DELTA-9-TETRAHYDROCANNABINOL SELECTIVELY ACTS ON CANNABINOID 1 (CB1) RECEPTORS IN SPECIFIC REGIONS OF THE DORSAL VAGAL COMPLEX TO INHIBIT EMESIS IN THE FERRET

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ABSTRACT

The aim of this study was to investigate the efficacy, receptor specificity and site of action of (delta)$^9$-tetrahydrocannabinol (THC) as an anti-emetic in the ferret. Pretreatment with THC (0.05-1 mg/kg, i.p.) dose-dependently inhibited the emetic actions of cisplatin (10 mg/kg, i.v.). The ED$_{50}$ for retching was approximately 0.1 mg/kg and for vomiting 0.05 mg/kg. A specific CB$_1$ receptor antagonist, SR 141716A (5 mg/kg, i.p.), reversed the effect of THC, while a CB$_2$ receptor antagonist, SR 144528 (5 mg/kg, i.p.), was ineffective, confirming the selectivity of action at CB$_1$ receptors. A low dose of THC applied to the surface of the brainstem was sufficient to inhibit the emesis induced by intragastric hypertonic saline. The site of action of THC in the brainstem was further assessed using Fos immunohistochemistry. Fos expression induced by cisplatin in the dorsal motor nucleus of the vagus and the medial subnucleus of the nucleus of the solitary tract (NTS), but not other subnuclei of the NTS, was significantly reduced by THC rostral to obex. At the level of the obex, THC reduced Fos expression in the area postrema and the dorsal subnucleus of the NTS. The highest density of CB$_1$ receptor immunoreactivity was found in the dorsal motor nucleus of the vagus and the medial subnucleus of the NTS. Lower densities were observed in the area postrema and dorsal subnucleus of the NTS. Caudal to obex there was moderate density of staining in the commissural subnucleus of the NTS. These results show that THC selectively acts at CB$_1$ receptors to reduce neuronal activation in response to emetic stimuli in specific regions of the dorsal vagal complex.

Keywords: area postrema, CB$_2$ receptor, dorsal motor nucleus of the vagus, nucleus of the solitary tract.
EMESIS IS AN IMPORTANT PROTECTIVE GASTROINTESTINAL REFLEX (11).

It is also an unpleasant and serious side-effect of chemotherapy for the treatment of cancer and is a symptom of many diseases including diabetes and HIV/AIDS (3, 22, 29). Cannabinoids are known to be effective anti-emetics in humans, although they are not widely used because of their side effect profile (34). In a previous study we showed in the ferret that cannabinoids are effective anti-emetics against the centrally acting opiate, morphine 6 glucuronide, through an action at CB₁ receptors (37). Other investigators have also found CB₁ receptor agonists reduce emesis in the ferret and the shrew (14, 33). An interesting extension of these observations was that in the ferret (37) and shrew (13) evidence was presented suggesting that an endogenous cannabinoid system may be present in the brainstem centers that modulate emesis, since specific CB₁ receptor antagonists potentiated the emetic response in the ferret and caused emesis when given alone to the shrew. These data are consistent with the hypothesis that the sites of CB₁ receptor distribution in the brainstem are the sites of neuronal activation in emesis and that cannabinoids will inhibit emesis at these sites. We have tested this hypothesis using Fos expression as a marker of neuronal activation (7, 9), in a system where we first characterized the behavioural effects of the cancer chemotherapeutic and emetic agent cisplatin and (delta)⁹-tetrahydrocannabinol (THC), a well characterized cannabinoid derived from the cannabis plant.

Diverse emetic stimuli are known to activate the dorsal vagal complex (DVC) of the medulla in two ways. The DVC comprises the area postrema, nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMNX) (24). Activation of the DVC through the bloodstream activates neurons of the area postrema and the dorsal medial parts of the NTS by direct contact with blood-borne factors through their leaky blood-brain barrier (16, 25, 30). Neuronal activation in the NTS occurs through afferent inputs from the vagus and splanchnic...
nerves carrying sensory information from the gastrointestinal tract (18, 26). The NTS is an important site of integration with higher centres including the cortex. The DMNX carries signals to initiate the motor program of reverse peristalsis which when coordinated with appropriate respiratory movements results in emesis (11).

The NTS is subdivided anatomically into subnuclei with differing functions (8, 19, 27). Primary visceral afferent fibers coming into the brain through the solitary tract, project to different subnuclei with a viscerotopic arrangement. Gastrointestinal afferent fibers project to the subnucleus gelatinosus, while the cardiovascular, respiratory and pulmonary afferent fibers project to the dorsal/dorsolateral, interstitial and ventral/ventrolateral subnuclei respectively. All visceral organ systems (except pelvic) also project to the commissural subnucleus in the caudal NTS, that becomes the medial subnucleus in the “intermediate” NTS (20). The ascending projections to forebrain autonomic nuclei arise mostly from the commissural and medial subnuclei of the NTS (26).

The ferret is a commonly used animal model for studies of emesis because of its sensitivity to emetics and the anatomic similarities to humans in the neuroanatomy of the brainstem and stomach (2, 21). We used cisplatin in this species to explore the anti-emetic actions of THC, to test if this was mediated by CB\(_1\) receptors. We also examined the patterns of neuronal activation before and after CB\(_1\) receptor antagonism to correlate them with the distribution of CB\(_1\) receptors determined by immunohistochemistry.
METHODS

Behavioural experiments

Adult male ferrets (900-1500g, Mustela putoris furo, Marshall Research Labs, NY) were used for all studies (n=4-5/group). These experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the University of Calgary Health Sciences Animal Care Committee. Animals were fasted overnight prior to behavioural experiments, but had free access to water.

Ferrets were anaesthetized with halothane and maintained at 1.5-2.0%. In the first treatment group, the vehicle used to dissolve THC (2% DMSO, 1% Tween 80 in physiological saline) was administered alone before the emetic agent, cisplatin. A small incision was made to expose the left jugular vein for the administration of cisplatin (10 mg/kg, i.v.). The incision was closed and then the unanaesthetized ferret was observed for up to 3h (1.5-3h; animals were observed for 1h after the last emetic episode) to count the number of episodes of retching (defined by rhythmic abdominal contractions with an open mouth) and vomiting (defined by retching with the expulsion of saliva and gastric juices). In the second treatment group, THC (0.05 to 1.0 mg/kg, i.p., Lipomed, Arlesheim, Switzerland) was administered 15 minutes before cisplatin. In the third treatment group, the selective CB₁ receptor antagonist, SR141716A (5 mg/kg, i.p., Sanofi Research), or the selective CB₂ receptor antagonist, SR144528 (5 mg/kg, i.p., Sanofi Research), was administered 15 minutes before THC (0.5 mg/kg, i.p. for SR144528 and 1.0mg/kg for SR141716A ), which was again administered 15 minutes before cisplatin.

One hour after the emetic episodes, ferrets from each treatment group were deeply anaesthetized with sodium pentobarbital (65mg/kg, i.p., Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada), and fixed by intracardiac perfusion with 1L/kg of phosphate buffered
saline followed by 1L/kg of 4% paraformaldehyde (pH 7.3). Brains were collected, fixed overnight at 4°C in the same fixative and processed for Fos immunoreactivity as described below. Only data from those ferrets that received 1 mg/kg THC were analysed for Fos expression.

**CB1 receptor immunoreactivity in the DVC**

Ferrets (n=4) were deeply anaesthetized with pentobarbital sodium (65 mg/kg i.p.), perfused fixed as above and the brains removed to be fixed overnight in 4% paraformaldehyde at 4°C. Brains were then transferred to 30% sucrose in 0.1 M phosphate buffer at 4°C overnight. Sections of brainstem were cut at 30 μm and floating sections were incubated in one of three CB1 receptor antibodies at 4°C for 48 hours. Antibodies used in this study were raised to the N terminus of the human CB1 receptor (1:500; Affinity BioReagents), and the N (Affinity BioReagents) and C terminus of the rat CB1 receptor (1:500; requests for this antibody may be directed to: kmackie@u.washington.edu) (17, 36). Specificity was confirmed by preincubation of the antibodies with the peptides used to raise the antibodies (10nM in diluted antibody). Sections were washed with 0.1% Triton X-100 (in phosphate buffered saline [PBS]) and incubated for 2h in donkey anti-rabbit Cy3 as the secondary antisera. Tissues were examined using a Zeiss Axioplan fluorescence microscope and photographed with a digital camera (Sensys, Photometrics, Tucson, AZ).

**Fos immunohistochemistry**

Sections of brainstem were cut at 30 μm and floating sections were incubated in 10% normal donkey serum (NDS) diluted in 0.1% Triton X-100 (in PBS) for 1 hour followed by
incubation in sheep anti-Fos (1:1000, Genosys Biotechnologies, Cambridge, UK, OA-11-824) in PBS with 0.1% triton and 10% NDS for 48 hours at 4°C. Sections were washed with in PBS and then incubated in biotinylated anti-sheep IgG secondary antibody solution for 60 minutes at room temperature. Staining was revealed using the avidin biotin complex technique (Vectastain Elite ABC Kit, Vector). Briefly, sections were incubated in ABC reagent for 60 minutes at room temperature and then in a solution containing 3,3’-diaminobenzidine (DAB, 0.5 mg/ml) for 30s-60s. Brainstem sections were then mounted on slides to dry for 24h, dehydrated for 4 minutes each in 50% ethanol, 70% ethanol, 95% ethanol 100% ethanol then cleared in xylene, after which they were mounted and viewed. The boundaries of the nuclei and subnuclei of the NTS were assessed in adjacent sections stained with cresyl violet based on our previous work (7-9).

The number of Fos immunoreactive neuronal nuclei were determined bilaterally for the area postrema, DMNX and for all subnuclei of the NTS at three levels along the rostrocaudal extent of the dorsal vagal complex, 0.5-1.0mm rostral and 0.5mm caudal to obex and at obex. Three sections per level per animal were counted and averaged (n=4 animals). Data were compared using ANOVA with Tukey post test (Prism, GraphPad Software Inc., San Diego, CA).

Central THC administration

Adult male ferrets were anaesthetized with Halothane and a tracheotomy was performed so that retching episodes did not interfere with anaesthetic administration. A blood pressure transducer was inserted and secured in the right carotid artery. The neck incision was then stapled closed around the tubes and the ferret turned onto its stomach. A midline incision was made over the base of the skull and blunt dissection through the neck muscle exposed the dura, which was cut open over the cisterna magna. Once the brainstem was exposed the Halothane
was lowered to 0.8% and the ferret was left for 10 minutes. During this time the level of
anaesthetic was monitored by jaw tone and corneal reflex. Microdroplets of 0.01mg/kg THC
(approximately 2.5 μl total) were applied to the surface of the brainstem followed by a 15 minute
wait (n=3 ferrets). In a separate control group (n=3) 2.5 μl of the vehicle used to dissolve THC
was applied to the brainstem surface. Hypertonic saline (2M) was administered directly into the
stomach through a feeding tube at 1ml/min over 10 minutes. Hypertonic saline was used
because it is a reliable emetic in the lightly anaesthetized ferret while injectable emetics such as
cisplatin are unreliable in this model. The ferret was then observed visually and by recordings of
blood pressure for 60 min.
RESULTS

Anti-emetic actions of THC in the ferret are mediated by CB₁ receptors

We first investigated whether the CB₁ receptor agonist THC was an anti-emetic using cisplatin to evoke emesis. Cisplatin administered to the ferrets, after the injection of the vehicle for THC (n=5), evoked 17.4 ± 5.5 episodes of retching and vomiting and 5.6 ± 2.1 episodes of vomiting alone in a 1 hour period (Figure 1A). With the administration of increasing doses of THC, the numbers of episodes of retching and vomiting were reduced, with the complete elimination of emetic episodes at a dose of 1 mg/kg THC (n=4, Figure 1A and B). To examine whether this was due to activation of CB₁ receptors, the specific CB₁ receptor antagonist, SR141716A was used. SR 141716A reversed the effects of THC. Thus SR 141716A increased the number of episodes of retching and vomiting evoked by cisplatin in the presence of THC to 10.8 ± 1.4 episodes of retching and 4.0 ± 0.8 episodes of vomiting (n=4, Figure 1C). When THC (n=3) or SR 141716A (n=3) were administered alone without cisplatin, no episodes of retching or signs of emesis were observed. Administration of SR 141716A with cisplatin evoked 16 ± 3.2 episodes of retching and vomiting and 4.3 ± 1.5 episodes of vomiting (n=3), which is not significantly different from cisplatin alone.

To further address the specificity of the response, we investigated the effects of the selective CB₂ receptor antagonist SR 144528. SR 144528 had no effect on the number of emetic episodes evoked by cisplatin in the presence of a submaximal dose of THC (n=3, 0.5 mg/kg). This dose which reduced the episodes of retching to 4.8 ± 1.0 was used as it allowed us to evaluate both a further reduction and also an increase in emetic episodes in the presence of SR 144528.
CB₁ receptor activation selectively reduced Fos expression in discrete subnuclei of the NTS, the DMNX and the area postrema

Cisplatin treatment induced Fos expression in specific regions of the DVC. Fos expression was highest in the area postrema and the medial subnucleus of the NTS at all levels of the DVC that were examined (Figure 2). Consistent Fos expression was also observed in the dorsal/dorsolateral, ventral/ventrolateral and intermediate subnuclei of the NTS, and the DMNX. Only few Fos immunoreactive nuclei were observed in the interstitial or gelatinosus subnuclei of the NTS. As shown in Figure 3, THC reduced Fos expression in the DVC evoked by cisplatin treatment in specific subnuclei of the NTS and the area postrema.

In the rostral DVC (0.5mm rostral to obex), THC treatment significantly reduced the number of Fos immunoreactive nuclei per section in the medial subnucleus of the NTS from 65.6 ± 10.6 with cisplatin to 26.0 ± 4.4 (Figure 4, p<0.05, n=4). This was reversed by pretreatment with the CB₁ receptor antagonist SR 141716A to 80.0 ± 9.3 Fos immunoreactive nuclei per section, which was not significantly different from cisplatin alone (p>0.05, n=5). Within the medial subnucleus of the NTS, the density of Fos expression was highest in the area immediately dorsal to the DMNX after cisplatin treatment. In the same sections, THC reduced Fos immunoreactivity in the DMNX from 19.0 ± 2.9 to 7.3 ± 2.1 and the reduction was reversed by SR 141716A to 12.5 ± 0.3 (Figure 4, p<0.05). The majority of nuclei with Fos immunoreactivity in the DMNX were those of smaller cells rather than the large vagal motor neurons. There were no significances between the Fos immunoreactivity in the subnucleus gelatinosus, the medial, dorsal/dorsolateral, ventral/ventrolateral, intermediate, or interstitial subnuclei at this level for the three treatments (data not shown).
At the obex (Figure 3), Fos expression in the area postrema in animals treated with cisplatin was reduced from $100.6 \pm 19.6$ to $42.0 \pm 11.6$ by THC treatment and then significantly increased to $135.8 \pm 33.7$ by SR 141716A (Figure 3, p<0.05). In the same sections, Fos immunoreactivity was reduced in the medial subnucleus of the NTS from $55.4 \pm 7.1$ to $42.8 \pm 3.0$ by THC, and following SR 141716A pretreatment, significantly increased to $66.5 \pm 4.1$ (p<0.05). Also around the obex, the number of Fos immunoreactive nuclei in the dorsal/dorsolateral subnucleus of the NTS was reduced from $7.5 \pm 1.1$ to $3.8 \pm 0.3$ by THC and the reduction significantly reversed by SR 141716A to $10.5 \pm 1.8$. Other subnuclei of the NTS at this level showed no significant changes in Fos immunoreactivity between treatments and, in contrast to more rostral regions, the DMNX showed no significant change in Fos immunoreactive nuclei (Figure 3).

In the caudal DVC, the only subnucleus that showed any significant changes between treatments was the ventral/ventrolateral subnucleus of the NTS where Fos expression was reduced from $4.0 \pm 0.8$ to $1.3 \pm 0.6$ nuclei by THC (Figure 4, p<0.05). Pretreatment with SR 141716A increased Fos to $3.3 \pm 0.7$ nuclei, but this did not reach significance (p>0.05).

**The regions of Fos expression induced by cisplatin that are sensitive to CB₁ receptor activation are also sites of CB₁ receptor expression**

At approximately 0.5-1.0mm rostral to the obex, CB₁ receptor immunoreactive terminals were concentrated in the DMNX and the medial subnucleus of the NTS (mn) with all three CB₁ receptor antisera (Figure 5.I). Only with the antisera to the N terminus of the human CB₁ receptor were fibers localized within the solitary tract (TS) itself (Figure 5.II). The majority of
the subnucleus gelatinosus of the NTS contains little CB₁ receptor immunoreactivity, as does the intermediate subnucleus of the NTS, the interstitial subnucleus of the NTS, and tissue immediately surrounding the TS. Moderate terminal immunoreactivity was seen with all CB₁ receptor antisera in the dorsal/dorsolateral subnucleus of the NTS and the most dorsomedial aspect of the subnucleus gelatinosus. Single immunoreactive neuronal perikarya were present in the dorsal/dorsolateral, interstitial, intermediate and the ventral/ventrolateral subnucleus of the NTS with the antisera to the N terminus of the human CB₁ receptor. The area of the medial subnucleus dorsolateral to the DMNX and medial to the TS shows less dense immunoreactivity. This is the position of the subnucleus centralis (6, 12), which has not been completely described in the ferret.

At the obex, CB₁ receptor immunoreactivity in the medial subnucleus is most dense close to the dorsal aspect of the DMNX and becomes less dense as it extends laterally toward the TS (Figure 5). Antisera to the human CB₁ receptor revealed neuronal perikarya in the interstitial, intermediate and ventral/ventrolateral subnuclei although there was not much terminal staining with any of the antisera at this level. The DMNX, dorsal/dorsolateral, and medial subnuclei of NTS and the subnucleus gelatinosus of NTS (which is much smaller at this level) show an even distribution of intense staining. The area postrema shows less dense immunoreactivity, but at higher magnification immunoreactive terminals are clearly present in this tissue.

Immediately caudal to obex, the distribution of immunoreactivity is much the same as in the obex (Figure 5). The sections show the same pattern of staining in the DMNX. Moderate staining appears in the commissural subnucleus of the NTS. There is less immunoreactivity in the area of the TS and the ventral/ventrolateral subnuclei, but they are not very well defined at this level.
**CB₁ receptor activation by THC occurs centrally in the brainstem to inhibit emesis**

A low dose of THC, which was ineffective against cisplatin in the unanaesthetized ferret (0.01 mg/kg), applied to the surface of the brainstem completely inhibited the emesis induced by intragastric hypertonic saline. Without THC application in control experiments, hypertonic saline induced 5.0 ± 1.2 episodes of retching and vomiting (Figure 6C, n=3, p<0.05). The mean rise in arterial blood pressure in the THC treated group was 8.7 ± 1.8 and the mean rise in arterial blood pressure was 62.0 ± 11.1 in the control group (Figure 6D, p<0.05). Episodes of retching and vomiting began to occur within an average of 4 minutes from the end of the hypertonic tonic saline infusion.
DISCUSSION

In the present study we have shown that THC dose-dependently reduced emesis induced by the chemotherapeutic agent cisplatin through an action at CB\textsubscript{1} receptors. The specific CB\textsubscript{2} receptor antagonist, SR 144528 was found to have no effect on the anti-emetic action of THC. The investigation of neuronal activation in the DVC demonstrated not only that THC treatment reduced Fos expression, but that this could be reversed using pharmacological blockade of the CB\textsubscript{1} receptor. In the DMNX, medial subnucleus of the NTS, dorsal/dorsolateral subnucleus of the NTS and the area postrema neuronal activation in response to emesis was significantly reduced by CB\textsubscript{1} receptor agonism and returned to control levels by CB\textsubscript{1} receptor antagonism. These same areas of the DVC showed dense CB\textsubscript{1} receptor immunoreactivity. Taken together, these data provide strong support for the idea that cannabinoid modulation of the emetic response to cisplatin occurs in the DVC of the brainstem at CB\textsubscript{1} receptors. These data are consistent with the hypothesis that endogenous cannabinoids are physiological regulators of vagal reflexes that include emesis.

Our results show that THC was effective at reducing cisplatin-induced emesis. Cisplatin induces an early phase of emesis by causing serotonin (5-HT) release from enterochromaffin cells in the gut. This response can be inhibited by 5-HT\textsubscript{3} receptor antagonists such as ondansetron (3, 18). THC has previously been shown to reduce emesis induced by cisplatin in the shrew through CB\textsubscript{1} receptors (14). Interestingly, a CB\textsubscript{1} receptor antagonist, SR 141716A, was able to induce emesis when administered alone in the shrew (13), possibly suggesting a high level of emetic tone controlled by endogenous cannabinoids in this species. The presence of endogenous cannabinoids modulating emesis was also suggested by the potentiation of the emetic response to morphine 6 glucuronide, by a CB\textsubscript{1} receptor antagonist AM 251 in the ferret.
(37). Why potentiation was not seen the present study when the CB₁ receptor antagonist was
given alone is not clear. Perhaps the response itself is at a maximum. Alternatively, it may be
that the centrally acting emetic, morphine 6 glucuronide, can be potentiated as its site of action is
in the brainstem whereas the peripheral emetic cisplatin activates pathways with a lesser degree
of endocannabinoid tone. It may be that tonic activation of CB₁ receptors occurs in discrete
subnuclei in the DVC since SR 141716A not only antagonized the reduction in Fos expression,
but raised it to levels higher than control in the AP, medial and dorsal/dorsolateral subnucleus of
the NTS. This selectivity is also shown where THC had no effect on Fos expression, such as the
intermediate and interstitial subnuclei of the NTS, where SR 141716A did not change the
baseline (emesis induced) Fos counts.

CB₁ receptor agonists are effective at reducing emesis induced by morphine 6
glucuronide, which acts centrally (37). Recent work by Simoneau et al. showed similar
behavioural findings using centrally acting opioids to induce emesis (33). The synthetic
cannabinoid WIN 55,212-2 reduced the number of emetic episodes in the ferret. This effect was
reversed using AM 251, while a CB₂ receptor antagonist (AM 630) had no effect. Behavioural
evidence showing effectiveness against both central and peripheral emetic agents suggests a
common central site of action for cannabinoids as anti-emetics. Consistent with this hypothesis
is the functional result that a dose of THC 100 fold lower than the effective dose to reduce
emesis in response to cisplatin applied directly to the brainstem surface abolished emesis.

The DVC has been implicated as the site in the brainstem of neuronal activation in
emesis (11, 18). Using Fos as a marker, various emetic stimuli have been found to activate
neurons in specific subnuclei of the NTS, the DMNX and the area postrema (4, 7, 9, 31, 39).
The NTS and DMNX have been interpreted as sites of integration of gastrointestinal primary
afferent activity with vestibular, cortical or other regions of the brain that lead to coordinated activation of the motor nuclei that initiate emesis (11, 18). The area postrema is thought to be the chemoreceptor trigger zone in emesis since neurons in this region are activated by circulating agents in the cerebrospinal fluid (5, 10, 30). There is also a primary afferent input to neurons in this region.

Previous work in the ferret and the cat shows a similar distribution of Fos immunoreactivity as that shown in the present study (7, 9, 31). The medial subnucleus of the NTS and the area postrema show the most extensive Fos expression after peripheral emetic stimuli in the ferret (9), and this expression was dependent on an intact vagus nerve (7). A model of fictive vomiting (where cats are decerebrated and paralysed) showed only minor differences in Fos immunoreactivity when compared with conscious treated cats (31). Similar to the ferret, Fos was detected in the area postrema, NTS (medial and subpostremal), intermediate reticular zone of the lateral tegmental field, nucleus retroambiguus, dorsal vagal and phrenic motor nuclei in this model.

Esophageal primary afferents synapse in the subnucleus centralis of the NTS in the rat. The area of the subnucleus centralis, ventral and medial to the TS, was found to have less dense CB₁ receptor immunoreactivity. Fos immunoreactive nuclei were also less dense in this region after emesis. Gastric primary afferents project to the subnucleus gelatinosus of the NTS where they synapse with NTS neurons or directly with dendrites of neurons from the DMNX (1). The possibility of the CB₁ receptor functioning directly at the terminals of the primary afferent fibers from the stomach and duodenum is discussed further below. Gastric projections also extend into the DMNX and NTS at the level of the obex and just caudal and rostral to it (26). THC was found to significantly reduce Fos immunoreactivity after cisplatin throughout the rostrocaudal
extent of the medial subnucleus of the NTS to a greater degree than changes in other subnuclei. This may be due to the presence of neurons in the medial subnucleus of the NTS that project to the forebrain and other brain areas for functions such as sensation (26).

Interstitial and intermediate subnuclei of the NTS receive input from primary afferents of the palate and pharynx in the rat (1). The lack of change in the expression of Fos in the interstitial and intermediate subnuclei in the ferret may be due to the low number of perikarya in these nuclei or their lack of involvement in the cisplatin-induced emetic stimulus. However, the presence of CB$_1$ receptor immunoreactive neuronal perikarya indicates a function for endogenous cannabinoids in response to sensory information from the pharynx and palate. These sites also represent potential postsynaptic sites of CB$_1$ receptor distribution, similar to what has been described for some lamina II neurons in the spinal cord (32). Despite the low number of neurons in the dorsal/dorsolateral subnucleus at the level of obex and ventral/ventrolateral subnucleus caudal to the obex the reduction of Fos immunoreactivity by THC and the reversal by SR 141716A was significant. These areas could be involved in the receiving the sensory feedback from the cardiovascular and respiratory systems during emesis (26).

The CB$_1$ receptor has been found to reduce neurotransmitter release at both excitatory and inhibitory synapses in the cerebellum and the hippocampus by several means. CB$_1$ receptor is a G-protein coupled receptor that can inhibit adenylate cyclase, inactivate A-type $K^+$ channels and inhibit transmitter release by the direct binding of the $\beta$/$\gamma$ subunit of the G-protein to the calcium channels in the postsynaptic membrane (23). Preliminary studies by Derbenev et al. show that CB$_1$ receptor activation reduces glutamatergic transmission to the DMNX in the brainstem of the rat (15). While the behavioural data and immunohistochemistry supports a site
of CB₁ action within the DVC, the site at which emetic inputs converge and emesis is initiated currently remains unknown.

The CB₁ receptor may reduce emesis at three sites indicated in Figure 7: 1) by an action on the terminals of the primary afferent fibers themselves, 2) by acting on the terminals of neurons of the NTS that project to the output neurons of the DMNX, 3) by inhibiting transmitter release from interneurons of the NTS. The possibility that CB₁ receptors are found on the dendrites of the DMNX that project up into the NTS could be inferred from the immunohistochemical data, and further ultrastructural studies are required to definitively identify the localization of CB₁ receptors in the DVC. However, the majority of electrophysiological evidence and electron microscopy have revealed CB₁ receptors in the brain are located at presynaptic sites (38). The interpretation of the three possibilities is based on the assumption that input to an unidentified group of neurons in the DMNX results in their activation and the initiation of the motor program in the stomach, via the vagus (18).

At the first putative site, THC activating CB₁ receptors would inhibit emesis by reducing afferent transmission. In this case, a reduction in Fos immunoreactivity would be shown in neurons downstream of synaptic connections. This is consistent with our data which showed a reduction in the NTS and DMNX. However, the lack of CB₁ receptor immunoreactivity in the subnucleus gelatinosus of the NTS would suggest that CB₁ receptor is not present on some vagal afferent fibers from the stomach. Similarly, in our previous work unilateral nodose ganglionectomy had no effect on the density of CB₁ receptor immunoreactivity in the ferret brainstem, although this result may have been confused by the presence of contralateral projection fibers (37). CB₁ receptor immunoreactive fibers are evident in the TS itself, but the origins and targets of these fibers are unknown.
At the second potential site of action, CB₁ receptors are present on the terminals of neurons within the NTS that receive inputs from the primary afferents. These may be inhibitory interneurons, which then reduce the activity of inhibitory NTS neurons that project to the DMNX. However, to date, the presence of interneurons, which transmit within the NTS and do not project to other brainstem nuclei, has not been demonstrated (35).

The third potential site, where CB₁ receptors are present are on the terminals of NTS neurons, which project to the DMNX or area postrema. The medial subnucleus of the NTS had enhanced Fos immunoreactivity after the emetic stimulation that was reduced by THC through a CB₁ receptor-mediated action. The downstream target of this activation, the DMNX, also showed a reduction in Fos immunoreactivity by THC. The area postrema was strongly excited in response to cisplatin. The dramatic reduction in Fos immunoreactivity that we have demonstrated after THC pretreatment may be due to a modulatory input to the area postrema from the NTS regulated by CB₁ receptor. The area postrema itself is required for the initiation of emesis, since animals with their area postrema ablated are incapable of vomiting in response to cisplatin (28). However, assuming that the area postrema is the sole site of action, then CB₁ receptors would have to be present directly on the area postrema neurons and this localization has not been observed.

THC clearly appears to reduce emesis by reducing the neuronal activation in the DVC in response to cisplatin. The complex neuronal circuitry for this reflex within the NTS is consistent with a nucleus of integrative capacity as opposed to a simple relay centre (26, 35). Thus the importance of demonstrating CB₁ receptor modulation of the emetic reflex goes beyond the regulation of emesis to the modulation of other vagovagal and vagospinal reflexes that control other important gastrointestinal and possibly other functions.
In summary, we have shown that THC dose-dependently inhibits emesis induced by cisplatin through an action at CB₁ receptors. The site of action of THC is in discrete nuclei of the DVC. These same areas are also the location of CB₁ receptor immunoreactivity. These data are consistent with the hypothesis that endogenous cannabinoids are physiological regulators of vagal reflexes including emesis.

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Reference List


FIGURE LEGENDS

Figure 1
THC reduces emesis induced by cisplatin. (A,B) Effect of vehicle (2% DMSO, 1% Tween 80 in PBS; n=4) or increasing concentrations of THC (0.05 to 1 mg/kg; i.p.; n=4 at each dose) administered 15 minutes prior to cisplatin (10 mg/kg iv) on episodes of retching (A) and vomiting (B) in ferrets. The specific CB₁ receptor antagonist SR141716A (5 mg/kg i.p.; n=4) administered 15 minutes prior to THC reversed the effects of THC (1 mg/kg i.p.) on cisplatin induced retching and vomiting (C). * indicates significant difference (p<0.05, ANOVA followed by Tukey post test comparison of groups) of THC/cisplatin from vehicle/cisplatin alone and from SR14176A/THC/cisplatin.

Figure 2
Fos expression in the area postrema (AP; A,B) and rostral dorsal vagal complex (0.5 mm rostral to obex; C,D) in ferrets treated with vehicle/cisplatin (A,C) or THC/cisplatin (B,D). Cisplatin (10 mg/kg; i.v.) treatment induced significant Fos expression in the AP, the medial subnucleus (mn) of the NTS and the DMNX (X). Fos expression in the subnucleus gelatinosus (sg) of the NTS was not significantly increased by cisplatin. THC pre-treatment (1 mg/kg; i.p.) reduced Fos expression in the AP and dorsal vagal complex (B,D). Scale bar: 50 μm.
Figure 3

Distribution of Fos expression and CB₁ receptor immunoreactivity in the area postrema (AP) and the different subnuclei of the dorsal vagal complex in ferrets treated with vehicle/cisplatin (10 mg/kg; i.v., ■; n=5), THC (1mg/kg; i.p) /cisplatin (□; n=4) or SR141716A (5mg/kg i.p.)/THC/cisplatin (■; n=4) at the level of obex. THC significantly reduced the number of Fos immunoreactive nuclei/section evoked by cisplatin in the dorsal/dorsolateral subnucleus (d/dln) of the NTS. SR141716A significantly reversed the effects of THC on cisplatin induced Fos expression in the AP, medial subnucleus (mn) and d/dln. No significant differences were observed in the intermediate (nI), interstitial (ni), ventral/ventrolateral subnuclei (v/vln) or the dorsal motor nucleus of the vagus (DMNX) at this level. These data are superimposed on the underlying CB₁ receptor immunoreactivity (slightly caudal to obex; rat C-terminal antibody). CB₁ shows dense labelling in the AP, the mn of the NTS and the DMNX. * indicates significant difference (p<0.05, ANOVA followed by Tukey post test comparison) of THC/cisplatin from vehicle/cisplatin alone and from SR14176A/THC/cisplatin and Y indicates significant difference (p<0.05) of THC/cisplatin from THC/cisplatin/SR141716A alone.

Figure 4

Number of Fos positive nuclei/section in specific subnuclei of the dorsal vagal complex (DVC) in ferrets treated with vehicle/cisplatin (10 mg/kg; i.v., ■; n=5), THC (1mg/kg; i.p) /cisplatin (□; n=4) or SR141716A (5mg/kg i.p.)/THC/cisplatin (■; n=4). In the rostral DVC (0.5mm rostral to the obex) THC treatment significantly reduced the number of Fos immunoreactive nuclei per section in only the medial subnucleus (mn) of the NTS (A) and the dorsal motor nucleus of the vagus (DMNX; B). This effect was reversed by the CB₁ receptor antagonist
SR141716A in both subnuclei. In the caudal DVC, THC treatment significantly reduced the number of Fos immunoreactive nuclei per section in the ventral/vetrolateral subnucleus (v/vln) only (C). SR141716A pretreatment did not reverse this effect. * indicates significant difference (p<0.05, ANOVA followed by Tukey post test comparison) of THC/cisplatin from vehicle/cisplatin alone and from SR14176A/THC/cisplatin and d indicates significant difference (p<0.05) of THC/cisplatin from vehicle/cisplatin alone.

**Figure 5**

I. Distribution of CB$_1$ receptor immunoreactivity in the dorsal vagal complex with human N-terminal (A-C), rat C-terminal (D-F) and rat N-terminal (G-I) CB$_1$ receptor antibodies 0.5mm caudal to the obex (A,D,G), at the level of the obex (B,E,H) and 0.5-1.0 mm rostral to the obex (C,F,I). The human N-terminal antibody gave dense CB$_1$ receptor immunoreactivity in the dorsal motor nucleus of the vagus (X) and medial subnucleus (mn) of the NTS. With this antibody we also observed immunoreactive neurons in the hypoglossal nucleus (XII). At rostral levels the subnucleus gelatinosus (sg) of the NTS was only weakly immunoreactive. The area postrema (AP) contained punctate immunoreactive terminals illustrated at higher magnification in figure 6. Both rat CB$_1$ receptor antibodies displayed similar patterns of immunoreactivity to that of the human however, the rat C-terminal antibody gave more pronounced immunoreactivity in the AP, while the neuronal immunoreactivity of the rat N-terminal antibody in the hypoglossal nucleus was less intense than seen with the human N-terminal receptor antibody. Scale bar: 100µm.

II. Higher magnifications of human N-terminal CB$_1$ receptor immunoreactivity in the area postrema (A) and regions adjacent to the solitary tract (B, C). A. Punctate immunoreactive terminals are evident in the AP. B. The TS shows immunoreactive fibers (open arrowhead) and
in the ventral/ventrolateral subnucleus of the NTS immunoreactive neuronal perikarya (closed arrowheads) were observed. C. Immunoreactive neurons were also evident in the intermediate (open arrowhead) and interstitial subnucleus (closed arrowhead). Scale bars: 50 μm.

Figure 6
Direct application of a low dose of THC to the brainstem inhibited emesis evoked by intragastric hypertonic saline. Arterial blood pressure traces begin after the end of the infusion of hypertonic saline in A. a control experiment where vehicle was applied (arrows indicate increases in blood pressure during retching episodes) and B. after a pretreatment with THC no increase in blood pressure was observed. C. Episodes of retching and vomiting were completely abolished in animals pretreated with THC in comparison with control animals. D. Ferrets pretreated with THC had a significantly lower increase in mean arterial blood pressure in response to the emetic stimulus than those in control experiments.

Figure 7
Potential sites of CB₁ receptor activation resulting in anti-emetic action consistent with studies of immunohistochemistry and neuronal activation. 1) CB₁ receptors on the terminals of primary afferent fibers from the stomach and duodenum could reduce the input indicating intestinal distress and reduce the resulting episodes of emesis. 2) CB₁ receptors on the terminals of interneurons within the NTS could reduce the input to the DMNX and therefore reduce emesis. 3) CB₁ receptors on the terminals of NTS projection neurons could modulate input from the area postrema or directly reduce excitatory transmission to the DMNX. See discussion for details. Figure adapted from Travagli et al. (35).
Figure 1.

A

Episodes of Retching

THC (mg/kg)

B

Episodes of Vomiting

THC (mg/kg)

C

Episodes of Retching

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