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Functional CB1 Receptors Are Broadly Expressed in Neocortical GABAergic and Glutamatergic Neurons

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Hill EL, Gallopin T, Férézou I, Cauli B, Rossier J, Schweitzer P, Lambollez B. Functional CB1 receptors are broadly expressed in neocortical GABAergic and glutamatergic neurons. *J Neurophysiol* 97: 2580–2589, 2007. First published January 31, 2007; doi:10.1152/jn.00603.2006. The cannabinoid receptor CB1 is found in abundance in brain neurons, whereas CB2 is essentially expressed outside the brain. In the neocortex, CB1 is observed predominantly on large cholecystikinin (CCK)-expressing interneurons. However, physiological evidence suggests that functional CB1 are present on other neocortical neuronal types. We investigated the expression of CB1 and CB2 in identified neurons of rat neocortical slices using single-cell RT-PCR. We found that 63% of somatostatin (SST)-expressing and 69% of vasoactive intestinal polypeptide (VIP)-expressing interneurons co-expressed CB1. As much as 49% of pyramidal neurons expressed CB1. In contrast, CB2 was observed in a small proportion of neocortical neurons. We performed whole cell recordings of pyramidal neurons to corroborate our molecular findings. Inhibitory postsynaptic currents (IPSCs) induced by a mixed muscarinic/nicotinic cholinergic agonist showed depolarization-induced suppression of inhibition and were decreased by the CB1 agonist WIN-55212-2 (WIN-2), suggesting that interneurons excited by cholinergic agonists (mainly SST and VIP neurons) possess CB1. IPSCs elicited by a nicotinic receptor agonist were also reduced in the presence of WIN-2, suggesting that neurons excited by nicotinic agonists (mainly VIP neurons) indeed possess CB1. WIN-2 largely decreased excitatory postsynaptic currents evoked by intracortical electrical stimulation, pointing at the presence of CB1 on glutamatergic pyramidal neurons. All WIN-2 effects were strongly reduced by the CB1 antagonist AM 251. We conclude that CB1 is expressed in various neocortical neuronal populations, including glutamatergic neurons. Our combined molecular and physiological data suggest that CB1 widely mediates endocannabinoid effects on glutamatergic and GABAergic transmission to modulate cortical networks.

INTRODUCTION

Cannabinoid substances act at CB1 receptors to impair brain functioning in a variety of cognitive and performance tasks, including memory, learning, and attention (Iversen 2003); and endogenous ligands for these receptors, the endocannabinoids, have emerged as transmitters regulating neuronal activity (Freund et al. 2003). CB1 is found throughout the brain and is present at a high density in neocortex and hippocampus (Herkenham et al. 1990). In these brain areas, CB1 immunoreactivity is mostly found on large cholecystikinin (CCK)-expressing interneurons but not vasoactive intestinal peptide

(VIP)- or somatostatin (SST)-expressing interneurons (Bodor et al. 2005; Katona et al. 1999; Tsou et al. 1999). The CB1 mRNA is expressed in pyramidal neurons (Marsicano and Lutz 1999; Matsuda et al. 1993), but CB1 immunoreactivity has often been undetected in these neurons (Bodor et al. 2005; Katona et al. 1999; Tsou et al. 1999). Although the presence of CB1 immunoreactivity at hippocampal excitatory presynaptic terminals has been recently reported (Katona et al. 2006; Kawamura et al. 2006), the extent of cortical CB1 expression in pyramidal neurons remains unclear.

Numerous electrophysiological studies have investigated the influence of cannabinoids in brain preparations. In neocortex and hippocampus, cannabinoids acting at CB1 depress inhibitory and excitatory synaptic transmission (Auclair et al. 2000; Bender et al. 2006; Davies et al. 2002; Fortin and Levine 2006; Piomelli 2003; Sjostrom et al. 2003, 2004). The use of CB1 knock-out mice further established the occurrence of functional CB1 on forebrain glutamatergic neurons (Domenici et al. 2006; Kawamura et al. 2006; Marsicano et al. 2003; Takahashi and Castillo 2006). Yet the CB1 expression patterns in glutamatergic neurons remain unclear, and this prompted us to investigate the expression of CB1 in pyramidal neurons of the neocortex.

Endocannabinoids act as retrograde messengers to elicit the phenomenon of depolarization-induced suppression of inhibition (DSI) (Wilson and Nicoll 2001). DSI has been observed in neocortex (Bodor et al. 2005; Trettel and Levine 2002, 2003; Trettel et al. 2004), and activation of cholinergic receptors is often required to obtain significant DSI (Martin and Alger 1999), implicating acetylcholine-responsive interneurons expressing CB1 to contribute to DSI (Trettel et al. 2004). Neocortical interneurons expressing cholinergic receptors typically co-express SST or VIP (Gulledge et al. 2006; Kawaguchi 1997; Porter et al. 1999), suggesting that these interneuron populations also possess CB1 in contradiction with the reported presence of CB1 mainly on large CCK neurons. Therefore we investigated the expression of CB1 in SST and VIP interneuron populations.

Cannabinoids also act at CB2, which is principally found outside the brain, but mediates part of cannabinoid effects on cerebellar granule cells (Skaper et al. 1996) and brain stem neurons (Van Sickle et al. 2005). Although CB2 is only present at low level in the cortex (Van Sickle et al. 2005), we probed its expression in parallel with that of CB1.

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We used single-cell reverse transcription-polymerase chain reaction (scPCR) to investigate the expression of CB1 and CB2 in neocortical neurons. scPCR enabled us to characterize receptor expression in identified single neurons, eliminating the problem of nonspecific or background labeling levels. We also performed whole cell recordings of excitatory and inhibitory synaptic transmission to corroborate our molecular findings. Both our scPCR and electrophysiological data indicate that CB1, but not CB2, is widely expressed in SST and VIP interneurons as well as pyramidal neurons. Our findings suggest a broad role for the cannabinoid system of transmitters to modulate cortical networks.

METHODS

Slice preparation

Wistar rats (14–21 days old) were decapitated, and 300- μ m-thick parasagittal sections of cerebral sensorimotor cortex were prepared as described previously (Cauli et al. 1997). The slices were incubated at room temperature (22°C) in artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgCl_2 , 26 NaHCO_3 , 20 glucose, and 5 pyruvate, which was bubbled with a mixture of 95% O_2 –5% CO_2 .

Drugs

All drugs and chemicals were obtained from Sigma (Saint Louis, MO) except tetrodotoxin (TTX), which was purchased from Latoxan (Valence, France). Drug concentrations were as follows: carbachol (CCh), 3 μ M; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μ M; D-AP5, 20 μ M; WIN-55212-2 (WIN-2), 2 μ M; AM 251, 2 μ M; 1-1-dimethyl-4-phenyl-piperazinium iodide (DMPP), 100 μ M; atropine, 5 μ M; kynurenate, 1 mM.

Whole cell recordings

Slices were transferred to a recording chamber placed under a microscope (Axioscop FS Zeiss, Germany) and superfused at 2 ml/min with ACSF at room temperature. Patch pipettes (5–7 M Ω), pulled from borosilicate glass, were filled with 8 μ l internal solution. Membrane potentials were not corrected for junction potential. For characterization of neuronal types, investigation of electrophysiological properties and scPCR, we used an internal solution containing (in mM) 144 K-gluconate, 3 MgCl_2 , 0.5 EGTA, and 10 HEPES and 2 mg/ml biocytin (Sigma). The pH was adjusted to 7.2 and osmolarity to 285/295 mosM. Whole cell recordings were made from layers I to V neurons selected under infrared videomicroscopy (Stuart et al. 1993) with a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) connected to a Digidata 1200B interface board (Axon Instruments). Signals were amplified and collected using the data-acquisition software P-Clamp-8.02 (Axon Instruments). Recorded neurons were characterized by their firing properties as regular spiking pyramidal, fast spiking, regular spiking nonpyramidal, or irregular spiking cells as previously established (Cauli et al. 1997, 2000; Kawaguchi 1993, 1995; Kawaguchi and Kubota 1993). Fast-spiking neurons, which are either insensitive or inhibited by cholinergic agonists (Gulledge et al. 2006; Kawaguchi 1997; Porter et al. 1999; Xiang et al. 1998), were discarded from the present study.

GABAergic transmission

Whole cell recordings were made from layers II/III or V pyramidal cells using an internal solution containing (in mM) 144 CsCl, 3 MgCl_2 , 10 EGTA, 10 HEPES, and 5 QX-314 and 2 mg/ml biocytin. Neurons were voltage clamped and held at –60 mV. To augment

interneuron activity and subsequently increase GABA release onto pyramidal neurons, we preapplied CCh (3 μ M) for ≥ 3 min and throughout the experiment. Inhibitory postsynaptic currents (IPSCs) were subsequently recorded in the presence of CNQX (10 μ M) and D-APV (20 μ M) to block glutamate transmission. IPSCs were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA) with a minimum threshold set at 7 pA. DSI was induced by a 2-s depolarization from –60 to 0 mV. For each neuron, DSI was attempted at least three times, and the mean DSI was calculated as the percentage of inhibition corresponding to the ratio between the average amplitude of IPSCs during the first 10 s after the induction protocol and the average amplitude 60 s before DSI. Following the DSI protocol, IPSCs were recorded for a 2 min period before applying WIN-2 (2 μ M) for a minimum of 5 min. The maximum effect of WIN-2 was usually observed about 8 min after the beginning of WIN-2 application. IPSC amplitude and inter-event interval were measured during a 2-min period at the maximum WIN-2 effect and compared with control condition. WIN-2 effects on CCh-induced IPSCs was also investigated in the presence of 2 μ M AM 251, which was applied for 10 min before WIN-2 application. In another set of experiments, the nicotinic agonist DMPP (100 μ M) was applied for 10 s in the presence of CNQX (10 μ M), D-APV (20 μ M), and muscarinic receptor antagonist atropine (5 μ M). DMPP-induced IPSCs were recorded in pyramidal neurons in the presence and absence of WIN-2 (2 μ M). The same protocol was repeated in slices pretreated with 2 μ M AM 251.

Glutamatergic transmission

We performed extracellular stimulation by using conventional glass electrodes (filled with ACSF) placed in layer II/III, ~ 250 μ m rostral to the recorded layer II/III pyramidal neuron. Stimulation (10–40 μ A, 0.2–0.5 ms) was delivered every 15 s using a stimulation isolation unit (Isolator-11, Axon Instruments). Whole cell recordings were made from layers II/III pyramidal cells using an internal solution containing (in mM): 144 Cs-Gluconate, 3 MgCl_2 , 10 EGTA, 10 HEPES, and 5 QX-314 and 2 mg/ml biocytin ($E_{\text{Cl}} = -78$ mV in these experimental conditions). Neurons were voltage clamped and held at –60 mV. We applied a hyperpolarizing voltage step (5 mV) before each stimulation to monitor passive electrical properties of the recorded cell as well as access resistance, which did not vary $>20\%$ during the analyzed recording period. Access resistance was not compensated. For each experiment, we applied 2 μ M WIN-2 for 15–25 min. For EPSCs analysis, the mean control amplitude (measured over a 2.5-min period prior to WIN-2 application, $n = 10$ stimulations) was compared with the mean EPSC amplitude recorded during the final 2.5 min of WIN-2 application. At the end of the experiment, we applied 1 mM kynurenate (5–10 min) to confirm the glutamatergic nature of the EPSC. The same protocol was repeated in slices pretreated with 2 μ M AM 251.

All values presented in this study are means \pm SE. Statistical analyses for these experiments were conducted using Statistica software (StatSoft, Tulsa, OK). Statistical significance was assessed with Student's paired *t*-test. *P* value <0.05 was considered statistically significant.

Single cell RT-PCR

We performed cytoplasm harvesting of the recorded neurons and reverse transcription essentially as previously described (Lambolez et al. 1992). After recording, the cell's cytoplasm was aspirated into the recording pipette by application of a gentle negative pressure while maintaining the tight seal. Harvesting of the nucleus was carefully avoided. The pipette was then delicately removed to allow outside-out patch formation. Then the content of the pipette was expelled into a test tube and reverse transcription was performed in a final volume of 10 μ l. We performed two steps of multiplex PCR as described (Cauli

et al. 1997). The cDNAs present in the 10 μ l reverse transcription reaction were first amplified simultaneously using primer pairs described in Table 1 (sense and antisense primers were positioned on 2 different exons, except for CB1, CB2 and SST intron). We added taq polymerase (2.5 Units, Qiagen GmbH, Hilden, Germany) and 20 pmol of each primer to the buffer supplied by the manufacturer (final volume: 100 μ l) and 20 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 35 s) of PCR were run. We then performed second rounds of PCR using 2 μ l of the first PCR product as template. In this second round, each cDNA was individually amplified using its specific primer pair by performing 35 PCR cycles (as described above). 10 μ l of each individual PCR reaction were then run on a 2% agarose gel using ϕ x174 digested by *Hae*III as molecular weight marker and stained with ethidium bromide.

Identification of the PCR products and testing of the scPCR protocol

We analyzed PCR-generated fragments obtained from each cell by fluorescence resonance energy transfer (FRET) between two adjacent oligoprobes (Table 1, purchased from Proligo, Paris, France) internal to the amplified sequence. The upstream probe was FITC-labeled at the 3' end (donor, excitation 470 nm) and the downstream probe Red705-labeled at the 5' end (acceptor, emission, 710 nm). FRET between the two fluorophores, which can only occur when both probes are hybridized to their cognate PCR fragment, was measured with a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) as described (Ferezou et al. 2002). The RT-PCR protocol was tested on 500 pg of total RNA purified from rat neocortex. All of the cDNAs were detected from 500 pg of neocortical total RNA; whereas the SST gene intron was detected from 10 ng of rat genomic DNA. The sizes of the PCR generated fragments were as predicted from published sequences (see Table 1) and their identity was confirmed by FRET between adjacent oligoprobes (as described in the preceding text).

Cellular morphology

We performed diaminobenzidine labeling of the recorded neurons filled with biocytin using the ABC elite kit (Vector Laboratories, Burlingame, CA). Images of neurons were captured using a Leica DMR light microscope (Leica Microsystems, Rueil-Malmaison, France) coupled with a Canon Powershot S45 digital camera (Canon France, Courbevoie, France).

RESULTS

Expression of CB1 mRNA in cortical neurons

We analyzed neocortical neurons by scPCR to investigate the expression of CB1 and CB2 mRNAs. The recorded neurons were characterized by their intrinsic firing patterns and mRNA expression profiles, and several neurons were randomly selected and assessed for morphology following biocytin labeling. In all neurons, we investigated the expression of the interneuronal markers: glutamic acid decarboxylase (GAD65 and GAD67), calbindin (CaB), calretinin (CR), neuropeptide Y (NPY), CCK, VIP, and SST, together with CB1 and CB2. In addition, for putative pyramidal cells we included the vesicular glutamate transporter vGLUT1 in the scPCR protocol to confirm their glutamatergic nature. Because CB1 and CB2 amplified sequences are intronless, a negative control for genomic DNA contamination (amplifying the SST gene intron) was always included to ascertain the mRNA origin of the CB1 amplified product (see METHODS). We tested genomic DNA contamination on 10 pyramidal neurons processed as described in METHODS except that reverse transcriptase was omitted. No PCR product was detected in this sample except in one cell positive for CB1, CB2, and the SST intron. This indicates that genomic DNA contamination occurred at a low frequency and was reliably detected by SST intron amplification. We discarded SST intron-positive cells from the present study (2.8%

TABLE 1. PCR primers and FRET probes

	Size	PCR Primers	FRET Probes
CB1	351	Sense, 42 : TACCATCACACAGACCTCCTC	3' FITC, 176: CAAGAAAAGATGACCGCAGGAGAC
No. X55812		Antisense, 373 : GTGAAGGTGCCAGTGTGAG	5' R705, 200 : ACTCCCCGTTGGTCCACG
CB2	434	Sense, 571 : CTCCTGGGCTGGCTTCTTT	3' FITC, 893 : TTTATGCCCTGCGGAGTGGA
No. AF176350		Antisense, 983 : GACTTTGGGGCTTCTTCTTTC	5' R705, 914 : AGATCCGCTCTGCTGCCCA
vGlut1	259	Sense ^d , 361 : GGCTCCTTTTTCTGGGGGTAC	3' FITC, 413 : GCCAAAAATTCGCAGCCAAC
No. U07609		Antisense ^d , 600 : CCAGCCGACTCCGTTCTAAG	5' R705, 434 : GGGTCTTTGGCTTTGCCATTG
Gad65	391	Sense ^a , 713 : TCTTTCTCCTGGTGCGCC	3' FITC ^c , 848 : TTTCTCTCAAGAGGGAGCTGCA
No. M72422		Antisense ^a , 1085 : CCCAAGCAGCATCCACAT	5' R705 ^c , 872 : CCTTGGGGATCGGAACAGACA
Gad67	600	Sense ^b , 713 : TACGGGGTTCGCACAGGTC	3' FITC ^c , 930 : CCTGGGGGAGCCATATCCAA
No. M76177		Antisense, 1294 : same as GAD65	5' R705 ^c , 951 : ATGTACAGCATCATGGCGGCTC
CaB	432	Sense ^b , 134 : AGGCACGAAAGAAGCTGGAT	3' FITC ^c , 263 : TCCTGCTGCTCTTTCGATGCC
No. M27839		Antisense ^b , 544 : TCCCACACATTTTGATTCCCTG	5' R705 ^c , 285 : GCAACTGAAGTCTCTGCGAGGAA
CR	309	Sense ^b , 142 : CTGGAGAAGGCAAGGAAAGGT	3' FITC ^c , 248 : TTGAGATGGCAGAGCTGGCG
No. X66974		Antisense ^b , 429 : AGGTTCATCATAGGGACGGTTG	5' R705 ^c , 269 : AGATCTTGCCAAACGAGAGAAT
NPY	359	Sense ^b , -45 : GCCCTAGCGAGAGATGACA	3' FITC ^c , 17 : AACGAATGGGGCTGTGTGGA
No. M15880		Antisense ^b , 292 : CAAGTTTCATTTCCCATCACCA	5' R705 ^c , 38 : TGACCCTCGCTCTATCCCTGC
SST	209	Sense ^b , 43 : ATCGTCCTGGCTTTGGGC	3' FITC ^c , 105 : GCAGAAGTCTCTGGCGGCTG
No. K02248		Antisense ^b , 231 : GCCTCATCTCGTCTCTGCTCA	5' R705 ^c , 126 : CACCGGGAAACAGGAAGTGGC
VIP	286	Sense ^b , 167 : TGCCTTAGCGGAGAAATGACA	3' FITC ^c , 199 : TGTCTCCAGAAATGCCAGGCA
No. X02341		Antisense ^b , 434 : CCTCACTGCTCCTCTTCCCA	5' R705 ^c , 221 : GCTGATGGAGTTTTCACCAGCG
CCK	216	Sense ^b , 174 : CGCACTGCTAGCCCCGATACA	3' FITC ^c , 198 : CCAGCAGGTCGCGAAAGCT
No. K01259		Antisense ^b , 373 : TTTCTCATTCGCGCTCTCC	5' R705 ^c , 218 : CCTCTGGCCGCATGTCCG
SST Intron	421	Sense, 143 : GGAAATGGCTGGGACTCGTC	Not analyzed
No. K02248		Antisense, 540 : AAACCATGGATGATAGGAAGTCGT	

Position 1 : 1st base of the start codon

FRET, fluorescence resonance energy transfer. ^aBochet et al. 1994; ^bCauli et al. 1997; ^cFerezou et al. 2002; ^dGallopin et al. 2006.

of sampled cells). Last, we tested mRNA contamination from surrounding tissue by placing a patch pipette into the slice without establishing a seal. Positive pressure was then interrupted, and following removal of the pipette its content was processed as described. No PCR product was obtained using this protocol ($n = 20$).

We focused on three main cell populations throughout the present study. Two populations were SST- and VIP-containing interneurons comprising a total of 149 cells. These neurons were found to express GAD and displayed regular spiking nonpyramidal or irregular spiking patterns. Histological analysis of 11 SST- and 26 VIP-expressing neurons demonstrated bipolar/bitufted or multipolar dendritic morphology. These electrophysiological and morphological properties were consistent with those described in previous reports (Bayraktar et al. 2000; Cauli et al. 1997; Kawaguchi and Kubota 1996; Porter et al. 1998; Somogyi et al. 1984; Wang et al. 2004). The third population consisted of 106 pyramidal neurons ($n = 39$

from layer II/III and $n = 67$ from layer V) that showed typical low-frequency regular-spiking patterns, expressed mRNA for vGluT1, and were negative for GAD. Histological analysis of 55 pyramidal neurons revealed a clear apical dendrite. Representative examples of the three cell populations studied are depicted in Fig. 1.

A majority (63%) of SST-expressing interneurons also expressed CB1 ($n = 62$; Fig. 2A). In contrast, CB2 was only found in 13% of SST-expressing interneurons. We also observed a high occurrence of CaB (48%) and a lower occurrence of CCK (32%) and VIP (21%) in SST interneurons, consistent with previous findings (Cauli et al. 2000; Gallopin et al. 2006; Gonchar and Burkhalter 1997; Kubota et al. 1994; Papadopoulos et al. 1987; Somogyi et al. 1984; Toledo-Rodriguez et al. 2005; Wang et al. 2004). Analysis of VIP-expressing interneurons revealed that 69% of these cells co-expressed CB1 but that only 4% co-expressed CB2 ($n = 100$; Fig. 2B). Consistent with previous findings (Cauli et al. 2000; Gallopin et al. 2006;

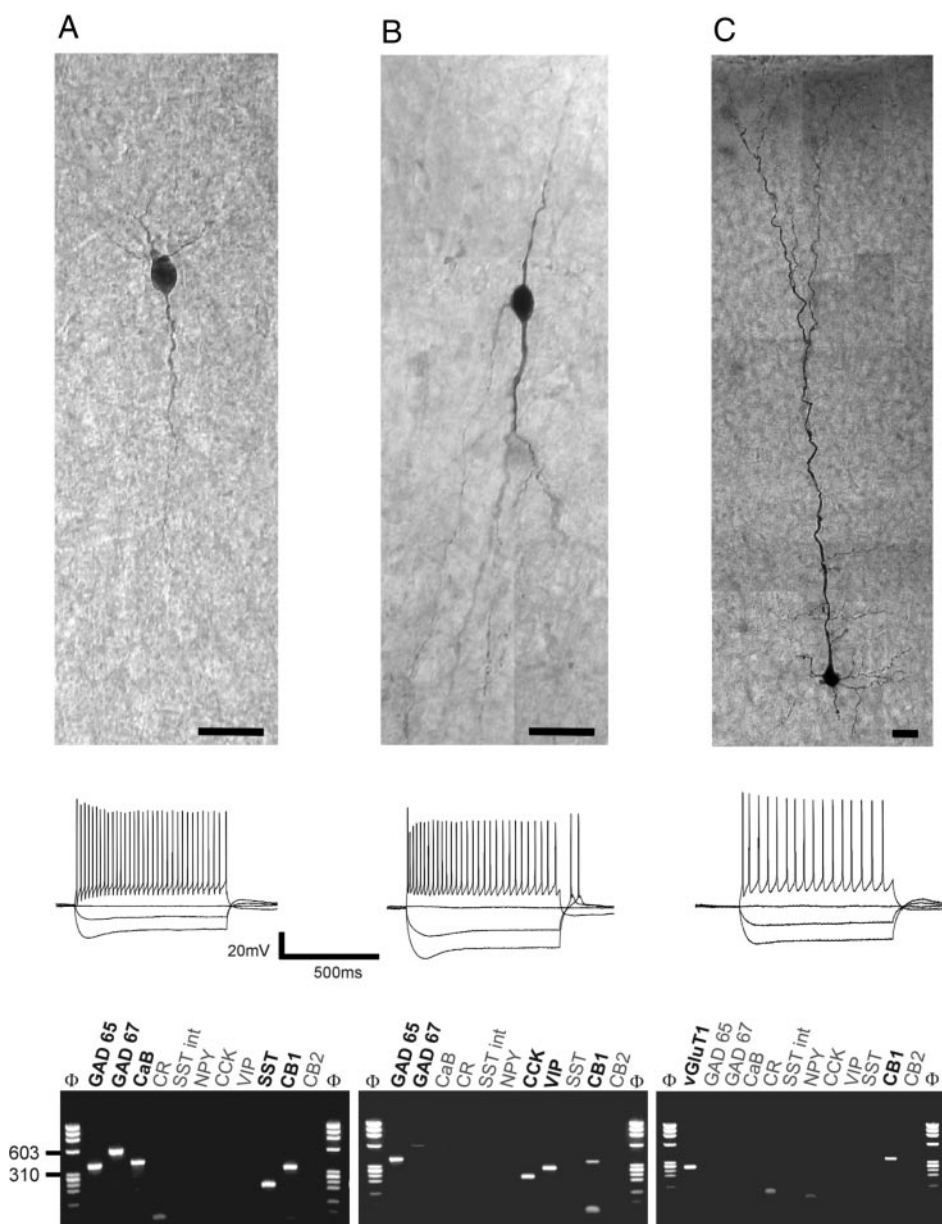


FIG. 1. Morphological, physiological, and molecular characterization of somatostatin (SST) interneurons, vasoactive intestinal polypeptide (VIP) interneurons and pyramidal cells. *A, top*: SST neurons showed a bitufted or multipolar morphology (note multiple dendrites emerging from the apical aspect of the soma). *Pial surface is upward and scale bar is 20 μ m (same in B and C).* *Middle*: responses of the same neuron recorded in current-clamp mode (holding potential, -60 mV) to hyperpolarizing and depolarizing current steps (-100 , -50 , and $+100$ pA). This neuron exhibited a regular spiking firing pattern. The agarose gel analysis of single-cell reverse transcription-polymerase chain reaction (scPCR) products (*bottom*) showed that this neuron expressed glutamic acid decarboxylase 65 and 67 (GAD65 and GAD67), calbindin (CaB), SST, and CB1 mRNAs. Positions of the 603- and 310-bp bands of ϕ x174/HaeIII marker are indicated. *B*: this biocytin-labeled neuron exhibited a bipolar morphology and a regular spiking discharge (*middle*) typical of VIP interneurons, and expressed GAD65 and 67, VIP, cholecystokinin (CCK), and CB1 mRNAs (*bottom*). *C*: pyramidal neurons demonstrated a prominent apical dendrite and basal lateral dendrites. This cell exhibited a typical slow regular firing pattern (*middle*) and expressed vGluT1 together with CB1 (*bottom*).

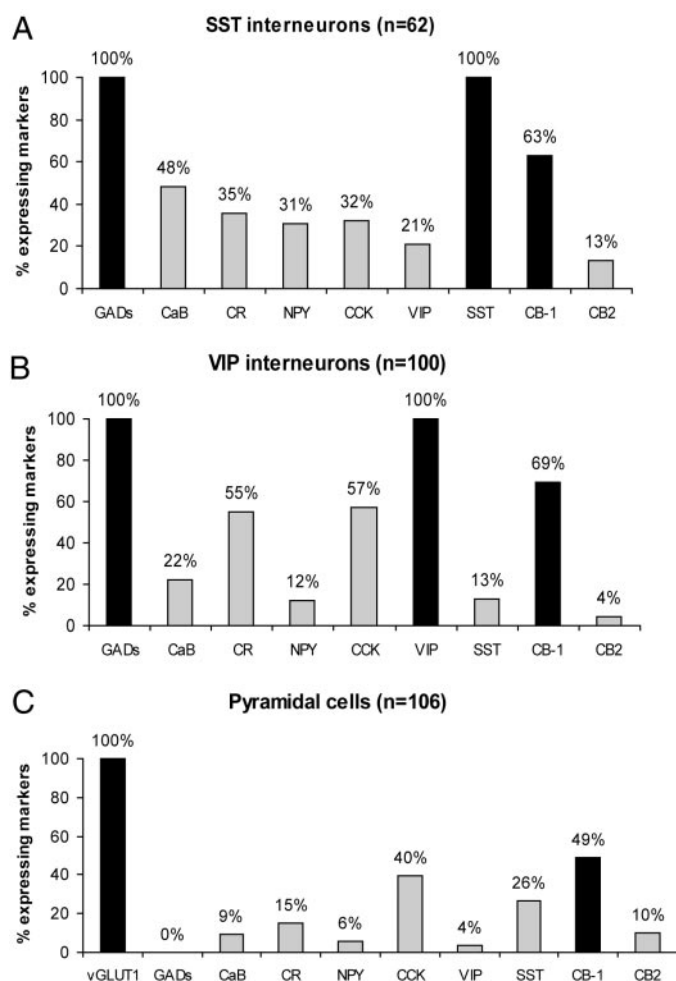


FIG. 2. Summary of scPCR. CB1 expression was probed in a total of 255 neocortical neurons comprising 62 SST-expressing interneurons, 100 VIP-expressing interneurons, and 106 pyramidal neurons. *A*: CB1 mRNAs were detected in 63% of SST-expressing interneurons that showed limited co-expression of VIP (21%) or CCK (32%). *B*: large proportion of VIP interneurons expressed CB1 (69%) as well as CCK (57%), whereas SST expression (13%) was low in these neurons. *C*: pyramidal neurons expressed vGlut1 but not GAD. They showed a high occurrence of CB1 mRNA (49%), followed by CCK and SST (40 and 26%, respectively). Note that 13 interneurons expressed both VIP and SST and were therefore included in both *A* and *B*.

Kubota et al. 1994; Papadopoulos et al. 1987; Toledo-Rodriguez et al. 2005), this neuronal population frequently expressed CCK (57%) and CR (55%). In contrast, VIP interneurons showed low expression of SST (13%). Analysis of pyramidal neurons revealed that 49% of these cells expressed CB1 ($n = 106$, Fig. 2*C*), with similar proportions in layer II/III (51%, $n = 20$ of 39) and in layer V (48%, $n = 36$ of 67). Only 10% of pyramidal neurons of either layers expressed CB2. As previously reported (Gallopín et al. 2006; Ong et al. 1994; Schiffmann and Vanderhaeghen 1991), pyramidal cells also expressed CCK (40%) and to a lesser extent SST (26%).

Consistent with its low abundance in the cortex (Van Sickle et al. 2005), the CB2 mRNA was rarely found in our sample of neocortical neurons. The expression of CB1 mRNA was only weakly correlated to that of CCK in our sample of 255 neurons (correlation coefficient: 0.19, Spearman rank correlation test, $P \leq 0.05$). Indeed, a large percentage of cells positive for CB1 were negative for CCK in each neuronal population (62, 38,

and 44% for SST, VIP, and pyramidal cells, respectively). Hence the expression of the CB1 mRNA is broader than suggested by immunocytochemistry (Bodor et al. 2005; Katona et al. 1999; Tsou et al. 1999) but consistent with in situ hybridization studies (Marsicano and Lutz 1999) and the conditional CB1 knock-out results (Domenici et al. 2006; Marsicano et al. 2003). Specifically, CB1 receptor mRNA expression extended to SST and VIP interneurons that can be activated by cholinergic agonists as well as to glutamatergic pyramidal neurons. We next tested whether functional CB1 receptors are translated from the mRNAs expressed in SST, VIP, and pyramidal neurons.

Cannabinoid actions on cholinergic responsive interneurons

Cholinergic agonists increase the frequency and amplitude of IPSCs in the neocortex (Blatow et al. 2003; Kawaguchi 1997; Xiang et al. 1998). These IPSCs are TTX sensitive (Xiang et al. 1998) and thus stem from SST and VIP interneurons that selectively show action potential firing on cholinergic stimulation (Gulledge et al. 2006; Kawaguchi 1997; Porter et al. 1999). Because we found that the majority of SST and VIP interneurons express CB1, we assessed the effect of CB1 agonists on cholinergic-responsive interneurons by applying 3 μ M of the cholinergic agonist carbamylcholine (carbachol, CCh) and recording IPSCs in pyramidal neurons in the presence of glutamate receptor antagonists (10 μ M CNQX and 20 μ M D-APV). We used two approaches to investigate CB1 modulation of synaptic transmission in these cell populations: we first delivered a DSI paradigm (see METHODS) to establish the participation of endocannabinoids, and second we applied the synthetic CB1 agonist WIN-2.

Application of CCh consistently elicited a tonic increase in IPSC frequency (interevent interval decreased from 312.9 ± 57.7 to 136.1 ± 30.4 ms; $P < 0.05$, Student's paired t -test) but insignificantly increased IPSC amplitude (from 18.1 ± 1.0 to 23.5 ± 2.9 pA; $P = 0.091$, Student's paired t -test) in eight of eight pyramidal cells tested, including six layer II/III neurons and two layer V neurons. In the continuous presence of CCh, delivery of DSI paradigms largely decreased the inhibitory activity recorded in four layer II/III pyramidal neurons tested (Fig. 3*A*). In these four pyramidal cells, CCh-elicited IPSC amplitude was reduced by $21 \pm 4\%$ on delivery of the DSI paradigm, suggesting that CB1 are present on CCh responsive cells and are physiologically activated by endocannabinoids. No DSI was observed in the remaining four cells. We further tested the presence of CB1 on cholinergic responsive neurons in the same neuronal sample ($n = 8$) by applying WIN-2 (2 μ M), which greatly reduced the CCh-evoked IPSCs (Fig. 3*B*). In seven of eight cells WIN-2 reduced the IPSC amplitude by $27 \pm 8\%$ (from 23.6 ± 2.5 to 16.0 ± 1.1 pA, $P < 0.05$, Student's paired t -test) and increased the interevent interval by $54 \pm 14\%$ (from 120.7 ± 13.4 to 191.5 ± 31.1 ms, $P < 0.05$, Student's paired t -test; Fig. 3*C*). In the remaining cell, from layer V, we observed no effect of the DSI paradigm and of WIN-2 application on IPSCs. The effect of WIN-2 was strongly reduced by the CB1 antagonist AM 251. Indeed, in slices pretreated with 2 μ M AM 251, WIN-2 decreased the amplitude of CCh-evoked IPSCs by $8 \pm 1\%$ (from 21.7 ± 1.7 to 20.0 ± 1.2 pA; $P < 0.05$, Student's paired t -test) and increased the interevent Interval by $11 \pm 5\%$ (from 123 ± 7 to

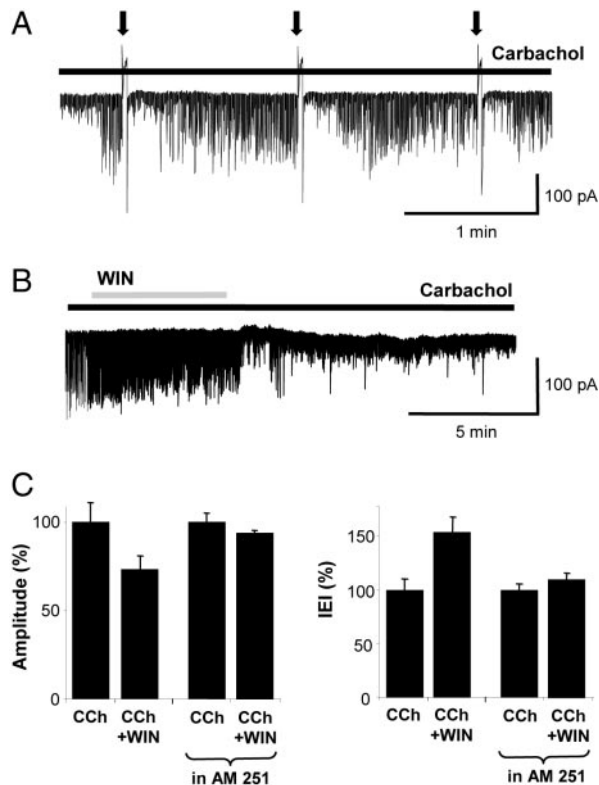


FIG. 3. Cannabinoids decrease carbachol (CCh)-elicited inhibitory postsynaptic currents (IPSCs) in pyramidal cells. *A*: application of $3 \mu\text{M}$ CCh largely increased inhibitory synaptic activity. Delivery of depolarization-induced suppression of inhibition (DSI) paradigms (denoted by arrows) transiently depressed the CCh-elicited IPSCs (mean amplitude reduction of 42% for the 3 DSI trials). *B*: in the same neuron, application of the CB1 agonist WIN-55212-2 (WIN-2; $2 \mu\text{M}$) decreased the amplitude of CCh-elicited IPSCs by 66%. *C*: on average, WIN-2 decreased the amplitude of CCh-elicited IPSCs by 27% and increased the interevent interval by 54% ($n = 7$). In the continuous presence of the CB1 antagonist AM 251 ($2 \mu\text{M}$), WIN-2 decreased the amplitude of CCh-elicited IPSCs by 8% and increased the interevent interval by 11% ($n = 5$).

$136 \pm 11 \text{ ms}$, $P = 0.11$, Student's paired t -test) ($n = 5$ layer II/III pyramidal cells, Fig. 3C). The pronounced effect of WIN-2 on CCh-evoked IPSCs suggests that functional CB1 and cholinergic receptors are co-expressed in SST and VIP interneurons.

Because CCh activates both muscarinic and nicotinic subtypes of cholinergic receptors, we also assessed cannabinoid effects on increased inhibitory activity elicited by the selective nicotinic agonist DMPP. In the neocortex, nicotinic receptors are principally located on VIP interneurons (Gulledge et al. 2006; Porter et al. 1999), a cell population that we found to express CB1. To minimize desensitization of the nicotinic response, we made two short (10 s) bath applications of $100 \mu\text{M}$ DMPP separated by $\geq 5 \text{ min}$ in two neuronal samples. In the control sample ($n = 6$ layer II/III pyramidal neurons), the second DMPP application was performed in the absence of WIN-2, whereas in the drug sample ($n = 9$ layer II/III pyramidal neurons), the second DMPP application was performed in the presence of WIN-2. The first application of DMPP largely increased IPSC amplitude (Fig. 4A). In the control sample, the response elicited by the second DMPP application was insignificantly decreased by $7 \pm 5\%$ (from 52 ± 1 to $48 \pm 1 \text{ pA}$, $P = 0.051$, Student's paired t -test) when compared with

the first DMPP application (Fig. 4B). In the presence of $2 \mu\text{M}$ WIN-2, however, the response elicited by the second DMPP application was significantly reduced by $41 \pm 5\%$ from 49.0 ± 2.1 to $28.7 \pm 0.6 \text{ pA}$ ($P < 0.0001$, Student's paired t -test; see Fig. 4, A and B). This effect of WIN-2 was strongly reduced by the CB1 antagonist AM 251. In slices pretreated with $2 \mu\text{M}$ AM 251, the response to the second DMPP application in the presence of $2 \mu\text{M}$ WIN-2 was only decreased by $9 \pm 1\%$ from 29.2 ± 3.3 to $26.5 \pm 2.9 \text{ pA}$ ($n = 3$ layer II/III pyramidal neurons, $P < 0.05$, Student's paired t -test; see Fig. 4B). The marked reduction of DMPP-evoked IPSC in the presence of WIN-2 confirms that CB1 is co-expressed with nicotinic receptors on VIP interneurons.

Cannabinoid actions on glutamatergic transmission

We examined the presence of functional CB1 on glutamatergic neurons by stimulating intracortical excitatory connections and recording EPSCs in layer II/III pyramidal neurons. The amplitude of stimulation-evoked EPSCs was greatly reduced in the presence of WIN-2 (Fig. 5). In nine cells tested, WIN-2 significantly decreased the EPSC mean amplitude by $38 \pm 7\%$, from 111 ± 3 to $69 \pm 3 \text{ pA}$ ($P \ll 0.0001$, Student's paired t -test). At the end of the experiment, application of the nonselective glutamate receptor antagonist kynurenatate sharply

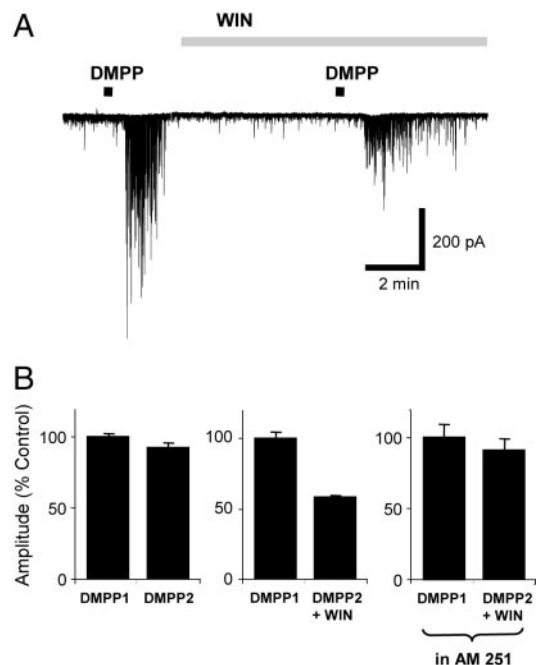


FIG. 4. WIN-2 decreases 1-1-dimethyl-4-phenyl-piperazinium iodide (DMPP)-elicited IPSCs in pyramidal cells. *A*: short application (10 s) of the selective nicotinic receptor agonist DMPP dramatically increased IPSCs. In the presence of $2 \mu\text{M}$ WIN-2, a 2nd application of DMPP was less effective to raise inhibitory activity (49% reduction of mean IPSC amplitude in this neuron). *B*, left: 2nd DMPP application (DMPP2) raised inhibitory activity to 93% ($n = 6$) of the level reached with the 1st application (DMPP1). *Middle*: in presence of $2 \mu\text{M}$ WIN-2, the 2nd DMPP application (DMPP2 in WIN) only raised inhibitory activity to 59% ($n = 9$) of the level obtained in absence of WIN-2 (DMPP1), indicating a depression of DMPP-elicited inhibitory activity by the CB1 agonist. *Right*: in slices pretreated with the CB1 antagonist AM 251 ($2 \mu\text{M}$), the 2nd DMPP application in presence of WIN-2 (DMPP2 in WIN) raised inhibitory activity to 91% ($n = 3$) of the level obtained in absence of WIN-2 (DMPP1).

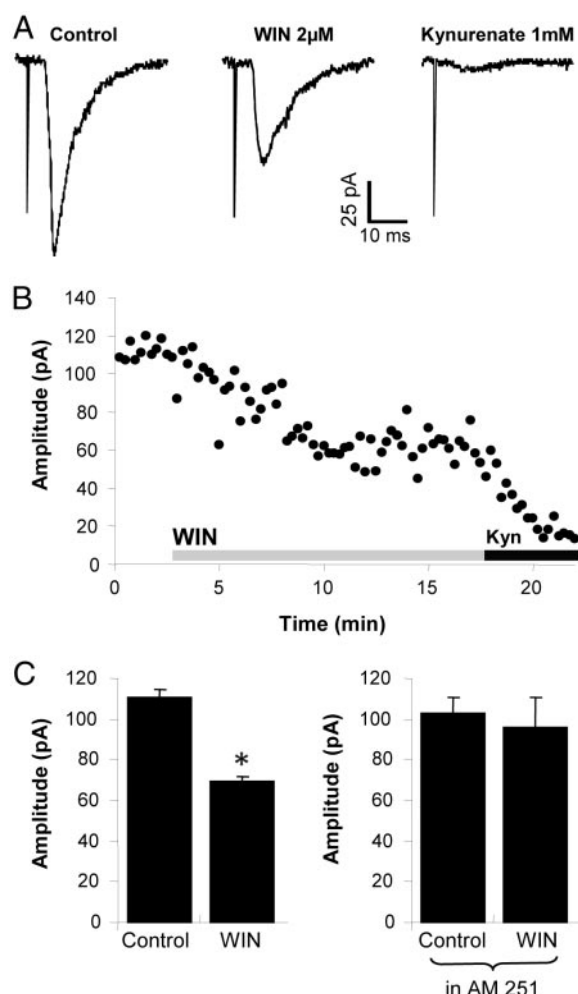


FIG. 5. Cannabinoids decrease electrically evoked glutamatergic transmission. *A*: excitatory postsynaptic currents (EPSCs) were evoked by stimulating locally with an electrode placed in layer II/III ~ 250 μm rostral to the recorded pyramidal neuron. Application of 2 μM WIN-2 gradually decreased the EPSC amplitude. Kynurenate was subsequently applied to confirm the glutamatergic nature of the recorded response. *B*: time course of the WIN-2 effect on EPSC amplitude (same neuron as in *A*). *C*: on average, WIN-2 decreased EPSC amplitudes by 38% in pyramidal neurons ($n = 9$). In slices pretreated with the CB1 antagonist AM 251 (2 μM), WIN-2 only decreased EPSC amplitudes by 7% ($n = 4$). *, $P < 0.0001$.

decreased the EPSC amplitude to $16 \pm 3\%$ of control value, confirming that the recorded postsynaptic current was essentially glutamatergic. The effect of WIN-2 was strongly reduced by the CB1 antagonist AM 251. In slices pretreated with 2 μM AM 251, WIN-2 insignificantly decreased the EPSC mean amplitude by $7 \pm 7\%$, from 102 ± 8 to 96 ± 14 pA ($n = 4$ layer II/III pyramidal cells, $P = 0.44$, Student's paired t -test). These results suggest that a functional CB1 is expressed in neocortical pyramidal neurons and is responsible for the WIN-2 effect on excitatory transmission.

DISCUSSION

In the present study, we have investigated the expression of CB1 and its role to modulate GABAergic and glutamatergic transmission in the neocortex. We found that the CB1 mRNA was frequently expressed in pyramidal neurons as well as in SST and VIP interneurons. Consistent with CB1 expression in

these cholinergic-responsive interneuron populations, IPSCs elicited by cholinergic agonists were depressed by the CB1 agonist WIN-2. Also in accord with CB1 expression in pyramidal neurons, WIN-2 depressed EPSCs evoked by intracortical stimulation. WIN-2 effects were strongly reduced by the CB1 antagonist AM 251. Our results indicate a broad functional expression of CB1 in both GABAergic and glutamatergic neurons of the neocortex.

Expression of CB1 in pyramidal neurons and multiple interneuron populations

We found that a substantial proportion of pyramidal neurons, SST interneurons and VIP interneurons express the CB1 mRNA. These neurons were clearly distinct from the large CCK interneurons that reportedly express high levels of CB1 (Katona et al. 1999; Marsicano and Lutz 1999; Tsou et al. 1999). Indeed, the peptides VIP and SST are typically absent from large CCK interneurons (Kubota and Kawaguchi 1997; Somogyi 1984), and high-CB1-expressing large CCK interneurons present electrophysiological hallmarks such as irregular spiking discharge (Galarreta et al. 2004) or occurrence of a low-threshold calcium spike (Bacci et al. 2004) that were rarely encountered in the presently studied neurons. Previous *in situ* hybridization studies have shown that multiple neuronal populations may express the CB1 mRNA in the neocortex, including principal neurons, albeit at lower levels than large CCK interneurons (Marsicano and Lutz 1999; Matsuda et al. 1993). Our present scPCR results demonstrate that pyramidal neurons from layers II/III and V, SST interneurons, and VIP interneurons indeed express the CB1 mRNA. The occurrence of the CB1 mRNA in these cell populations is probably underestimated by scPCR, due to its detection limit (around 25 molecules of mRNA) and because only part of the cellular mRNA is harvested in the patch pipette (Tsuzuki et al. 2001). This is exemplified in other studies where the expression of various G-protein-coupled somatodendritic receptors was detected by scPCR in only a fraction of the neurons showing functional expression of these receptors (Ferezou et al. 2006; Gallopin et al. 2006). This suggests that the proportions of pyramidal, SST and VIP neurons expressing CB1 are larger than presently found by scPCR, consistent with the robust effects of WIN on postsynaptic currents we observed in this study.

Because of the intense binding of CB1 radioligands, the CB1 protein is believed to be one of the most abundant G-protein-coupled receptors in the brain (Herkenham et al. 1990). This intense binding, abolished in CB1 knock-out mice (Zimmer et al. 1999), is relatively uniform in the neocortex (Herkenham et al. 1990), a distribution inconsistent with immunodetection of the CB1 protein mainly in large CCK cells (Bodor et al. 2005; Katona et al. 1999; Tsou et al. 1999). A plausible explanation to this discrepancy is that bivalent binding of antibodies is much stronger than monovalent binding (Hornick and Karush 1972; Mattes 1997; Turner 2002) and, as a consequence, the affinity of antibodies sharply drops with the density of their antigens, especially for immobile antigens (Kaufman and Jain 1992) found in fixed tissues. The resulting threshold effect may only allow detection of dense CB1 expression sites under the stringent conditions used to ensure specific immuno-histochemical staining. Therefore we propose that the CB1 protein

is present in pyramidal neurons as well as SST interneurons and VIP interneurons, albeit at lower levels than in large CCK interneurons, as suggested by their cognate CB1 mRNA expression levels (Marsicano and Lutz 1999; Matsuda et al. 1993). This is supported by the recent observation that the CB1 protein is detected in excitatory axon terminals at asymmetrical synapses in the forebrain using immuno-electron microscopy (Katona et al. 2006; Kawamura et al. 2006).

CB1 effects on cholinergic responsive interneurons

We found that CB1 activation depressed GABAergic IPSCs elicited by cholinergic agonists in pyramidal neurons. This effect was strongly reduced by the CB1 antagonist AM 251. The different sensitivities of neocortical interneuron subtypes to cholinergic agonists have been established in previous reports. Cholinergic agonists elicit a marked excitation of SST and VIP interneurons accompanied by firing of action potentials (Gulledge et al. 2006; Kawaguchi 1997; Porter et al. 1999), whereas “fast spiking” and “late spiking” interneurons are either insensitive or inhibited by cholinergic agonists (Gulledge et al. 2006; Kawaguchi 1997; Porter et al. 1999; Xiang et al. 1998). In large CCK interneurons, cholinergic agonists elicit a sequence of hyperpolarization-depolarization that usually does not trigger the firing of action potentials (Kawaguchi 1997) or an inhibitory response (Gulledge et al. 2006). Therefore the CCh-induced IPSCs recorded in the present study originated principally from SST and VIP interneurons. Hence our results indicate that the depressing effect of WIN-2 on CCh-induced IPSCs is due to activation of CB1, consistent with the scPCR detection of CB1 mRNAs in SST and VIP interneurons.

It is now well established that DSI is mediated by a retrograde action of endogenously formed cannabinoids (Wilson and Nicoll 2001), and DSI has been described in neocortex (Bodor et al. 2005; Trettel and Levine 2003). Recent data obtained in neocortex also indicated that GABAergic interneurons depolarized by muscarinic agonists provide the majority of DSI-susceptible inputs to pyramidal neurons (Trettel et al. 2004). Our data showing that delivery of a DSI paradigm depressed CCh-induced IPSCs confirm these results and indicate that CB1 expressed by SST and VIP interneurons are physiologically activated by endogenously released cannabinoids. Whereas both SST and VIP interneurons are depolarized by muscarinic receptor agonists (Kawaguchi 1997), only VIP interneurons are depolarized by nicotinic receptor agonists (Porter et al. 1999). The observation that WIN-2 depressed IPSCs elicited by application of a nicotinic receptor agonist further confirms that functional CB1 receptors are expressed in VIP interneurons.

CB1 effects on glutamatergic transmission

Although CB1 immunoreactivity has often been undetected in pyramidal neurons of the forebrain (Bodor et al. 2005; Katona et al. 1999; Tsou et al. 1999), molecular and functional data indicate that the CB1 receptor is indeed expressed in these neurons as recently shown on hippocampal excitatory axon terminals using immuno-electron microscopy (Katona et al. 2006; Kawamura et al. 2006). The present molecular data

indicate that CB1 is expressed in neocortical pyramidal neurons from both layers II/III and V. We corroborated our molecular findings by testing the effects of the CB1 agonist WIN-2 on intracortical glutamatergic inputs onto layer II/III pyramidal neurons. We found that the reduction of excitatory synaptic transmission by WIN-2 was strongly reduced by the CB1 antagonist AM 251, indicating that functional CB1 receptors are indeed present on neocortical pyramidal neurons. These results are consistent with numerous physiological reports demonstrating direct CB1 effects on intracortical glutamatergic connections originating from pyramidal neurons of both layers II/III and V (Auclair et al. 2000; Bender et al. 2006; Fortin and Levine 2006; Sjostrom et al. 2003, 2004). Therefore we propose that functional CB1 receptors are widely expressed in neocortical pyramidal neurons and account for the cannabinoid effects on excitatory transmission in this brain region.

Modulation of the neocortical network by CB1

Given the low occurrence of the CB2 mRNA observed in the present study, it is likely that CB1 mediates most of the cannabinoid effects on neocortical neurons. It is established that cannabinoids act at CB1 to hyperpolarize large CCK interneurons (Bacci et al. 2004) and to decrease their GABAergic output (Galarreta et al. 2004). The present results indicate that CB1 mediates cannabinoid effects on other cell types, including pyramidal glutamatergic neurons. In light of our data, CB1 appears to play a major role to modulate cortical activity by acting on both glutamatergic and GABAergic neurons. We therefore propose that endocannabinoids acting at CB1 modulate the synaptic output of several neuronal types to influence neocortical networks. Depolarization of pyramidal neurons triggers the production of endocannabinoids, which act as retrograde messengers to inhibit GABA release (DSI) and to elicit short- or long-term depression of glutamate release (Diana and Marty 2004; Gerdeman and Lovinger 2003). It is likely that neocortical DSI involves endocannabinoid actions on axon terminals of large CCK interneurons and of cholinergic responsive SST and VIP interneurons. Similarly, endocannabinoids acting at CB1 inhibit glutamate release from axon terminals of layers II/III and V pyramidal neurons in the neocortex.

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