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Cellular localization of cannabinoid receptors and activated G-proteins in rat anterior cingulate cortex

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Abstract

Cannabinoid receptors are found in moderate density throughout the cerebral cortex. The anterior cingulate cortex (ACC) is of particular interest due its high level of cannabinoid receptors and role in behaviors known to be modulated by cannabinoids. These studies were conducted to determine the cellular localization of cannabinoid receptors and to compare the level of cannabinoid receptor binding with receptor-mediated G-protein activity in the rat ACC. Either ibotenic acid or undercut lesions were made in ACC, and brains were processed for [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS autoradiography. Both cannabinoid receptors and receptor-activated G-proteins were highest in laminae I and VI of ACC in control tissue. Although similar levels of receptor binding were found in these laminae, significantly higher levels of receptor-activated G-proteins were found in lamina VI. Ibotenic acid lesions that destroyed ACC neurons decreased [^{3}H]WIN 55,212-2 binding by 60–70% and eliminated WIN 55,212-2-stimulated [^{35}S]GTPγS binding. In contrast, deafferentation of the ACC with undercut lesions had no significant effect on cannabinoid receptor binding or G-protein activation. These results indicate that cannabinoid receptors in laminae I and VI of the ACC are located on somatodendritic elements or axons intrinsic to the ACC. In addition, differences in the relative levels of cannabinoid binding sites and activated G-proteins between cortical laminae indicate that the efficiency of cannabinoid receptors for G-protein activation may vary within a specific brain region.

Keywords: [^{35}S]GTPγS autoradiography; WIN 55,212-2; Deafferentation; Excitotoxin

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Introduction

Cannabinoid agonists such as delta^9-tetrahydrocannabinol (Δ^9-THC) produce psychoactive effects, antinociception, memory impairment and inhibition of motor activity [1,2]. Many studies have described the localization of cannabinoid receptors in subcortical regions that contribute to these effects, but relatively little is known regarding the cellular localization of cortical cannabinoid receptors. Furthermore, although the distribution of cannabinoid binding sites in brain has been reported, studies have shown that receptor binding levels do not always correlate with receptor activity. The efficiency of cannabinoid receptors for activation of G-proteins varies by brain region, and differs from the efficiency for other G-protein-coupled receptors to activate G-proteins [3,4]. However, studies to date have examined cannabinoid receptor efficiency in membrane homogenates prepared from grossly dissected brain areas and have not addressed potential differences within a discrete region.

Cannabinoid receptor binding and G-protein activity in the anterior cingulate cortex (ACC) are of interest for several reasons. Cannabinoid receptor density in the ACC is moderately high, as demonstrated by both receptor binding [5–7] and immunohistochemistry [8,9]. This receptor density is correlated with high levels of cannabinoid receptor-mediated G-protein activity [4,10]. The ACC is a component of the rostral limbic system and is involved in affective and cognitive behaviors, which have been reviewed in detail [11]. Functions that are of particular interest in relation to the cannabinoid system include motor, motivational and antinociceptive effects. The possibility that the ACC is involved in these effects of cannabinoids is supported by studies showing increased cerebral blood flow in the cingulate cortex of subjects given Δ^9-THC infusions [12].

The combined use of cannabinoid receptor binding and agonist-stimulated [^{35}S]GTPγS autoradiography provides an opportunity to examine the relationship between cannabinoid receptors and activation of G-proteins in specific brain regions. In the present studies, these techniques have been combined with specific lesions of the ACC, including ibotenic acid and undercut lesions, to examine the relationship between cannabinoid binding sites and G-protein activation and to determine the cellular localization of cannabinoid receptors in the ACC.

Methods

Materials

Male Long Evans rats (350–400 g) were purchased from Harlan Laboratories (Indianapolis, IN). [^{35}S]GTP$^{\gamma}$S (1250 Ci/mmol) and [^{3}H]WIN 55,212-2 (45.5 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). WIN 55,212-2 was purchased from Research Biochemicals International (Natick, MA) and GDP was purchased from Sigma Chemical Co. (St. Louis, MO). Reflections® autoradiography film and Hyperfilm βmax were purchased from New England Nuclear Corp. and Amersham Life Sciences (Arlington Heights, IL), respectively. All other chemicals were obtained from Sigma or Fisher.

Surgery

Ibotenic acid or deafferentation lesions were made as previously described [13]. Rats were anesthetized (0.3 ml chloropent/100 g body weight), and lesions were placed according to stereotaxic coordinates. For ibotenic acid lesions, two injections of ibotenic acid (3–3.5 μl per injection of 10 μg ibotenic acid/μl 0.9% saline) were placed +1.0 and +0.4 mm anterior to bregma, 0.5 mm lateral to the midline, and 2.4 mm ventral to the cortical surface (N = 7). Unilateral undercut lesions were made by passing a scalpel blade 1.6 mm lateral to the midline, 4.5 mm ventral to the cortical surface of the brain extending from 1.7 mm anterior to bregma to 1.0 mm posterior to bregma (N = 6). Coronal knife cuts were made at the rostral and caudal extents of the knife cut. Two weeks later, rats were decapitated, brains were removed and frozen in isopentane at −30 °C. Twenty micron coronal sections through the cingulate cortex were cut on a cryostat at −20 °C and mounted on gelatin-coated slides. In addition, 50 μM sections were collected at appropriate intervals for thionin staining. Slides were desiccated at 4 °C overnight and stored desiccated at −80 °C until assay.

Receptor autoradiography

Slides were stored at −20 °C overnight before processing for cannabinoid receptor autoradiography. For assay, slides were brought to room temperature under cool air, then incubated in 20 mM HEPES buffer containing 0.5% bovine serum albumin (BSA) and 1 mM $MgCl_2$ (pH 7.0) (HEPES/BSA/$MgCl_2$) at 30 °C for 20 minutes. Slides were transferred to HEPES/BSA/$MgCl_2$ containing 1 nM [^{3}H]WIN 55,212-2 at 30 °C for 80 minutes. Nonspecific binding was assessed by including 1μM WIN 55,212-2 in the incubation mixture. Slides were then rinsed four times for 10 minutes each at 25 °C in HEPES/BSA/$MgCl_2$, and 30 seconds in deionized H_2O on ice. Slides were dried under a cool stream of air, then exposed to Hyperfilm [^{3}H] in cassettes containing a [^{3}H] microscale for 4 weeks.

[^{35}S]GTPγS autoradiography

Slides were brought to room temperature using a cool air dryer, then incubated in 50 mM Tris buffer (pH 7.4) with 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl and 0.5% BSA (TME buffer + BSA) for 10 minutes at 25 °C. Slides were then incubated in TME buffer + BSA with 2 mM GDP for 15 minutes at 25 °C, followed by 10 μM WIN 55,212-2, 0.04 nM [^{35}S]GTPγS and 2 mM GDP in assay buffer at 25 °C for 2 hours. 10 μM WIN55,212-2 has been shown to produce maximal stimulation of [^{35}S]GTPγS binding in brain that is antagonized by addition of SR141716A [10,14]. Basal binding was determined in the absence of agonist. Slides were rinsed twice for 2 minutes each in 50 mM Tris buffer (pH 7.4) at 4 °C, then for 30 seconds in deionized H_2O at 4 °C. Slides were dried overnight, then exposed to Reflections® film for 48 hours. Each cassette contained a [^{14}C] microscale for densitometric analysis.

Data analysis

Specific criteria were required for inclusion of brains in densitometric analyses. Thionin-stained sections were used to verify that 1) ibotenic acid ablated brains showed complete neurodegeneration in

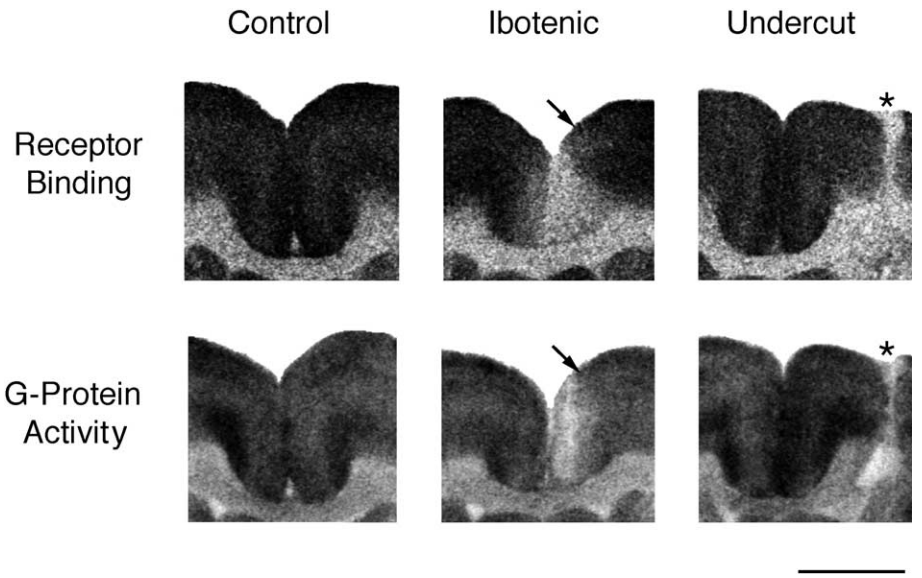

Fig. 1. Brain sections showing unablated (control) ACC, ibotenic acid and deafferentation lesions in the ACC. The asterisk indicates the site of the undercut lesion and the point of ibotenic acid injection is marked by the arrow. Decreases in [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding in ibotenic acid ablated brains are evident, whereas no changes in binding are seen following deafferentation. (bar = 1.5 mm)

the measurement field and 2) ACC of deafferentation brains did not exhibit tissue damage. Four ibotenic acid ablated and six deafferented brains met these criteria. Films were digitized with a Sony XC-77 video camera and analyzed using the NIH Image program for Macintosh computers. Densitometric measurements were made by placing a standard rectangular sample area over lamina I or VI of areas 24a and 24b at the level of the lesion shown in Fig. 1 and as depicted in previous studies [15]. [^{35}S]GTPγS binding values are expressed as fmol [^{35}S]GTPγS/mg protein and values were corrected for [^{35}S] based upon incorporation of [^{35}S] into sections of frozen brain paste [16]. Agonist-stimulated [^{35}S]GTPγS and receptor binding values were converted to fmol or pmol/mg protein based on specific activity of the isotopes and the ratio of mg protein/mg tissue. Data are reported as mean values ± standard error (S.E.) of triplicate sections of brains from 4–6 animals. Statistical significance was determined by analysis of variance followed by a post-hoc analysis with the non-paired two tailed Student's T-test using JMP (SAS Institute, Cary, NC) using the mean binding from each animal as an individual determination.

Results

An analysis of cannabinoid receptor binding and cannabinoid-activated G-proteins in the ACC was performed by comparing autoradiograms of [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding (Fig. 1). Both [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding were dense in the ACC, although binding levels were not as high as in the lateral caudate

putamen and globus pallidus in the same sections. A laminar distribution of [^{3}H]WIN 55,212-2 binding was evident in the ACC, with the greatest binding densities in laminae I and VI. This same laminar distribution was found for WIN 55,212-2-stimulated [^{35}S]GTPγS binding (Fig. 1). [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding were measured in areas 24a and 24b of the ACC. Area 24a is located ventral to area 24b and the two regions can be distinguished based on cytoarchitecture [17].

Control binding was measured in laminae I and VI of areas 24 a and b in unablated ACC. Data from areas 24 a and b were pooled because similar results were obtained in both regions. [^{3}H]WIN 55,212-2 binding did not significantly differ between laminae I and VI (1.27 ± 0.04 and 1.11 ± 0.06 pmol/mg, respectively). In contrast, the level of WIN 55,212-2-stimulated [^{35}S]GTPγS binding was significantly higher in lamina VI than lamina I (6.20 ± 0.65 vs. 3.72 ± 0.49 fmol/mg ($p = 0.01$)).

Brains from excitotoxin and deafferentation ablated animals were examined to determine the cellular localization of cannabinoid receptors and receptor-activated G-proteins in the ACC. Separate measurements were made in areas 24a and 24b of the ACC, although similar results were obtained in both regions (Table 1). Control values were measured in the contralateral ACC of deafferented brains, because no differences in binding were found between this hemisphere and unablated controls. Ibotenic acid injection destroyed neurons in the ACC, as seen in thionin stained sections. Visual inspection of sections revealed that [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding were dramatically reduced in laminae I and VI of ibotenic acid ablated ACC compared to control tissue (Fig. 1). Densitometric analysis confirmed that [^{3}H]WIN 55,212-2 binding was reduced by at least 60% in ibotenic acid ablated ACC. Moreover, WIN 55,212-2-stimulated [^{35}S]GTPγS binding was effectively eliminated in the ACC of brains from ibotenic acid ablated

Table 1
Cannabinoid Receptor Binding and Receptor-Activated G-Proteins in Rat Anterior Cingulate Cortex

	AREA 24a lamina I		AREA 24b lamina I	
	[^{3}H]WIN	Net WIN-Stim [^{35}S]GTPγS	[^{3}H]WIN	Net WIN-Stim [^{35}S]GTPγS
CONTROL	1.35 ± 0.06	3.31 ± 0.54	1.27 ± 0.07	4.00 ± 0.62
UNDERCUT	1.37 ± 0.06	3.61 ± 0.43	1.20 ± 0.09	4.46 ± 0.58
IBOTENIC	0.31 ± 0.01 *	0.17 ± 0.48 *,‡	0.40 ± 0.01 *	−0.28 ± 0.31 *,‡
	AREA 24a lamina VI		AREA 24b lamina VI	
	[^{3}H]WIN	Net WIN-Stim [^{35}S]GTPγS	[^{3}H]WIN	Net WIN-Stim [^{35}S]GTPγS
CONTROL	1.15 ± 0.08	6.25 ± 69	1.13 ± 0.07	6.10 ± 0.62
UNDERCUT	1.10 ± 0.08	5.49 ± 0.66	0.98 ± 0.06	5.74 ± 0.45
IBOTENIC	0.45 ± 0.01 *	0.23 ± 0.20 *,‡	0.55 ± 0.03 *	1.13 ± 0.28 *

[^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding in the anterior cingulate cortex of rats that received undercut or ibotenic acid lesions. Laminae I and VI of areas 24a and 24b were analyzed using computer-assisted densitometry and resulting values are expressed in pmol/mg protein for [^{3}H]WIN 55,212-2 and fmol/mg protein for [^{35}S]GTPγS binding. Data represent triplicate sections from six control and undercut lesion brains and four ibotenic acid lesion brains.

* $p < 0.005$ different from control.

‡ not significantly different from basal.

animals (Table 1). In fact, WIN 55,212-2-stimulated [^{35}S]GTPγS binding in ablated brains was not significantly different from basal [^{35}S]GTPγS binding in lamina I of areas 24 a and b and lamina VI of area 24 a ($p > 0.6$). Basal [^{35}S]GTPγS binding was also reduced in ibotenic acid ablated brains. Reductions in basal [^{35}S]GTPγS binding were greatest in area 24b, where basal binding decreased by 47% ($p < 0.005$) and 34% ($p \leq 0.001$) in laminae I and VI, respectively. Basal [^{35}S]GTPγS binding in area 24a was decreased by 34% ($p \leq 0.01$) and 25% (NS) in laminae I and VI, respectively.

Examination of brains from animals with undercut lesions confirmed that the cortex was deafferented without direct damage to ACC. No changes in [^{3}H]WIN 55,212-2 or WIN 55,212-2-stimulated [^{35}S]GTPγS binding were seen in sections from ablated cortices as compared to unablated tissue (Fig. 1). This was confirmed by densitometric analysis (Table 1); no significant differences were measured in [^{3}H]WIN 55,212-2 or WIN 55,212-2-stimulated [^{35}S]GTPγS binding

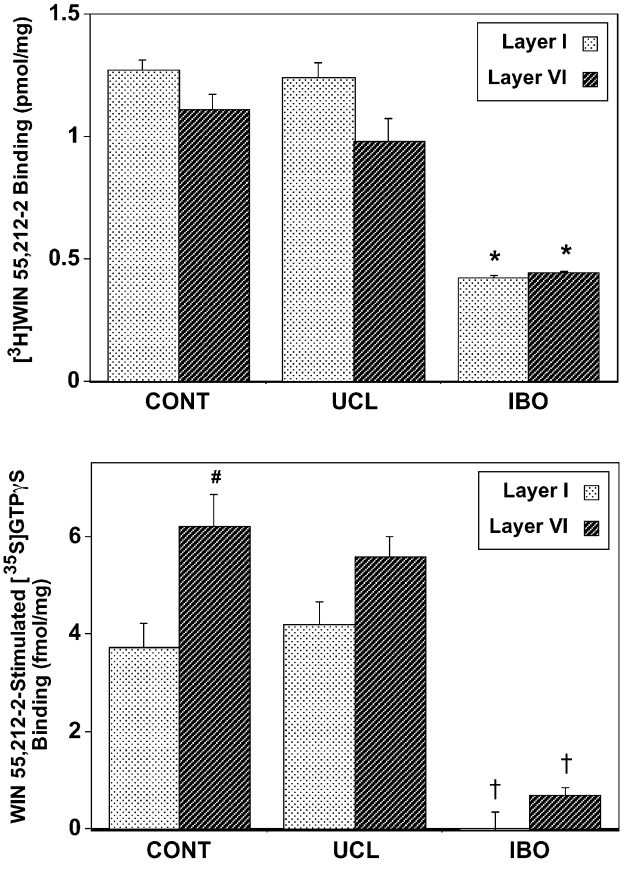

Fig. 2. Graphs summarizing the densitometric data obtained from control and undercut (UCL) and ibotenic acid (IBO) ablated ACC. [^{3}H]WIN 55,212-2 (top) and WIN 55,212-2-stimulated [^{35}S]GTPγS (bottom) binding are pooled from areas 24a and 24b, because no significant differences were found between these regions. (* $p \leq 0.0001$ vs. control; † $p \leq 0.005$ vs. control; # $p \leq 0.01$ vs. lamina I).

in lamina I or VI of areas 24a or 24b of the ACC in deafferented ACC compared to the unablated hemisphere.

Densitometric data pooled from areas 24 a and b are summarized graphically in Fig. 2. Reductions in [^{3}H]WIN 55,212-2 binding are apparent following ibotenic acid ablation. The loss of WIN 55,212-2-stimulated [^{35}S]GTPγS binding after ibotenic acid ablation is also evident. In addition, higher WIN 55,212-2-stimulated G-protein activity, in the absence of differences in receptor binding, is seen in lamina VI compared to lamina I in each case.

Discussion

The highest densities of [^{3}H]WIN 55,212-2 binding and WIN 55,212-2-stimulated [^{35}S]GTPγS binding were found in laminae I and VI of the ACC. Interestingly, the levels of cannabinoid receptors and receptor-mediated G-protein activity did not always correlate. Comparison of [^{3}H]WIN 55,212-2 and WIN 55,212-2-stimulated [^{35}S]GTPγS binding in control tissue shows that cannabinoid receptors in lamina VI of ACC produce a higher level of G-protein activity than those in lamina I. The results of lesion experiments indicate that cannabinoid receptors are localized on somatodendritic elements or axons intrinsic to the ACC, since ibotenic acid lesions reduced cannabinoid receptor binding and G-protein activation, whereas deafferentation lesions did not affect cannabinoid binding levels. It is unlikely that the lack of effect of deafferentation on cannabinoid binding was due to an incomplete lesion because this same ablation procedure reduced mu opioid receptor binding and G-protein activation in the ACC [15].

One goal of this study was to compare cannabinoid receptor binding with receptor-activated G-proteins in the ACC. As expected, the laminar distribution of [^{3}H]WIN 55,212-2 binding corresponded with that of WIN 55,212-2-stimulated [^{35}S]GTPγS binding. Interestingly, although the levels of [^{3}H]WIN 55,212-2 binding were similar in laminae I and VI, significantly higher levels of receptor-activated G-proteins were found in lamina VI. We have previously calculated the ratio of activated G-proteins to receptor B_{max} values using saturation binding assays in membrane homogenates to determine a measure of receptor efficiency [18]. However, it is difficult to calculate efficiency in this study because the autoradiographic assays utilized a single concentration of [^{3}H]WIN 55,212-2 or [^{35}S]GTPγS. Nonetheless, comparison of the data obtained from lamina I versus lamina VI indicates that receptors in lamina VI activate approximately twice as many G-proteins as those in lamina I. Previous studies have shown that cannabinoid receptor efficiency is generally low in the cortex compared with other brain regions [3]. However, the present results demonstrate greater efficiency of cannabinoid receptors in lamina VI that may be more comparable to areas with higher efficiency, such as thalamus and brainstem. The functional consequences of higher receptor efficiency are not clear, but indicate that activation of cannabinoid receptors in lamina VI produces greater amplification of the signal transduction response than activation of receptors in lamina I.

Another interesting result of this study was that ibotenic acid lesions that reduced cannabinoid binding by approximately 60% essentially eliminated cannabinoid-mediated G-protein activation as measured by WIN 55,212-2-stimulated [^{35}S]GTPγS binding. There are several interpretations for the more dramatic decrease in cannabinoid-stimulated [^{35}S]GTPγS binding compared to cannabinoid receptor binding following ibotenic acid lesions. Receptor binding may detect newly synthesized

receptors or desensitized/internalized receptors that do not couple to effectors. The process of receptor internalization, with accompanying G-protein uncoupling, is particularly relevant under conditions of downregulation, which would occur during neuronal degeneration or chronic agonist treatment. For example, autoradiographic analysis of chronic Δ^9-THC treated brains showed that profound desensitization of cannabinoid receptors was associated with moderate losses in receptor binding [19]. Moreover, uncoupling of mu opioid receptor-activated G-proteins after chronic treatment of rats with heroin was actually accompanied by an increase in mu receptor binding [20]. Alternatively, some cannabinoid receptors may be localized to non-neuronal elements such as glia, although electronmicroscopy studies do not support this possibility [9]. Another possibility is that some [^{3}H]WIN 55,212-2 binding sites activate signal transduction mechanisms that are not detected using agonist-stimulated [^{35}S]GTPγS binding. Agonist-stimulated [^{35}S]GTPγS binding is thought to primarily recognize activated $G\alpha_i/G\alpha_o$, due to the abundance of these G-proteins in brain and their guanine nucleotide exchange properties. Cannabinoid receptors may couple to other types of G-proteins such as $G\alpha_s$ [21,22], which may not be detected in the [^{35}S]GTPγS assay. An interesting possibility is that ibotenic acid ablation may lead to release of endocannabinoids, which could desensitize remaining CB1 receptors. Studies have shown that the endocannabinoid 2-arachidonoyl glycerol is increased in the brain following head injury [23], suggesting that neuronal damage produced by ibotenic acid could elicit this same response.

The aminoalkylindole WIN 55,212-2 was used for the autoradiographic studies because it is a full agonist for cannabinoid receptor activation of G-proteins [24]. However, recent studies indicate that putative non-CB1 binding sites exist in the brain and activate G-proteins, as measured using agonist-stimulated [^{35}S]GTPγS binding [25]. This possibility could affect the interpretation of the present results in that possibly not all of the [^{3}H]WIN 55,212-2 binding sites identified are cannabinoid CB1 receptors. Since WIN 55,212-2 was used for both receptor and [^{35}S]GTPγS autoradiography, the comparison of receptor binding and receptor-activated G-proteins is not complicated by this issue. Furthermore, the laminar distribution of [^{3}H]WIN 55,212-2 binding sites is similar to that reported by Herkenham et al. (1991) using [^{3}H]CP 55,940. This suggests that either most of the [^{3}H]WIN 55,212-2 binding sites in the ACC are cannabinoid CB1 receptors, or that non-CB1 [^{3}H]WIN 55,212-2 binding sites exhibit the same laminar distribution in ACC as CB1 receptors.

The present study demonstrates that CB1 receptors are localized on somatodendritic elements and/or axons intrinsic to the ACC. Pyramidal neurons from which ACC projections originate are found in laminae V and VI of the ACC. Apical and basal dendrites from these neurons terminate in laminae I and V/VI, respectively, so it is likely that a population of cannabinoid receptors is localized on these dendrites. Electron microscopic analysis of cannabinoid receptor immunoreactivity in the primate brain has demonstrated postsynaptic localization of cannabinoid receptors in the cerebral cortex, with immunolabeling of dendrites and dendritic spines [9], consistent with this hypothesis. These data, as well as the present results, indicate that cannabinoid receptors may modulate the output of ACC projection neurons, as well intrinsic ACC activity. Studies using in situ hybridization and immunohistochemistry for CB1 receptors have shown that cannabinoid receptors and mRNA are located on both pyramidal and non-pyramidal neurons in the ACC [9,26,27]. CB1 mRNA has been localized primarily in cells in laminae II-III and V-VI of the cerebral cortex [26,27], indicating that cannabinoid receptors are not expressed exclusively by projection neurons. Moreover, CB1 receptors are expressed by GAD65 positive neurons in the cortex [28]. In light of

these data and the present results, it appears that a population of CB1 receptors may also be found on ACC interneurons.

The results of this study demonstrate that cannabinoid receptors in laminae I and VI of the ACC are localized on somatodendritic elements or axons intrinsic to the ACC. Differences in the levels of receptor binding and G-protein activity between laminae I and VI indicate that cannabinoid receptors in lamina VI produce greater G-protein activation, although the functional consequences of this finding are not clear. Further studies are necessary to fully characterize the relationship between cannabinoid receptors and G-protein activation in the ACC.

Conclusion

Cannabinoid receptors in laminae I and VI of the ACC are localized on somatodendritic elements and/or intrinsic axons. Moreover, differences in the levels of receptor binding and G-protein activity between laminae I and VI in control ACC and following ibotenic acid lesion further support the concept that receptor binding sites and levels of G-protein activity may not always correlate. However, the functional consequences of these differences within cingulate cortex are not yet clear.

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