

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*-[(1*S*)-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.⁹⁻¹³ Being adult

Received July 19, 2016; final revision received September 20, 2016; accepted October 24, 2016.

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The online-only Data Supplement is available with this article at <http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.116.014793/-/DC1>.

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DOI: 10.1161/STROKEAHA.116.014793

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 pathologies^{15–19} and its reported capacity to modulate neural stem cell proliferation^{20,21} as well as in vitro and ex vivo neuroblast migration²² in physiological conditions. The most relevant cannabinoid receptors are cannabinoid type-1 receptor (CB1R), mainly expressed by neurons,²³ and cannabinoid type-2 receptor (CB2R), mostly located in microglia and, interestingly, in neurogenic niches of both embryonic and postnatal brain.^{24–26} Consistent with its expression, selective activation of CB2R stimulates proliferation of neural stem/progenitor cells in culture and in the SVZ of adult mice.^{27–30} However, the role of CB2R and the mechanisms by which it regulates neurogenesis in pathological situations such as stroke are still unknown. 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-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) was from Tocris Bioscience. The CB2R-selective antagonist SR144528 (*N*-[1*S*]-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 μ L.

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.³¹ 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

tissue sections ([online-only Data Supplement](#)). Image acquisition was performed as described in the [online-only Data Supplement](#).

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 μ mol/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 \pm 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

bulb.³³ However, after cerebral ischemia, some SVZ neuroblasts migrate toward the injured cortex through the corpus callosum (CC).^{9,34,35} The expression of CB2R and its role in neuroblast migration *in vitro* and *ex vivo* toward the olfactory bulb^{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

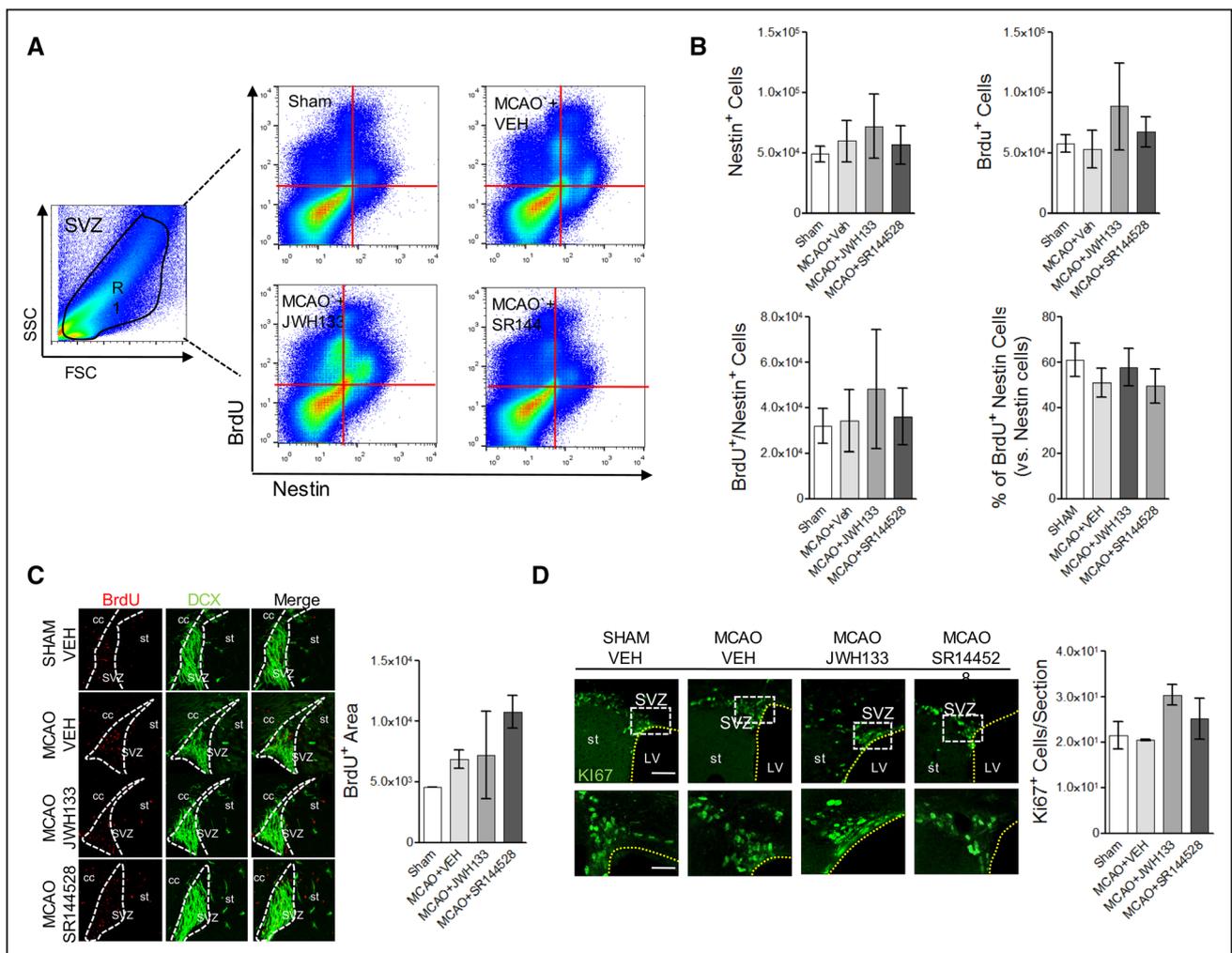


Figure 1. Subventricular zone (SVZ) cell proliferation after ischemia is not affected by cannabinoid type-2 receptor (CB2R) pharmacological modulation. **A**, Representative dot plots of ipsilateral SVZ cells double-stained for nestin and 5-bromo-2'-deoxyuridine (BrdU), isolated from SVZ 7 d after surgery. **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 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), or SR144528 (*N*-[(1*S*)-*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 μ m. **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 μ m (n=4 per group). Data are means \pm SEM. Data were compared by a nonparametric, 1-way Kruskal–Wallis ANOVA with Dunn post hoc testing.

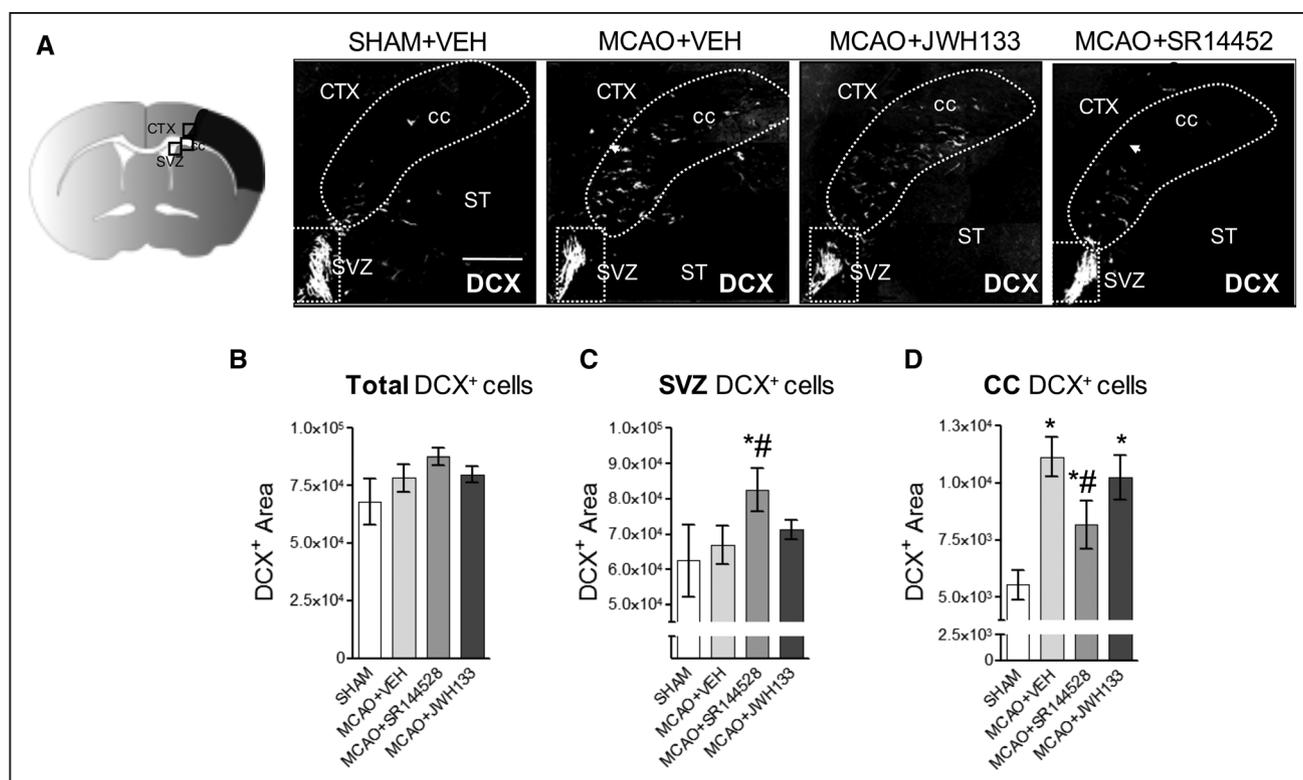


Figure 2. Cannabinoid type-2 receptor modulates neuroblast migration from subventricular zone (SVZ) to the injured cortex. **A**, Schematic representation showing the poststroke neuroblast migration area (left) and doublecortin (DCX) immunostaining (green), 14 d after surgery, in ipsilateral SVZ (dotted white square), corpus callosum (CC, dotted red region), and cortex (Ctx) of mice treated with vehicle (VEH), JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran), or SR144528 (*N*-[(1*S*)-*endo*-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide). Scale bar, 100 μ m. **B–D**, Area (arbitrary units) occupied by DCX staining represented as total (**B**), SVZ (**C**), or CC (**D**) 14 d after surgery (* $P < 0.05$ vs sham; # $P < 0.05$ vs middle cerebral artery occlusion [MCAO]-VEH; $n = 5$ per group). Data are means \pm 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.³⁷ 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

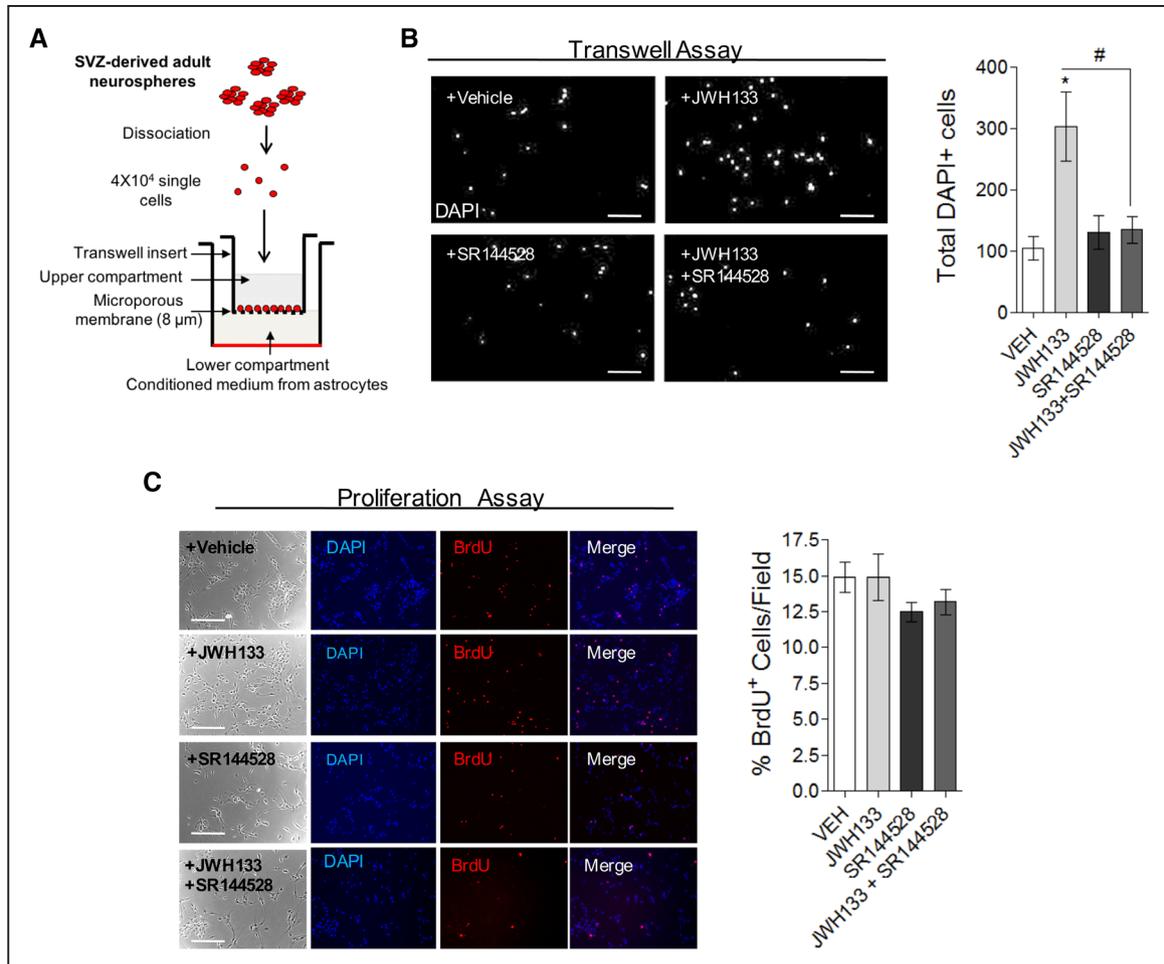


Figure 3. Cannabinoid type-2 receptor modulates neural progenitor cells (NPCs) migration in vitro. **A**, Schematic representation of the migration assay of NPC dissociated from adult neurospheres of mice. **B**, Representative images of in vitro migration assay after treatment with vehicle (VEH; DMSO), JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; 1 $\mu\text{mol/L}$), SR144528 (*N*-[1*S*]-*endo*-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; 2 $\mu\text{mol/L}$), or the combined treatment (1 $\mu\text{mol/L}$ JWH133+2 $\mu\text{mol/L}$ SR144528). Cells migrated onto the bottom side of the transwell membrane were stained with the nuclear marker 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (gray). Scale bar, 50 μm . **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 \times . Data are means \pm SEM. **C**, Representative images of in vitro proliferation assay after NPCs treatment with vehicle (DMSO), JWH133 (1 $\mu\text{mol/L}$), SR144528 (2 $\mu\text{mol/L}$), or the combined treatment (1 $\mu\text{mol/L}$ JWH133+2 $\mu\text{mol/L}$ 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 μm . **Right**, Quantification of proliferating cells as % of DAPI/BrdU $^+$ cells/field. Each assay was done in duplicate at least 3 \times . Data are means \pm SEM. Data were compared by a nonparametric, 1-way Kruskal-Wallis ANOVA with Dunn post hoc testing.

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.³⁸ 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.^{9,39,40} In physiological situations, adult neurogenesis occurs in the SVZ of the lateral ventricles and in the subgranular zone of the dentate gyrus.^{41,42} Notably, CB2R has been demonstrated to modulate adult neurogenesis.^{27–30} 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,⁴³ CB2R pharmacological treatment was initiated 48

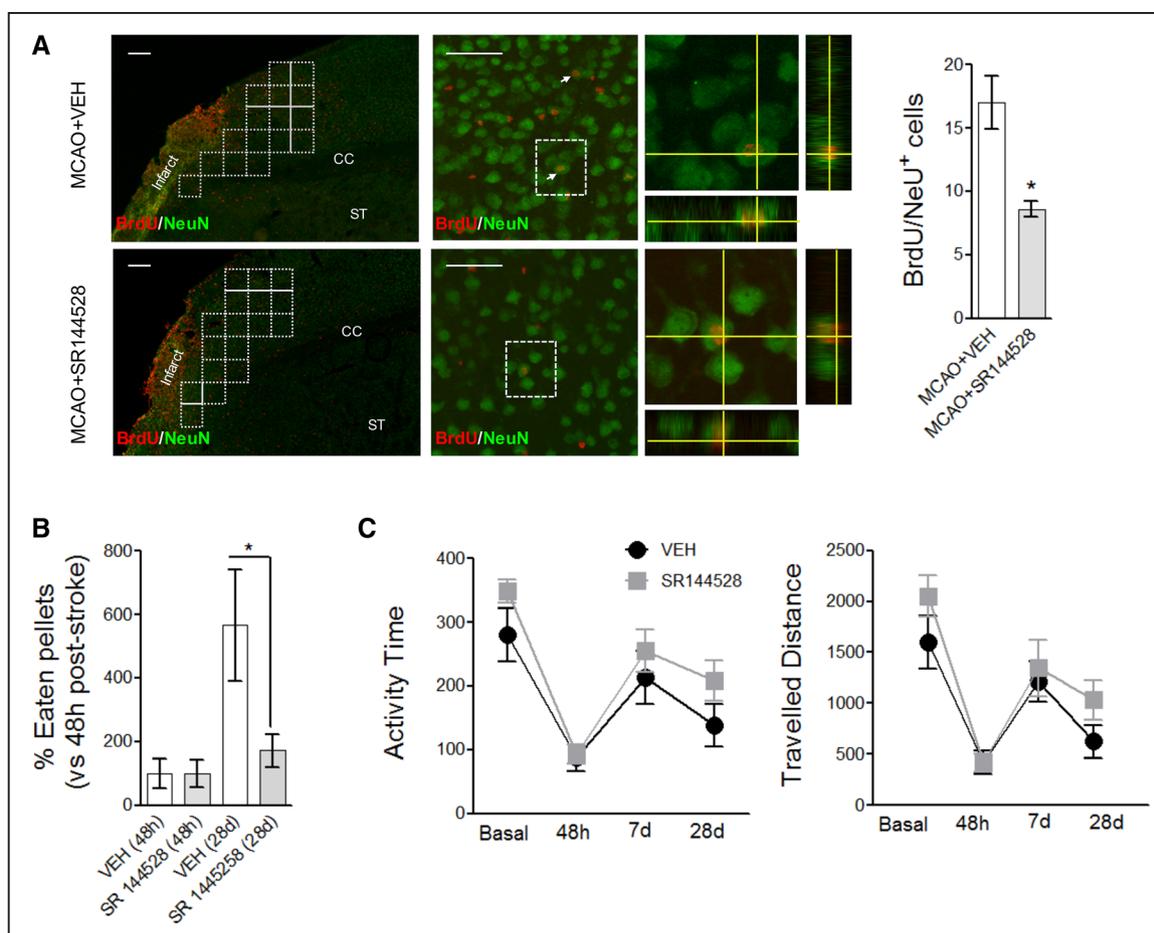


Figure 4. Cannabinoid type-2 receptor antagonist SR144528 (*N*-[(1*S*)-*endo*-1,3,3-trimethylbicyclo-[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) reduces the number of new neurons in the peri-infarct area and impairs spontaneous recovery after stroke. **A**, 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 μ m. **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 posts ischemic 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 \pm SEM. Data were compared by a nonparametric 2-tailed Mann–Whitney U test.

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.⁴⁴ 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.⁴⁴ 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.^{27–30} 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,^{22,36} resulting in an increased endocannabinoid tone that could not be further increased by the agonist. Cannabinoids have been reported to

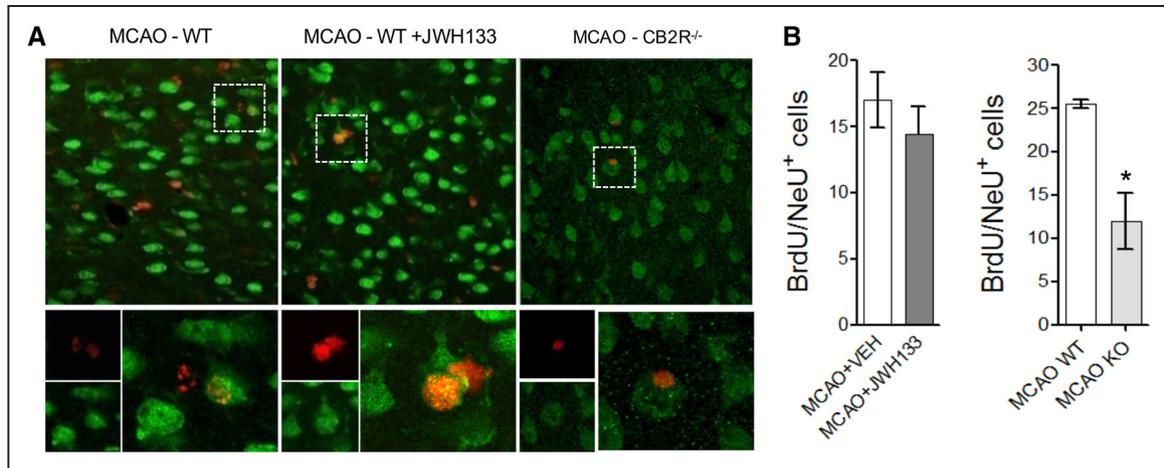


Figure 5. Effect of cannabinoid type-2 receptor (CB2R) modulation on the number of new neurons in the peri-infarct area. **A**, Representative confocal images of 5-bromo-2'-deoxyuridine (BrdU) and the neuronal marker NeuN in the peri-infarct region of vehicle, JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran)-treated and CB2R^{-/-} middle cerebral artery occlusion (MCAO) mice 28 d after occlusion. Scale bar, 50 μ m. **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.

repress angiogenesis,⁴⁵ an effect 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 proneurogenic 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.

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).⁴⁹ An interesting possibility that remains to be studied is that a decreased endocannabinoid tone in the aged brain could account for aging-induced

reduced neurogenesis. In line with this notion, it has been reported that CBR2 agonists induce a more pronounced effect in SVZ-derived neurogenesis in older animals.²⁹ These observations open novel lines of investigation on drug therapies targeting CB2R for the recovery of the injured brain in aged patients.

Summary

Our study demonstrates an indispensable role of CB2R in the migration of neuroblasts and subsequent neurogenesis in the peri-infarct cortex after experimental stroke in mice. Our findings also suggest the existence of an endocannabinoid tone responsible for this process that might be modulated in specific circumstances such as aging, thus posing novel therapeutic possibilities to improve the outcome of aged patients and reduce their disabilities in the chronic phase of stroke.

Sources of Funding

This work was supported by grants from Spanish Ministry of Economy and Competitiveness SAF2012-33216 (Dr Moro), SAF2015-68632-R (Dr Moro), CSD2010-00045 (Dr Moro) and SAF2014-52225 (Dr Lizasoain), from Instituto de Salud Carlos III and cofinanced by Fondo Europeo de Desarrollo Regional (FEDER) Una manera de hacer Europa PI15-00310 (Dr Galve-Roperh) and RETICS RD12/0014/0003 (Dr Lizasoain), and from Regional Madrid Government S2010/BMD-2336 (Drs Moro and Galve-Roperh) and S2010/BMD-2349 (Dr Lizasoain).

Disclosures

None.

References

- Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. *Neuron*. 2010;67:181–198. doi: 10.1016/j.neuron.2010.07.002.
- National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med*. 1995;333:1581–1587.
- Berkhemer OA, Fransen PJ, Beumer D, van den Berg LA, Lingsma HF, Yoo AJ, et al; MR CLEAN Investigators. A randomized trial of intraarterial treatment for acute ischemic stroke. *N Engl J Med*. 2015;372:11–20. doi: 10.1056/NEJMoa1411587.
- Campbell BC, Mitchell PJ, Kleinig TJ, Dewey HM, Churilov L, Yassi N, et al; EXTEND-IA Investigators. Endovascular therapy for ischemic stroke with perfusion-imaging selection. *N Engl J Med*. 2015;372:1009–1018. doi: 10.1056/NEJMoa1414792.
- Saver JL, Goyal M, Bonafe A, Diener HC, Levy EI, Pereira VM, et al; SWIFT PRIME Investigators. Stent-retriever thrombectomy after intravenous t-PA vs. t-PA alone in stroke. *N Engl J Med*. 2015;372:2285–2295. doi: 10.1056/NEJMoa1415061.
- Goyal M, Demchuk AM, Menon BK, Eesa M, Rempel JL, Thornton J, et al; ESCAPE Trial Investigators. Randomized assessment of rapid endovascular treatment of ischemic stroke. *N Engl J Med*. 2015;372:1019–1030. doi: 10.1056/NEJMoa1414905.
- Sarti C, Rastenyte D, Cepaitis Z, Tuomilehto J. International trends in mortality from stroke, 1968 to 1994. *Stroke*. 2000;31:1588–1601.
- Sacco RL. Risk factors, outcomes, and stroke subtypes for ischemic stroke. *Neurology*. 1997;49(5 suppl 4):S39–S44.
- Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med*. 2002;8:963–970. doi: 10.1038/nm747.
- Thored P, Arvidsson A, Cacci E, Ahlenius H, Kallur T, Darsalia V, et al. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells*. 2006;24:739–747. doi: 10.1634/stemcells.2005-0281.
- Zhang R, Zhang Z, Wang L, Wang Y, Gousev A, Zhang L, et al. Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. *J Cereb Blood Flow Metab*. 2004;24:441–448. doi: 10.1097/00004647-200404000-00009.
- Wang X, Mao X, Xie L, Sun F, Greenberg DA, Jin K. Conditional depletion of neurogenesis inhibits long-term recovery after experimental stroke in mice. *PLoS One*. 2012;7:e38932. doi: 10.1371/journal.pone.0038932.
- Jin K, Wang X, Xie L, Mao XO, Greenberg DA. Transgenic ablation of doublecortin-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice. *Proc Natl Acad Sci U S A*. 2010;107:7993–7998. doi: 10.1073/pnas.1000154107.
- Greenberg DA. Neurogenesis and stroke. *CNS Neurol Disord Drug Targets*. 2007;6:321–325.
- Fernández-López D, Faustino J, Derugin N, Wendland M, Lizasoain I, Moro MA, et al. Reduced infarct size and accumulation of microglia in rats treated with WIN 55,212-2 after neonatal stroke. *Neuroscience*. 2012;207:307–315. doi: 10.1016/j.neuroscience.2012.01.008.
- Zarruk JG, Fernández-López D, García-Yébenes I, García-Gutiérrez MS, Vivancos J, Nombela F, et al. Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke*. 2012;43:211–219. doi: 10.1161/STROKEAHA.111.631044.
- Mechoulam R. Discovery of endocannabinoids and some random thoughts on their possible roles in neuroprotection and aggression. *Prostaglandins Leukot Essent Fatty Acids*. 2002;66:93–99. doi: 10.1054/plef.2001.0340.
- Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, et al. The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis*. 2006;22:257–264. doi: 10.1016/j.nbd.2005.11.004.
- Schmidt W, Schäfer F, Striggow V, Fröhlich K, Striggow F. Cannabinoid receptor subtypes 1 and 2 mediate long-lasting neuroprotection and improve motor behavior deficits after transient focal cerebral ischemia. *Neuroscience*. 2012;227:313–326. doi: 10.1016/j.neuroscience.2012.09.080.
- Galve-Roperh I, Chiurchiù V, Díaz-Alonso J, Bari M, Guzmán M, Maccarrone M. Cannabinoid receptor signaling in progenitor/stem cell proliferation and differentiation. *Prog Lipid Res*. 2013;52:633–650. doi: 10.1016/j.plipres.2013.05.004.
- Katona I, Freund TF. Multiple functions of endocannabinoid signaling in the brain. *Annu Rev Neurosci*. 2012;35:529–558. doi: 10.1146/annurev-neuro-062111-150420.
- Oudin MJ, Gajendra S, Williams G, Hobbs C, Lalli G, Doherty P. Endocannabinoids regulate the migration of subventricular zone-derived neuroblasts in the postnatal brain. *J Neurosci*. 2011;31:4000–4011. doi: 10.1523/JNEUROSCI.5483-10.2011.
- Hillard CJ. Role of cannabinoids and endocannabinoids in cerebral ischemia. *Curr Pharm Des*. 2008;14:2347–2361.
- Maccarrone M, Guzmán M, Mackie K, Doherty P, Harkany T. Programming of neural cells by (endo)cannabinoids: from physiological rules to emerging therapies. *Nat Rev Neurosci*. 2014;15:786–801. doi: 10.1038/nrn3846.
- Patel KD, Davison JS, Pittman QJ, Sharkey KA. Cannabinoid CB(2) receptors in health and disease. *Curr Med Chem*. 2010;17:1393–1410.
- Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, et al. Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Res*. 2006;1071:10–23. doi: 10.1016/j.brainres.2005.11.035.
- Palazuelos J, Ortega Z, Díaz-Alonso J, Guzmán M, Galve-Roperh I. CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling. *J Biol Chem*. 2012;287:1198–1209. doi: 10.1074/jbc.M111.291294.
- Molina-Holgado F, Rubio-Araiz A, García-Ovejero D, Williams RJ, Moore JD, Arévalo-Martín A, et al. CB2 cannabinoid receptors promote mouse neural stem cell proliferation. *Eur J Neurosci*. 2007;25:629–634. doi: 10.1111/j.1460-9568.2007.05322.x.
- Goncalves MB, Suetterlin P, Yip P, Molina-Holgado F, Walker DJ, Oudin MJ, et al. A diacylglycerol lipase-CB2 cannabinoid pathway regulates adult subventricular zone neurogenesis in an age-dependent manner. *Mol Cell Neurosci*. 2008;38:526–536. doi: 10.1016/j.mcn.2008.05.001.
- Palazuelos J, Aguado T, Egia A, Mechoulam R, Guzmán M, Galve-Roperh I. Non-psychoactive CB2 cannabinoid agonists stimulate neural progenitor proliferation. *FASEB J*. 2006;20:2405–2407. doi: 10.1096/fj.06-6164fje.
- Ballesteros I, Cuartero MI, Moraga A, de la Parra J, Lizasoain I, Moro MA. Stereological and flow cytometry characterization of leukocyte

- subpopulations in models of transient or permanent cerebral ischemia. *J Vis Exp*. 2014;(94):e52031. doi: 10.3791/52031.
32. Díaz-Alonso J, Aguado T, Wu CS, Palazuelos J, Hofmann C, Garcez P, et al. The CB(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the Ctip2/Satb2 transcriptional regulation axis. *J Neurosci*. 2012;32:16651–16665. doi: 10.1523/JNEUROSCI.0681-12.2012.
 33. Lois C, García-Verdugo JM, Alvarez-Buylla A. Chain migration of neuronal precursors. *Science*. 1996;271:978–981.
 34. Zhang R, Zhang Z, Zhang C, Zhang L, Robin A, Wang Y, et al. Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. *J Neurosci*. 2004;24:5810–5815. doi: 10.1523/JNEUROSCI.1109-04.2004.
 35. Gotts JE, Chesselet MF. Migration and fate of newly born cells after focal cortical ischemia in adult rats. *J Neurosci Res*. 2005;80:160–171. doi: 10.1002/jnr.20434.
 36. Oudin MJ, Hobbs C, Doherty P. DAGL-dependent endocannabinoid signalling: roles in axonal pathfinding, synaptic plasticity and adult neurogenesis. *Eur J Neurosci*. 2011;34:1634–1646. doi: 10.1111/j.1460-9568.2011.07831.x.
 37. Zhang RL, Zhang ZG, Zhang L, Chopp M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience*. 2001;105:33–41.
 38. Felling RJ, Song H. Epigenetic mechanisms of neuroplasticity and the implications for stroke recovery. *Exp Neurol*. 2015;268:37–45. doi: 10.1016/j.expneurol.2014.09.017.
 39. Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol*. 2002;52:802–813. doi: 10.1002/ana.10393.
 40. Parent JM. Injury-induced neurogenesis in the adult mammalian brain. *Neuroscientist*. 2003;9:261–272.
 41. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. *Nature*. 2002;415:1030–1034. doi: 10.1038/4151030a.
 42. Altman J. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol*. 1969;137:433–457. doi: 10.1002/cne.901370404.
 43. Moraga A, Pradillo JM, Cuartero MI, Hernández-Jiménez M, Osés M, Moro MA, et al. Toll-like receptor 4 modulates cell migration and cortical neurogenesis after focal cerebral ischemia. *FASEB J*. 2014;28:4710–4718. doi: 10.1096/fj.14-252452.
 44. Moraga A, Pradillo JM, García-Culebras A, Palma-Tortosa S, Ballesteros I, Hernández-Jiménez M, et al. Aging increases microglial proliferation, delays cell migration, and decreases cortical neurogenesis after focal cerebral ischemia. *J Neuroinflammation*. 2015;12:87. doi: 10.1186/s12974-015-0314-8.
 45. Velasco G, Sánchez C, Guzmán M. Endocannabinoids and Cancer. *Handb Exp Pharmacol*. 2015;231:449–472. doi: 10.1007/978-3-319-20825-1_16.
 46. Butti E, Bacigaluppi M, Rossi S, Cambiagli M, Bari M, Cebrían Silla A, et al. Subventricular zone neural progenitors protect striatal neurons from glutamatergic excitotoxicity. *Brain*. 2012;135(pt 11):3320–3335. doi: 10.1093/brain/aww194.
 47. Sütterlin P, Williams EJ, Chambers D, Saraf K, von Schack D, Reisenberg M, et al. The molecular basis of the cooperation between EGF, FGF and eCB receptors in the regulation of neural stem cell function. *Mol Cell Neurosci*. 2013;52:20–30. doi: 10.1016/j.mcn.2012.10.006.
 48. Ribeiro Xavier AL, Kress BT, Goldman SA, Lacerda de Menezes JR, Nedergaard M. A distinct population of microglia supports adult neurogenesis in the subventricular zone. *J Neurosci*. 2015;35:11848–11861. doi: 10.1523/JNEUROSCI.1217-15.2015.
 49. Huttner HB, Bergmann O, Salehpour M, Rácz A, Tatarishvili J, Lindgren E, et al. The age and genomic integrity of neurons after cortical stroke in humans. *Nat Neurosci*. 2014;17:801–803. doi: 10.1038/nn.3706.

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

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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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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL MATERIALS AND METHODS

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 described¹, a model that induces cortical infarcts. Therefore, animals with striatal affection 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 control².

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 infarcted 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) = $\sum \text{InfArea}_i / \text{SwellingIndex}_i$, ContrVol (Contralesional Hemisphere Volume) = $\sum \text{ContrArea}_i$ and $\text{SwellingIndex}_i = \text{IpsArea}_i / \text{ContrArea}_i$.

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 transcardially 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 μ m) per animal spaced 300 μ m apart (from bregma +1.70 mm to bregma 0.02 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 μ m) per animal spaced 300 μ m apart (from bregma +1.70 mm to bregma 0.02 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 μ m x 450 μ m, 0.20 mm²), 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 μ 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 μ m) per animal spaced 300 μ m apart (from bregma +1.70 mm to bregma 0.02 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- μ 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 Cytotfix/Cytoperm Kit, BrdU Flow Kits BD Biosciences). Finally, cells were washed and resuspended in 300 μ 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 described³. 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 described³.

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 cm² 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 described⁶.

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), JWH1333 (1 µM), SR144528 (2 µM) or JWH 1333 (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 injury⁷. 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 neuroinflammation⁸. 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 place⁹. 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^{8,10}, we found similar results in wild-type (WT) vs. CB2R^{-/-} mice 28 days after ischemia (Suppl. fig. IE).

SUPPLEMENTAL REFERENCES

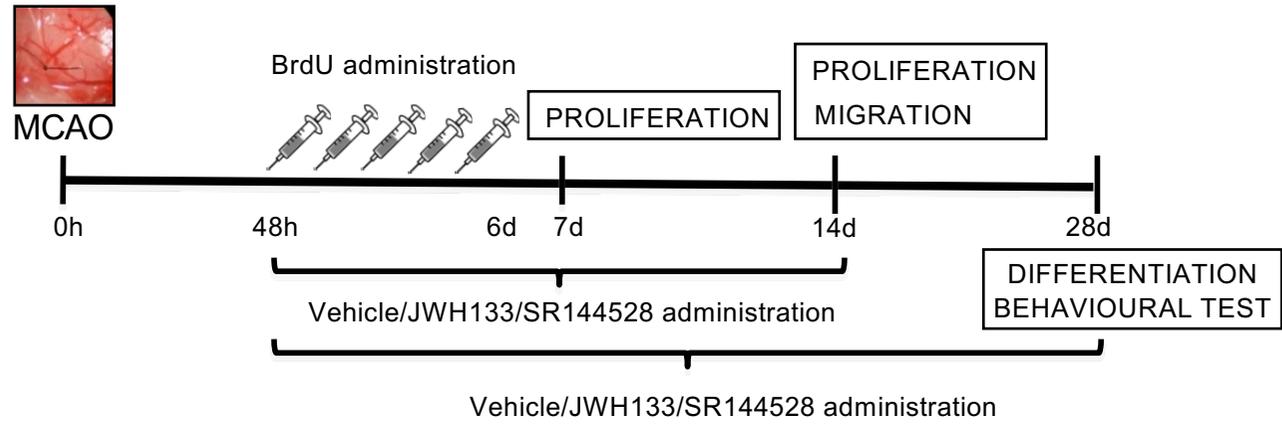
1. Ballesteros I, Cuartero MI, Moraga A, de la Parra J, Lizasoain I, Moro MA. Stereological and flow cytometry characterization of leukocyte subpopulations in models of transient or permanent cerebral ischemia. *J Vis Exp*. 2014;94.
2. Buckley NE, McCoy KL, Mezey E, Bonner T, Zimmer A, Felder CC, Glass M, Zimmer A. Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB(2) receptor. *Eur. J. Pharmacol.* 2000;396:141-149.
3. Diaz-Alonso J, Aguado T, Wu CS, Palazuelos J, Hofmann C, Garcez P, et al. The cb(1) cannabinoid receptor drives corticospinal motor neuron differentiation through the ctip2/satb2 transcriptional regulation axis. *J Neurosci.* 2012;32:16651-16665.
4. Ferron SR, Andreu-Agullo C, Mira H, Sanchez P, Marques-Torrejon MA, Farinas I. A combined ex/in vivo assay to detect effects of exogenously added factors in neural stem cells. *Nat Protoc.* 2007;2:849-859.
5. Azari H, Rahman M, Sharififar S, Reynolds BA. Isolation and expansion of the adult mouse neural stem cells using the neurosphere assay. *J Vis Exp*. 2010;45.
6. Hurtado O, Moro MA, Cardenas A, Sanchez V, Fernandez-Tome P, Leza JC, et al. Neuroprotection afforded by prior citicoline administration in experimental brain ischemia: Effects on glutamate transport. *Neurobiol Dis.* 2005;18:336-345.
7. Moraga A, Pradillo JM, Cuartero MI, Hernandez-Jimenez M, Oses M, Moro MA, et al. Toll-like receptor 4 modulates cell migration and cortical neurogenesis after focal cerebral ischemia. *FASEB J.* 2014;28:4710-4718.
8. Zarruk JG, Fernandez-Lopez D, Garcia-Yebenes I, Garcia-Gutierrez MS, Vivancos J, Nombela F, et al. Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke.* 2012;43:211-219.
9. Hossmann KA. The two pathophysiologies of focal brain ischemia: Implications for translational stroke research. *J Cereb Blood Flow Metab.* 2012;32:1310-1316.
10. Murikinati S, Juttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, et al. Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. *FASEB J.* 2010;24:788-798.

SUPPLEMENTAL FIGURES

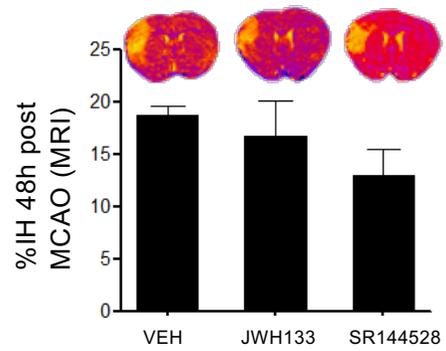
Supplemental figure I. Experimental design and infarct volume after administration of the CB2R agonist JWH133 and the antagonist SR144528 48 h after MCAO (A) Experimental protocol outline. 48h after MCAO, infarct volume was determined by MRI and all mice were allocated in three experimental groups randomly. 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.

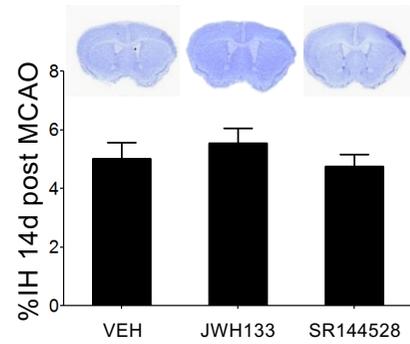
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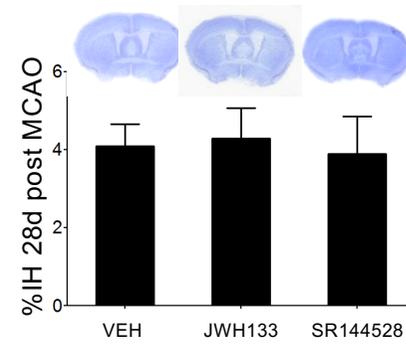
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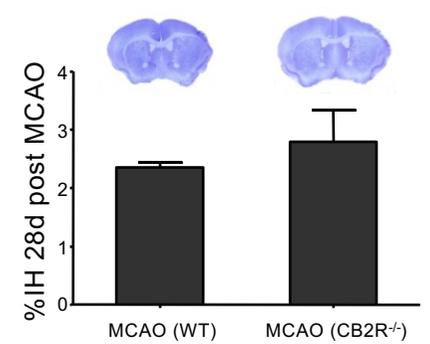
C



D



E



Suppl. fig. I