Cannabinoid Type-2 Receptor Drives Neurogenesis and Improves Functional Outcome After Stroke

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Background and Purpose—Stroke is a leading cause of adult disability characterized by physical, cognitive, and emotional disturbances. Unfortunately, pharmacological options are scarce. The cannabinoid type-2 receptor (CB2R) is neuroprotective in acute experimental stroke by anti-inflammatory mechanisms. However, its role in chronic stroke is still unknown.

Methods—Stroke was induced by permanent middle cerebral artery occlusion in mice; CB2R modulation was assessed by administering the CB2R agonist JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) or the CB2R antagonist SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) once daily from day 3 to the end of the experiment or by CB2R genetic deletion. Analysis of immunofluorescence-labeled brain sections, 5-bromo-2′-deoxyuridine (BrdU) staining, fluorescence-activated cell sorter analysis of brain cell suspensions, and behavioral tests were performed.

Results—SR144528 decreased neuroblast migration toward the boundary of the infarct area when compared with vehicle-treated mice 14 days after middle cerebral artery occlusion. Consistently, mice on this pharmacological treatment, like mice with CB2R genetic deletion, displayed a lower number of new neurons (NeuN+/BrdU+ cells) in peri-infarct cortex 28 days after stroke when compared with vehicle-treated group, an effect accompanied by a worse sensorimotor performance in behavioral tests. The CB2R agonist did not affect neurogenesis or outcome in vivo, but increased the migration of neural progenitor cells in vitro; the CB2R antagonist alone did not affect in vitro migration.

Conclusions—Our data support that CB2R is fundamental for driving neuroblast migration and suggest that an endocannabinoid tone is required for poststroke neurogenesis by promoting neuroblast migration toward the injured brain tissue, increasing the number of new cortical neurons and, conceivably, enhancing motor functional recovery after stroke. (Stroke. 2017;48:204-212. DOI: 10.1161/STROKEAHA.116.014793.)

Key Words: chronic phase ▪ neurogenesis ▪ neurons ▪ recovery ▪ sensorimotor function ▪ stem cells ▪ stroke

Stroke is a leading cause of death and disability worldwide, affecting millions of lives every year.1 Thrombolysis with tissue-type plasminogen activator is the only approved pharmacological treatment for the acute phase of stroke,2 only aided by intra-arterial thrombectomy, an effective alternative for a subset of patients as shown by several randomized clinical trials.3–6 Despite the reduced therapeutic opportunities, advances in prevention and healthcare have produced a progressive decrease in stroke mortality in the past few decades.7 A direct consequence is a high number of stroke survivors having a range of motor, cognitive, and psychiatric impairments.8 This highlights the need of novel therapies able to enhance neuroplasticity and brain recovery and to improve functional outcome of stroke survivors. Importantly, studies using experimental stroke models demonstrated that cerebral ischemia promotes the proliferation of neural progenitor cells (NPCs) located in the subventricular zone (SVZ) and the migration of newly generated neuroblasts toward the ischemic boundary region, where they can differentiate into mature neurons to replace lost neurons, form new astrocytes that contribute to the formation of glial scar, or release neurotrophic factors that contribute to tissue repair.9–11 Being adult...
neurogenesis a limited process in humans,14 enhancement of this endogenous process might serve as a therapeutic strategy to improve functional outcome of stroke survivors. In this sense, the endocannabinoid system could be a promising candidate, given its well-established protective role in different brain pathologies15–19 and its reported capacity to modulate sense, the endocannabinoid system could be a promising can
to improve functional outcome of stroke survivors. In this
to alleviate the chronic phase of stroke, this study aimed at
this endogenous process might serve as a therapeutic strategy
studies. Therefore, as a possible strategy to alleviate the chronic phase of stroke, this study aimed at
determining the impact of CB2R on neurogenesis by examining
cell proliferation, migration, and differentiation processes after experimental stroke induced by distal middle cerebral artery occlusion (MCAO) in mice.

Materials and Methods

Reagents
The CB2R-selective agonist JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6H-dibenzo[b,d]pyran) was from Tocris Bioscience. The CB2R-selective antagonist SR144528 ((N-[(1S)-endo-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) was from Merck Millipore. JWH133 and SR144528 were dissolved in DMSO:Tween:PBS (1:1:18), and total volume injected intraperitoneally into each animal was 200 μL.

Animals
Experiments were performed in male C57BL/6 mice of 10 to 12 weeks obtained from The Jackson Laboratories. Animals were kept in a room with controlled temperature and a 12-hour dark/light cycle and fed with standard food and water ad libitum. All experimental protocols adhered to Animal Welfare Committee of the Universidad Complutense (following EU directives 86/609/CEE and 2003/65/CE) and were conducted and reported according to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. A total number of 105 mice were used. No animals were excluded from the study.

MCAO in Mice
Mice were subjected to permanent focal cerebral ischemia through the distal occlusion of MCA as previously described.19 Further details are provided in the online-only Data Supplement.

Infarct Outcome Determination
Infarct outcome was assessed by magnetic resonance imaging 48 hours after MCAO or by Nissl staining (0.1% cresyl violet) 14 and 28 days. Analysis was performed as described in the online-only Data Supplement.

Tissue Processing and Immunofluorescence
Brain tissue was processed from mice after 14 and 28 days after MCAO as described in the online-only Data Supplement. Immunofluorescence was performed on free-floating sections and

Quantification of In Vivo Neuroblast Migration, Newly Generated Neurons, and Proliferative Cells
Assessment of in vivo number of proliferating cells, neuroblast migration toward the injured cortex, and number of newly generated neurons in the ischemic cortex was performed as described in the online-only Data Supplement.

Ex Vivo Flow Cytometry From SVZ-Derived NPCs
SVZ-derived NPCs obtained 7 days after surgery in sham and MCAO mice were characterized as described in the online-only Data Supplement.

Behavioral Tests
To evaluate fine motor performance in MCAO mice, mice were subjected to the staircase pellet-reaching test.32 Detailed procedure is described in the online-only Data Supplement. Additional characterization of general motor activity, exploration, and coordination was performed by using ActiTrack (open field test).32 All tests were recorded to avoid interferences with the behavior of the animals.

Cell Cultures
Adult mouse SVZ-derived neurospheres and cortical primary astrocyte cultures were prepared as described in the online-only Data Supplement.

NPCs Proliferation and Migration Assays
Dissociated SVZ-derived neurospheres were treated with 5-bromo-2′-deoxyuridine (BrdU) (10 μmol/L), plated for 48 h and analyzed as described in the online-only Data Supplement. In vitro NPCs migration was assessed as described in the online-only Data Supplement.

Statistics
Results are expressed by mean±SEM of the indicated number of experiments. Data were compared by a nonparametric, 1-way Kruskal–Wallis ANOVA with Dunn post hoc testing to compare >2 groups, and by a nonparametric 2-tailed Mann–Whitney test to compare 2 groups. All differences were considered statistically significant at P<0.05.

Results

SVZ Proliferation After Stroke Is Not Affected by the Subacute Pharmacological Modulation of CB2R
Given that CB2R is expressed neural progenitors both in vivo and in culture,22,27,28,30 we explored its role on SVZ cell proliferation after stroke by using a chronic treatment with either JWH133 or SR144528 starting 48 hours after MCAO to avoid acute actions of CB2R that could affect the size of the lesion (Figure I and Results in the online-only Data Supplement). Using flow cytometry of ex vivo ipsilateral SVZ-isolated cells stained with BrdU (administered days 2–6 after MCAO), similar numbers of nestin+, BrdU+, and proliferating BrdU+/nestin+ cells were observed in the different experimental groups 7 days after MCAO (Figure 1A and 1B). Similarly, 14 days after MCAO, immunohistochemical studies in the SVZ did not reveal significant differences in the total number of BrdU+ cells (Figure 1C) or Ki67+ cells (Figure 1D).

CB2R Modulates Neuroblast Migration Toward the Injured Cortex
Under normal conditions, neuroblasts generated in the SVZ migrate through the rostral migratory stream to the olfactory
However, after cerebral ischemia, some SVZ neuroblasts migrate toward the injured cortex through the corpus callosum (CC)\(^\text{33}\). The expression of CB2R and its role in neuroblast migration in vitro and ex vivo toward the olfactory bulb\(^\text{22,36}\) suggest a role of this receptor in migration of neuroblasts toward the injured cortex after ischemia in vivo. Indeed, although 14 days after ischemia no changes were observed in the total area occupied by doublecortin\(^+\) in the different experimental groups (Figure 2A), significant differences were found in the number of neuroblasts when SVZ and CC were analyzed separately. Thus, after MCAO, doublecortin immunoreactivity was increased in the ipsilateral CC of all 3 treated groups compared with sham-operated mice, indicating an enhanced ectopic neuroblast migration toward the damaged cortex after stroke (Figure 2A through 2D). Interestingly, the CB2R antagonist SR144528 significantly increased the number of neuroblasts in the SVZ (Figure 2B) while producing a decrease at CC when compared with MCAO vehicle-treated group (Figure 2D), thus supporting that CB2R sustains neuroblast migration toward the lesion 14 days after MCAO (Figure 2A, 2C and 2D), an effect inhibited by the CB2R antagonist leading to neuroblast accumulation at the SVZ. These results are in line with the trend toward increase observed in the number of BrdU\(^+\) cells at the SVZ 14 days after MCAO on SR144528 treatment (Figure 1C).

In animals treated with the CB2R agonist JWH133, no effect was observed when compared with MCAO vehicle-treated group (Figure 2A through 2D) although doublecortin\(^+\) staining was increased versus sham group at the CC, strongly suggesting the existence of an endogenous activation of CB2R after ischemia, which could conceivably not be further

**Figure 1.** Subventricular zone (SVZ) cell proliferation after ischemia is not affected by cannabinoid type-2 receptor (CB2R) pharmacological modulation. A, Representative dot plots of ipsilateral SVZ cells double-stained for nestin and 5-bromo-2′-deoxyuridine (BrdU), isolated from SVZ 7 d after surgery. B, Total numbers of nestin\(^+\); BrdU\(^+\) and BrdU\(^+/\)nestin\(^+\) cells, and percentage of nestin\(^+\) cells in the ipsilateral SVZ (n=5–7 per group). C, Representative confocal micrographs of BrdU (red) in combination with doublecortin (DCX, green) and quantification of BrdU areas (right; arbitrary units) in the ipsilateral SVZ of mice treated with vehicle (VEH), JWH133 ((6αR,10αR)-3-(1,1-dimethylbutyl)-6α,7,10,10α-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), or SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide), 14 d after surgery. Scale bar, 50 μm. D, Representative micrographs and quantification (right) of proliferating Ki67\(^+\) cells in the ipsilateral SVZ of mice treated with VEH, JWH133, or SR144528 14 d after middle cerebral artery occlusion (MCAO) or sham procedure. Bottom, Magnification of Ki67\(^+\) positive cells within the dotted box. Scale bar, 50 μm (n=4 per group). Data are means±SEM. Data were compared by a nonparametric, 1-way Kruskal–Wallis ANOVA with Dunn post hoc testing.
increased by the receptor agonist but that was clearly blocked by the receptor antagonist.

**CB2R Activation Promotes Neuroblast Migration In Vitro**

To gain further insight into the role of CB2R in neuroblast migration without the presence of an endogenous endocannabinoid tone, we performed cell culture experiments using a modified Boyden transwell chamber assay (Figure 3A) with adult mouse SVZ-derived NPCs seeded in the upper compartment and astrocyte-conditioned medium in the lower one. The CB2R agonist JWH133 increased NPC migration toward the lower compartment, an effect that was CB2R dependent as it was abolished by the antagonist SR144528 (Figure 3B). Treatment of NPCs with JWH133 did not affect the percentage of DAPI/BrdU+ cells at 48 hours compared with vehicle-treated group (Figure 3C), thus ruling out that CB2R-induced increase in neuroblast migration was because of enhanced cell proliferation.

**CB2R Antagonist SR144528 Reduces the Number of BrdU+ Neurons in the Peri-Infarct Area and Impairs Spontaneous Recovery After Stroke**

Neuroblast migration toward the injured cortex is known to lead to the appearance of newly born neurons in the peri-infarct area. To determine whether the decrease in migration caused by the CB2R antagonist SR144528 negatively affects the number of new neurons in damaged cortical areas, new generated neurons were analyzed in peri-infarct cortex 28 days later. Consistent with our previous results, SR144528 significantly decreased the number of new neurons (BrdU+/NeuN+ cells) in the perilesional area compared with the MCAO vehicle-treated group (Figure 4A). Similar to mice treated with the CB2R antagonist, CB2R−/− mice also showed a significant reduction in the number of new neurons located in the boundary of the injured cortex (Figure 5). Consistent with the data on in vivo migration analysis, the CB2R agonist JWH133 did not affect the number of newborn neurons in the cortex compared with vehicle-treated mice (Figure 5).

Because neurogenesis is thought to participate in recovery after stroke, we next explored whether the reduction in the number of new neurons with SR144528 correlates with a worse neurological sensorimotor function using the staircase test 28 days after the ischemic insult. Compared with 48 hours post ischemia performance, treatment with SR144528 abolished the spontaneous recovery observed in the vehicle-treated group, as demonstrated by a reduction in the number of eaten pellets (Figure 4B). SR144528-treated MCAO mice displayed similar general motor activity and travelled distance in the open field test compared with the MCAO vehicle-treated mice.
at all time points studied (Figure 4C), discarding other effects of the drug on general performance.

**Discussion**

The final consequence of stroke is patient death or disability characterized by severe physical, cognitive, and psychiatric impairments, associated to a high sociosanitary burden. Importantly, a slow but consistent recovery of patients with stroke can be observed in the clinical practice over weeks and months. The genesis of new neurons from neural stem/progenitor cells could be one of the mechanisms involved in this partial recovery. In this study, we demonstrate that subacute pharmacological blockade of CB2R inhibits stroke-induced neurogenesis by reducing the migration of neuroblasts toward the injured cortex, effect that could support a role of CB2R in poststroke spontaneous recovery.

In recent years, there has been a growing interest in the development of novel neuroreparative strategies capable of enhancing brain recovery and improving functional outcome of stroke survivors. Neurorepair after cerebral ischemia includes processes such as restructuration of cortical circuits adjacent to lesion site, angiogenesis, synaptic plasticity, formation of the glial scar, and even the migration of neuroblasts toward the ischemic boundary and the formation of newborn neurons. In physiological situations, adult neurogenesis occurs in the SVZ of the lateral ventricles and in the subgranular zone of the dentate gyrus. Notably, CB2R has been demonstrated to modulate adult neurogenesis. Therefore, CB2R could also be an important player in poststroke-induced neurogenesis. To test this hypothesis, given that a positive correlation exists between lesion size and poststroke neurogenesis, CB2R pharmacological treatment was initiated 48

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**Figure 3.** Cannabinoid type-2 receptor modulates neural progenitor cells (NPCs) migration in vitro. A, Schematic representation of the migration assay of NPC dissociated from adult neurospheres of mice. B, Representative images of in vitro migration assay after treatment with vehicle (VEH; DMSO), JWH133 (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; 1 \( \mu \)mol/L), SR144528 (N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; 2 \( \mu \)mol/L), or the combined treatment (1 \( \mu \)mol/L JWH133+2 \( \mu \)mol/L SR144528). Cells migrated onto the bottom side of the transwell membrane were stained with the nuclear marker 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (gray). Scale bar, 50 \( \mu \)m. Right, Quantification of the total number of migrating cells stained with DAPI 48 h after seeding. Each assay was done in duplicate at least 3×. Data are means±SEM. C, Representative images of in vitro proliferation assay after NPCs treatment with vehicle (DMSO), JWH133 (1 \( \mu \)mol/L), SR144528 (2 \( \mu \)mol/L), or the combined treatment (1 \( \mu \)mol/L JWH133+2 \( \mu \)mol/L SR144528) 48 h after BrdU was added to the medium. Cells were stained with the nuclear marker DAPI (blue) and with an anti-5-bromo-2´-deoxyuridine (BrdU) antibody (red). Scale bar, 50 \( \mu \)m. Right, Quantification of proliferating cells as % of DAPI/BrdU+ cells/field. Each assay was done in duplicate at least 3×. Data are means±SEM. Data were compared by a nonparametric, 1-way Kruskal–Wallis ANOVA with Dunn post hoc testing.
hours after MCAO to avoid differences in lesion size because of neuroprotective effects of CB2R during the acute phase (in the online-only Data Supplement).

No significant changes were detected in the number of proliferating (BrdU+ or Ki67+) cells in the SVZ at 7 or 14 days after experimental stroke versus sham mice. The possibility that our experimental design could be omitting those cells proliferating in the first hours after stroke may be ruled out by our recent report showing similar results when proliferating SVZ cells are determined by fluorescence-activated cell sorter 1 to 2 days after MCAO. Moreover, the number of proliferating SVZ cells was unaffected by pharmacological modulation of CB2R using either a selective agonist or an antagonist, in contrast with previous observations showing CB2R-induced proliferation of cultured neural stem/progenitor cells and in adult mice SVZ. This apparent lack of effect of ischemia and/or CB2R activation on proliferation may be because of the fact that newly generated cells have migrated away from the area. In this context, although no differences were detected among the different experimental groups in the amount of neuroblasts determined as total area occupied by doublecortin staining after MCAO, we found that the amount of neuroblasts in the CC of ischemic mice was remarkably greater than that found in sham-operated mice, further supporting that ischemia induces neuroblast migration into the CC. More importantly, although treatment with the CBR2-selective agonist did not cause any significant effect versus untreated ischemic mice, the CB2R-selective antagonist decreased doublecortin+ area at the CC while increasing that at the SVZ, indicating that neuroblast migration was severely impaired after CB2R inhibition thereby causing the accumulation of these cells at the SVZ. The lack of effect of the CB2R agonist may be because of an increase in endocannabinoid levels on injury in agreement with previous data in the literature, resulting in an increased endocannabinoid tone that could not be further increased by the agonist. Cannabinoids have been reported to

Figure 4. Cannabinoid type-2 receptor antagonist SR144528 (N-(1S)-endo-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide reduces the number of new neurons in the peri-infarct area and impairs spontaneous recovery after stroke. A, Representative confocal images of 5-bromo-2´-deoxyuridine (BrdU) and the neuronal marker NeuN in the peri-infarct region of vehicle (VEH; top) and SR144528-treated (bottom) mice 28 d after middle cerebral artery occlusion (MCAO). Orthogonal projection of NeuN and BrdU colocalizations (top, clear colocalization; bottom, false colocalization). Scale bar, 50 µm. Right, Quantification of double BrdU+/NeuN+ immunoreactive cells; *P<0.05 vs MCAO VEH; n=5 to 6 per group. Left, Explanatory figure of the image capture for cell quantification. B, Behavioral analysis of VEH and SR144528-treated MCAO mice 28 d after surgery using the staircase test. Data are represented as the percentage of pellets retrieved according to the 48 h postischemic performance; *P<0.05 vs MCAO VEH; n=6 to 7 per group. C, General motor activity of vehicle and SR144528-treated MCAO mice at different time points after occlusion using the open field test measured as activity time (left) and travelled distance (right; n=6–7 per group). Data are means±SEM. Data were compared by a nonparametric 2-tailed Mann–Whitney U test.
represents further studies.

In our experimental setting, endogenous CB2R activation through the rostral migratory stream to the olfactory bulb.48 Both survival and migration of newly generated neuroblasts and fibroblast growth factor signaling has been reported to cooperate with CBRS in the control of NSC proliferation.47 The lack of effect of the CB2R agonist on proliferation is very likely because of the absence in our experimental conditions of growth factors; indeed, previous reports of CB2R-induced NPC proliferation were performed with epidermal growth factor/fibroblast growth factor-treated progenitors,27,30 and epidermal growth factor and fibroblast growth factor signaling has been reported to cooperate with CBRS in the control of NSC proliferation.47 Our results demonstrate the important role of CB2R in post-stroke-induced neuroblast migration. However, we cannot discard that other cells may contribute to this promigratory effect in vivo. Specifically, it has been described that microglia at the SVZ and the rostral migratory stream, with features of alternatively activated microglia, seems to support both survival and migration of newly generated neuroblasts through the rostral migratory stream to the olfactory bulb.48

In our experimental setting, endogenous CB2R activation might underlie the skewing of microglia toward a phenotype that fosters the migration of neuroblasts, an effect that deserves further studies.

As a result of the migration of neuroblasts toward the lesion, mouse brains exposed to MCAO show the presence of new neurons (BrdU+/NeuN+) in the peri-infarct area 28 days after the ischemic insult. Very importantly, and in agreement with the decreased migration, treatment with the CB2R antagonist significantly reduced the number of newborn neurons, whereas the agonist did not cause any effect. Again, this supports the existence of an endocannabinoid tone mediating neurogenesis in vivo after stroke, which cannot be further enhanced by an exogenous agonist, in agreement with previous data in the literature about physiological adult neurogenesis.22,29 Importantly, our pharmacological data are supported by the fact that, similar to the treatment with a selective antagonist, animals with genetic deletion of CB2R also displayed a significant decrease in the number of new neurons generated in the injured cortex of CB2R-deficient mice compared with wild-type controls (in the online-only Data Supplement).

Conceivably, neurogenesis after stroke might serve to form new neurons that could integrate into the injured cortex and contribute to brain functional recovery. In this context, our results demonstrate that CB2R antagonist-induced reduction in the number of new neurons was concomitant to a worsened neurological sensorimotor function 28 days after ischemic insult, which was not because of a decrease in general performance. These findings collectively demonstrate a beneficial role for CB2R in the chronic phase of ischemic injury that could be mediated, at least in part, by fostering neuroblast migration toward the injured cortex and the subsequent generation of new neurons, which may then contribute to spontaneous recovery observed after stroke. This adds to the neuroprotective and anti-inflammatory effects of CB2R in the acute phase of stroke. Interestingly, we have recently reported that aged mice show a substantially decreased neurogenesis,44 in agreement with evidence reporting that neurogenesis is negligible in the human neocortex after ischemic stroke in aged patients (average age of 70 years).45 An interesting possibility that remains to be studied is that a decreased endocannabinoid tone in the aged brain could account for aging-induced effects that theoretically could lead to an impairment of the neurogenic response. However, the inhibitory action of SR144528 in our setting suggests that this effect is not playing a major role in our experimental setting. On the contrary, SVZ endocannabinoid production exerts a neuroprotective action through CB1R46 that could add to the pro-neurogenic response described herein via CB2R.

To further explore the impact of CB2R modulation on cell migration and proliferation, we used an in vitro migration assay. Consistent with the expression of CB2R expression in neural progenitors,28,29 the CB2R agonist JWH133 increased NPC migration. The effect was CB2R dependent as it was blocked by the CB2R antagonist SR144528. The CB2R antagonist on its own did not affect migration, supporting the existence of an in vivo endocannabinoid tone responsible for stroke-induced migration. Activation of CB2R in this system did not modify neural progenitor proliferation, thus ruling out that increased migration could be because of increased proliferation. The lack of effect of the CB2R agonist on proliferation is very likely because of the absence in our experimental conditions of growth factors; indeed, previous reports of CB2R-induced NPC proliferation were performed with epidermal growth factor/fibroblast growth factor-treated progenitors,27,30 and epidermal growth factor and fibroblast growth factor signaling has been reported to cooperate with CBRS in the control of NSC proliferation.47

Our results demonstrate the important role of CB2R in post-stroke-induced neuroblast migration. However, we cannot discard that other cells may contribute to this promigratory effect in vivo. Specifically, it has been described that microglia at the SVZ and the rostral migratory stream, with features of alternatively activated microglia, seems to support both survival and migration of newly generated neuroblasts through the rostral migratory stream to the olfactory bulb.48

In our experimental setting, endogenous CB2R activation might underlie the skewing of microglia toward a phenotype that fosters the migration of neuroblasts, an effect that deserves further studies.

**Figure 5.** Effect of cannabinoid type-2 receptor (CB2R) modulation on the number of new neurons in the peri-infarct area. A, Representative confocal images of 5-bromo-2′-deoxyuridine (BrdU) and the neuronal marker NeuN in the peri-infarct region of vehicle, JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran)–treated and CB2R−/− middle cerebral artery occlusion (MCAO) mice 28 d after occlusion. Scale bar, 50 µm. B, Quantification of double BrdU+/NeuN+ immunoreactive cells; *P<0.05 vs MCAO vehicle (VEH) or wild-type (WT); n=5 to 6 per group.
Disclosures
None.

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Stroke. 2017;48:204-212; originally published online November 29, 2016; doi: 10.1161/STROKEAHA.116.014793

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Middle cerebral artery occlusion (MCAO) in mice

All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. Mice were anesthetized with isoflurane 1.5-2% in a mixture of 80% air/20% oxygen, and body temperature was maintained at physiological levels with a heating pad during the surgical procedure and anaesthesia recovery. Mice were subjected to permanent focal cerebral ischemia through the distal occlusion of middle cerebral artery (MCA) as previously described\(^1\), a model that induces cortical infarcts. Therefore, animals with striatal affectation would not be included. In the present study, no animals were excluded as all of them showed infarcts limited to the cortical area. Mice in which the MCA was exposed but not occluded served as sham-operated controls. Following surgery, individual animals were returned to their cages with free access to water and food.

Physiological parameters were not significantly different among the different groups studied. All data analysis for immunofluorescent measurements and behaviour tests were performed in a double-blind manner by an investigator who was given no information about the experimental groups.

Experimental design

The in vivo experimental design used is illustrated in Supplemental figure IA. In brief, 2 days after occlusion, mice were treated with the CB2R agonist JWH133 (1.5 mg/Kg), the CB2R antagonist SR144528 (5mg/Kg) or vehicle until they were killed at 7, 14 or 28 days after MCAO to determine, respectively, NPC proliferation, neuroblast migration and neuronal differentiation. In some experiments, CB2R\(^{-/-}\) (C57BL/6) male mice were used with their wild-type littermates as control\(^2\).

To label proliferating NPCs, 5-bromo-2′-deoxyuridine (BrdU; Sigma Aldrich) was administered i.p. (50 mg/Kg) once daily from day 2 to 6 after MCAO.

Infarct outcome determination

Fourteen and 28 days after MCAO infarct outcome was assessed by Nissl staining (0.1% cresyl violet). Brain sections were digitally scanned (Hp Scanjet G4050), and analysed using ImageJ 1.44I (NIH, Bethesda, MD, USA). With the observer masked to the experimental conditions, the areas of infarced tissue (InfArea), the whole ipsilateral hemisphere (IpsArea) and the whole contralateral hemisphere (ContrArea) were delineated for each slice. Then, infarct volume, expressed as % of the hemisphere that is infarcted (%IH), is calculated using the formula: %IH = InfVol/ContrVol*100, where InfVol (Infarcted Tissue Volume) = ΣInfArea/SwellingIndex, ContrVol (Contralesional Hemisphere Volume) = ΣContrArea, and SwellingIndex = IpsArea/ContrArea.

In addition, infarct size was determined by T2W MRI 48 hours after MCAO using a BIOSPEC BMT 47/40 (Bruker, Ettlingen, Germany). Infarct volume was calculated using the ImageJ software (NIH, USA) from the T2-weighted images.

Tissue processing and immunofluorescence

Fourteen and/or 28 days after MCAO, mice were anesthetized with isoflurane and perfused transcardiacally with 0.1 M phosphate buffer followed by 4% PFA in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed overnight and placed in 30% sucrose for 2 days. Coronal series sections (30 μm) were cut on a freezing microtome (Leica SM2000R) and stored in cryoprotective solution. Immunofluorescence was performed on free-floating sections that were incubated overnight at 4°C with the following primary antibodies: mouse anti-mouse NeuN (1:500, Millipore), goat anti-doublecortin (DCX) (1:250, SantaCruz) and rabbit anti-mouse Ki67 (1:500, Abcam). For BrdU staining, free-floating sections were pre-treated with 2 N HCl for 30 minutes at 37°C and then incubated overnight at 4°C with rat anti-mouse 5-bromo-2′-deoxyuridine (BrdU; 1:200; Abcam).
The secondary antibodies used were goat anti-mouse biotin (Vector Laboratories) in combination with Alexa-488 streptavidin (Molecular Probes), donkey Cy3 anti-mouse (Vector Laboratories) and donkey Alexa-488 anti-goat (Invitrogen). Controls performed in parallel without primary antibodies showed very low levels of nonspecific staining.

Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710) and image analysis was performed with the ZEN2009 software (Zeiss). All co-localization images were confirmed by orthogonal projection of z-stack files. Image quantification was performed with ImageJ Software (NIH).

Quantification of in vivo neuroblast migration, newly generated neurons and proliferative cells
To assess the number of proliferating cells (Ki67\(^+\) and BrdU\(^+\) cells), 6 serial sections (30 \(\mu\)m) per animal spaced 300 \(\mu\)m apart (from bregma +1.70 \(\text{mm}\) to bregma 0.02 \(\text{mm}\)) were used. Confocal images covering the complete ipsilateral and contralateral SVZ from these 6 sections were taken at 20X, and cell proliferation was estimated by densitometry of Ki67 and/or BrdU SVZ positive area by using ImageJ software.

In vivo neuroblast migration analysis toward injured cortex was performed by densitometry of the neuroblast marker DCX in both ipsilateral and contralateral hemispheres. For that, 6 serial sections (30 \(\mu\)m) per animal spaced 300 \(\mu\)m apart (from bregma +1.70 \(\text{mm}\) to bregma 0.02 \(\text{mm}\)) were used. A systematic random sampling of the area covering the SVZ, the corpus callosum and region adjacent to the border of the infarcted cortex was carried out by dividing this area into squares (450 \(\mu\)m x 450 \(\mu\)m, 0.20 \(\text{mm}^2\)), the first of which was positioned at the dorsolateral corner of the lateral ventricle. Images of DCX positive area were taken in these squares by moving from the dorsolateral corner of the lateral ventricle along the X/Y axis a fixed distance (450 \(\mu\)m). Photomicrographs of DCX\(^+\) neuroblast staining were taken in these squares with a laser-scanning confocal imaging system (Zeiss LSM710) and DCX densitometric analysis was performed with ImageJ.

To assess the number of newly generated neurons in the ischemic cortex (NeuN\(^+\)/BrdU\(^+\) cells) 28 days after MCAO, 5 serial sections (30 \(\mu\)m) per animal spaced 300 \(\mu\)m apart (from bregma +1.70 \(\text{mm}\) to bregma 0.02 \(\text{mm}\)) were used. BrdU\(^+\)/NeuN\(^+\) cells were counted by taking at 40X a total of 16-18 confocal images per hemisphere and section covering the peri-infarct cortex. Co-localization of BrdU and NeuN was confirmed by orthogonal projection of z-stack files.

Ex vivo flow cytometry from SVZ-derived NPCs
Seven days after surgery, sham and MCAO mice were killed and brains were rapidly removed. SVZs were dissected, placed in ice-cold PBS and dissociated into a single cell suspension as described for neurosphere cultures. Cell suspensions were filtered on 40-\(\mu\)m nylon mesh strainers and centrifuged at 300g for 10 min at room temperature. Next, cells were fixed, permeabilised and stained with anti-BrdU-APC and anti-Nestin-PE according to manufacturer’s instructions (BD Cytofix/Cytoperm Kit, BrdU Flow Kits BD Biosciences). Finally, cells were washed and resuspended in 300 \(\mu\)l FACS Flow (BD Pharmingen); isotype controls (Miltenyi) were run in parallel. Whole suspensions were examined in a FACSCalibur flow cytometer using CellQuest software (BD Pharmingen) and data were analysed using FlowJo software (Tree Star Inc).

Behavioural tests
Staircase pellet-reaching test: To evaluate fine motor performance in MCAO mice treated either with vehicle or the CB2R antagonist SR144528, mice were subjected to the staircase pellet-reaching test as previously described\(^3\). The staircase apparatus (Campden Instruments, Model 80301) is formed by two stairs with eight steps each on which two rewarding food pellets can be placed. Habituation to sucrose-flavoured food
pellets was performed for 3 consecutive days, and then mice were habituated to the apparatus for the next 2 days by placing some pellets along the central corridor and stairs. During the training phase (5 days), right stairs were filled with two pellets per stair, and mice were challenged to reach pellets that had been placed exclusively in the stairs for 5 min. In two additional test sessions, animals were challenged to reach pellets placed in the five lowest steps (4-8), which need the use of a paw to be reached (note that the three upper steps can be reached with the tongue and thus are not useful to assess paw skilled reaching ability). The total pellets retrieved from these lowest steps were compared between the groups.

**Open field**: Additional characterization of general motor activity, exploration, and coordination was performed by using ActiTrack (open field test) as described\(^3\).

**Cell cultures**

Adult mouse SVZ-derived neurospheres were prepared as previously described\(^4,5\). Adult male C57BL/6 mice (8-10 weeks old) were deeply anesthetized by isoflurane inhalation and killed by decapitation. Brains were removed and placed on ice-cold Basal Medium Eagle (BME, Gibco) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Adjacent tissue to the anterior part of lateral ventricles was carefully dissected out with a scalpel and dissociated into a single cell suspension with enzymatic (papain) and mechanical digestion and filtered through a 40-µm strainer. SVZ-derived cells were plated in complete Neurocult proliferation medium (Stem Cell Technologies) supplemented with EGF (20 ng/ml), bFGF (10 ng/ml) and heparin (2 µg/ml) and seeded in T-25 cm\(^2\) flasks (Corning). In each flask, cells from a single animal were seeded. Passage of neurospheres was carried out with mechanical and enzymatic dissociation by Accutase when reached approximately 100-150 microns of diameter (every 7 days). Adult mice neurospheres were used at passages 3 to 6.

Cortical primary astrocyte cultures were prepared from postnatal P1 C57BL/6 mice as previously described\(^6\).

**Neurosphere proliferation assay**

To analyse the effect of CB2 receptor in proliferation, dissociated SVZ-derived neurospheres were treated with vehicle, JWH133 (1µM), SR144528 (2 µM) or JWH133 (1µM) + SR144528 (2 µM), BrdU (10 μM) was added to Complete Neurocult proliferation medium (without factors) and plated for 48 h. Thereafter, neurospheres were washed with PBS and fixed in 4% paraformaldehyde and stained with anti-BrdU antibody after DNase I treatment to expose BrdU. The number of BrdU\(^+\) cells was determined as percentage of total number of DAPI\(^+\) (Invitrogen) nuclei per view field.

**NPCs migration assay**

In vitro NPCs migration was assessed by using a 48-well modified Boyden chamber assay with a 12-micron nucleopore polyvinylpyrrolidone-free polycarbonate filter membrane (coated with 100µg/ml poly-D-Lysine and 15µg/ml laminin) separating the upper compartment from the bottom chamber. Accutase-dissociated NPCs were resuspended in Neurocult proliferation medium (without factors), treated with vehicle (DMSO), JWH133 (1 µM), SR144528 (2 µM) or JWH 133 (1 µM) + SR144528 (2 µM) and then, 40,000 single cells were seeded in the upper compartment of the transwell. In the lower compartment, astrocytes-conditioned medium was used to enhance NPC migration. Forty-eight hours after treatment, non-migrated cells located in the upper surface of the insert were scraped off. Then, filter membrane was fixed for 10 min with 4% p-formaldehyde, stained with DAPI and mounted bottom-side up on glass slides. Cell migration was determined by counting total number of DAPI\(^+\) cells in the whole filter membrane. Each experiment was done in triplicate; a total of three independent experiments were performed.
SUPPLEMENTAL RESULTS
Administration of CB2R agonist or antagonist in the sub-acute phase after stroke does not influence long-term infarct outcome

Stroke-induced neurogenic response can be affected by the severity of brain ischemia and the extent of brain injury. We have previously demonstrated that a single dose of the CB2R-selective agonist JWH133, administered 10 min or 3 h after MCAO, exerts a neuroprotective effect by decreasing both infarct volume and neuroinflammation. Therefore, to avoid CB2R-dependent neuroprotection in the acute phase after MCAO, we delayed the administration of pharmacological treatments to 48 h after MCAO (Suppl. fig. IA), when most acute events which influence the final injury size have already taken place. As expected, similar infarct volumes were observed in all experimental groups by MRI before the treatment, at 48 h (Suppl. fig. IB), and by Nissl staining 14 and 28 days after the delayed treatment with the CB2R-selective agonist JWH133 or the CB2R-selective antagonist SR144528 (Suppl. fig. IC-D). In line with previous observations, we found similar results in wild-type (WT) vs. CB2R<sup>−/−</sup> mice 28 days after ischemia (Suppl. fig. IE).

SUPPLEMENTAL REFERENCES

SUPPLEMENTAL FIGURES
Supplemental figure I. Experimental design and infarct volume after administration of the CB2R agonist JWH133 and the antagonist SR144528 48 h after MCAO (A) Experimental protocol outline. 48h after MCAO, infarct volume was determined by MRI and all mice were allocated in three experimental groups randomly. Two days after surgery, all mice received BrdU injection (50mg/Kg/day, i.p.) and continued once daily until day 6. Simultaneously with BrdU injection, treatment with...
CB2R agonist (JWH133; 1.5 mg/Kg), CB2R antagonist (SR144528; 5 mg/Kg) or vehicle (DMSO:Tween:PBS) was started. Mice were sacrificed 14-28 days after MCAO. (B) Infarct volume determined by MRI 48h after MCAO before mice were allocated in the 3 experimental groups. (C-D) Administration of JWH-133 or SR144528 had no effect at 14 (C) or 28 days (D) after MCAO on %IH compared with sham-operated mice.