as a control for region-specific effects. Placement of the micropipette in the vlPAG was then adjusted to obtain the correct anatomical position and until a functional connection was achieved. The effects of bicuculline are transient and reversed after 30 min, and a further 30 min washout period was allowed. After which the response of nociceptive neurons in the TCC to electrical stimulation of the dura mater and mechanical stimulation of the ophthalmic dermatome were tested before and after microinjection of drug intervention in the vlPAG for 45 min. Because of the small volume of drug applied (100 nl each time) and the transient nature of the neuronal changes, several experimental paradigms related to these endocannabinoid ligands were tested in each animal, with at least a 30 min washout allowed between test scenarios and no

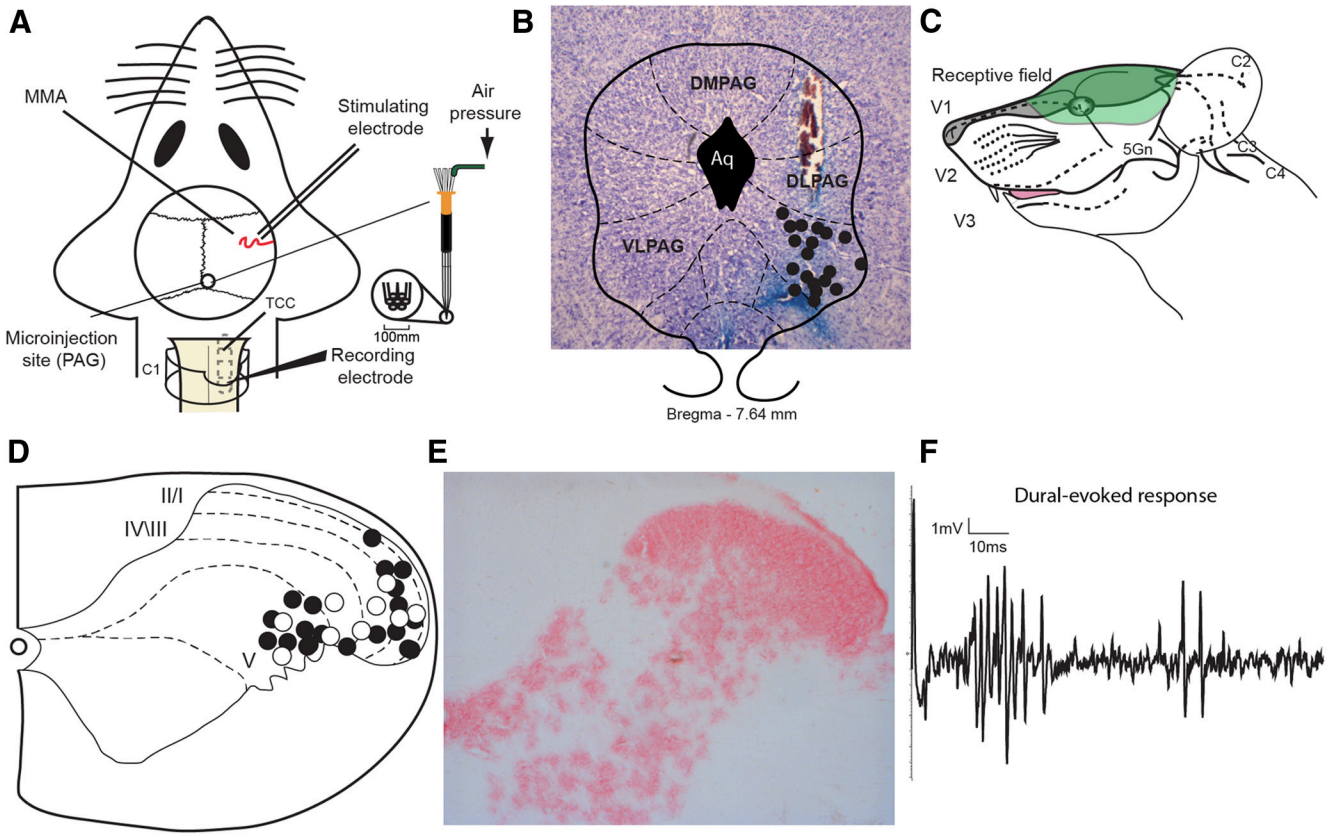

Figure 1. Overview of the experimental setup and neuronal characteristics. ***A***, The basic experimental setup with dural stimulation and recording in the TCC, and modulating the descending contribution of the vlPAG by direct microinjection of compounds. ***B***, The location of all microinjection sites within the vlPAG according to the template from Paxinos and Watson (1998) over an example Pontamine Sky Blue injection site in the vlPAG. All neurons studied were WDR with cutaneous receptive field in at least the first (ophthalmic) division of the trigeminal nerve, but also with corneal and sometimes cutaneous receptive field from the second (mandibular) region of the trigeminal nerve (***C***). The green shading represents an example of receptive characterization in an animal. ***D***, The location of recording sites in the TCC of nociceptive neurons receiving convergent input from the dura mater and facial receptive field, predominantly in laminae I–II and V. These locations were reconstructed from lesions (●) or from microdrive readings (○) and an original lesion site is included in ***E***. ***F***, An original tracing from a typical unit responding to dural stimulation with latencies in the Aδ-fiber and C-fiber range. 5GN, trigeminal ganglion; Aq, aqueduct; DLPAG, dorsolateral PAG; DMPAG, dorsomedial; V1, first trigeminal (ophthalmic) division; V2, second trigeminal (mandibular) division; V3, third trigeminal (maxillary) division.

more than four different microinjections applied in each animal, and this was spread over at least 4 h. The reliability of these responses is illustrated by the lack of significant effect on test responses of vehicle administration repeated up to four times. In studies that used intravenous injection there was only one intervention in each animal.

Data analysis. Data collected for Aδ-fibers represent the normalized data for the number of cells firing over a 10 ms time period in the region 3–20 ms poststimulation over the 20 collections, and expressed as mean ± SEM. Spontaneous activity was measured in cell firings per second (Hz). ANOVA for repeated measures with Bonferroni *post hoc* correction for multiple comparisons applied was used to measure whether there was a significant effect across the 45 min time course. If Mauchly's test of sphericity was violated we made appropriate corrections to degrees of freedom according to Greenhouse–Geisser (Field, 2005). 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 (SPSS, v16.0). One-way ANOVA was used to compare receptive field responses across different groups. Statistical significance was set at $p < 0.05$.

Drugs. The infusion of anesthetic was via the femoral catheter. Bicuculline methiodide, 0.4 mM, pH 6.0, and a 2% solution of Pontamine Sky Blue (both Sigma-Aldrich) were diluted in aqueous solution as reported previously (Bartsch et al., 2004). In the same study naratriptan (10 mg/ml, ~25 mM with Ki ~ 20 nM at 5-$HT_{1B/1D}$ receptors; Cumberbatch et al., 1998) in the vlPAG was effective at inhibiting dural nociceptive neurons (Bartsch et al., 2004), and similar concentration ranges were therefore used for molecules in this study with similar binding affinity data. Anandamide (10 mg/ml, Ki ~ 89 nM and ~ 371 nM for CB_1 and CB_2 receptors, respectively) and ACPA (Ki = 2.2 nM for CB_1 receptor, diluted to 5 mM concentration; both from Tocris Bioscience) came prepared in a soya oil:water (1:4) water-soluble emulsion (Tocrisolve) and diluted in an aqueous solution. GR127935 hydrochloride (Tocris Bioscience) was dissolved in an aqueous solution, 20 mM for microinjection or a concentration of 0.1 mg/ml for intravenous injection, as previously shown (Goadsby and Knight, 1997). SR141716, 21 mM (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide), a gift from National Institute of Mental Health, was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). WIN55,212 (Tocris Bioscience, Ki ~ 62.3 nM and ~ 3.3 nM for CB_1 and CB_2 receptors, respectively), 18 mM, was dissolved in DMSO.

Additionally we conducted a series of control electrophysiological experiments with the solvents used to dissolve the test compounds, aqueous solution, Tocrisolve, and DMSO, to test for effects on TCC neurons after injection in the vlPAG.

Results

Recordings were made from 31 neurons in 28 rats (all WDR) responsive to dural stimulation and with cutaneous receptive fields restricted to the first (ophthalmic, V1) division of the trigeminal nerve, as well as from ophthalmic corneal brush, with some overlap into the second (mandibular) division of the trigeminal nerve (Fig. 1*C*). Neurons were found in mainly superficial (laminae I–II) and deep layers (laminae V and VI) of the dorsal horn of the TCC at a range of depth, 450–1005 μm; for

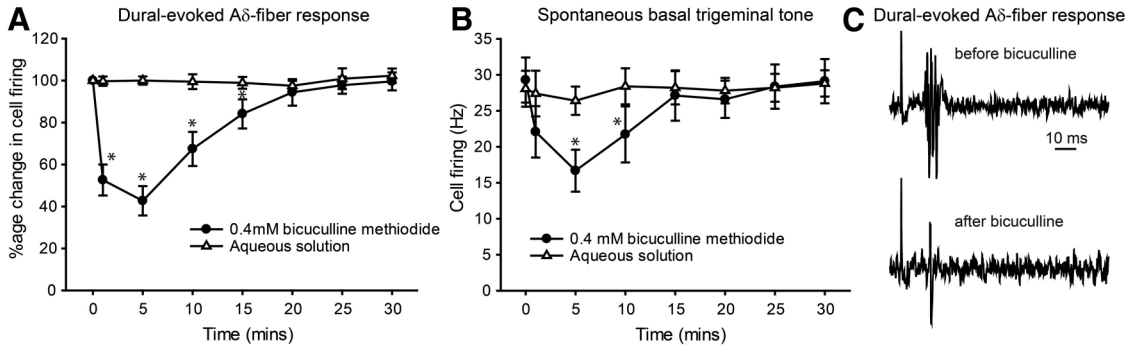

Figure 2. Summary of changes in dural-evoked neuronal firing in the TCC in response to microinjection of the $GABA_A$ receptor antagonist bicuculline methiodide into the vlPAG. Dural-evoked Aδ-fiber activity in the TCC is stable and not significant across 30 min of observations with aqueous solution (△) microinjection in the vlPAG. After microinjection of bicuculline methiodide (●) into the vlPAG there is a significant inhibition of evoked firing in the TCC of (***A***) Aδ-fiber neurons and (***B***) basal spontaneous trigeminal tone. ***C***, Original tracing from a dural-evoked Aδ-fiber neuronal response before and after bicuculline that is significantly inhibited. Data are presented as mean ± SEM; *$p < 0.05$ significance when compared with an average of the three baselines, using Student's paired *t* test.

recording sites see Figure 1*D* and *E*. Units had an average baseline firing latency after dural stimulation of 13 ± 0.7 ms for Aδ-fiber responses (range 4–24 ms). While data for C-fiber responses were recorded within the range of 20–80 ms, we only saw specific stimulation-linked firing in two units and these responded with a latency of ~0.5 ms; therefore there is not sufficient power to perform statistical analysis of these responses (an example of evoked neuronal firing can be seen in Fig. 1*F*). The mean ongoing spontaneous firing rate was 28.8 ± 4 Hz (range 5.5–67.6 Hz) with the majority of neurons responding between 10 and 20 Hz; this is within the same range as that demonstrated in previous studies (Knight et al., 2002; Bartsch et al., 2004; Akerman et al., 2007).

Recordings for control experiments were made from 15 neurons in five rats responsive to dural stimulation, all classified as WDR and with cutaneous receptive fields restricted to the first (ophthalmic) division of the trigeminal nerve, and the cornea. Microinjection of an aqueous solution (Aδ-fiber, $F_{(2.24,8.96)} = 0.481$, $p = 0.84$), Tocrisolve (Aδ-fiber, $F_{(2.92,11.66)} = 1.32$, $p = 0.28$) and DMSO (Aδ-fiber, $F_{(2.49,9.94)} = 0.621$, $p = 0.73$) had no significant effect on Aδ-fiber responses and spontaneous activity mediated responses of trigeminal second-order neurons.

Bicuculline injection into the vlPAG

To identify functional inhibitory projections from the vlPAG to the TCC and to indicate that the correct region of the vlPAG had been localized, as demonstrated previously (Knight et al., 2003), in some rats the $GABA_A$ receptor antagonist, bicuculline (0.4 mM), was microinjected into the vlPAG ($n = 25$). Injection of bicuculline caused inhibition of firing of Aδ-fibers (maximally by 57%, $F_{(3.3,79.8)} = 19.5$, $p = 0.001$) in the TCC and spontaneous activity (maximally by 43%, $F_{(3.3,78.7)} = 4.7$, $p = 0.004$; Fig. 2*A–C*). Bicuculline microinjection also caused a nonsignificant transient decrease in mean arterial blood pressure.

Activation of CB_1 receptor in vlPAG

Anandamide, an endogenous endocannabinoid that activates both CB_1 and CB_2 receptors, as well as having known activity at GPR55 and TRPV1 channels, had no significant effect on dural-evoked neuronal responses in the Aδ-fiber latency range ($F_{(2.2,11.1)} = 1.1$, $p = 0.38$, $n = 6$) or on baseline spontaneous activity ($F_{(1.6,8.0)} = 0.486$, $p = 0.6$, $n = 6$), when directly microinjected into the vlPAG. WIN55,212 is an alternate cannabinoid receptor agonist that activates both CB_1 and CB_2 receptors, with no reported activity at other receptor subtypes. With microinjection of WIN55,212, dural-evoked neuronal responses in the Aδ-fiber range were significantly inhibited over each time point to 45 min ($F_{(7,63)} = 4.2$, $p = 0.001$), with the maximum inhibition after 10 min of 19% ($t_{(9)} = 4.51$, $p = 0.001$, $n = 10$; Fig. 3*A*). These responses returned to baseline levels after 45 min. Spontaneous activity was also inhibited across the time points ($F_{(3.1,27.5)} = 3.3$, $p = 0.03$), from 10 to 15 min and maximally after 10 min with a 34.2 ± 11% inhibition ($t_{(9)} = 2.5$, $p = 0.03$, $n = 10$) similar to dural-evoked responses (Fig. 3*A–C*). Microinjection of WIN55,212 0.5–1 mm dorsal or lateral to the border of the PAG did not significantly affect dural-evoked Aδ-fiber responses ($F_{(2.6,10.2)} = 0.51$, $p = 0.66$), or spontaneous activity ($F_{(3.2,12.7)} = 0.29$, $p = 0.84$).

When a specific CB_1 receptor antagonist, SR141716, was given 5 min before treatment with the cannabinoid receptor agonist and the response of trigeminal neurons to dural stimulation repeated over 45 min there was no significant change in the Aδ-fiber response ($F_{(2.83,14.12)} = 1.01$, $p = 0.412$) or from spontaneous activity ($F_{(2.7,13.7)} = 2.8$, $p = 0.85$; Fig. 3*A–C*), implying the effects of WIN55,212 were reversed. There were no significant changes in the receptive fields for cutaneous V1 pinch ($F_{(2,10)} = 0.70$, $p = 0.52$, $n = 6$), cutaneous V1 brush ($F_{(2,10)} = 3.63$, $p = 0.1$, $n = 6$), and V1 corneal brush ($F_{(2,10)} = 0.77$, $p = 0.49$, $n = 6$) across the cohort of control, cannabinoid agonist pretreatment, and cannabinoid agonist and antagonist treatment trials (Fig. 3*D*).

The response after SR141716 alone over the 45 min was also not significant for Aδ-fiber firing ($F_{(2.39,11.9)} = 1.29$, $p = 0.32$, $n = 6$) but spontaneous activity was significantly increased ($F_{(3.0,14.8)} = 3.7$, $p = 0.04$, $n = 6$), specifically at 5 and 10 min, compared with baseline control responses.

Activation of a specific CB_1 cannabinoid receptor and interaction with the $5\text{-}HT_{1B/1D}$ receptors in the vlPAG

While WIN55,212 was able to inhibit the effects of dural-evoked trigeminovascular activation, and these effects appear to be specific to the CB_1 receptor, it does lack specificity and is not as potent as other selective CB_1 receptor agonists. On the other hand, ACPA is a potent and highly specific CB_1 receptor agonist, compared with the dual action of WIN55,212, and the responses on trigeminal neuronal firing to dural stimulation were examined over 45 min. Dural-evoked neuronal responses with Aδ-fiber latencies over the course of the experiment were significantly inhibited ($F_{(7,56)} = 3.14$, $p = 0.007$, $n = 9$; Fig. 4*A*, *E*)

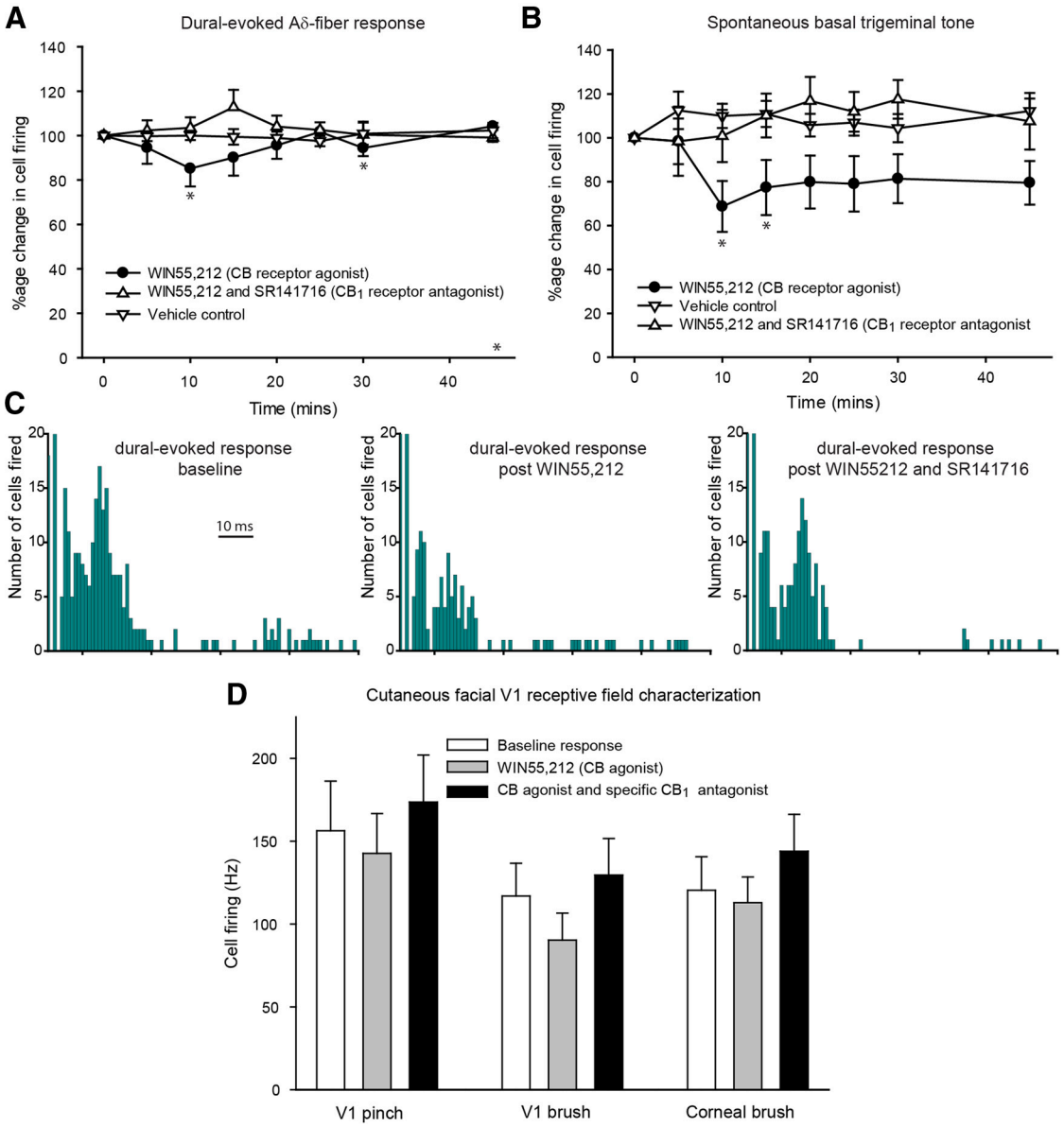

Figure 3. Summary of changes in dural-evoked neuronal firing in the TCC in response to microinjection of a cannabinoid receptor agonist, WIN55,212, into the vlPAG. ***A***, Dural-evoked Aδ-fiber neuronal activity in the TCC was stable after microinjection of vehicle (DMSO) into the vlPAG across 45 min (▽). After microinjection of WIN55,212 (●) into the vlPAG there was significant inhibition of evoked firing in the TCC of neurons with Aδ-fiber latency. These responses were significantly reversed by the specific CB_1 receptor antagonist, SR141716 (△). This was also the case with spontaneous neuronal firing in the TCC (***B***). WIN55,212 significantly inhibited responses and SR141716 was able to reverse this effect. ***C***, Example of poststimulus histogram (cumulative over 20 dural stimulations) identifying baseline Aδ-fiber responses that are inhibited by WIN55,212 application; a response that is reversed by coapplication with SR141716. In each group poststimulus histograms are taken at the 10 min time point after drug intervention. ***D***, Activation of the facial receptive field with cutaneous V1 pinch and V1 brush or V1 corneal brush was not significant across the treatment groups of baseline: WIN55,212 and WIN55,212 and SR141716. Data are presented as mean ± SEM; $*p < 0.05$ significance when compared with an average of the three baselines using Student's paired *t* test.

with maximal inhibition at 15 min of 19% ($t_{(8)} = 3.33, p = 0.01$). There was also significant inhibition of background spontaneous activity ($F_{(2.7,21.3)} = 3.6$, $p = 0.04$; Fig. 4*B*) across the 45 min, maximally after 5 min by 19.1%. However, there were no significant changes in any of the cutaneous receptive fields tested, cutaneous V1 brush ($t_{(8)} = 0.81$, $p = 0.44$, $n = 9$), cutaneous V1 pinch ($t_{(8)} = 0.5$, $p = 0.63$, $n = 9$), and V1 corneal brush ($t_{(8)} = 0.41$, $p = 0.69$, $n = 9$; Fig. 4*D*).

When the $5\text{-HT}_{1B/1D}$ receptor antagonist, GR127,935, was microinjected 5 min before treatment with ACPA and the response of trigeminal neurons to dural stimulation repeated, the ACPA-induced inhibition was reversed and no significant effect was seen in neuronal activity in Aδ-fiber latencies ($F_{(2.5,12.5)} = 0.85$, $p = 0.48$, $n = 6$; Fig. 4*C*,*E*) and on spontaneous activity ($F_{(2.9,14.4)} = 1.13$, $p = 0.37$, $n = 6$). Cutaneous receptive fields were also unaffected. Five minutes after GR127,935 hydrochloride alone, before ACPA microinjection, dural-evoked Aδ-fiber neuronal firing was significantly increased ($t_{(5)} = 2.7, p < 0.05, n = 6$), and no other changes on trigeminovascular responses were observed.

GR127,935 (0.1 mg/kg^{-1}) was also given intravenously 5 min before microinjection of ACPA into the vlPAG microinjection. There was no effect of GR127,935 alone on Aδ-fiber responses and spontaneous activity, but it did reverse the inhibition caused by ACPA on neuronal Aδ-fiber firing ($F_{(3.2,16.0)} = 0.83$, $p = 0.50$, $n = 6$; Fig. 4*C*). Baseline spontaneous activity was also not signif-

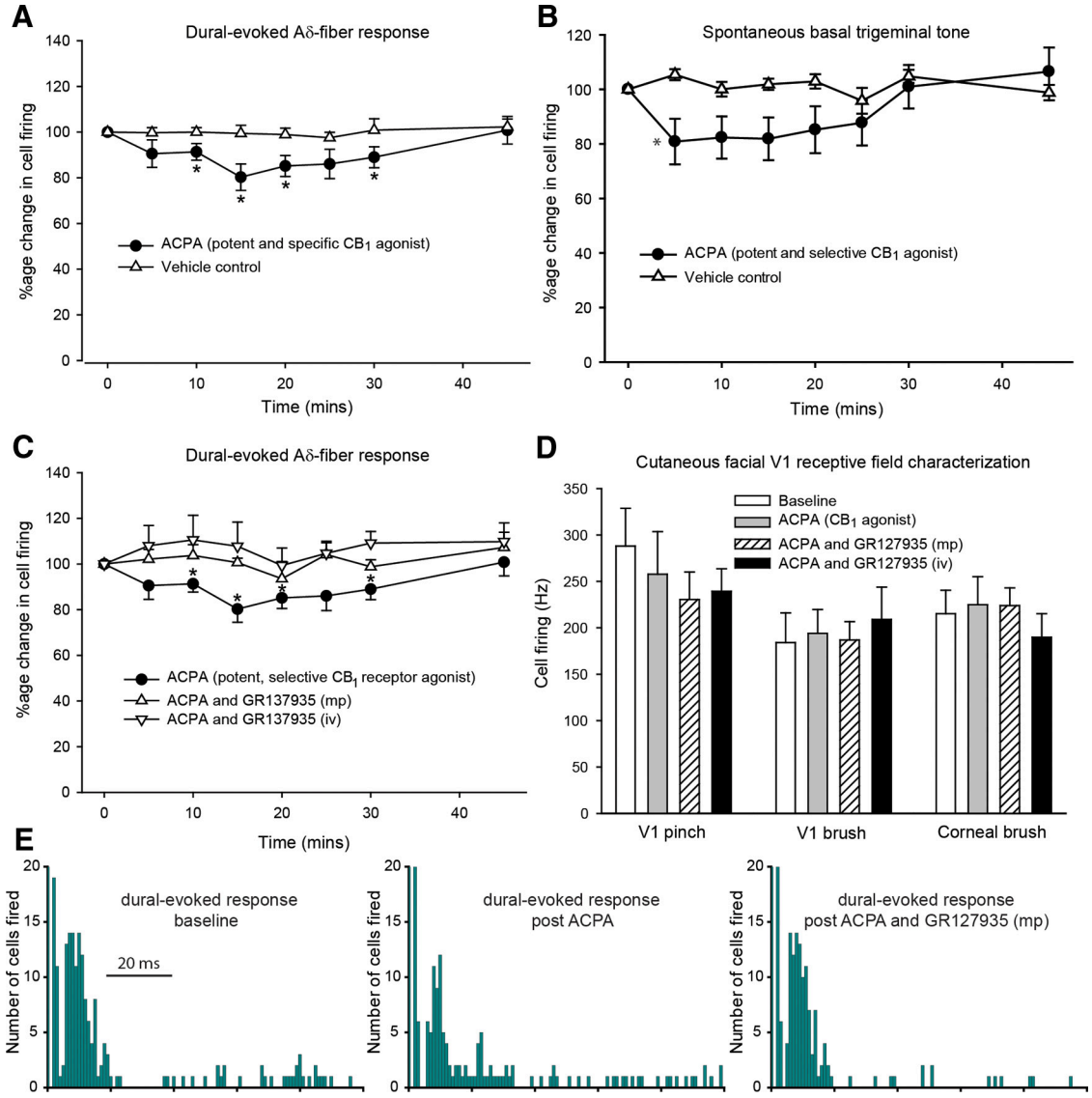

Figure 4. Summary of changes in dural-evoked neuronal firing in the TCC in response to microinjection of a specific CB_1 receptor agonist (ACPA) and reversal with a 5-$HT_{1B/1D}$ receptor antagonist (GR127935), into the vlPAG. Dural-evoked Aδ-fiber neuronal activity in the TCC was stable after microinjection of vehicle (Tocrisolve) into the vlPAG across 45 min (△). ***A***, After microinjection of ACPA in the vlPAG there was significant inhibition of evoked firing in the TCC of neurons with Aδ-fiber latency (●). Basal spontaneous trigeminal tone was also significantly reduced (***B***). ***C***, Responses to ACPA were significantly reversed with prior treatment with either intravenous administration (▽) or microinjection (△) of GR127925. ***D***, Activation of the facial receptive field with cutaneous V1 pinch and V1 brush or V1 corneal brush was not significant across the treatment groups of baseline, ACPA and ACPA/GR127935 (intravenous; iv) or ACPA/GR137935 (micropipette; mp), respectively. ***E***, Example of poststimulus histogram (cumulative over 20 dural stimulations) identifying baseline Aδ-fiber responses that are inhibited by ACPA application, a response that is reversed by co-microinjection with GR137935. In each group poststimulus histograms are taken at the 15 min time point after drug intervention. Data are presented as mean ± SEM; $*p < 0.05$ significance when compared with an average of the three baselines or a single baseline for the receptive field, using Student's paired *t* test.

icant ($F_{(2.5,12.7)} = 0.95$, $p = 0.43$, $n = 6$) and cutaneous receptive fields were unaffected.

Discussion

The PAG, via connections with the RVM (Fields and Heinricher, 1985), contributes to the control of pain transmission in the spinal cord dorsal horn (Fields et al., 1983). Endocannabinoids are involved in this process, through activation of the CB_1 receptor, and descending modulation of nociceptive neuronal firing at the spinal level (Meng et al., 1998; Palazzo et al., 2001; Finn et al., 2003; Meng and Johansen, 2004; Maione et al., 2006). In this study the hypothesis that endocannabinoid mechanisms in the vlPAG can also contribute to the descending modulation of dural trigeminovascular nociceptive traffic was tested in anesthetized rats. The potent and highly specific CB_1 receptor agonist, ACPA, and the less specific CB agonist, WIN55,212, locally applied to the vlPAG, attenuated the dural-evoked Aδ-fiber neuronal activation in the TCC, at a similar level of response (~20%) found previously with naratriptan (Bartsch et al., 2004). Further, the effects of WIN55,212 were reversed by a specific CB_1 receptor antagonist applied directly in the vlPAG. There was no effect of CB_1 receptor activation on either innocuous or noxious ophthalmic (V1) cutaneous receptor field activation or V1 corneal activation. ACPA and WIN55,212 also caused a significant inhibition of basal trigeminal neuronal tone over 45 min. These studies show for the first time that specific CB_1 receptor activation in the vlPAG attenuates dural-evoked nociceptive

Aδ-fiber neuronal firing and basal trigeminal tone in the TCC. The data imply that the endocannabinoid system may contribute to the descending modulation of trigeminovascular nociceptive traffic through the brainstem, which is hypothesized to play a role in migraine pathophysiology (Akerman et al., 2011).

Previous studies indicate that the PAG-RVM pathway provides descending control of only noxious cutaneous pinch-evoked C-fiber responses at the spinal level (Waters and Lumb, 1997, 2008), with innocuous inputs and spinal tone unaffected. The PAG's descending control of trigeminovascular responses, similar to responses at the spinal level, have no effect on cutaneous innocuous inputs. CB_1 receptor activation in the vlPAG did not affect innocuous V1 cutaneous receptive field and V1 corneal brush responses in the TCC, and previous studies with naratriptan similarly show no effects on innocuous V1 corneal brush (Bartsch et al., 2004). However, there are differences in the way the PAG-RVM pathway provides descending modulatory control of noxious inputs and basal neuronal tone. In this study dural-evoked Aδ-fiber TCC neuronal responses and basal spontaneous trigeminal tone were significantly inhibited by specific CB_1 receptor activation, but there was no effect on the noxious V1 cutaneous receptive field. Sample size of C-fiber-responsive neurons was not sufficient for statistical testing. However, in previous studies both noxious Aδ-fiber and C-fiber dural-evoked, and basal spontaneous trigeminal neuronal responses were inhibited by electrical (Knight and Goadsby, 2001) or chemical manipulation in the vlPAG (Knight et al., 2002; Bartsch et al., 2004). Noxious heat applied to the V1 cutaneous receptive field was unaffected by vlPAG naratriptan (Bartsch et al., 2004). The PAG provides inhibitory control of dural nociceptive Aδ-fiber and C-fiber trigeminovascular neurons, but has no effect on noxious V1 cutaneous inputs of either Aδ-fiber or C-fiber latency, whereas only C-fiber noxious cutaneous responses at the spinal level are modulated by the PAG.

Interestingly, anandamide, an endogenous endocannabinoid, which acts at CB_1 and CB_2 receptors, as well as TRPV1 ion channels, had no effect on dural nociceptive trigeminovascular activation. However, previous studies indicate that the effects of anandamide acting via CB_1 receptors or TRPV1 ion channels, in the midbrain PAG, are limited by enzymatic degradation by FAAH (Kawahara et al., 2011). Blockade of FAAH activity unmasks the inhibition and excitation of presynaptic glutamatergic transmission mediated via the CB_1 receptors and TRPV1, respectively. We believe that rapid enzymatic degradation of anandamide in the vlPAG is likely to explain its lack of effect here.

These studies demonstrate that trigeminal neurons do not always respond in the same way as spinal neurons with respect to somatosensory modulation. In this case the vlPAG's descending inhibitory control of somatosensory nociceptive inputs at the spinal and trigeminal levels seems to differ. These differences are further highlighted pharmacologically by the response to systemic application of $5\text{-}HT_{1B/1D}$ receptor agonists. Noxious, mechanical trigeminal neuronal responses are inhibited by naratriptan while noxious, mechanical spinal dorsal horn neuronal responses are unaffected (Cumberbatch et al., 1998). Despite these differences an interaction between endocannabinoid and serotonergic receptor systems in providing descending modulation of nociceptive inputs may also be shared by both neuronal populations. In the present study, in the vlPAG, the CB_1 receptor-mediated trigeminovascular responses are modulated by the serotoninergic system, specifically via the $5\text{-}HT_{1B/1D}$, triptan receptor. Previous studies in the brainstem dorsal raphe have shown that changes in firing of serotonergic neurons, particularly in the chronic constriction injury model of neuropathic pain, are modulated by CB_1 receptor activation (Palazzo et al., 2006; Haj-Dahmane and Shen, 2009). These data taken together highlight that within the brainstem, endocannabinoid and serotonergic neurons can modulate the effects of either system in the way both spinal and trigeminal nociceptive inputs are processed.

It is not known how these separate transmitter systems are able to modulate the effects of either system; however, it is possible they modulate descending projections to spinal and trigeminal neurons in a similar way. Triptans in the PAG are thought to act by inhibiting GABAergic and glutamatergic transmission, probably by preventing their release from nerve terminals (Jeong et al., 2008). Likewise, endocannabinoids are described as "synaptic circuit breakers" (Katona and Freund, 2008), acting as retrograde neurotransmitters in the PAG and RVM, inhibiting GABAergic and glutamatergic transmission by preventing the release of transmitters from nerve terminals, via activation of CB_1 receptors (Vaughan et al., 1999, 2000). This synaptic mechanism may also explain the inhibitory effects on basal trigeminal tone that both naratriptan and CB_1 receptor activation have in the PAG, by blocking the known tonic release of GABA and glutamate from nerve terminals, where they provide descending modulatory control of trigeminovascular neurons. Furthermore, the specific CB_1 receptor antagonist, SR141716, alone causes an increase in spontaneous firing, indicating the inhibitory effects of likely endocannabinoid-mediated tone involved in controlling trigeminovascular neuronal transmission.

Drugs that inhibit trigeminovascular dural nociception have been shown to be predictive of therapeutic efficacy in migraine. These new data, alongside our previous work using systemic administration of endocannabinoids (Akerman et al., 2004, 2007), provide evidence that specific CB_1 receptor activation may be therapeutic in migraine. Furthermore, some part of this action may be through descending modulation from the vlPAG to trigeminal neurons. The use of endocannabinoids and molecules that activate the CB_1 receptor as therapeutics are known to have the potential for overuse, and this may therefore serve as a limitation in their development for pain indications. However, the inhibitory actions of $5\text{-}HT_{1B/1D}$ receptor antagonists on the CB_1 responses in the vlPAG provide interesting clinical implications into the pharmacology of the therapeutic action of triptans in migraine. The acute anti-migraine triptan action may, in part, be acting via the modulation of endocannabinoidergic neurons, potentially in the brainstem, and descending control of trigeminovascular nociceptive transmission may therefore occur via an interaction between endocannabinoid and serotonergic receptor systems. Further evidence is necessary to dissect the exact mechanism of the endocannabinoid–triptan interaction before definitive conclusions can be made.

Brainstem modulation of trigeminovascular nociceptive transmission is thought to be involved in the pathophysiology of migraine (Akerman et al., 2011), and these data provide support for the argument that endocannabinoids, through brainstem mechanisms, may contribute to the modulation of trigeminovascular nociceptive transmission. Activation of CB_1 receptors in the vlPAG was able to modulate and inhibit trigeminovascular nociceptive processing of inputs generated from the dura mater, which are hypothesized to be involved in

the mechanism of pain in migraine. It is known already that there is dysfunction in the regulation of endocannabinoids (Sarchielli et al., 2007; Cupini et al., 2008; Rossi et al., 2008) in other primary headache patients, contributing to lower levels systemically. Furthermore, using nitroglycerin, a migraine trigger, in animals, increases activity of endocannabinoid enzymes that break down endogenous endocannabinoids, in the midbrain, where the PAG is located (Greco et al., 2010). These data point to an involvement of endocannabinoids in headache disorders, and this may be through changes in the midbrain vlPAG, contributing to altered trigeminovascular nociceptive processing, believed to be involved in migraine mechanisms.

In summary, the data show that endocannabinoid mechanisms are involved in the descending modulatory control of trigeminovascular nociceptive transmission from the brainstem, a mechanism hypothesized to contribute to the pathophysiology of migraine. Additionally, these effects may imply that endocannabinoids could be therapeutic in migraine, and as the CB_1 receptor agonist responses are reversed by a triptan receptor antagonist, when injected directly into the vlPAG, this interaction may suggest they are already involved in the mechanism of action of triptans. Clinically, the data may offer the promise of an interesting avenue for therapeutic development, although with agonist approaches there is a theoretical potential for cannabinoid agonist overuse. This may limit the development of targeted therapeutics despite a potential efficacy in the clinic.

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