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Arachidonylethanolamide (AEA) activation of FOS proto-oncogene protein immunoreactivity in the rat brain

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Abstract

It is thought that the physiological actions of endogenous cannabinoid arachidonylethanolamide (AEA), as well as exogenous cannabinoids such as Δ^9-tetrahydrocannabinol (THC), are mediated by two subtypes of cannabinoid receptors, CB1 and CB2, which have recently been characterized. Injection of AEA leads to alterations in motor behavior and endocrine function. While these phenomena have been well characterized, the neuronal substrate of AEA's actions remains undetermined. In this study, FOS immunoreactivity (FOSir) was used to map rat brain nuclei that are responsive to a single intracerebroventricular injection of AEA. The results showed that FOSir was induced in several nuclei including the bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), central nucleus of the amygdala (Ce), periaqueductal gray area (PAG), dentate gyrus in the hippocampus (Dg), paraventricular nucleus of the thalamus (PVA), median preoptic nucleus (MnPO), periventricular nucleus (Pe), caudate putamen (CPU) and the ependymal lining of the ventricles. The pattern of activation identified correlates, in part, with the distribution of CB receptors. At the same time, a new subset of nuclei, without demonstrable CB receptors, have been shown to respond to an AEA challenge. Activation of these nuclei is consistent with the physiological effects of AEA. These findings provide valuable information on the response to AEA at the level of neuronal activation and provide the basis for a broader understanding of the possible role of CB receptors in the modulation of motor and endocrine function associated with the use of exogenous cannabinoids, such as marijuana. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: FOS; Immunocytochemistry; Arachidonylethanolamide; Marijuana; Cannabinoid receptor

1. Introduction

Arachidonylethanolamide (AEA), a lipid-soluble compound, has been identified as the endogenous ligand for the cannabinoid (CB) receptor [14]. AEA was originally isolated from porcine brains, and recently has been isolated from both human and rat brains [15]. Systemic injection of cannabinoids has been shown to produce analgesia [25] and altered motor function [33]. Direct injection of AEA into the striatum also results in an alteration in motor function [35]. Administration of AEA to rats has been shown to produce various biological effects, including anti-nociception, hypothermia, hypomotility, and catalepsy [2,34]. Although physiological effects of AEA treatment have been demonstrated, the neuronal pathways associated with AEA's actions have not yet been defined.

Marijuana is one of the most commonly abused drugs available today. The use of marijuana is associated with several socially desirable effects, such as an associated 'high' or sense of euphoria accompanied by uncontrollable laughter, alteration in the sense of time, introspection, and a dream-like state. Marijuana has also been shown to be responsible for several detrimental health effects, including respiratory and cardiovascular abnormalities, as well as the characteristic 'amotivational syndrome' [23]. The major psychoactive component of marijuana is Δ^9-tetrahydrocannabinol (THC), which acts via CB receptors.

Two subtypes of CB receptors, CB1 and CB2, have been identified. Both the endogenous ligand, AEA, and the external stimulus, THC, have been shown to bind both receptor subtypes with different affinities [1]. The CB2 receptor is found in abundance within the immune system, specifically in B-lymphocyte-rich areas of the spleen, lymph nodes, and Peyer's patches, but is absent in the T-lymphocyte-predominant areas within these organs [26].

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In contrast, CB1 is found primarily in the central nervous system with a sparse peripheral distribution in the adrenal gland, heart, lung, testes, uterus, ovaries, bone marrow, thymus and tonsils [16]. Within the CNS, CB1 receptors have been found in the outflow nuclei of the basal ganglia (substantia nigra pars reticulata and the globus pallidus), the hippocampus, and the cerebellum [20,27] which are expected to be the sites where both AEA and THC exert their neuronal actions. The distribution of these receptors corresponds to the regions in which AEA has been isolated in the brain including the hippocampus, cerebellum, striatum, and thalamus [15]. By determining the neuronal substrates and pathways associated with AEA's actions, we may better understand the functional role these receptors play in the psychoactive effects produced by an external stimulus such as THC.

Over the past decade, FOS expression has been used as an anatomical marker for neuronal activation in the CNS. FOS, the proto-oncogene nuclear protein product of the immediate early gene, c-*fos*, is low or absent at the basal level [30], but can be rapidly and transiently induced by various pharmacological, electrical, and physiological stimuli [2,6–8,19,29]. Using immunocytochemistry techniques, FOS immunoreactivity (FOSir) has been used successfully for neuronal activation mapping at the single-cell resolution [7,8]. Since the FOS–JUN complex is a transcriptional factor, the detection of FOS expression can also provide information about subsequent gene regulation. Several studies have demonstrated that FOS expression can be used to investigate the neuronal substrates and pathways in the CNS [8,9,17]. Recently, Wegner et al. [39] reported that AEA treatment results in the activation of FOS expression in the hypothalamic paraventricular nucleus (PVN), and that the increase of FOS expression is temporally related to an increase in corticotropin releasing factor (CRF) synthesis.

In this study, we have mapped the expression of FOS immunoreactivity following an acute intracerebroventricular (ICV) injection of AEA to provide a neuron-based explanation for the physiological effects of AEA and to explore the relationship between CB receptor distribution and the sites of AEA neuronal action. By doing so, we can present a holistic view of the neuronal substrates and pathways involved in AEA's actions in the rat brain and may help to explain the mechanisms by which cannabinoids, such as THC, produce their biological effects.

2. Materials and methods

2.1. Cannulation surgery

Twenty-four adult male Harlan Sprague–Dawley rats were acclimatized on a 12 h:12 h light–dark schedule for one week. The animals were then anesthetized with ketamine (80 mg/kg)/xylazine (8 mg/kg) for implantation of a 20 gauge guide cannula into the right ventricle at 0.3 mm posterior to the bregma, 1.3 mm lateral to the midline, and 4.5 mm below the skull surface. The cannula was anchored to the skull with two stainless steel screws and cranioplastic cement. A dummy cannula (26 gauge) was inserted into the guide cannula, and the animals were allowed to recover from surgery for 7–10 days. During the post-operative period, the dummy cannula was removed and re-inserted once a day to condition the animals to injection.

2.2. Animal treatments

At the end of the recovery period, the animals ($n = 4$) were randomly assigned into six groups. Based on previously unpublished dose response trials, two animals from each group were injected (i.c.v.) with either 10 μl (16.7 μg/μl) AEA (Sigma, St. Louis, MO) or control vehicle [18:1:1 saline/ethanol/Alkamuls EL620 (Rhone-Poulenc, Cranbury, NJ)] for 5 min, 2 h, 4 h, 8 h, 16 h and 24 h prior to being overdosed with 0.5 ml pentobarbital (i.p.).

2.3. Animal perfusion and brain preparation

At the end of treatment, the animals were perfused transcardially using 250 ml isotonic saline followed by 500 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. The brains were removed, post-fixed for an additional 2 h at 4°C, and cryoprotected in 30% sucrose in PB at 4°C until the brains sank. The brains were frozen on powdered dry ice and stored at −80°C until microdissection.

2.4. FOS immunocytochemistry

Coronal sections (40 μm) were cut on a freezing microtome (Miles, Lansing, MI) and soaked in Tris-buffered saline (TBS), pH 7.6, for at least 1 h to remove the fixative. The tissues were then treated with 0.2% Triton X-100 (Sigma, St Louis, MO) in TBS for 30 min at room temperature (RT), followed by two washes with TBS for 15 min each. The sections were then stored in PB at 4°C until immunocytochemical staining with FOS antiserum was performed. The sections were incubated with anti-FOS antiserum (AB-2, Oncogene Science, Uniondale, NY), diluted 1:200 in normal goat serum, at 4°C for 40 h with gentle shaking on an orbital shaker. The sections were then washed with TBS four times for 15 min each with gentle shaking at RT. The sections were then incubated with biotinylated goat anti-rabbit IgG (Vectastain ABC kit,

Table 1
Distribution of FOS immunoreactivity in the rat brain after an ICV injection of AEA compared with control vehicle-injected rat

Neuroanatomical area	Control	Treatment
Telencephalon		
Cingulate cortex	0	0
Infralimbic cortex	0	0
Frontal parietal cortex	0	0
Piriform cortex	0	0
Entorhinal cortex	0	0
Temporal cortex	0	0
Claustrum	0	0
Amygdala	0	0
Medial nucleus	0	0
Central nucleus	0	+ + +
Lateral nucleus	0	0
Basolateral nucleus	0	0
Hippocampus	0	+
Dentate gyrus, hippocampus	0	+ +
Anterior olfactory nucleus	N/A	N/A
Nucleus accumbens	0	0
Caudate putamen	0	+
Globus pallidus	0	0
Septum		
Lateral nucleus	N/A	N/A
Medial nucleus	N/A	N/A
Diagonal band	N/A	N/A
Subfornical organ	0	0
Bed nucleus stria terminalis	0	+ + +
Median preoptic area	0	+ + + +
Diencephalon		
Hypothalamus		
Anterior hypothalamus	0	0
Supraoptic nucleus	0	+
Paraventricular nucleus	0	+ + +
Periventricular nucleus	0	+ + +
Arcuate nucleus	0	0
Median eminence	0	+
Ventromedial nucleus	0	0
Dorsomedial nucleus	0	0
Lateral hypothalamus	0	0
Medial mammillary nucleus	0	0
Thalamus		
Paraventricular nucleus	0	+ + +
Mediodorsal nucleus	0	+
Intermediodorsal nucleus	0	0
Laterodorsal nucleus	0	+
Central medial nucleus	0	+
Paracentral nucleus	0	0
Rhomboid nucleus	0	0
Reuniens nucleus	0	0
Ventromedial nucleus	0	+
Zona incerta	0	0
Habenula	0	+
Mesencephalon		
Interpeduncular complex	0	0
Supramammillary nucleus	0	0
Substantia nigra	0	0
Ventral tegmental area	0	0
Periaqueductal gray	+	+ + +
Anterior pretectal nucleus, dorsal	0	0
Olivary pretectal nucleus	0	0
Superior colliculus	0	0
Metencephalon		
Locus coeruleus	0	0
Dorsal raphe	0	0
Solitary nucleus	0	0

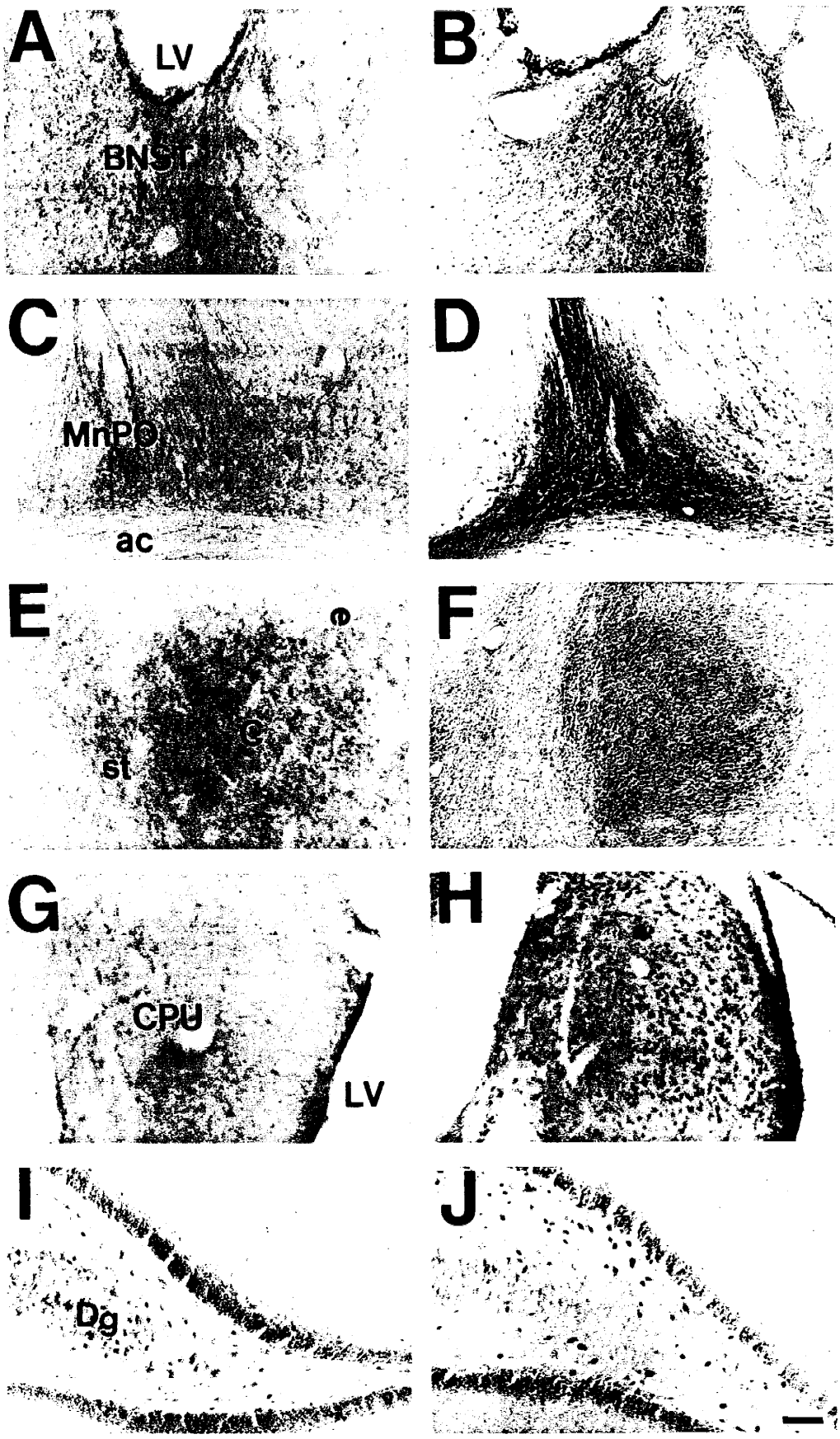
Density: + + + +, very dense; + + +, dense; + +, moderately dense; +, light; 0, not detectable; N/A, not available.

Vector Laboratories, Burlingame, CA), diluted 1:300 in TBS. The sections were washed two times with TBS for 15 min each at RT, and two more times using TBS (pH 9.6). After these four washes, the sections were incubated in avidin–biotin–peroxidase (ABC) in TBS (pH 9.6) for 2 h at RT with gentle shaking. The sections were then washed four times using TBS for 10 min each at RT with gentle shaking. Finally, the tissues were incubated at RT for 5–10 min in a solution in which glucose oxidase was used to generate hydrogen peroxide to initiate the ABC reaction using glucose coupled with 3,3′-diaminobenzidine tetrahydrochloride (DAB) which produces a black-green stain. After FOS staining, the sections were mounted on slides, air dried, counter-stained with methyl-green, dehydrated through graded alcohols into Hemo-De® (Fisher Scientific, Springfield, NJ) and cover-slipped with Permount® (Fisher Scientific, Springfield, NJ). The FOS-stained cells were counted against the methyl-green stained cells. This procedure was repeated at least three times using members of a separate group of animals ($n = 4$) which were perfused 2 h after injection. Two controls for

Table 2
Time course of FOS activation in various nuclei in the rat brain

	5 min	2 h	4 h	8 h	16 h	24 h
Controls	0	0	0	0	0	0
Caudate putamen	0	+ +	+	0	0	0
Paraventricular hypothalamic nucleus	0	+ + +	+	+ or + +	0	0
Periventricular nucleus	0	+ + +	+	+ +	0	0
Bed nucleus stria terminalis	+	+ + +	+	0	0	0
Central nucleus of amygdala	0	+ + +	+	0	0	0
Paraventricular thalamic nucleus	0	+ + +	+ +	+	0	0
Periaqueductal gray	0	+ + +	+	0	0	0
Ependymal lining	0	+ + +	+ + +	+ +	0	0
Median preoptic area	0	+ + +	+	+	0	0
Dentate gyrus of the hippocampus	0	+ + +	+	0	0	0

Density: + + + +, very dense; + + +, dense; + +, moderately dense; +, light; 0, not detectable.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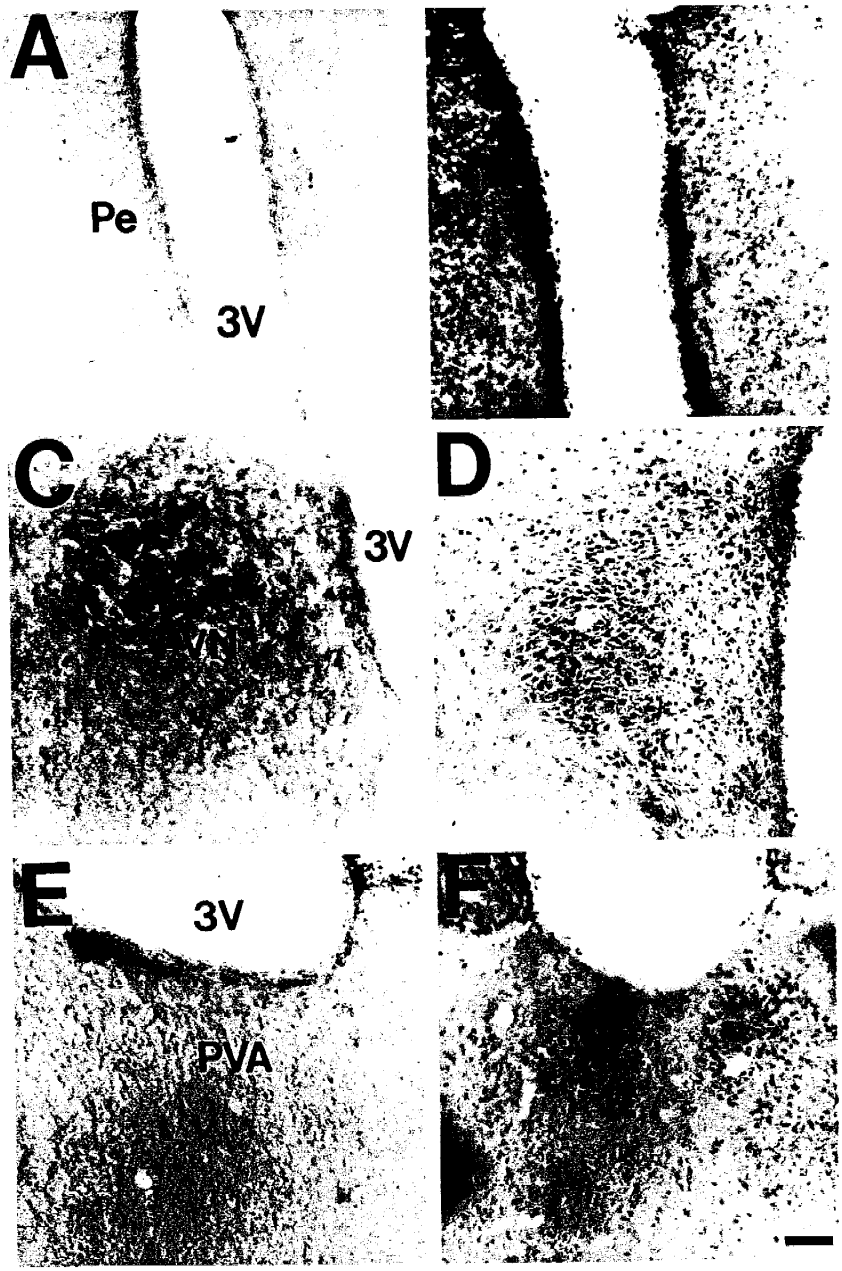

Fig. 2. Representative photomicrographs showing induction of FOS immunoreactivity in the rat diencephalon after injection with AEA. Induction seen in the periventricular nucleus (Pe) (B), the paraventricular nucleus (PVN) (D) and the paraventricular thalamic nucleus (PVA) (F) compared to a rat given control vehicle (A, Pe; C, PVN; E, PVA). Abbreviations: 3 V, third ventricle. Plane of section relative to bregma: −1.8 mm for Pe, −1.8 mm for PVN and −2.3 mm for PVA. Scale bar = 100 μm.

specificity of immunostaining were used: (1) incubation in non-immune rabbit serum in place of the anti-FOS anti-sera; and (2) anti-FOS anti-sera pre-absorbed with the synthetic peptide-2 (Oncogene Science, Uniondale, NY) at 10 fold concentration of AB-2 overnight at 4°C prior to use in the primary incubation.

Fig. 1. Representative photomicrographs showing induction of FOS immunoreactivity in the rat telencephalon after injection with AEA. Induction seen in the bed nucleus of the stria terminalis (BNST) (B), median preoptic nucleus (MnPO) (D), central amygdala (Ce) (F), dorsomedial caudate putamen (CPU) (H), and dentate gyrus of the hippocampus (Dg) (J) compared to a rat given control vehicle (A, BNST; C, MnPO; E, Ce; G, CPU; I, Dg). Abbreviations: st, stria terminalis; ac, anterior commissure. Plane of section relative to bregma: 0.2 mm for BNST, −2.3 mm for Ce and CPU, −3.0 mm for MnPO and −3.30 mm for Dg. Scale bar = 100 μm.

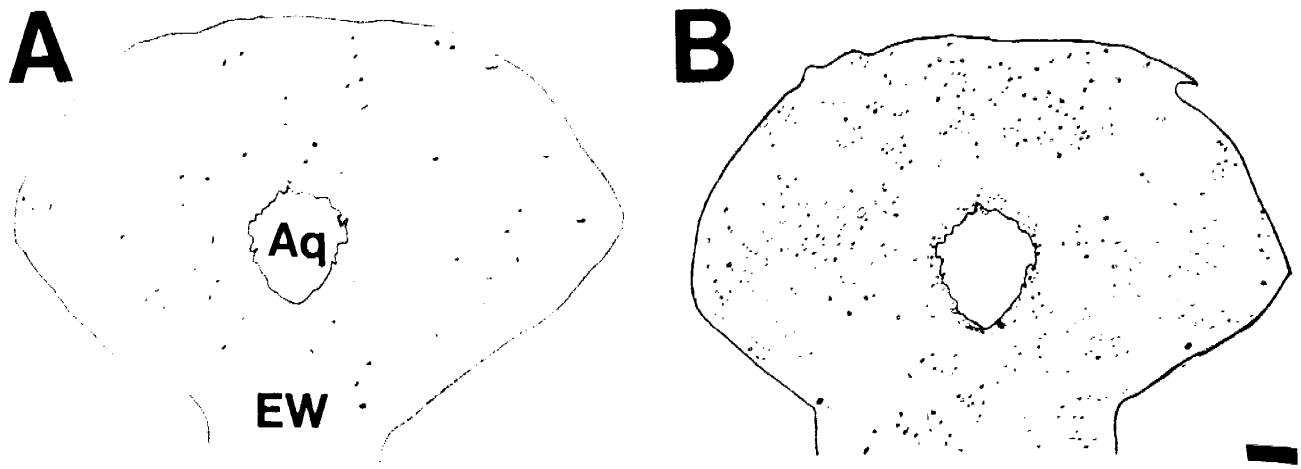

Fig. 3. AEA induction of FOS immunoreactivity in the periaqueductal gray (PAG). Camera lucida drawing shows FOS immunoreactivity induction in the PAG (B) after injection with AEA compared with a rat given control vehicle (A). Abbreviations: Aq, aqueduct; EW, Edinger-Wesphal nucleus. Plane of section relative to bregma: −6.3 mm.

2.5. Determination of FOS immunoreactivity

In order to determine the number of cells showing FOSir in various nuclei, two technicians counted FOS immunoreactive cells vs. non-reactive methyl green-stained cells from matched sections of three animals. The mean of FOSir positive cells was reported in both Tables 1 and 2.

3. Results

3.1. Pattern of FOS activation

3.1.1. Telencephalon

Within the telencephalon, FOS immunoreactivity was not detectable in the cerebral cortex in the basal state. The central nucleus of the amygdala (Ce), the hippocampus (Hi), and the dentate gyrus (Dg) within this region showed a clear elevation in FOS expression following an acute injection of AEA. A similar response was noted in the bed nucleus of the stria terminalis (BNST) and the median preoptic nucleus (MnPO) (Table 1 and Fig. 1A–J).

3.1.2. Diencephalon

Within the hypothalamus and thalamus of the diencephalon, several nuclei responded to an acute injection of AEA. In the hypothalamus, both the periventricular nucleus (Pe) and the paraventricular hypothalamic nucleus (PVN) showed a clear elevation in FOS expression over control; the supraoptic nucleus (SON) and median emi-

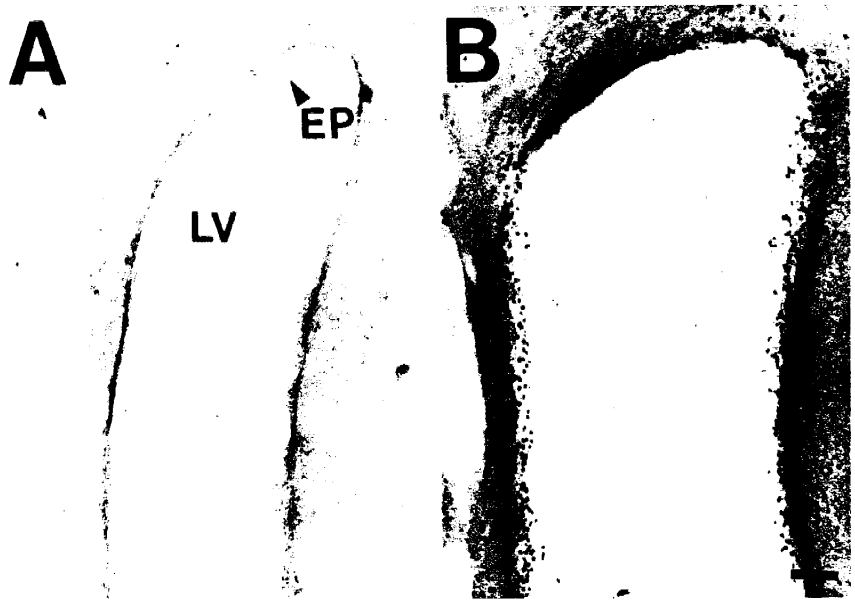

Fig. 4. Representative photomicrographs showing AEA induction of FOS immunoreactivity (B) in the ependymal lining (EP) of the lateral ventricle (LV) compared to a rat given control vehicle (A). A similar pattern of activation was seen in the ependymal lining of the 3rd and 4th ventricles as well as their associated ducts. Plane of section relative to bregma: 0.2 mm. Scale bar: 100 μm.

nence (ME) showed light FOSir compared with the animals given control vehicle. In the thalamus, the paraventricular thalamic nucleus (PVA) demonstrated a very dense sensitivity to AEA treatment, while the mediodorsal (MD), laterodorsal (LD), central medial (CM), and ventromedial (VM) nuclei, and the habenula (Hb) showed only a light sensitivity to AEA treatment (Table 1 and Fig. 2A–F).

3.1.3. Mesencephalon

The periaqueductal gray (PAG) was the only area of the mesencephalon in which an elevation in FOS expression was observed in the AEA-treated animals (Table 1 and Fig. 3A–B).

3.1.4. Metencephalon

In both AEA-treated and control animals, virtually no FOSir was detected within the metencephalon, including the locus coeruleus, dorsal raphe and solitary nucleus (Table 1).

3.2. Time course of FOS activation

As shown in Table 2, an acute AEA injection induced a distinct time-dependent pattern of FOSir in several nuclei of the brain, including the BNST, PVN, Ce, PAG, Hi, Dg, PVA, MnPO, Pe, CPU and the ependymal lining (EP) of all four ventricles and associated ducts (Fig. 4). Virtually no FOSir was detected in these nuclei in rats treated with control vehicle alone. In animals sacrificed 5 min after injection, FOSir in the BNST was minimal. The pre-absorption experiment confirmed the specificity of the FOS activation. All of the positive nuclei reached a maximal level of FOS expression 2 h after injection; however, several nuclei showed a variation in the duration of activation. Peak activation of FOSir in the BNST, CPU, Ce, PAG and Dg was seen 2 h after injection and leveled off at 4 h. Activation of FOSir in the PVA, MnPO and ependymal lining peaked at 2 h after injection but persisted for up to 8 h. The PVN and Pe showed a biphasic pattern, with peak FOSir occurring at 2 h, followed by a drop at 4 h, and a second rise at 8 h.

4. Discussion

The results of this study provide a neuroanatomical map of the various pathways within the rat brain which show neuronal activation in response to an acute challenge with AEA, the endogenous ligand for the CB receptor. Following an acute ICV injection of AEA, expression of the FOS protein was induced in several brain areas, including the BNST, PVN, SON, ME, Ce, PVA, MD, LD, CM, VM, PAG, Hb, Hi, Dg, MnPO, Pe, CPU and the ependymal linings of the brain. All these nuclei, except the PVN and Pe, demonstrated a maximal response in FOSir at 2 h and a gradual diminution in response within 6–8 h. In the case of the PVN and Pe, a maximal response was noted at 2 h with a slight decrease at 4 h; however, 8 h after injection, there was a second smaller response. One explanation of these findings may be that the PVN and Pe are directly activated in response to an acute AEA challenge, and then secondarily reactivated by another group of neuroanatomically linked nuclei during the recovery phase. Further investigation is needed to test this hypothesis.

A dense distribution of CB1 receptors has been identified in the caudate putamen (CPU). THC has been shown to induce FOS expression in the dorsomedial striatum [28], which is consistent with our results showing AEA induced FOSir in the dorsal–caudal portion of the CPU (Fig. 1H). CB1 receptors have also been found in the globus pallidus, the substantia nigra and the cerebellum. However, neither THC [28] nor AEA in this study induced FOSir in these areas.

The hippocampus, including the dentate gyrus, is one of the regions with the densest distribution of CB1 receptors. We demonstrated FOSir in this area in response to AEA; however, THC-induced FOSir in the hippocampus is reportedly absent [28]. The hippocampus has been shown to be involved in both short- and long-term memory [38]. Exogenous cannabinoids, such as THC, have been shown to hinder memory function in the rat [24]. Similarly, CB1 receptor antagonists have been shown to enhance memory function [36]. To date, AEA has not been shown to alter memory; however, the role of CB1 receptors in memory, as suggested by the papers mentioned above, along with AEA induction of FOSir in the dentate gyrus of the hippocampus does not allow one to discount AEA as a memory-altering agent.

CB1 receptors have also been shown to be expressed in the nucleus accumbens (NAc) [26,27]. Low doses of THC failed to induce FOS expression in the NAc, however, a high dose (10 mg/kg) of THC did induce FOSir [28]. In this study, AEA-induced FOSir was not demonstrated in this region.

The lack of complete correlation between the distribution of the CB1 receptors and FOSir induced by both THC and AEA is similar to previous studies by our lab and others suggesting a mismatch between receptor distribution and FOS activation in the CNS [7,17]. The densest distribution of CB1 receptors is found within the basal ganglia, cerebellum, and hippocampus. While FOS induction by AEA was noted in the striatum and dentate gyrus, the response to AEA in other nuclei does not correlate with the reported CB1 receptor distribution.

However, there has been some correlation demonstrated between FOS activation and the physiological effects of AEA. For example, neuronal activation within the periaqueductal gray has been associated with analgesia [3]. Induction of FOSir in this area was identified in this study

after injection of AEA, and may account for these analgesic effects. Additionally, direct administration of AEA into the striatum causes turning behavior in mice [35].

One function common to several of the AEA-activated nuclei includes the response to stress. The Ce, PVN, Dg, PVA, and EP have all been shown to respond to stressful stimuli, which directly or indirectly increases the levels of CRF. The Ce, along with the BNST, serves as an integral part of the amygdaloid complex that participates in the modulation of various endocrine functions [13]. Similarly, induction of FOSir in the dentate gyrus has been noted in response to increased cortisol levels, suggestive of a negative feedback mechanism [22,37]. Recent studies have demonstrated FOS expression in the PVA and the Hb in response to the stress of morphine withdrawal in rats. The PVA has been also reported to be involved in conditioning to drug exposure [4,5].

One of the areas demonstrating dense FOSir induced by AEA in this study is the median preoptic area (MnPO). The MnPO is a sexually dimorphic nucleus with a role in neuroendocrine function and reproductive behavior [18]. Sexual behavior has been shown to induce FOS expression in the MnPO, BNST, and Ce in male rat [11]. A neuronal network between the MnPO and periaqueductal gray (PAG) has been reported to play a functional role in reproductive behavior [32]. AEA induction of FOSir in the MnPO, BNST and Ce, as shown in this study, suggests that AEA-dependent pathways may be related to male sexual behavior and the reproductive system.

The ependyma has also been shown to express FOSir [31] and increased levels of CRF in response to stressful/noxious stimuli [10,21]. CRF is a potent stimulator produced by the PVN, which activates the HPA axis to produce both ACTH and corticosterone. Recent studies have shown that administration of AEA significantly increases ACTH and CRF levels. This finding is consistent with the expression of FOSir within the PVN following AEA administration, as seen in this study and a previously published report [39]. Taken together, these data indicate that the PVN could be the site in which a marked increase in ACTH and corticosterone is initiated by AEA [40].

Overall, this study provides a neuroanatomical map of rat brain nuclei activated following an acute challenge with AEA, and supplies important information in understanding the physiological effects of AEA, the endogenous ligand of CB receptors. Some of these effects can be attributed to the activation of brain regions expressing CB receptors such as the Hi, Dg and the CPU. However, several areas that do not express CB receptors, including the EP, MnPO, Ce, PVN, Hb, PVA, Pe, and the BNST could also play a functional role associated with the actions of AEA. In summary, the nuclei activated by AEA consisted of, but were not restricted to, areas expressing CB receptors. To fully understand the significance of the neuronal pathways affected by AEA administration further investigation is needed; however, this study provides a valuable insight into the neuronal substrates associated with AEA, and possibly with exogenous cannabinoids, such as marijuana.

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