Research Report

Cannabinoid CB2 receptors: Immunohistochemical localization in rat brain

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Abbreviations:
CB2, cannabinoid receptor 2
CB1, cannabinoid receptor 1
Cnr, cannabinoid receptor
Cnrs, cannabinoid receptors
EPCS, endocannabinoid physiological control system
eCBs, endocannabinoids

ABSTRACT

Brain expression of CB2 cannabinoid receptors has been much less well established and characterized in comparison to the expression of brain CB1 receptors. Since CB2 receptors are intensely expressed in peripheral and immune tissues, expression in brain microglia has been anticipated. Nevertheless, we now describe expression of CB2-receptor-like immunoreactivity in brain in neuronal patterns that support broader CNS roles for this receptor. Two anti-CB2 affinity purified polyclonal antibodies were raised in rabbits immunized with peptide conjugates that corresponded to amino acids 1–33 and 20–33. Western blot analyses revealed specific bands that were identified using these sera and were absent when the sera were preadsorbed with 8.3 μg/ml of the immunizing peptides. These studies, and initial RT-PCR analyses of brain CB1 and CB2 mRNAs, also support the expression of brain CB2 receptor transcripts at levels much lower than those of CB1 receptors. CB2 cannabinoid receptor mRNA was clearly expressed in the cerebellum of wild type but not in CB2 knockout mice. CB2 immunostaining was detected in the interpolar part of spinal 5th nucleus of wild type but not in CB2 knockout mice, using a mouse C-terminal CB2 receptor antibody. Immunohistochemical analyses revealed abundant immunostaining for CB2 receptors in apparent neuronal and glial processes in a number of rat brain areas. Cerebellar Purkinje cells and hippocampal pyramidal cells revealed substantial immunoreactivity that was absent when sections were stained with preadsorbed sera. CB2 immunoreactivity was also observed in olfactory tubercle, islands of Calleja, cerebral cortex, striatum, thalamic nuclei, hippocampus, amygdala, substantia nigra, periaqueductal gray, paratrochlear nucleus, paralemniscal nucleus, red nucleus, pontine nuclei, inferior colliculus and the parvocellular portion of the medial vestibular nucleus. In-vitro, CB2 immunoreactivity was also present in hippocampal cell cultures. The multifocal expression of CB2 immunoreactivity in glial and neuronal patterns in a number of brain regions suggests reevaluation of the possible roles that CB2 receptors may play in the brain.

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1. Introduction

Research on the molecular and neurobiological basis of the physiological and neurobehavioral effects of marijuana and cannabinoids lagged behind those of other natural addictive products like opium and tobacco because of lack of specific molecular tools and technology. Now, significant and rapid progress has transformed marijuana–cannabinoid research into mainstream science with the cloning of genes encoding cannabinoid receptors (Cnrs) and generation of cannabinoid receptor knockout mice. Furthermore, these advancements in marijuana and cannabinoid research indicate the existence of a previously unknown, but elaborate and ubiquitous endocannabinoid physiological control system (EPCS) in the human body and brain whose role is unfolding. This remarkable progress includes identification of genes encoding cannabinoid receptors (Cnrs) (Chakrabarti et al., 1995; Matsuda et al., 1990; Munro et al., 1993), isolation of endocannabinoids (eCBs) (Devane et al., 1992; Hanus et al., 2001; Mechoulam and Parker, 2003; Onaivi et al., in press; Porter et al., 2002; Sugiura et al., 1995; Tsou et al., 1998) and entourage ligands (Ben-Shabat et al., 1998), and functional identification of transporters and enzymes for the biosynthesis and degradation of these endogenous substances, which, thus, represent the EPCS (for a review, see Onaivi et al., 2002). While CB1 turned out to be one of the most abundant neuromodulatory receptors in the brain, both CB1 and CB2 receptors are widely distributed in peripheral tissues with CB2 cannabinoid receptors particularly enriched in immune tissues (Berdyshev, 2000; Suigiura and Waku, 2000, Wilson and Nicoll, 2001). Despite this wealth of information and major advances, little information is available about the CB2 cannabinoid receptors that have been generally referred to as peripheral Cnrs because CB2 receptor has been found primarily in cells of the immune system. While a number of laboratories have not been able to detect the presence of CB2 in healthy brains (Carlisle et al., 2002; Chakrabarti et al., 1995; Derocq et al., 1995; Galiegue et al., 1995; Griffin et al., 1999; Shatz et al., 1997; Sugiura et al., 2000), there has been demonstration of CB2 expression in rat microglial cells (Kearn and Hilliard, 1997), in cerebral granule cells (Skaper et al., 1996), in mast cells (Facci et al., 1995; Samson et al., 2003), in adult rat retina (Lu et al., 2000) and induction of CB2 receptor expression in the rat spinal cord of neuropathic but not inflammatory chronic pain models (Zhang et al., 2003). Additional reports indicate that CB2 receptors are expressed by a limited population of microglial cells in normal healthy and neuritic plaque-associated glia in Alzheimer's disease brain (Benito et al., 2003; Nunez et al., 2004; Pazos et al., 2004), human brain capillaries and microvessels (Golech et al., 2004), CB2 receptors expressed in the brains of macaque model of encephalitis induced by simian immunodeficiency virus (Benito et al., 2005), and human astrocytes expressed in both CB1 and CB2 cannabinoid

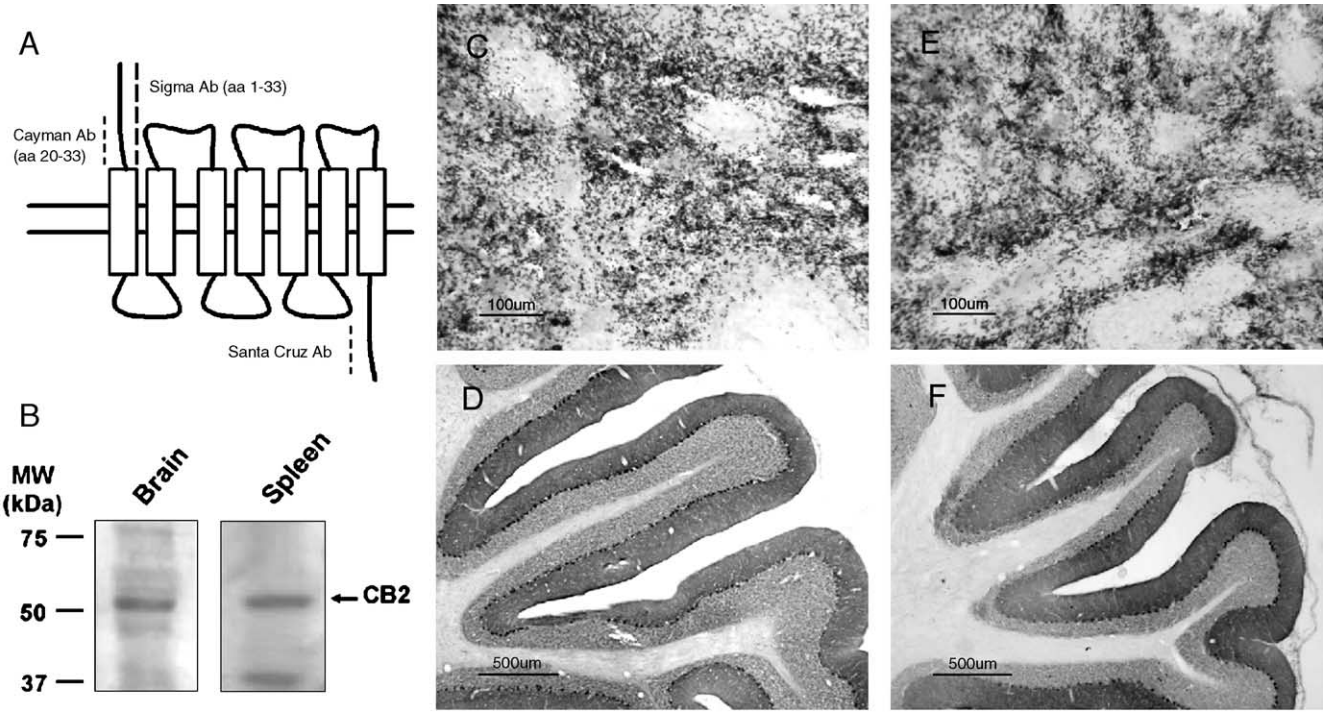

Fig. 1 – Characterization of three different anti-CB2 antibodies. A: Diagram showing the locations of CB2 epitopes to generate three different antibodies from Cayman, Sigma, and Santa Cruz. B: Western analyses of proteins separated on 4–20% SDS-PAGE gels along with size standards Cayman antibody visualized with enhanced chemiluminescence. Lysates prepared from the rat spleen and brain regions noted at the top of the figure. C to F: Comparison of staining patterns of two CB2 different antibodies. C: iCB2 immunostaining using CB2 antibody from Cayman in a section of rat spleen. D: iCB2 immunostaining using CB2 antibody from Cayman in a section of rat cerebellum. E: iCB2 immunostaining using CB2 antibody from Sigma in a section of rat spleen. F: iCB2 immunostaining using CB2 antibody from Sigma in a section of rat cerebellum.

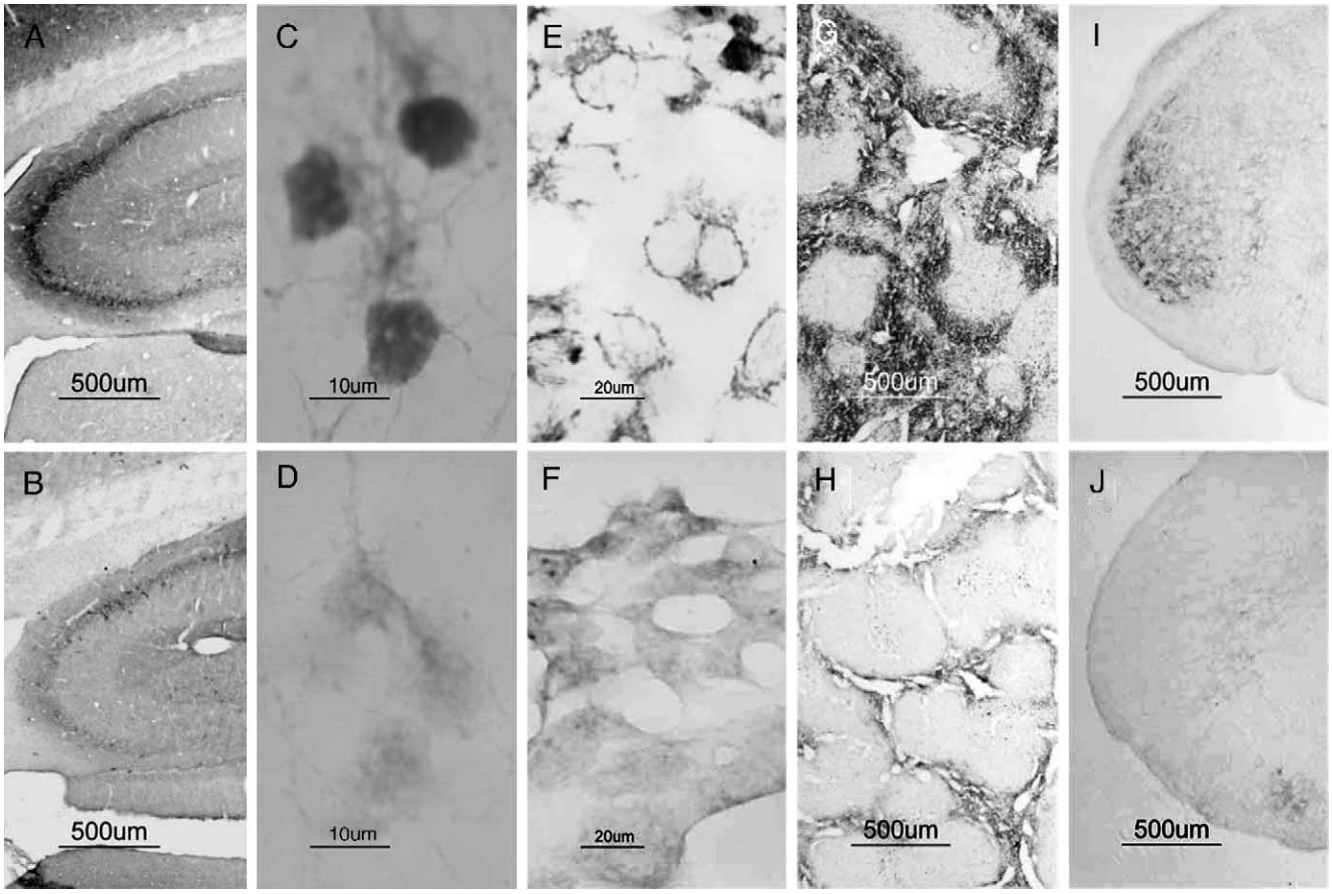

Fig. 2 – Specificity of anti-CB2 antibodies. A: iCB2 immunostaining using CB2 antibody from Cayman in a section through hippocampus CA2 region, with intense staining in pyramidal cells. B: Lack of iCB2 in a section stained in parallel to (D) with primary antibody preadsorbed using 8.3 μg/ml of the CB2 sequence peptide used to produce the antiserum. C: Bright-field photomicrograph of CB2 immunostaining in neuronal cells in primary hippocampus cultures. D: Lack of iCB2 in neuronal cells of primary hippocampus cultures with primary antibody preadsorbed using 8.3 μg/ml of the CB2 sequence peptide used to produce the antiserum. E: Bright-field photomicrograph of CB2 immunostaining in HEK 293 cells transfected with pcDNA 3.1 CB2. F: Lack of iCB2 in HEK 293 cells with pc DNA 3.1 mock transfection. G: CB2 immunostaining in the spleen of wild type mouse. H: Lack of iCB2 in the spleen of CB2 knockout mouse. I: CB2 immunostaining in the interpolar part of spinal 5th nucleus of wild type mouse brain. J: Lack of iCB2 in the interpolar part of spinal 5th nucleus of CB2 knockout mouse brain.

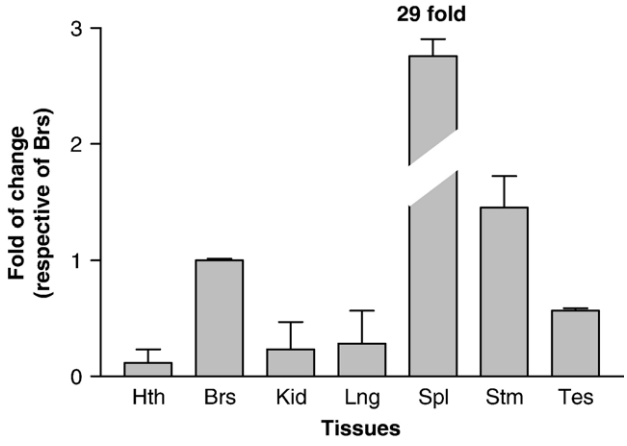

Fig. 3 – CB2 mRNA expression in different tissues. Real time RT-PCR found lower expression of CB2 mRNA in brain stem (Brs), hypothalamus (Hth), stomach (Stm), testis (Tes), lung (Lng), and kidney (Kid), in comparison with high level expression in spleen (Spl). Only the tissues with CB2 expression are shown.

receptors (Sheng et al., 2005). Despite these studies indicating that CB2 receptors might be present in the CNS, the expression of CB2 cannabinoid receptors in the brain has been much less well established and characterized in comparison to the expression of abundant brain CB1 receptors. However, our initial RT-PCR analyses of brain CB1 and CB2 mRNAs supporting (Onaivi et al., in press) brain expression of CB2 receptor gene transcripts prompted us to investigate the localization of CB2 cannabinoid receptors in rat brain slices using two CB2 affinity purified polyclonal antibodies raised in rabbits immunized with peptide conjugates that corresponded to amino acids 1–33 and 20–33. Our results indicate wide spread expression of CB2 receptors in the brain, and, in-vitro, CB2 immunoreactivity (iCB2) was detected in hippocampal NSE-positive neuronal cells. Immunohistochemistry localization revealed abundant CB2 immunostaining in apparent neuronal and glial processes in a number of brain areas. Cerebellar Purkinje cells and hippocampal pyramidal cells revealed substantial immunoreactivity that was absent when sections were stained with preadsorbed sera.

2. Results

2.1. *CB2 receptor immunohistochemical and gene expression analysis in the brain*

Immunoblots from mouse brain and spleen lysates revealed a major band of approximately 53 kDa, with other visible bands around 37 kDa and 75 kDa, similar to those observed recently (Van Sickle et al., 2005). The use of three anti-CB2 affinity purified polyclonal antibodies with peptide conjugates corresponding to different amino acid terminals and yielding similar patterns of staining indicated further specificity of CB2 receptor localization. The first two different CB2 antibodies (one from Cayman, another from Sigma) showed very similar staining patterns in both the rat spleen (Figs. 1C and E) and cerebellum (Figs. 1D and F) sections, supporting their specificity for CB2 receptors. As a positive control, the specific staining of CB2 expression in the follicular B cell areas on spleen section confirmed the specificity of the antibodies. Most of the photomicrographs shown in this paper are from experiments using Cayman CB2 antibody which was used recently to detect CB2 receptors in human and mouse atherosclerotic plaques heart sections (Steffens et al., 2005). iCB2 was observed in many parts of the rat CNS. Most was found in patterns consistent with nerve cell bodies, neuronal processes, and glial cells and its process. Staining patterns in each of the four rat brains studied in detail were essentially identical: the patterns described below correspond to the distribution and labeling intensities found in each animal (Figs. 5A to F). As these animals were perfused and their circulatory system was previously washed, it is clear that blood cells are absent. A 3rd CB2 receptor antibody (from Santa Cruz) was utilized for the CB2 knockout mice and their wild type controls. This 3rd antibody appeared more specific for mouse CB2 receptors and has origin different from the first two antibodies described above (Fig. 1A).

Advanced WU-Blast2 search (http://dove.embl-heidelberg.de/blast2/) using the immunizing peptide sequence and standard parameters against a non-redundant protein data yielded only CB2 sequence. However, the estimated molecular weight is different from the expected 40 kDa predicted for the post-translationally unmodified rat CB2 receptor based on extrapolation from its cDNA coding sequence (Griffin et al., 2000). This is probably due to variations in the method, in the glycosylation level of the protein, N-terminal processing of the protein, or anomalous mobility of CB2 receptor in SDS-PAGE. Additional control for the immunohistochemical analysis included the preadsorption, and co-incubation of the antibody with the immunizing protein (8.3 μg/ml) resulted in the abolition of any detectable staining in the rat hippocampus (Figs. 2A and B), hippocampal cell culture (Figs. 2C and D), HEK 293 cells transfected with pc DNA 3.1 CB2 (Fig. 2E) and lack of iCB2 in HEK cells with pc DNA 3.1 mock transfection (Fig. 2F). Using the CB2 knockout mice, CB2 immunostaining was present in the spleen of wild type but not in the spleen of CB2 knockout

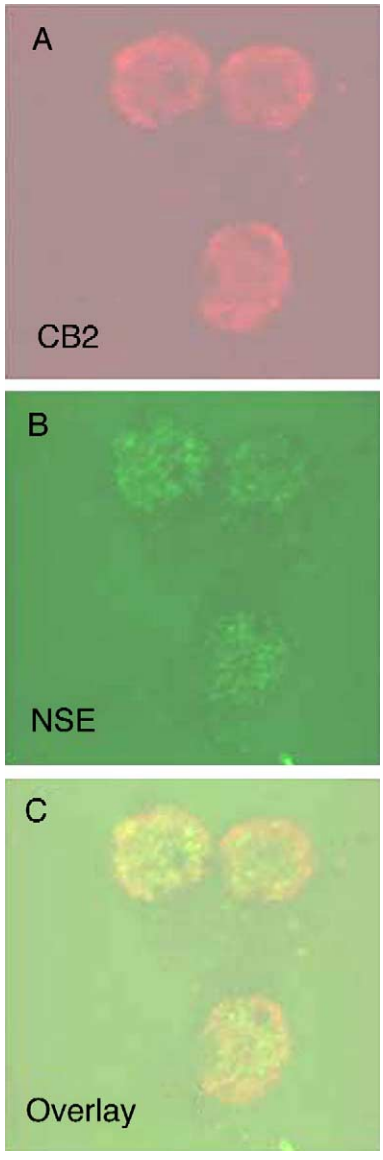

Fig. 4 – Confocal immunofluorescence image of CB2 (A), neuron marker NSE (B), and overlay (C).

mice. CB2 immunostaining was detected in the interpolar part of spinal 5th nucleus of wild type but not CB2 knockout mice (Figs. 2I and J). As an additional control, using in situ hybridization, we demonstrate that CB2 cannabinoid receptor mRNA was clearly expressed in the cerebellum of wild type and not in the CB2 knockout mice. CB2 sense probe did not detect signals in the cerebellum of wild type mice. Antisense cRNA probe did not detect signals around the Purkinje cells in the CB2 knockout mice. However, strong signals were detected around the Purkinje cells of the cerebellum in the wild type mice (data not shown). Specifically, these studies and our initial RT-PCR analyses of brain CB2 mRNAs of naive and drug-treated mice (Onaivi et al., in press) revealed brain CB2 receptor gene transcript expression at levels much lower than those of the spleen, further indicating the presence of CB2 receptors in brain. Quantification of CB2 mRNA was carried out by real time PCR using Tagman mouse CB2 probes (ABI pre-designed expression assay) and mouse tissue cDNA as templates. CB2 mRNA was detected in limited regions of brain, such as

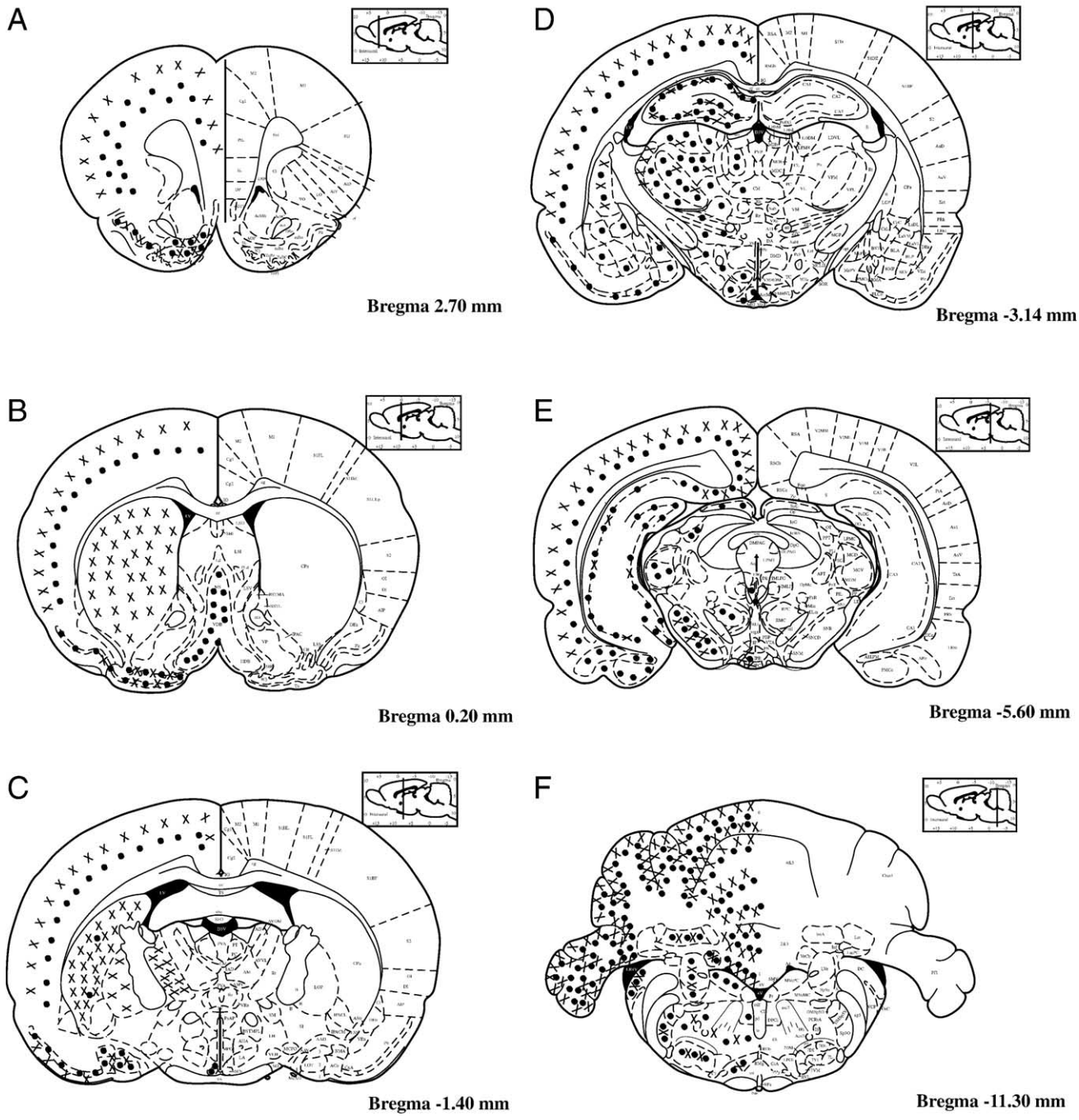

Fig. 5 – Schematic representation of iCB2-immunopositive structures in the rat brain corresponding to rostrocaudal levels (with respect to bregma) +2.70 mm (A), +0.20 mm (B), –1.40 mm (C), –3.14 mm (D), –5.60 mm (E), –11.30 mm (F). Closed circles indicate immunopositive perikarya. X represents immunoreactive fibers and nerve terminals.

brain stem and hypothalamus, but not in olfactory bulb, cortex, hippocampus, striatum, thalamus, mid brain, and spinal cord. The highest expression of CB2 is in spleen which is 29 fold respective of brain stem. Lower expression of CB2 mRNA was also observed in stomach, testis, lung, and kidney (Fig. 3). We could not find CB2 mRNA in heart, intestine, liver, and skeletal muscle. Using double labeling sequentially, tissues were first labeled with the CB2 receptor antibody followed by a neuron marker, neuron-specific enolase (NSE). Confocal immunofluorescence image of iCB2, NSE on neurons, and overlay of CB2 receptor on neurons illustrates the presence of CB2 receptors on hippocampal neurons in culture (Fig. 4).

2.2. *Olfactory bulb and tubercle*

The most prominent staining in the olfactory tubercle was in the anterior olfactory nucleus (Fig. 6A), which was relatively densely stained in its medial parts (Fig. 6B). Immunoreactive process and neurons were observed in piriform cortex (Figs. 6C, E, and F): the islands of Calleja contained numerous labeled neuronal processes and scattered iCB2 neurons (Figs. 6D and G).

2.3. *Cerebral cortex*

CB2-immunopositive staining was observed throughout the different regions of cerebral cortex that we analyzed, including orbital cortex, visual cortex, motor cortex, and auditory cortex. The cell bodies and apical dendrites of pyramidal neurons in the layers III and V are heavily stained (Figs. 7A–C). Less heavily stained neurons can be seen throughout the cortex.

2.4. *Hippocampus and thalamus*

A moderate to dense CB2 immunostaining (Figs. 8A–C) could be observed in pyramidal neurons of hippocampal allocortex, being more pronounced in CA2 and CA3 (Fig. 8B) and less intense in the subiculum (Fig. 8C). Some interneurons in the stratum oriens and stratum radiatum are immunoreactive for CB2. Some glial cells also appeared to be immunostained.

There were both cell-body-like and processes-like CB2 immunostaining in thalamus. Most of thalamic nuclei showed cell-body-like staining. There were clearly labeled processes in the reticular thalamic nucleus with dense plexus of CB2-like-immunoreactive fibers (Figs. 8D–F). However, CB2-immunopositive staining was observed in cell bodies in ventral posterior thalamic nucleus (Fig. 8E). The size of these cells is around 10 × 20 μm (Fig. 8F). The prominent CB2 cell body staining was found in lateral posterior thalamic nucleus, posterior thalamic nuclear group, and paracentral thalamic nucleus. There were also lightly stained cell body in paraventricular thalamic nucleus and mediodorsal thalamic nucleus. Comparing to thalamus, the CB2 staining in hypothalamus is much limited. Only ventromedial hypothalamic nucleus and arcuate hypothalamic nucleus showed some CB2-positive cell bodies.

2.5. *Midbrain, pons and medulla*

Moderately dense iCB2-positive cell bodies were found in the periaqueductal gray (Fig. 9A). The most intensely stained areas in the midbrain were the paratrochlear nucleus (Fig. 9B), paralemniscal nucleus (Fig. 9C), and red nucleus (Figs. 10A and B). In pons, the most prominent area

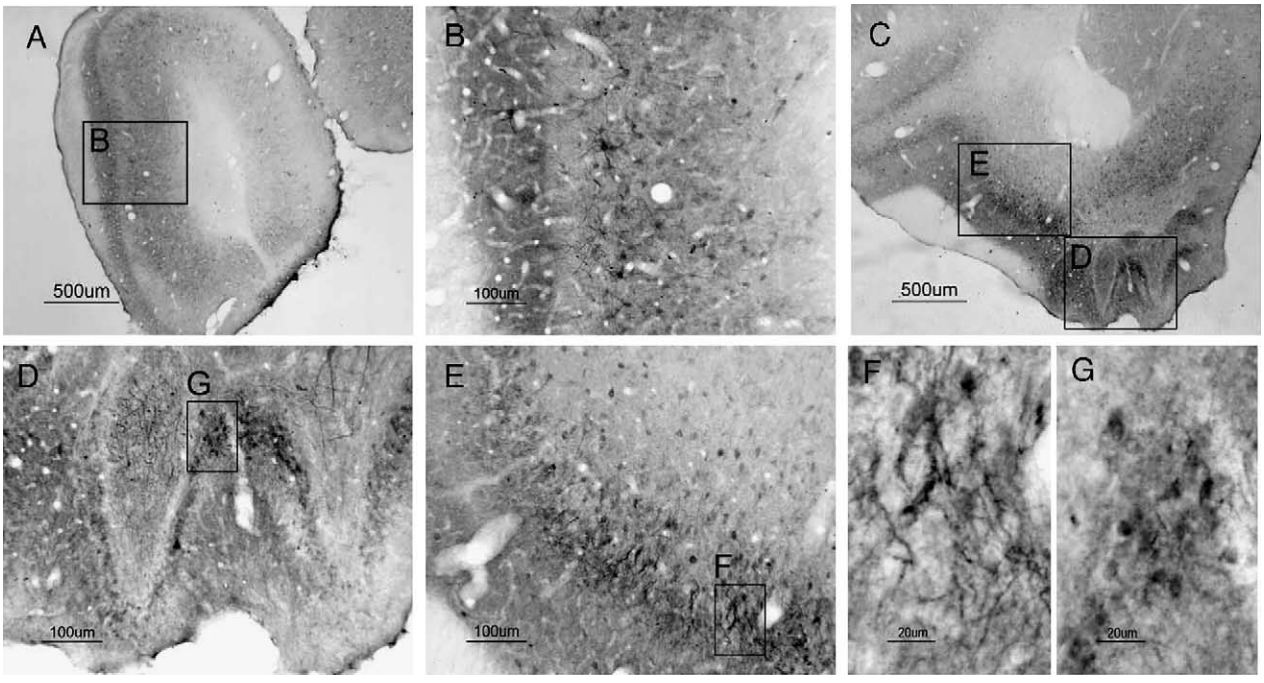

Fig. 6 – iCB2 in (A and C) olfactory tubercle (lower-magnification view), (B) medial part of anterior olfactory nucleus, (E and F) piriform cortex (higher-magnification view), and (D and G) islands of Calleja (higher-magnification view).

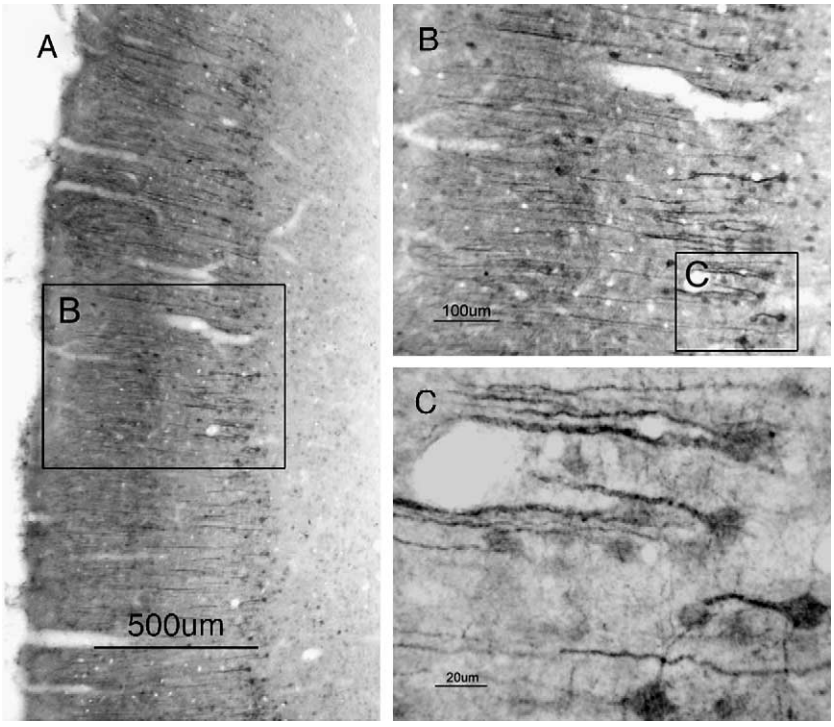

Fig. 7 – iCB2 in cortex, (A) low-magnification view and (B and C) higher-magnification view.

of CB2-positive staining is located in pontine nucleus (Figs. 10E and F). The iCB2-positive staining outlined the shape of the pontine nucleus (Fig. 10E). The densely stained process looks like astrocyte or microglial processes (Fig. 10F).

Substantia nigra pars reticulata showed CB2-like immunoreactivity with clear staining in the cell body, and some neuronal processes are visible (Figs. 10A, D, 11A–C). The CB2-positive neurons were bigger than 20 μm (Fig. 11C). The

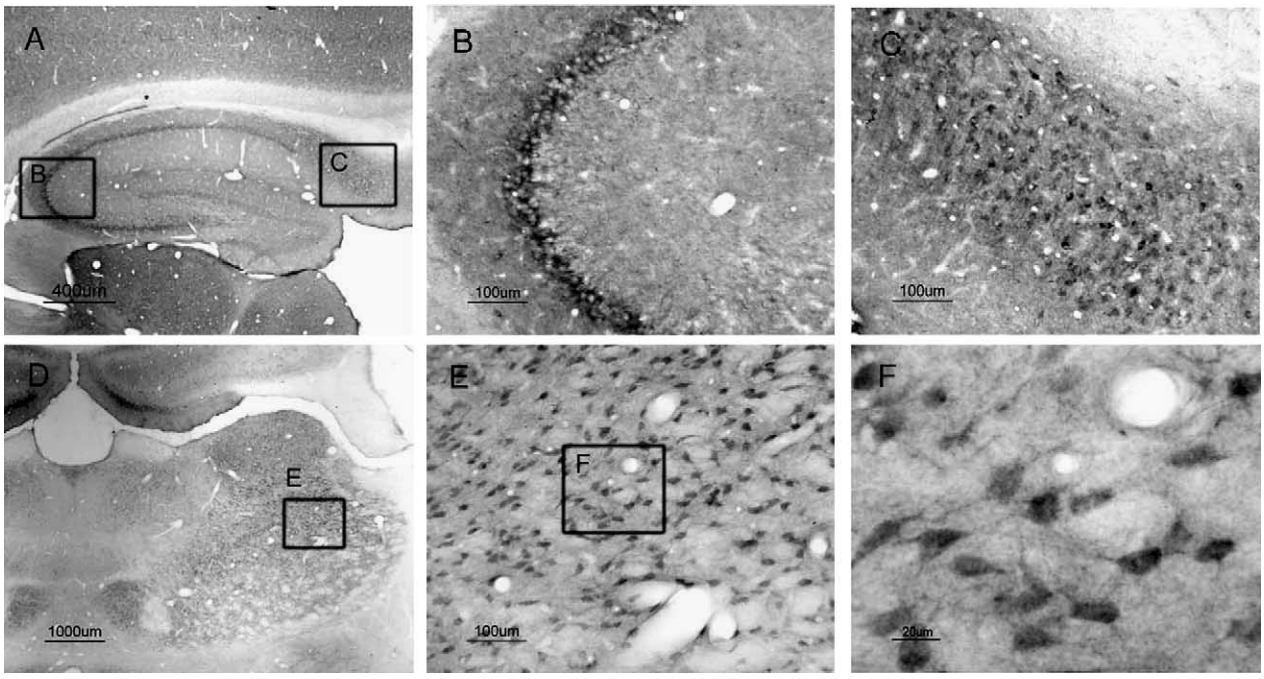

Fig. 8 – iCB2 in hippocampus and thalamus. (A) Low-magnification view of hippocampus, (B) CA2 and CA3 region (higher-magnification view), and (C) subiculum (higher-magnification view). (D) Low-magnification view of thalamus, (E and F) ventral poster thalamic nucleus (higher-magnification view).

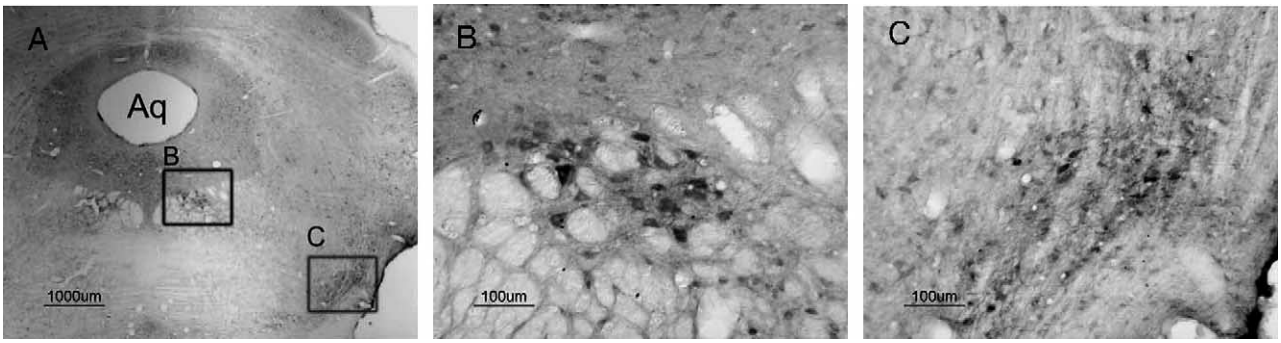

Fig. 9 – iCB2 in midbrain. (A) Low-magnification view, (B) paratrochlear nucleus (higher-magnification view), and (C) paralemniscal nucleus (higher-magnification view).

geniculate nucleus and interpeduncular nucleus expressed moderately dense iCB2 in cell bodies.

In inferior colliculus, cell body and process staining are observed in dorsal nucleus of lateral lemniscus (Figs. 12A and B). Different shapes of cell bodies, some with triangular-shaped patterns of CB2 immunoreactivity, are seen in the inferior colliculus (Figs. 12C and D), although glial localizations cannot be ruled out.

In medulla, the profuse staining of the medial vestibular nucleus is the parvicellar part, with moderately dense CB2-like immunoreactivity (Figs. 13A and B). There were some scattered cell body staining in facial nerve and its root (Fig. 13C). In the dorsal medulla, the prominent staining is observed in dorsal cochlear nucleus (Figs. 13A, C, and E) with both of positive cell bodies and processes (Fig. 13E). The relatively scattered large neuronal cell bodies were seen in parvocellular reticular nucleus (Figs. 13B and F), nucleus of spinal tract trigeminal nerve, oral part (Fig. 13A), and lateral vestibular nucleus (Fig. 13A). The intense iCB2 staining outlined the facial nucleus (Figs. 13D and G).

2.6. *Cerebellum*

The CB2-positive staining was distributed over all of the cerebellar lobules. Purkinje cell bodies were intensely stained, and their dendrites in the molecular layer were moderately stained (Figs. 14A–C). The molecular layer contained numerous heavy stained fine puncta (Fig. 14C).

3. Discussion

As accumulating evidence from our studies and those of others indicate that CB2 cannabinoid receptors may be present in the brain but has not been systematically characterized, our main goal was to localize CB2

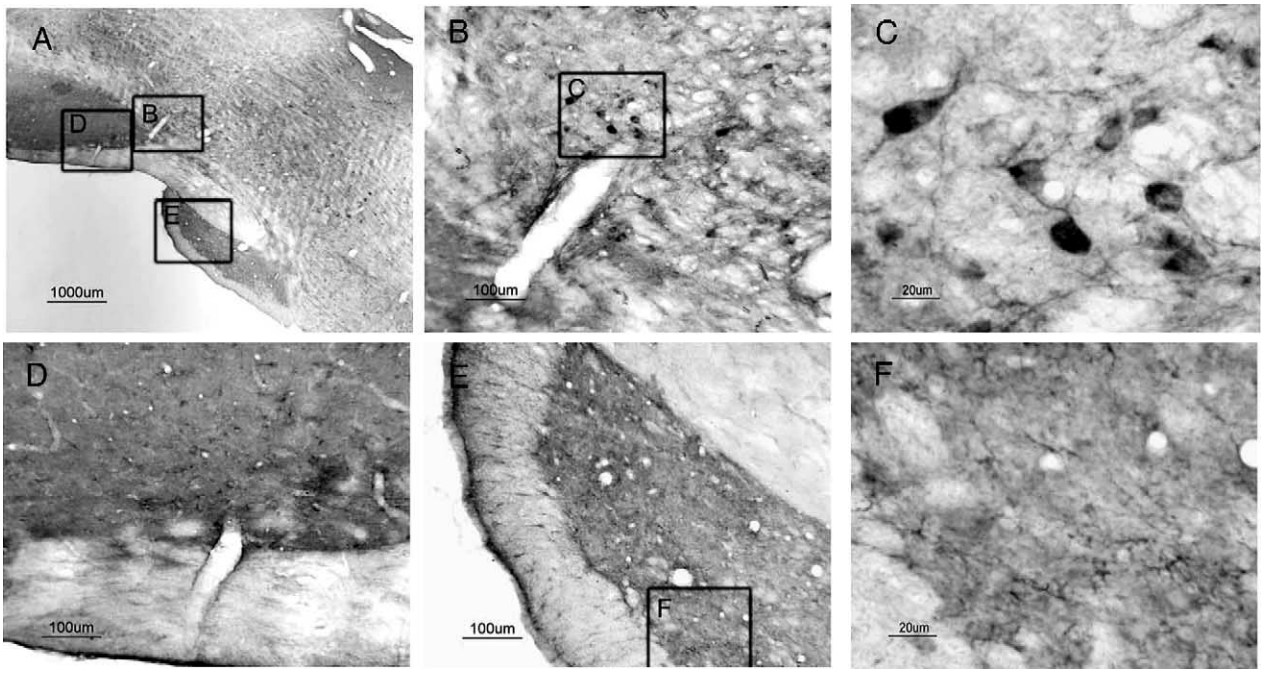

Fig. 10 – iCB2 in midbrain and pons (sagittal section). (A) Low-magnification view, (B and C) red nucleus (higher-magnification view), (D) substantia nigra, reticular part, and (E and F) pontine nuclei (higher-magnification view).

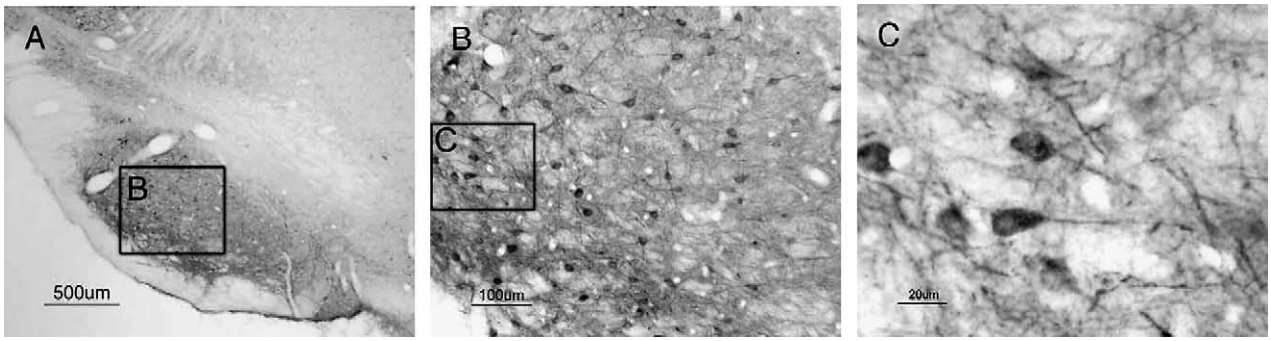

Fig. 11 – iCB2 in substantia nigra, (A) low-magnification view (B) and (C) higher-magnification view.

cannabinoid receptors and their distribution patterns in the rat brain. An initial publication of data from this work has appeared in abstract form (Gong et al., 2005, Onaivi et al., in press). Numerous previous studies for over a decade since the cloning of CB2 cannabinoid receptors have failed to detect the presence of CB2 cannabinoid receptors in the brain (Carlisle et al., 2002; Chakrabarti et al., 1995; Derocq et al., 1995; Galiegue et al., 1995; Griffin et al., 2000; Shatz et al., 1997; Sugiura et al., 2000), and little is known about its neuronal function. With the availability of specific probes and primers, we detected the presence of CB1 and CB2 mRNAs in the brains of rats and mice using RT-PCR (Onaivi et al., in press). In the present studies, we show that CB2 receptors are present in various regions of the adult rat brain. The two specific antibodies used in this study were obtained from different sources designed from two different epitopes on the CB2 receptor, and they showed very similar staining patterns in spleen and rat brain areas examined. As an example, we showed here the similar patterns of immunostaining in the spleen and cerebellum. Furthermore, in primary hippocampus cultures, CB2-positive expression was detected in neuron-specific enolase (NSE)-positive cells. In the Western blots, using lysates prepared from the rat spleen and brain regions showed the same bands at the predicted molecular weight which is in agreement with the expected molecular weight of CB2 receptor. Immunohistochemical analyses revealed abundant CB2 immunostaining in apparent neuronal and glial cells in a number of brain areas. Cerebellar Purkinje cells and hippocampal pyramidal cells revealed substantial immunoreactivity that was absent when sections were stained with preadsorbed sera. CB2 immunoreactivity was also observed in olfactory tubercle, islands of Calleja, cerebral cortex, striatum, thalamic nuclei, hippocampus, amygdala, substantia nigra, periaqueductal gray matter, paratrochlear nucleus, paralemniscal nucleus, red nucleus, pontine nuclei, inferior colliculus, and the parvocellular portion of the medial vestibular nucleus in the rat brain. The immunopositive expression was detected

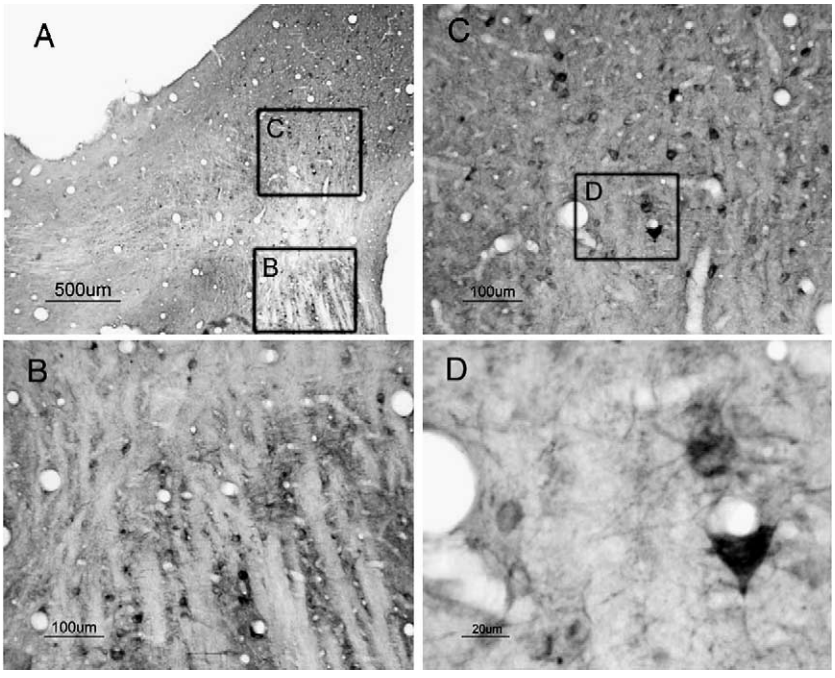

Fig. 12 – iCB2 in inferior colliculus (sagittal section). (A) Low-magnification view, (B) dorsal nucleus lateral lemniscus, and (C and D) inferior colliculus (higher-magnification view).

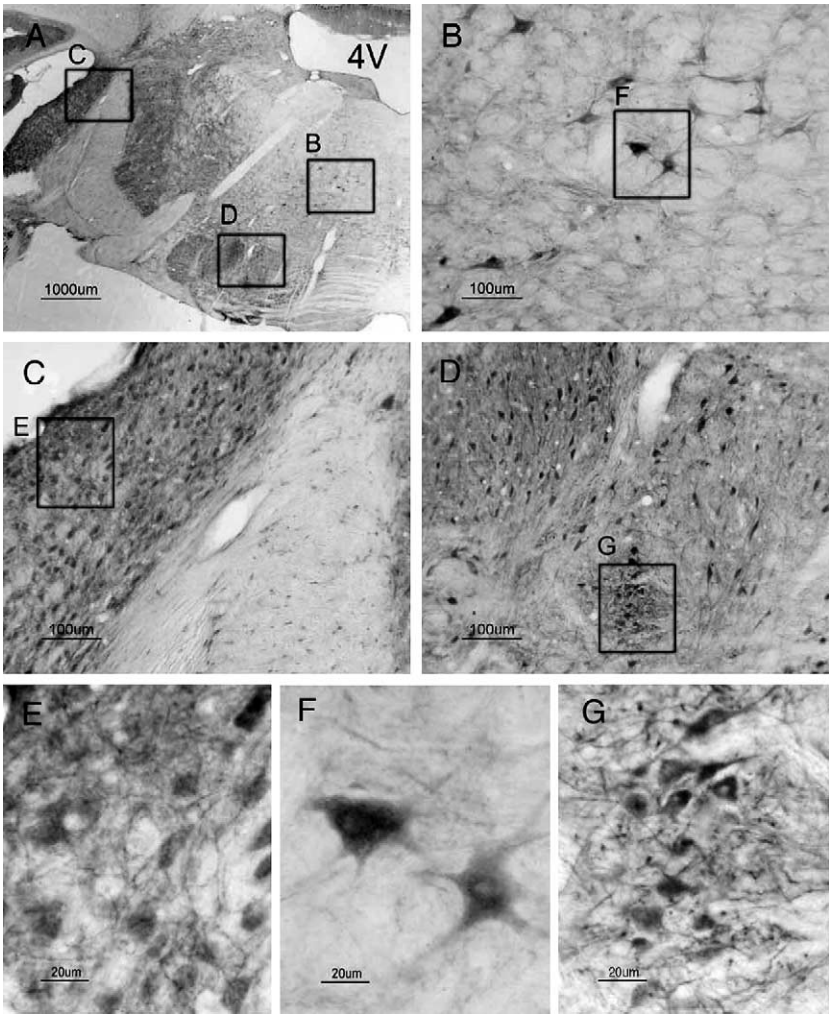

Fig. 13 – iCB2 in medulla. (A) Low-magnification view, (B and F) parvocellular reticular nucleus (higher-magnification view), (C and E) dorsal cochlear nucleus (higher-magnification view), and (D and G) facial nucleus (higher-magnification view).

in perikarya and in neuronal processes as well as in glial cells. Previous studies have shown intense CB1 receptor immunoreactivity in hippocampal formation, basal ganglia, and the molecular and granular layers of the cerebellum with moderate staining in the olfactory bulb, piriform cortex, cerebral cortex, and many thalamic nuclei (Pettit et al., 1998; Tsou et al., 1998). Our data indicate that CB1 and CB2 immunoreactivities may share localization in same brain structures with possible differences in the distribution patterns in neuronal elements where they are localized. For example, these previous studies (Pettit et al., 1998; Tsou et al., 1998) and our data show that both CB1 and CB2 receptors are localized in the cerebellum, where CB1 was seen in the molecular layer and granular layers, whereas

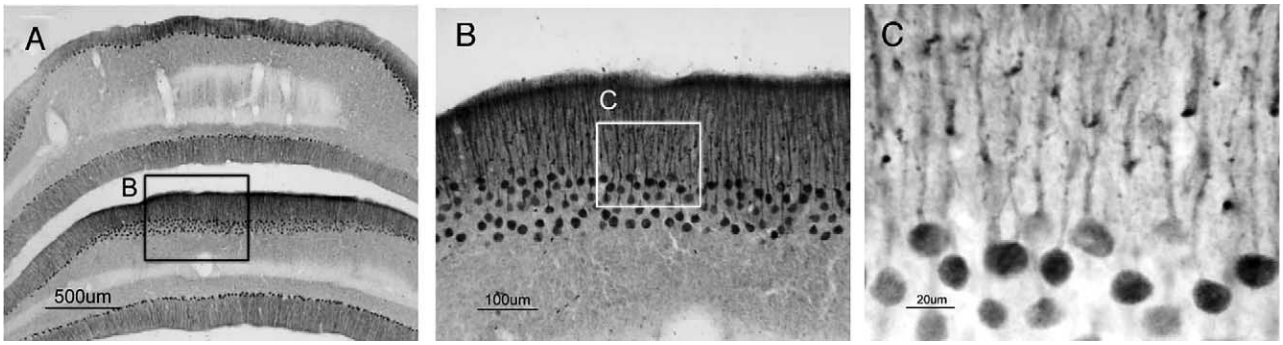

Fig. 14 – iCB2 in cerebellum, (A) low-magnification view and (B and C) higher-magnification view.

CB2 receptors as reported here were observed in Purkinje cells and the molecular layer. The data also indicate that the CB2 receptor antibodies utilized are sensitive for the histological identification and discrimination of CB2 receptor expression. In spite of the accumulating evidence using in-situ hybridization, mRNA probes, specific CB2 antibodies, and data from the current study indicating the expression of CB2 receptors in the brain, much less is known about the specific cell types in which CB2 receptors are found, and its subcellular localization in these cells in the brain remains to be determined.

The new knowledge from this study and those of other recent studies (Onaivi et al., in press; Van Sickle et al., 2005) that CB2 receptors are present in brain raises many questions about the possible roles that CB2 receptors may play in the nervous system. These results therefore extend the previous evidence that CB2 receptors are playing an important role in immune function to other putative neuronal function by their apparent presence in neuronal processes that remains to be determined. While the behavioral and physiological effects of CB2 receptor activation are the subject of a different report, the immunohistochemical localization of CB2 receptors reported here when compared to that of known CB1 receptor distribution in the brain may be an indication of putative functional role of CB2 cannabinoid receptors in the brain and spinal cord. Therefore, both CB1 and CB2 receptors seem likely to work both independently and/or cooperatively in differing neuronal populations to regulate important physiological activities in the central nervous system. While definitive electron microscopic evidence is needed to precisely determine the subcellular localization of CB2 receptors, from the data presented here and from the distribution patterns in the cerebellum, it can be postulated that the presence of CB2 immunoreactivity in Purkinje cell bodies and dendrites may be predicted to be mainly post-synaptic localization. This is because the dendritic trees arising from the Purkinje cells are positive for CB2 immunoreactivity. However, these dendrites have been shown not to be immunostained for CB1 receptors, confirming the presynaptic localization of CB1 receptors, while, as demonstrated in this study, the cell bodies of the Purkinje cells are immunoreactive for CB2 but negative for CB1 receptors as noted by others (Pettit et al., 1998; Tsou et al., 1998), again confirming the established presynaptic localization of CB1 receptors in the brain. Another possibility is that CB2 cannabinoid receptors may be synthesized in the cell body of the Purkinje cells to be transported to another location. It has been documented that CB1 receptors are present in the axons coming from the basket cells located in the molecular layer that forms the basket cells around the basal area of the Purkinje (Pettit et al., 1998; Tsou et al., 1998). Therefore, the apparent neuronal cell bodies and dendritic CB2 cannabinoid receptor immunoreactivity suggest that CB2 receptors may have post-synaptic localization. The recent report by Van Sickle et al. (2005) on the identification and functional characterization of brainstem cannabinoid CB2 receptors is consistent, in part, with our findings and with other recent reports of brain CB2 receptor expression (Pazos et al., 2004; Onaivi et al., in press). However, a focus on brainstem localization and antiemetic functions of brain cannabinoid CB2 receptors by the report of Van Sickle et al. (2005) may understate the importance of CB2 receptors in the other brain areas in which we and others have identified CB2 mRNA and protein. Nevertheless, the distribution of CB2 immunoreactivity reported here may provide novel targets for the effects of cannabinoids in the nervous system beyond neuro-immuno-cannabinoid activity.

4. Experimental procedures

4.1. *Animals and preparation of tissue*

Sprague–Dawley rats were anesthetized with pentobarbital (97.2 mg/kg i.p.), briefly perfused transcardially with saline and then with 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) for 5 min. Brains and spleens were dissected, post-fixed in buffered paraformaldehyde for 2 h at room temperature, equilibrated with 30% sucrose in phosphate buffer at 4 °C, frozen, and cut into coronal or sagittal 20–40 μm sections using a sliding microtome. Experiments confirmed to National Institutes for Health *Guidelines for the Care and Use of Laboratory Animals* that sought to minimize both the number of animals used and any suffering that they might experience.

4.2. *Immunohistochemistry of brain slices*

Immunohistochemistry used floating sections and an avidin-biotinylated peroxidase method (Skaper et al., 1996; Hsu et al., 1981). Sections were incubated with 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min at room temperature to inhibit endogenous peroxidase, washed three times with PBS, incubated for 1 h in 3% normal goat serum in Tris-buffered saline (TBS), pH 7.4 at 22 °C, incubated in primary CB2 antibody obtained from Sigma-Aldrich (MO, USA) and Cayman Chemicals (MI, USA) diluted 1:300 in TBS containing 3% normal goat serum for 24 h at 4 °C, rinsed, incubated for 1 h at 22 °C in 1:200 dilution of biotinylated goat anti-rabbit (Vector, Burlingame, CA, USA) for 1 h, rinsed, incubated with ABC reagent for 1 h (Vector), rinsed, and then incubated with diaminobenzidine for color deposition. Some control sections were processed as described above except for the addition of 8.3 μg/ml of the blocking peptide with primary antisera. Other control sections were stained without the primary antibody. Sections were mounted on coated slides, dehydrated, cover-slipped, viewed and imaged using Zeiss and Leitz microscope and a Nikon digital camera, immunoreactive elements plotted onto the atlas depictions of Paxinos and Watson (1982), and images edited using photoshop (vCS; Adobe systems). As additional control, iCB2 of brain sections from CB2 receptor knockout mice and wild type controls were also analyzed using the C-terminal CB2 receptor antibody obtained from Santa Cruz (CA, USA).

4.3. *Hippocampal cultures*

Primary cultures were prepared from the hippocampi of embryonic Sprague–Dawley rats killed at gestation day 15 after mechanical trituration in calcium- and magnesium-free Hanks' balanced salt solution (pH 7.4) containing 10 g/l glucose at 4 °C. Cells were pelleted by centrifugation, resuspended, and ca. 5×10^5 cells were plated onto coverslips precoated with 0.002 g/l poly-lysine in 0.5 ml of Dulbecco's modified Eagle medium with 10% heat activated fetal bovine serum, 1×10^3 mol/l pyruvate, 4.2×10^3 mol/l sodium bicarbonate, 20×10^3 mol/l HEPES, and 3 g/l bovine serum albumin. Cultures were maintained at 37 °C in a humidified atmosphere of

5% CO_2 and 95% air and examined for CB2 immunoreactivity on the day after plating.

4.4. *Immunofluorescence staining*

Coverslips containing primary cultures were washed with PBS, incubated for 60 min in PBS containing 2% normal serum and 0.3% Triton X-100, and then incubated overnight at 4 °C in PBS containing 2% normal serum, 0.3% Triton X-100, and 1:100 dilution of the rabbit anti-CB2 serum and 1:1000 mouse anti-Enolase (NSE) monoclone antibody (BD Biosciences). Coverslips were washed in PBS, incubated for 60 min in PBS containing 0.3% Triton X-100 and 1:500 dilutions of anti-rabbit IgG to Alexa Fluor 594 and anti-mouse IgG to Alexa Fluor 488, washed in PBS, mounted onto glass slides using fluoromount, and viewed using LSM 410 confocal microscope (Zeiss).

4.5. *Western analysis of CB2 cannabinoid receptor immunoreactivity*

Rats were killed by decapitation, brain regions dissected, tissues homogenized in 50 mM Tris–HCl (pH 7.4, 4 °C) containing 150 mM NaCl, 0.25% SDS, 0.25% sodium deoxycholate, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin, homogenates centrifuged at 14,000 rpm for 20 min at 4 °C, supernatants centrifuged for 10 min, and the supernatants from the second centrifugation assessed for protein concentrations using Bradford assays (Bio-Rad). For Western analyses, 50 μg aliquots of supernatant proteins were electrophoresed using 4–20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated in 5% nonfat milk (5%) in Tris-buffered saline (25 mM Tris, 0.15 M NaCl, pH 7.2) containing 0.05% Tween-20 (milk–TBS-T) overnight at 4 °C, incubated for 2 h at room temperature with CB2 antibody diluted 1:500 in milk–TBS-T, washed with milk–TBS-T, incubated in horseradish-peroxidase-conjugated anti-rabbit IgG for 30 min at 22 °C, and rinsed with milk–TBS-T. Immunoreactivity was detected using Amersham ECL detection system. To establish a control, some blots were incubated with primary antisera that had been preadsorbed overnight at 4 °C with 8.3 μg/ml of the immunizing peptides. Similar protocol was used for the spleen lysates.

4.6. *CB2 receptor mRNA detection from RNA isolation, cDNA synthesis, and Taqman real-time RT-PCR quantification*

Total RNA was extracted from mouse and rat peripheral tissues and brain regions using RNAzolB (Tel-Test, Inc. Friendwood, TX) protocol. Single strand cDNA was synthesized from total RNA using SuperScript™ One-Step RT-PCR System (Invitrogen, Carlsbad, CA). For quantitative real-time PCR assays, Taqman gene expression assays were ordered from Applied Biosystems (Foster City, CA) for CB1 (Mm0043261_s1) and CB2 (Mm00438286_m1) that were used to quantify CB1 and CB2 expression levels in mouse brain and peripheral tissues using the single strand cDNA as templates. A similar assay was used for the selected rat brain and peripheral tissue CB2 gene expression.

Two-step PCR reactions used default settings (ABI 7900 HT PCR; Applied Biosystems, Foster City, CA). We used the methods in user bulletin #2 (ABI Prism 7700 Sequence Detection System) and Prism-3 programs (GraphPad Software, Inc, San Diego, CA) used for graphical and statistical analyses.

4.7. *Transfection of CB2 in HEK 293 cells*

HEK 293 cells were transfected with pcDNA3.1/CB2 or pcDNA 3.1/KEPI as a negative control. Transfection employed DNA preparations with OD_{260}/OD_{280} ratios 1.75. HEK 293 cells grown to confluence in flasks in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) containing 10% fetal calf serum (Life Technologies), 2 mM L-glutamine (Invitrogen Corporation), and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies) were split 1:2, harvested the next day using trypsin/EDTA, centrifuged (200 × *g*) for 10 min at 4 °C, washed with sterile HEBS (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose), recentrifuged, and resuspended in 4 °C HEBS. Suspended cells (0.9 ml) were transfected by electroporation at 300 V/1100 μF in 400-mm gene pulser cuvettes (Bio-Rad) containing 20 μg of plasmid DNA and 500 μg of fish sperm DNA (Roche Diagnostics, Mannheim, Germany) using a geneZAPPER 450/2500 (IBI, New Haven, CT). The transfected cells were then suspended in Dulbecco's modified Eagle's medium followed by distribution into six-well plates containing coverslips. Cells were grown at 37 °C for 48 h in a water-saturated atmosphere of 95% air and 5% CO_2 and were quickly washed twice with 2 ml of PBS, fixed by the addition of 1 ml/well of 4% paraformaldehyde in PB, and incubated at room temperature for 30 min. The immunostaining procedure of transfected HEK 293 cells was same as that of brain sections.

4.8. *In situ hybridization*

Biotin labeled RNA probes were used for in situ hybridization. The full length of human CB2 gene was subcloned from pcDNA3.1/CB2 (UMR cDNA resource center, Rolla, MO) into pBluescript II at the restriction sites of *Eco*RI and *Xho*I. The pcBluescript II/CB2 was linearized with *Xho*I (Anti-sense probe) or *Eco*RI (sense probe). The CB2 riboprobes were synthesized by incubating for 60 min at 37 °C. One microgram linearized plasmid in 2 μl 10× transcription buffer, 1 μl RNase inhibitor, 2 μl Biotin RNA labeling Mix containing 1 mM ATP/GTP/CTP, 650 μM UTP, 350 μM biotin–UTP (Roche Applied Science, Germany), 40 U T7 (anti-sense probe), or T3 RNA polymerase (sense probe) in a final volume of 20 μl. The reaction mixture was subsequently incubated for 15 min at 37 °C with 1 U RNase-free DNase I. The riboprobes were precipitated using LiCl and ethanol. The CB2 probes were diluted in 100 μl TE.

Coronal cerebellum sections (20 μm) of wild type and CB2 knockout mouse were cut in a cryostat microtome. All solutions were prepared in deionized H_2O treated with 0.1% (V/V) diethylpyrocarbonate and autoclaved. Sections were incubated with 1% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min at room temperature to inhibit endogenous peroxidase, washed three times with PBS. Sections were fixed by immersion in 4% paraformaldehyde in PBS, pH 7.4, and then briefly rinsed twice with PBS. After treatment with Proteinase K, sections were refixed in 4% paraformaldehyde. The sections then were acetylated by immersion in 0.1 M triethanolamine containing 0.25% acetic anhydride, permeabilized by 1% Triton X-100, and rinsed twice with PBS. Prehybridization was carried out at 4 °C overnight with prehybridization solution (50% formamide, 4× SSC, 0.5× Denhardt's solution, 100 mM DTT, 250 μg/ml yeast tRNA, and 250 μg/ml salmon sperm DNA). For hybridization, the sections were incubated in a prehybridization solution containing 1 μg/ml of cRNA probe, incubated at room temperature overnight on a shaker. Sections were immersed sequentially in 0.2× SSC twice and buffer 1 (0.1 M Tris pH 7.5, 0.15 M NaCl) twice. The sections were incubated with ABC reagent for 1 h (Vector), rinsed, and then incubated with diaminobenzidine for color deposition.

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