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Defective Adult Neurogenesis in CB1 Cannabinoid Receptor Knockout Mice

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ABSTRACT

Pharmacological studies suggest a role for CB1 cannabinoid receptors (CB1R) in regulating neurogenesis in the adult brain. To investigate this possibility, we measured neurogenesis by intraperitoneal injection of bromodeoxyuridine (BrdU), which labels newborn neurons, in wild-type and CB1R-knockout (CB1R-KO) mice. CB1R-KO mice showed reductions in the number of BrdU-labeled cells to ~50% of wild-type (WT) levels in dentate gyrus and subventricular zone (SVZ), suggesting that CB1R activation promotes neurogenesis. To test this further, WT mice were given the CB1R antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride (SR141716A) before measuring neurogenesis with BrdU. SR141716A paradoxically increased the number of BrdU-labeled cells by ~50% in SVZ; another CB1R antagonist, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide (AM251), had a similar effect. To investigate this discrepancy, SR141716A was given to CB1R-KO mice, in which it still stimulated neurogenesis, indicating involvement of a non-CB1 receptor. Action at one such non-CB1, SR141716A-sensitive site, the VR1 vanilloid receptor, was tested by administering SR141716A to VR1-KO mice, in which the ability of SR141716A to enhance neurogenesis was abolished. Thus, CB1 and VR1 receptors both seem to have roles in regulating adult neurogenesis.

Cannabinoid drugs such as Δ^9-tetrahydrocannabinol, the principal psychoactive constituent of marijuana, act via signaling pathways consisting of endogenous cannabinoids (endocannabinoids) and their receptors. Among endocannabinoids, the best characterized are arachidonylethanolamide or anandamide (AEA) and 2-arachidonylglycerol (2-AG), although additional candidates have been proposed. Two G-protein-coupled cannabinoid receptors, designated CB1R and CB2R, have been cloned (Howlett et al., 2002). CB1R are activated by Δ^9-tetrahydrocannabinol, AEA, 2-AG, and the synthetic cannabinoid agonist Win 55,212-2, and are blocked by SR141716A. CB2R are insensitive to SR141716A and are blocked instead by SR144528. CB1R and CB2R also differ in distribution: CB1R are localized primarily to neurons and are most abundant in brain, whereas CB2R are found principally on non-neuronal cells. Pharmacological studies suggest the existence of additional receptors for cannabinoid agonists and antagonists. For example, AEA and SR141716A bind to the VR1 vanilloid receptor (Zygmunt et al., 1999), and AEA and cannabidiol bind to an "abnormal cannabidiol" receptor expressed on endothelial cells (Jarai et al., 1999; Breivogel et al., 2001).

Endogenous cannabinoid signaling pathways have been implicated in a broad range of physiological functions, including memory, coordination, vasoregulation, thermoregulation, inflammation, and pain. Pharmacological and gene-knockout studies also point to a role for endocannabinoid signaling in promoting neuronal survival after cerebral ischemia or trauma (Mechoulam et al., 2002). Thus, Win 55,212-2 reduces neuronal loss from global ischemia and infarct size after focal ischemia (Nagayama et al., 1999), 2-AG attenuates traumatic cerebral injury (Panikashvili et al., 2001), and cerebral infarcts are increased in volume in CB1R-KO mice (Parmentier-Batteur et al., 2002). The brain's response to

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ABBREVIATIONS: AEA, anandamide; 2-AG, 2-arachidonylglycerol; CB1R, CB1 cannabinoid receptor; CB2R, CB2 cannabinoid receptor; WIN 55,212-2, [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone]; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide); KO, knockout; DG, dentate gyrus; HU-210, (6a*R*,10a*R*)-3-(1,1′-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9-methanol; MeAEA, methanandamide; BrdU, bromodeoxyuridine; Dcx, doublecortin; WT, wild-type; SVZ, subventricular zone; ERK, extracellular signal-regulated kinase.

ischemia also involves up-regulation of neuronal CB1 receptors (Jin et al., 2000) and increased production of the cannabinoid-related compound phosphatidylethanolamine, which modulates the inflammatory response to ischemia (Franklin et al., 2003). Therefore, endogenous cannabinoid signaling mechanisms may represent a key component of cell-survival programs mobilized in the injured brain.

In addition to their neuroprotective effects, cannabinergic systems may also have an important role in brain development (Fernandez-Ruiz et al., 2000). For example, the expression of endocannabinoids and cannabinoid receptors in brain is developmentally regulated (Fernandez-Ruiz et al., 2000), and cannabinoids promote the survival of oligodendrocyte progenitors (Molina-Holgado et al., 2002), consistent with the growth-promoting effect of endocannabinoids on hematopoietic progenitors. Neurogenesis, or the birth of new neurons, continues to occur beyond development and into adulthood, and several lines of evidence suggest that cannabinoid signaling may be involved in this process as well. First, CB1R are highly expressed in regions of the songbird brain—the higher vocal center and robust nucleus of the archistriatum—in which neurogenesis persists in adulthood in connection with song acquisition and production (Soderstrom and Johnson, 2000). Second, CB1R are also found in the subgranular zone of the mammalian hippocampal dentate gyrus (DG), which constitutes one of two principal neuroproliferative zones of the adult mammalian brain (Morales and Backman, 2002). Third, cannabinergic signaling mediates certain effects of fibroblast growth factor-2, a major neurogenesis-promoting factor (Williams et al., 2003).

At least one previous pharmacological study has addressed the possible role of CB1R in neurogenesis, and the results contradict those suggesting a positive effect of cannabinoids on neurogenesis. In in vitro experiments, the cannabinoid agonists AEA, 2-AG, and HU-210 inhibited neurite formation and expression of neuronal lineage markers in a variety of cell-culture systems, and these effects were blocked by SR141716A (Rueda et al., 2002). In vivo, the metabolically stable AEA analog methanandamide (MeAEA) produced an SR141716A-sensitive increase in the number of newborn (BrdU-immunopositive), non-neuronal (neuronal nuclear antigen NeuN-immunonegative) cells in the rat DG without affecting the total number of BrdU-labeled cells, which was interpreted as evidence for a CB1R-mediated impairment in neurogenesis. We now report the results of studies conducted using CB1R-KO mice (Ledent et al., 1999) in an effort to help clarify the role of cannabinoid signaling pathways in adult neurogenesis.

Materials and Methods

Drugs. SR141716A was provided by Dr. Kevin Gormley (National Institute on Drug Abuse, Bethesda, MD) and AM251 was purchased from Tocris (Ellisville, MO).

Animals. $CB1R^{+/-}$ heterozygous mice bred for at least five generations on a CD1 background were provided by Dr. Catherine Ledent (Ledent et al., 1999) and used to breed the mice for this study. VR1-KO and wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Experiments were conducted according to the *Guide for Care and Use of Laboratory Animals* as adopted and promulgated by the United States National Institutes of Health.

BrdU Labeling and Cell Counting. BrdU (50 mg/kg; Sigma) was dissolved in saline and administered i.p., twice daily at 8-h intervals, for 3 consecutive days, and mice were killed 1 week later. Brain sections were stained with mouse monoclonal anti-BrdU (2 μg/ml; Roche, Indianapolis, IN) and biotinylated goat-anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA), and staining was visualized with diaminobenzidine and H_2O_2 as described in detail elsewhere (Jin et al., 2003a). BrdU-positive cells in subgranular zone and SVZ were counted blindly in three to seven diaminobenzidine-stained, 50-μm coronal sections per animal, spaced 200 μm apart. Cells were counted under high power on a Nikon E800 microscope with Magnifire digital camera, and the image was displayed on a computer monitor. Results were expressed as the average number of BrdU-positive cells per animal.

Fluorescence Immunohistochemistry. Immunohistochemistry was performed as described in detail elsewhere (Parmentier-Batteur et al., 2002). The primary antibodies were affinity-purified rabbit polyclonal anti-CB1 (Dr. Ken Mackie, University of Washington, Seattle, WA), affinity-purified goat polyclonal anti-doublecortin (Dcx) (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse monoclonal anti-BrdU (2 μg/ml; Roche). The secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Vector Laboratories), fluorescein isothiocyanate-conjugated donkey anti-goat IgG, and rhodamine-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). Controls included omitting primary or secondary antibodies.

Statistics. Data were expressed as mean ± S.E.M. from at least three animals. Student's t test (single comparisons) or analysis of variance and post hoc Bonferroni's test (multiple comparisons) were used for statistical analysis, with $p < 0.05$ considered significant.

Results

Neurogenesis in CB1R-KO Mice. The brains of WT, but not KO, mice express CB1R mRNA (detected by reverse transcription-polymerase chain reaction), CB1R protein (demonstrated by Western blotting), and CB1R-immunoreactive neurons, endothelial cells, and smooth muscle cells (revealed by immunohistochemistry) (Parmentier-Batteur et al., 2002). To compare basal neurogenesis in WT and KO mice, BrdU was given for 3 days, and the number of BrdU-immunopositive cells was counted in DG and SVZ. CB1R gene knockout reduced BrdU-positive cell counts by ~50% in DG and SVZ (Fig. 1A, Table 1), suggesting that signaling via CB1R promotes cell proliferation under basal conditions in these regions. Consistent with this formulation, BrdU-labeled cells in DG and SVZ of WT mice expressed CB1R (Fig. 1B). Cells labeled by BrdU could represent stem cells undergoing proliferation without neuronal differentiation (self-renewal), or progenitors differentiating into non-neuronal cells, such as astrocytes. To ascertain the phenotype of newborn cells labeled by BrdU under these conditions, some BrdU-labeled brain sections through DG and SVZ were also stained with antibodies against Dcx, which is expressed selectively in immature neurons. In line with prior findings (Jin et al., 2003b), most BrdU-immunopositive cells in DG and SVZ also stained for Dcx, indicating their neuronal lineage (Fig. 1C).

Drug Effects on Neurogenesis in Wild-Type and CB1R-KO Mice. The observation that neurogenesis is decreased in CB1R-KO mice points to a role for CB1R in neurogenesis and led us to predict that CB1R antagonist drugs would have a similar effect. Therefore, WT mice were treated with the CB1R antagonist SR141716A for the same 3 days during which BrdU was administered, at a daily dose (1

mg/kg) that blocks CB1R-mediated effects on brain neurons in vivo (Nagayama et al., 1999). To our surprise, rather than reducing neurogenesis, SR141716A had no effect on BrdU labeling in DG and increased BrdU labeling by ~50% in SVZ. At least two interpretations of the discrepancy between this result and that obtained in the KO studies are possible. First, SR141617A could act at another (non-CB1/non-CB2) receptor site; if so, its effect would be expected to persist in CB1R-KO mice. On the other hand, SR141716A might increase BrdU labeling by acting as a CB1R partial agonist (Smith et al., 2000). In this case, the effect of SR141716A should be lost in CB1R-KO mice.

To distinguish these possibilities, SR141716A was given to CB1R-KO mice. SR141716A stimulated BrdU incorporation into DG, and its ability to increase BrdU labeling in SVZ was preserved, as was that of the structurally related CB1R antagonist AM251 (Howlett et al., 2002) (Table 1), indicating involvement of a non-CB1 receptor.

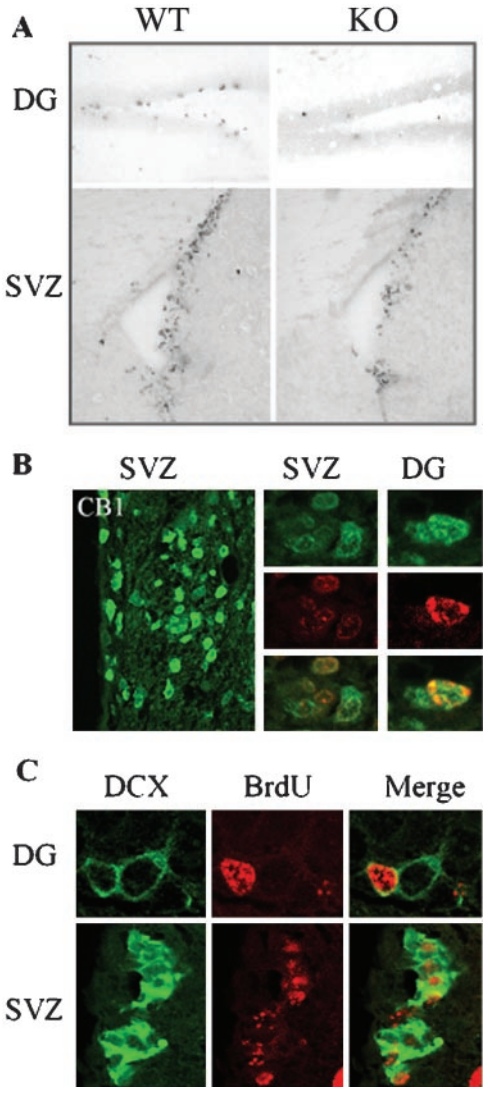

Fig. 1. BrdU labeling, CB1R expression, and colocalization of BrdU with the immature neuronal marker Dcx in DG and SVZ. A, WT and CB1R-KO mice were given BrdU as described under *Materials and Methods*, and BrdU incorporation into newborn cells (black puncta), which was reduced in KO mice, was detected by immunohistochemistry. B, in WT mice, CB1R expression (green) and BrdU labeling (red) were colocalized within the same cells (bottom right) in SVZ and DG, demonstrating that CB1R are suitably located to influence neurogenesis. C, Dcx expression (green) and BrdU labeling (red) were colocalized within the same cells (merge, right) in DG and SVZ, indicating that BrdU-labeled cells were of neuronal lineage.

TABLE 1

BrdU labeling of newborn cells in DG and SVZ of CD1 WT and CB1R-KO mice

Data are expressed as the average number of BrdU-labeled cells counted ± S.E.M., with the number of animals examined in parentheses.

	DG		SVZ	
Condition	WT	CB1R-KO	WT	CB1R-KO
Basal	23 ± 1 (4)	11 ± 2 (3)†	203 ± 14 (3)	109 ± 5 (3)†
SR141716A	20 ± 2 (3)	43 ± 5 (4)*†	305 ± 13 (3)*	329 ± 21 (4)*
AM251	N.D.	40 ± 1 (4)*	N.D.	328 ± 15 (4)*

* $P < 0.05$ compared with basal.
† $P < 0.05$ compared with WT (Student's t test or analysis of variance and post hoc Bonferroni's test).
N.D., not determined.

Neurogenesis in VR1-KO Mice. Next we tested the possible role of the VR1 vanilloid receptor (Zygmunt et al., 1999), also designated transient receptor potential vanilloid channel 1, in the neurogenesis-promoting effect of SR141716A. VR1 antagonists inhibit the pro-apoptotic effect of VR1 receptor activation in neuroblastoma and lymphoma cells (Maccarrone et al., 2000). To investigate the involvement of VR1 receptors in the regulation of neurogenesis by SR141716A, SR141716A was given to VR1-KO mice (Caterina et al., 2000). Because VR1-KO mice were bred on a C57BL/6 background and CB1R-KO mice on a CD1 background, we first retested the effect of SR141716A on C57BL/6 WT mice. It was interesting that whereas SR141716A increased BrdU labeling of SVZ cells to a similar extent in CD1 (Table 1) and C57BL/6 (Table 2) WT mice, its effects in DG were strain-dependent. In contrast to the lack of effect of the drug on BrdU incorporation into DG of CD1 mice, SR141716A increased labeling in DG of C57BL/6 mice about 2-fold. VR1 knockout abolished the neurogenesis-promoting effect of SR141716A in both DG and SVZ (Table 2), supporting the participation of VR1 in SR141716A-induced neurogenesis.

Discussion

The major finding of this study is that both CB1R and VR1 regulate adult neurogenesis in vivo, as measured by the increased incorporation of BrdU into cells that are located in neuroproliferative zones of the brain and that express neuronal lineage marker proteins. Neurogenesis is impaired in mice lacking CB1R, implying that endogenous signaling through this receptor promotes basal levels of neurogenesis in vivo. The CB1R antagonist drugs SR141716A and AM251,

TABLE 2

BrdU labeling of newborn cells in DG and SVZ of C57BL/6 WT and VR1-KO mice

Note that the background strain is different here than in Table 1. Data are expressed as the average number of BrdU-labeled cells counted ± S.E.M., with the number of animals examined in parentheses.

	DG		SVZ	
Condition	WT	VR1-KO	WT	VR1-KO
Basal	19 ± 3 (3)	33 ± 2 (3)†	190 ± 7 (3)	216 ± 6 (3)†
SR141716A	45 ± 3 (3)*	35 ± 3 (3)	313 ± 33 (3)*	189 ± 5 (3)*†

* $P < 0.05$ compared with basal.
† $P < 0.05$ compared with WT (Student's t test).

however, also increase neurogenesis. Because this effect occurs in both wild-type and CB1R-KO mice, it must involve a site other than the CB1R. A likely candidate site is the VR1 receptor, because the neurogenesis-promoting effect of SR141716A is lost in VR1-knockout mice. We interpret these results to indicate that activation of CB1R and blockade of VR1 both promote neurogenesis.

How do these findings relate to those reported by Rueda et al. (2002)? Those authors described a variety of cannabinoid effects in a range of neural cell culture models (primary cortical neurons, HNSC.100 human neural stem cell line, and human CB1R-transfected PC-12 cells), generally involving the inhibition by AEA, and reversal of inhibition by SR141716A, of the development of neuronal features such as neurite extension and expression of mature neuronal marker proteins. The endpoint examined, therefore, was not the birth of new neurons but their subsequent differentiation toward a neuronal phenotype. In addition, only in the PC-12 neurite-extension assays were drugs that can distinguish between CB1R and other AEA- and SR141716A-sensitive receptors (i.e., 2-AG, HU-210) employed. Finally, the same cannabinoid receptor-mediated mechanisms found in the intact brain cannot always be reproduced in cell culture systems (Nagayama et al., 1999). Because the in vitro studies reported by Rueda et al. (2002) addressed different endpoints and were conducted in different experimental systems than those we used, their results and ours are not necessarily in conflict. In their in vivo studies, Rueda et al. (2002) found that MeAEA, an AEA analog, increased the number of BrdU-positive/NeuN-negative cells in DG and that this effect was blocked by SR141716A. MeAEA also reduced the percentage (but not the number) of BrdU-positive/NeuN-positive cells in relation to the total number of BrdU-positive cells. These finding were taken as evidence for CB1R-mediated inhibition of neurogenesis in DG, but other interpretations are possible. First, induction by MeAEA and inhibition by SR141716A does not establish that an effect is caused by CB1R activation, as discussed above. Second, because drugs were given for 2 weeks after the last dose of BrdU, the findings may reflect effects on cell survival rather than cell proliferation. Third, because MeAEA and SR141716A had no effect on the number of BrdU-positive/NeuN-positive cells in DG, the MeAEA-induced decrease in the percentage of BrdU-positive cells that coexpressed NeuN is more likely to be caused by stimulation of non-neuronal cells than inhibition of neuronal cell survival. This would be in line with previous evidence that cannabinoids promote the survival of oligodendrocyte progenitors (Molina-Holgado et al., 2002).

Although we focused on direct neuronal effects of cannabinoid signaling systems in neurogenesis, cannabinoids can also act on non-neuronal cells, and these can influence neurogenesis as well. For example, radial glia seem to function as neuronal progenitors (Anthony et al., 2004), endothelial cells have been proposed to mediate the induction of neurogenesis by vascular endothelial growth factor (Louissaint et al., 2002), and microglia may regulate the migration and differentiation of neuronal precursors (Aarum et al., 2003).

The neurogenesis-promoting effect of CB1R that we report has certain features that merit consideration. First, because we measured neurogenesis by labeling with BrdU and coexpression of Dcx, we assayed only the initial stages of neurogenesis—proliferation and early differentiation of neuronal precursors. Thus, our results do not address how CB1R might influence subsequent events, such as neuronal migration or further developmental maturation. Second, although BrdU labeling is not selective for cells of neuronal lineage, the cells labeled with BrdU in DG and SVZ in this study are probably primarily neurons. We showed previously that these cells express a variety of neuronal markers (Jin et al., 2001, 2002a,b,c), and most BrdU-labeled cells detected in the present study expressed Dcx, a microtubule-associated protein localized to somata and processes of migrating and differentiating neurons. We noted that the effects of SR141716A were more prominent in SVZ than DG. Prior examples exist of inter-regional differences in neurogenesis, including the finding that several growth factors stimulate neurogenesis in DG and SVZ to different extents (Jin et al., 2002b, 2003b; Kuhn et al., 1997). Moreover, it has been proposed that DG and SVZ contain different populations of precursor cells with different proliferative capacities (Seaberg and van der Kooy, 2002).

We do not know which downstream signaling pathways couple CB1R (or VR1) stimulation to neurogenesis in vivo. However, in vitro studies implicate phosphatidylinositol 3-kinase and extracellular receptor-activated kinase (ERK) in the cytoproliferative and survival-promoting effects of cannabinoids on non-neuronal cells (Galve-Roperh et al., 2002; Molina-Holgado et al., 2002). In the same CB1R-KO mice used in our study, cannabinoid activation of ERK (Derkinderen et al., 2003) and p38 mitogen-activated protein kinase (Derkinderen et al., 2001) in hippocampus is abolished, indicating that ERK and p38 mitogen-activated protein kinase are linked to CB1R-mediated responses. Finally, inhibition of ERK activation interferes with neurogenesis in cultured SVZ cells (Learish et al., 2000) and cortical progenitors (Menard et al., 2002), and the former effect involves a defect in cell proliferation.

In addition to the well known effects of growth factors, a variety of drugs has been shown to influence adult neurogenesis. These include excitatory amino acid receptor antagonists (Bernabeu and Sharp, 2000), antidepressants (Santarelli et al., 2003), lithium (Chen et al., 2000), nitric oxide donors (Zhang et al., 2001), phosphodiesterase inhibitors (Zhang et al., 2002), and statins (Chen et al., 2003). Together with the finding that neurogenesis can be regulated by cannabinoids, these observations imply that a broad range of pharmacological approaches may exist through which to modify neurogenesis for therapeutic purposes. For example, drug-induced enhancement of neurogenesis has been associated with improved outcome from brain ischemia (Zhang et al., 2001, 2002). The feasibility of pharmacotherapeutic stimulation of neurogenesis will depend on a number of currently unresolved issues, including the extent to which newborn neurons can replace or otherwise restore the function of damaged brain and whether different drug modulators of neurogenesis target different populations of neuronal precursor cells or different stages of neurogenesis.

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