The Cannabinoid *WIN55212-2* Promotes Neural Repair After Neonatal Hypoxia–Ischemia

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Background and Purpose—The endocannabinoid system has been involved in the modulation of neural stem cells proliferation, survival and differentiation as well as in the generation of new oligodendrocyte progenitors in the postnatal brain. The present work aims to test the effect of the synthetic Type 1 and Type 2 cannabinoid receptor agonist WIN55212-2 on these processes in the context of neonatal rat brain hypoxia–ischemia (HI).

Methods—P7 Wistar rats were subjected to HI and treated either with WIN55212-2 (1 mg/kg) or vehicle twice daily for 7 days after HI and euthanized at 1, 2, 7, 14, or 28 days to explore white matter injury progression and the neurogenic response in the subventricular zone after HI.

Results—Our findings reveal that WIN55212-2 promotes remyelination of the injured external capsule, increasing the number of NG2+ early oligodendrocyte progenitors 7 days after HI in this area and the number of APC+ mature oligodendrocytes in the injured striatum 14 and 28 days after HI. WIN55212-2 also increases cell proliferation and protein expression of the neuroblast marker doublecortin in the subventricular zone 7 days after neonatal HI as well as the number of newly generated neuroblasts (5-bromodeoxyuridine+/doublecortin+ cells) in the ipsilateral striatum 14 days after HI.

Conclusions—Our results suggest that the activation of the endocannabinoid system promotes white and gray matter recovery after neonatal HI injury. **(*Stroke*. 2010;41:2956-2964.)**

Key Words: differentiation ■ encephalopathy ■ neuroblast ■ neurorepair ■ perinatal asphyxia ■ proliferation ■ restoration ■ stroke ■ white matter areas

In the last years, cannabinoids have emerged as promising neuroprotective agents in several animal paradigms of acute and degenerative brain damage.[1] Most neuroprotective effects of cannabinoids result from the activation of cannabinoid Type 1 (CB1R) and Type 2 (CB2R) receptors in neural and immune cells. Besides, the stimulating effect of cannabinoids on proliferation, survival, and differentiation of neural progenitor cells[2–6] provides interesting prospects for long-term neural repair after acute brain damage.

Neonatal hypoxic–ischemic (HI) encephalopathy is a prevalent condition in newborns that derives from acute and unpredictable episodes of perinatal asphyxia. The generation of periventricular white matter injury observed in rodent and human newborns after neonatal HI encephalopathy[7] has been correlated with a selective vulnerability of late oligodendrocyte progenitors (OLPs) to neonatal HI.[8] The subsequent alteration of the postnatal oligodendrogenesis and myelination has been clinically associated with some severe neurological sequelae of neonatal HI encephalopathy in human newborns.[9]

Recent studies have revealed a sustained proliferative and neurogenic response to neonatal HI in the subventricular zone (SVZ) that is followed by the migration of the newly generated cells toward the injured brain areas, where they can become phenotypically mature cells.[10,11]

To our knowledge, the effect of cannabinoids on the long-term neural recovery in animal models of neonatal HI encephalopathy has not been studied yet. Therefore, the aim of our study is to investigate the effect of WIN55212-2, a nonselective CBR agonist that crosses the blood–brain barrier,[12] on white and gray matter recovery after neonatal brain HI in rats.

Materials and Methods

Neonatal HI Animal Model

Cerebral HI was induced in 7-day-old Wistar rats (Harlan Laboratories, Barcelona, Spain) by a permanent unilateral section of the left common carotid artery followed by systemic hypoxia as described.[13]

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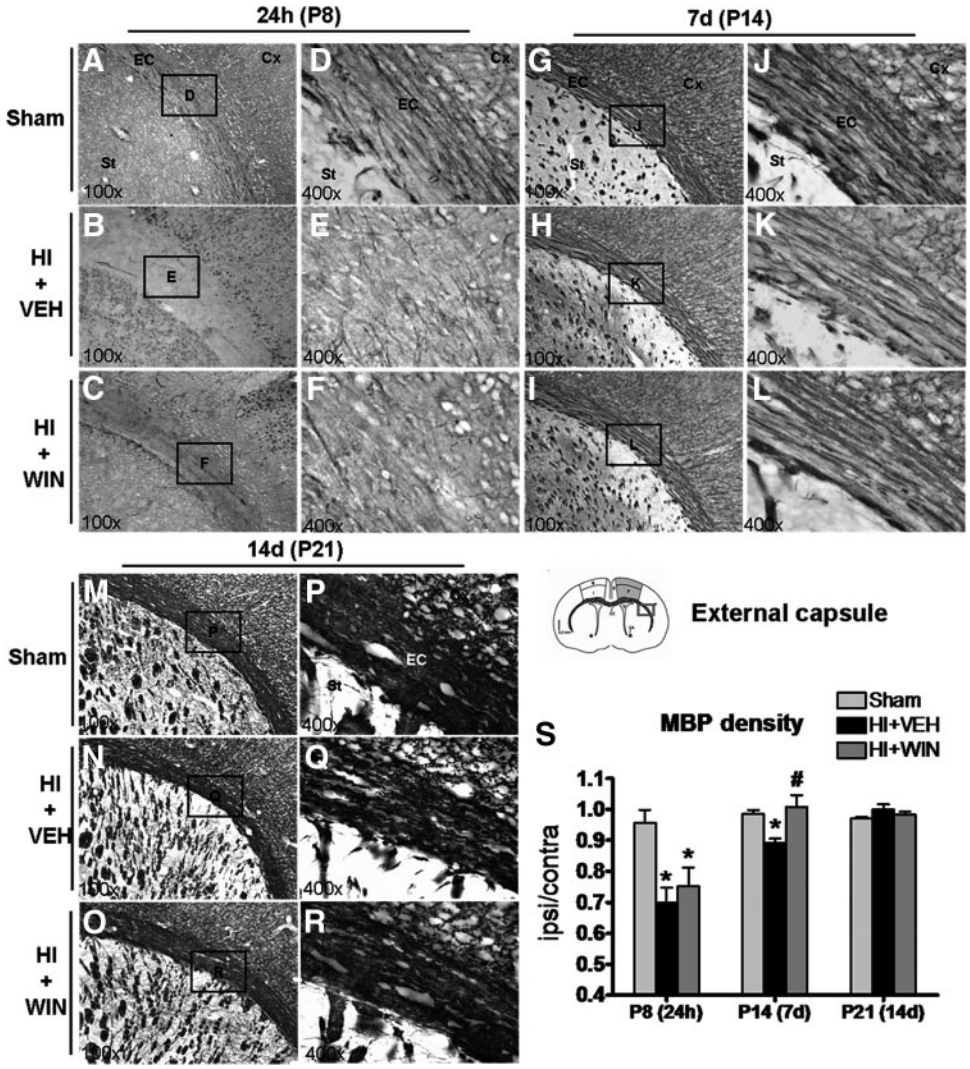


Figure 1. Effect of WIN55212-2 (WIN) on white matter remyelination after neonatal HI. A–F, S, MBP density was substantially reduced in the ipsilateral EC 24 hours after neonatal HI (P8). n=5 to 7, *$P<0.05$ versus sham. G, H, J, K, S, Vehicle-treated rats (HI+VEH) showed a partial restoration of MBP density 7 days after HI (P14). n=5 to 7, *$P<0.05$ versus sham. M, N, P, Q, S Vehicle-treated rats showed a complete MBP restoration 14 days after HI (P21) n=5 to 7. I, L, S, WIN led to a full recovery of MBP density as soon as 7 days after HI (P14), n=5 to 7, #$P<0.05$ versus HI+VEH. O, R, S, Restoration of MBP density was preserved in WIN-treated rats 14 days after HI. n=5 to 7.

Experimental Groups

Animals received WIN55212-2 (1 mg/kg) or vehicle by subcutaneous injections given twice daily until euthanasia or for a maximum of 7 days after HI (Postnatal Day [P] 7 to 14); the first injection given immediately after HI.

5-Bromo-2′-deoxyuridine (BrdU 50 mg/kg) was injected intraperitoneally twice daily from days 5 to 7 after HI (P12 to P14). Thereafter, cell proliferation and neurogenesis were quantified on days 7, 14, or 28 after HI (P14, P21, or P35).

Quantification of Brain Injury

Brain edema was quantified by MRI 24 hours after HI as described.[14] Animals were euthanized at 1, 2, 7, 14, or 28 days after HI. A minimum of 10 equidistant serial sections throughout the brain were stained with cresyl violet for the semiquantitative histopathologic evaluation in P14 brains. The rating scale for injury was as described.[15]

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

The dorsolateral striatal projection of the SVZ was dissected and total RNA was extracted using the RNeasy MinElute Kit (Qiagen). Quantitative reverse transcriptase–polymerase chain reaction was performed as described.[16] Specific primers for rat genes were designed using Primer Express software (Applied Biosystems; Supplemental Table I; available at http://stroke.ahajournals.org).

Western Blotting

The protein levels of doublecortin (Dcx) were determined in the SVZ 7 days after HI (P14). Total protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunodetection was performed by standard procedures.[16] The membranes were probed with goat anti-Dcx antibody (1:1000; Santa Cruz Biotechnology) and mouse anti-β-actin (1:5000; Sigma-Aldrich). Specific signals were quantified by densitometric analysis using ImageJ (National Institutes of Health).

Immunohistochemistry and Immunofluorescence

For the staining of myelin basic protein (MBP), free-floating sections were incubated with rat anti-MBP (1:1000; Abcam), treated following standardized immunohistochemical procedures, and developed by accurately time-controlled exposure to diaminobenzidine.[17,18] Double-label immunofluorescence was performed on free-floating

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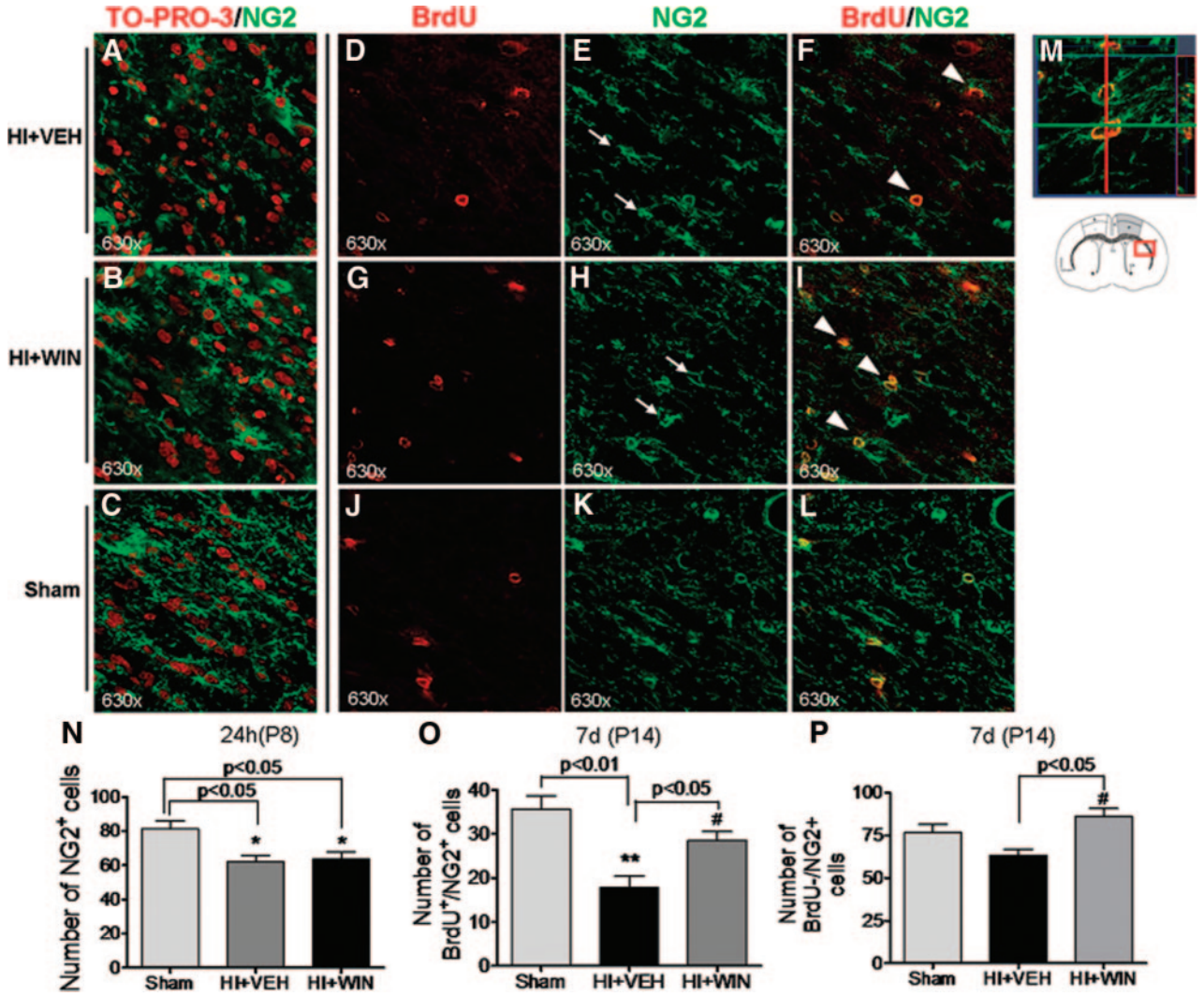


Figure 2. WIN55212-2 (WIN) increases the generation of new OLPs in the injured EC after neonatal HI. A–C, N, HI induced a significant reduction in the number of early OLPs (NG2+ cells) in the ipsilateral EC at 24 hours. n=5 to 7, **P*<0.05 versus sham. D–L, O, P, WIN increased the number of both BrdU+/NG2+ (white arrowheads) and BrdU−/NG2+ (white arrows) OLPs in the ipsilateral EC (HI+WIN) 7 days after HI (P14) as compared with vehicle-treated rats (HI+VEH). n=5 to 7, **P*<0.05 versus sham; ***P*<0.01 versus sham; #*P*<0.05 versus HI+VEH. M, Single-cell confocal z-stack showing BrdU and NG2 cell colocalization.

sections as described.[19] The following primary antibodies were used: rat anti-BrdU and mouse anti-OX42 (1:100 and 1:400; ABD Serotec), rabbit anti-CB2R and mouse anti-adenomatous polyposis Coli (APC; 1:300 and 1:1000; Calbiochem), goat anti-Dcx (1:400; Santa Cruz Biotechnology Inc), mouse anti-neuronal nuclei (NeuN), and mouse anti-NG2 (1:100 and 1:400; Chemicon).

Cell Quantification and Optical Density Measurements

The number of Dcx+, BrdU+/Dcx+, NG2+, BrdU+/NG2+, BrdU−/NG2+, BrdU+/APC+, and BrdU−/APC+ cells was quantified on digitalized confocal images captured from a minimum of 3 serial sections (2 to 3 fields of view per section; Zeiss LSM 710). Three-dimensional colocalization of protein markers was validated in confocal z-stack series.

The optical density of MBP was measured in the ipsilateral and contralateral external capsule 24 hours, 7 days, and 14 days after HI (P8, P14, and P21). Digitalized images were captured from 3 different serial 50-mm sections spaced 250 mm apart. A minimum of 4 images per section and brain region was captured from 3 serial sections to reach a total of 12 images per brain. All the images were transformed into binary and measured using the ImageJ 1.41s software. Contralateral MBP density in the external capsule was used as internal controls for normalization of MBP density in each slice, and data are expressed as the mean of ipsilateral/contralateral ratios of MBP density obtained from the individual rats.

Unbiased Stereology

The total volume of the dorsolateral striatal extension of the SVZ was estimated by the application of the Cavalieri principle on 10 serial sections per brain (50-mm thickness, 250 mm apart; bregma 2.2 mm to −0.7 mm). The morphological criteria used for the consistent delineation of the SVZ are described elsewhere.[20] Stereological estimation of the total number of BrdU+ cells in the dorsolateral extension of the SVZ was performed using the optical fractionator method.[21] The specific parameters used for stereological sampling and quantification of BrdU+ cells are summarized in Supplemental Table II.

Statistical Analysis

Data were expressed as mean±SEM. Comparisons between animal groups were performed using unpaired Student *t* test or 1-way analysis of variance with the Student-Newman Keuls post hoc test for multiple comparisons. Comparisons of semiquantitative scores

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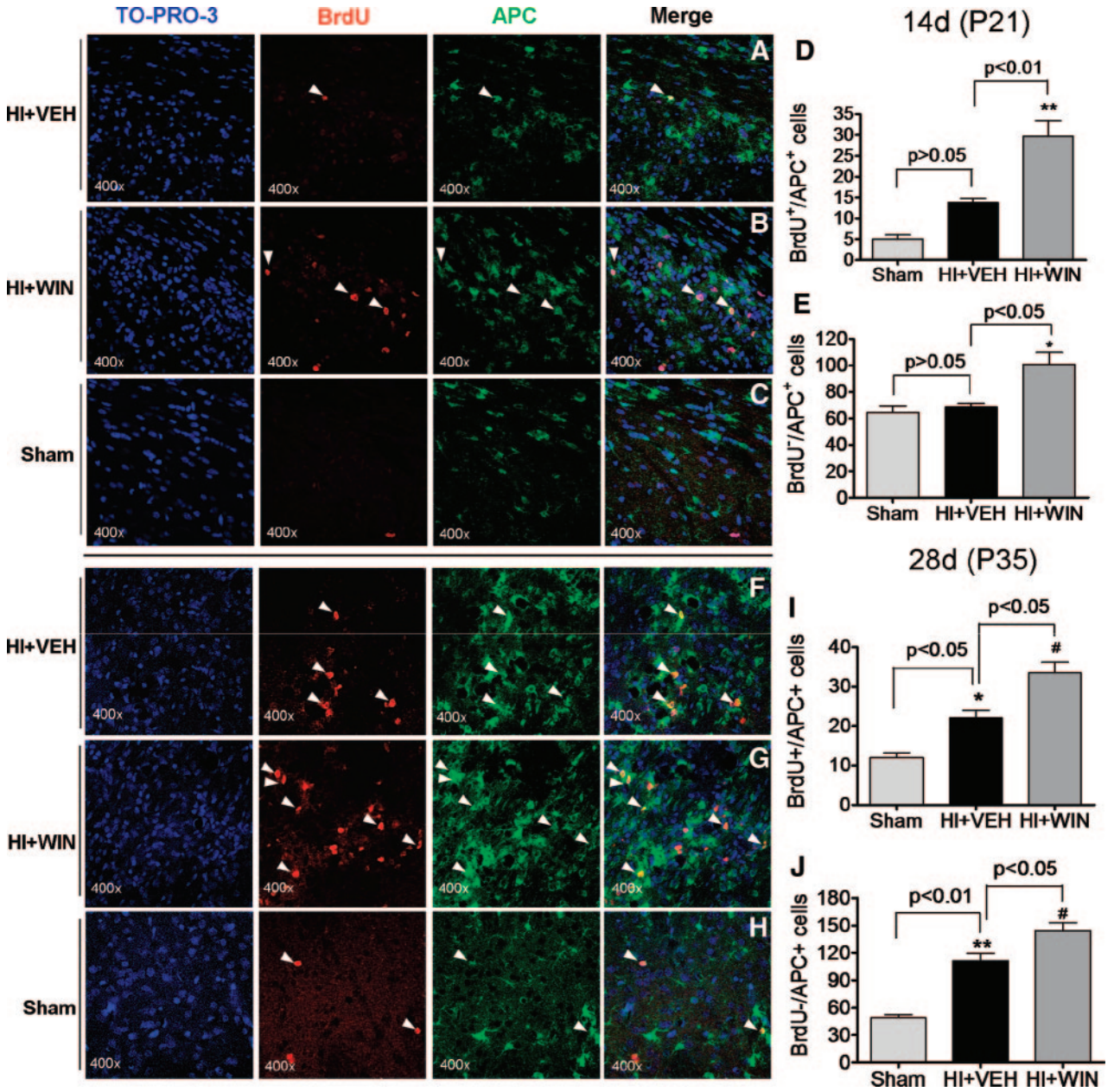


Figure 3. WIN55212-2 (WIN) increases long-term OL replacement in the injured dorsal striatum after neonatal HI. A, B, D, E, WIN increased the number of both BrdU+/APC+ (white arrowheads) and BrdU−/APC+ mature OLs in the injured striatum 2 weeks after HI (P21; HI+WIN). n=5 to 7, ***P*<0.01 versus HI+VEH; **P*<0.05 versus HI+VEH. F, G, I, J, Both BrdU+/APC+ (white arrowheads) and BrdU−/APC+ mature OLs persisted in higher numbers in the dorsal striatum of the WIN-treated animals 4 weeks after HI (P28).

were analyzed with nonparametric Mann–Whitney *U* est. Differences were considered significant at $P<0.05$.

Results

Effect of WIN55212-2 on White Matter Recovery After Neonatal HI

Exposure of neonates to HI caused a significant decrease in both the density of the myelin marker MBP and the number of early OLPs (NG2+ cells) in the ipsilateral external capsule (EC) 24 hours after HI (P8; Figure 1A–F, S and Figure 2A–C, N). This was followed by a partial restoration of MBP density in the injured EC 7 days after HI (P14; Figure 1J, K, S) and by a complete recovery 1 week after (P21; Figure 1P, Q, S). More importantly, the treatment with WIN55212-2 accelerated this process with a complete restoration of MBP density as soon as 7 days after HI (P14; Figure 1L, S). Moreover, this effect was maintained at 14 days (P21; Figure 1R, S), indicating a sustained effect of WIN55212-2 on the spontaneous myelin recovery.

Effect of WIN55212-2 on OLP Generation and Differentiation and on OL Survival

Treatment with WIN55212-2 increased the number of NG2+ cells in the EC of WIN55212-2-treated animals 7 days after neonatal HI (Figure 2D–L, O, P), indicating that this drug increases the number of newly generated early OLPs in this region.

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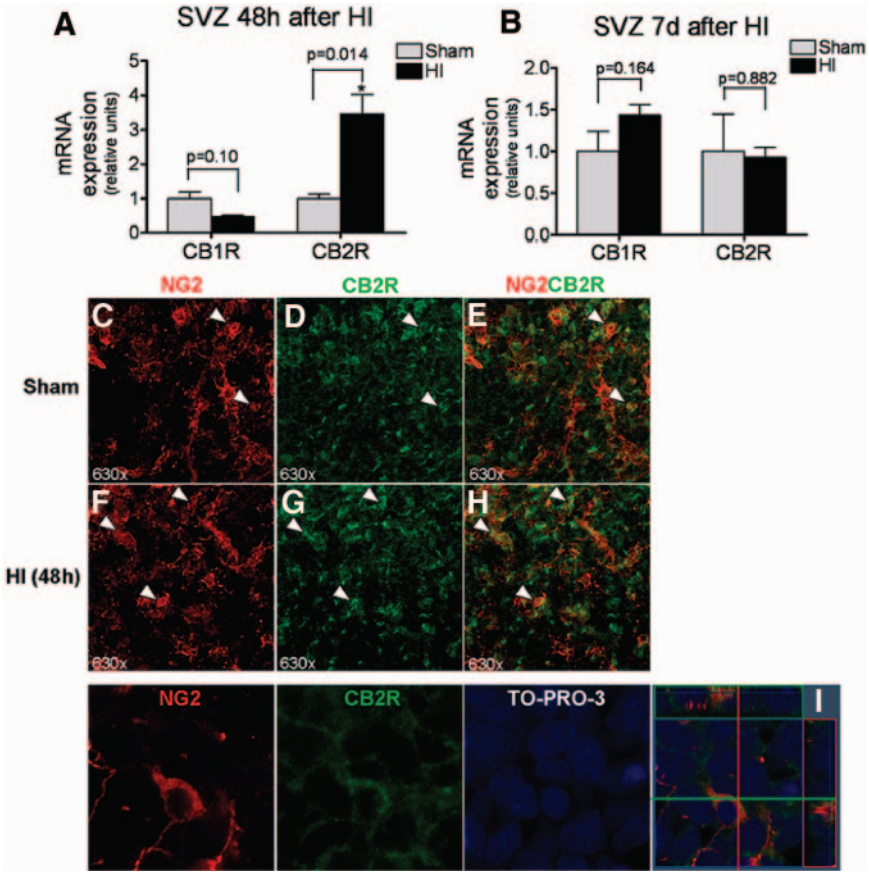


Figure 4. Expression of CBRs in the neonatal SVZ after HI. A, B, CBR1 and CBR2 mRNA expression is detected in the postanal SVZ at P9 and P14. mRNA expression of CB2R was transiently induced in the ipsilateral SVZ (SVZext) 48 hours after HI (P9; HI versus sham, Student *t* test $P=0.014$; n=4). C–H, I, Early OL progenitors (NG2+) express CB2R protein in the SVZ of both sham and HI rats at P9.

In addition, WIN55212-2 increased the number of mature APC+ OLs in the ipsilateral boundary of the EC and the injured dorsal striatum 14 days (Figure 3A–E) and 28 days after HI (P35; Figure 3F–J). These data indicate that the early NG2+ OLPs generated during the treatment with WIN55212-2 were able to survive and differentiate into a mature OL phenotype (APC+), enhancing the long-term OL replacement after neonatal HI.

Effect and Targets of WIN55212-2 on SVZ Cell Proliferation After Neonatal HI

To identify the source of the new OLPs generated during the treatment with WIN55212-2, we explored both CBR expression and the effect of this drug on cell proliferation in the dorsolateral projection of the SVZ, a neurogenic region closely associated to the injured EC. In noninjured neonatal rats, mRNA expression of CBR1 and CBR2 was detected in the SVZ 48 hours and 7 days after neonatal HI (P9 and P14). Neonatal HI led to a transient induction of CB2R mRNA expression in the ipsilateral SVZ at P9 that was no longer evident at P14 (Figure 4A–B). Double-immunofluorescence confocal analysis showed that CB2R is localized in NG2+ early OLPs in the SVZ of noninjured rats (Figure 4C–I).

Regarding proliferation, at the end of the treatment with WIN55212-2 (P14), the total number of BrdU+, proliferating cells was significantly increased in the dorsolateral striatal projection of the ipsilateral SVZ (144 373±7427 versus 182 570±10 335 cells in HI+vehicle and HI+WIN55212-2, respectively; Figure 5G, I). WIN55212-2 also increased SVZ cell proliferation in noninjured rats at P14 (Figure 5G), indicating a stimulating role of CBR activation on neural stem cell proliferation in the postnatal rat brain under normal conditions. Interestingly, the number of BrdU+ cells did no longer differ between vehicle- and WIN55212-2-treated animals at later time points (P21 and P35; Figure 5G), suggesting migration out from the SVZ of the WIN55212-2-induced proliferating cells and in agreement with the subsequent increase in the number of NG2+ OLPs and APC+ mature OLs observed in the injured EC.

Effect of WIN55212-2 on Neuroblast Generation, Survival, and Differentiation After Neonatal HI

We studied whether WIN55212-2 affects SVZ neuroblast generation, survival, and differentiation after HI. First, WIN55212-2 induced an increased protein expression of the neuroblast marker Dcx in the ipsilateral SVZ when compared with those in control and vehicle-treated animals at P14 (Figure 5H). This effect correlated well with a subsequent increase in the number of both total Dcx+ cells and BrdU+/Dcx+ cells in the injured dorsolateral striatum 7 days after the end of the treatment with WIN55212-2 (P21; Figure 6A–D, H). However, this effect was no longer observed 2 weeks later (P35; Figure 6F, G, I). The reduction at late times in BrdU+/Dcx+ neuroblasts could be due to an increased number of newly generated (BrdU+/NeuN+) neurons, thus reflecting differentiation and survival of the newly generated neuroblasts 4 weeks after the end of the treatment with

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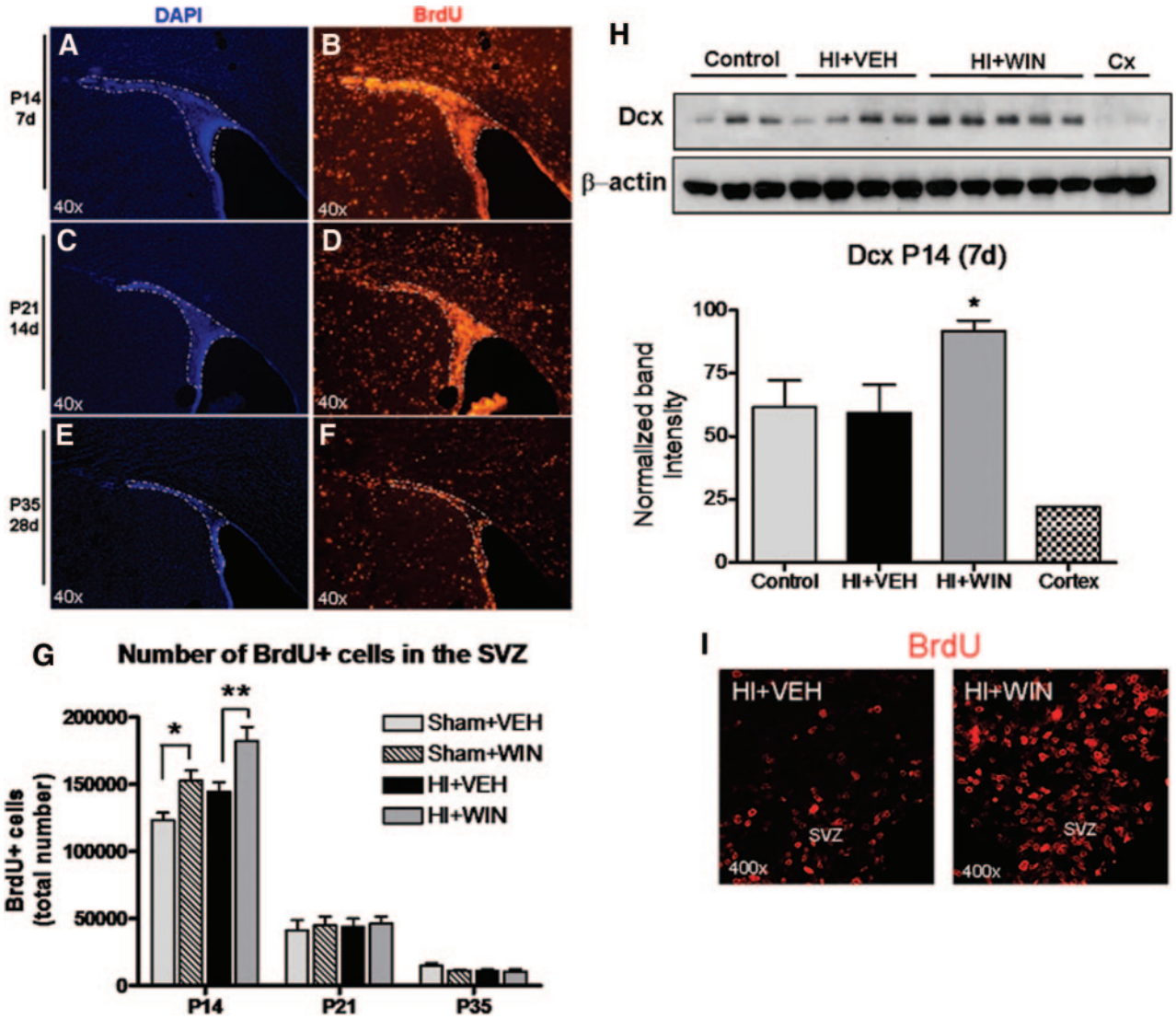


Figure 5. WIN55212-2 (WIN) stimulates SVZ cell proliferation after neonatal HI. A–F, Delimitation of the dorsolateral projection of the SVZ in 4′,6-diamidino-2-phenylindole (DAPI) and BrdU-stained sections for stereological estimations at 1, 2, and 4 weeks after neonatal HI (P14, P21, and P35). G, I, WIN increased SVZ cell proliferation 1 week after neonatal HI (P14; 144 373±7427 in HI+VEH versus 182 570±10 335 in HI+WIN; Student *t* test $P=0.008$; n=7). WIN also increased the number of BrdU+ cells in the SVZ of sham-operated animals at this time. The stimulant effect of WIN on SVZ cell proliferation was no longer evident at 2 and 4 weeks after HI (P21 and P35). H, Representative Western blot showing increased protein expression of the neuroblast marker Dcx in the SVZ after the treatment with WIN. n=3 to 5, $^*P<0.05$. Homogenates from ipsilateral neocortex (Cx) were used as negative controls.

WIN55212-2 (P35). However, we found that newly generated mature neurons (BrdU+/NeuN+ cells) were scarce or absent in the injured striatum of both vehicle- and WIN55212-2-treated animals (Figure 6J, K), and only a few BrdU+/NeuN+ cells were detected in the proximities of the ipsilateral SVZ (Figure 6L). Together, these observations indicate a limited long-term viability and differentiation of the newly generated striatal neuroblasts 4 weeks after the end of the treatment with WIN55212-2.

Effect of WIN55212-2 on Neuroprotection After Neonatal HI

WIN55212-2 did not affect the size of brain edema on T2-weighted imaging sequences when measured 24 hours after HI (P8; Supplemental Figure IA–B), in agreement with our previous observations,[14] thus discarding that early neuroprotective effects might be affecting subsequent studies. Also, WIN55212-2 did not affect core temperature, body weight, or behavioral signs during the treatment (data not shown).

The histopathologic evaluation 7 days after HI (P14) showed numerous pyknotic cells either in small clusters or covering large areas in regions of neocortex and striatum initially affected by early brain edema in vehicle-treated animals (Supplemental Figure IC–E). The treatment with WIN attenuated brain injury in these neocortical and striatal regions, reducing cell degeneration and resulting in a significant decrease of the total histopathologic score (Supplemental Figure IF–I). These observations confirm both the absence of an early biasing protective effect of WIN55212-2 and the late protection previously observed in this animal model.[14]

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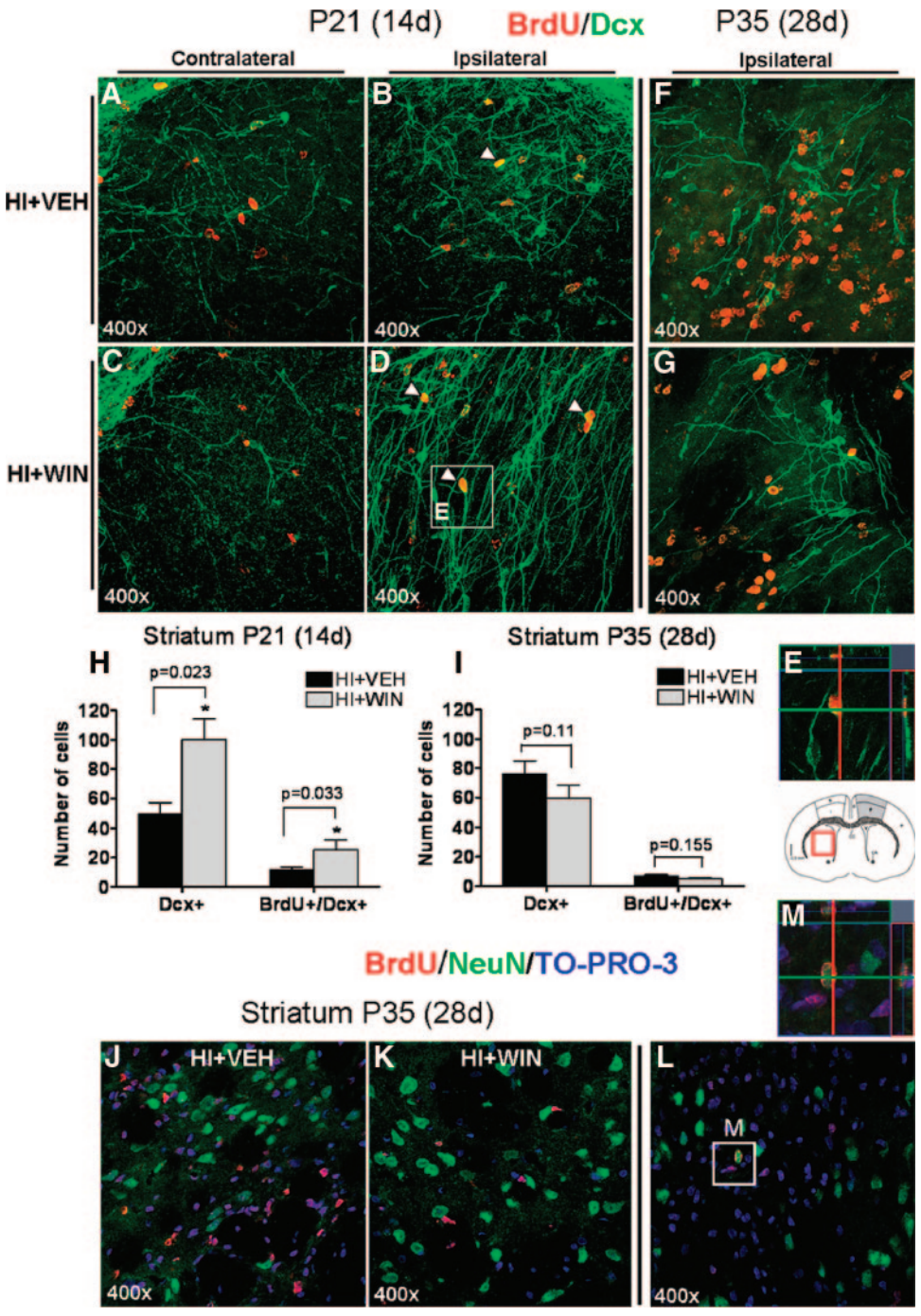


Figure 6. Effect of WIN55212-2 (WIN) on neuroblast generation after neonatal HI. A, B, Increased presence of Dcx+ migratory neuroblasts in the injured striatum 2 weeks after neonatal HI (P21). C, D, H, WIN increased the number of Dcx+ and BrdU+/Dcx+ (white arrowheads) cells in the injured striatum 2 weeks after neonatal HI. F, G, I, The number of Dcx+ and BrdU+/Dcx+ cells in the injured striatum was similar in vehicle- and WIN-treated rats 4 weeks after HI, being BrdU+/Dcx+ cells considerably reduced from P21 to P35 in both groups. n=7 (B versus F arrowheads, D versus G arrowheads, H versus I). J, K, L, Newly generated neurons (BrdU+/neuronal nuclei [NeuN]+ cells) were scarce or absent in the area of striatal injury at P35 (J, K), whereas few BrdU+/NeuN+ neurons could be detected in the proximities of the SVZ in both experimental groups (L). E, M, Orthogonal visualization of single-cell colocalization of BrdU and Dcx (E) and BrdU and NeuN (M) in confocal z-stacks.

Discussion

We have explored the effect of the mixed cannabinoid agonist WIN55212-2 in neurorestorative mechanisms after neonatal HI injury. Our data show that this compound exerts a remarkable acceleration of the recovery of HI-injured white matter, concomitant to an increase in OLP proliferation and maturation and in OL survival. This a very interesting effect of WIN55212-2 considering the direct correlation existing between the severity of periventricular white matter injury early after neonatal HI and the posterior appearance of permanent neurological and behavioral deficits in the affected newborns.[22]

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Although the ability of WIN55212-2 to increase MBP protein expression in the EC has been observed in the healthy postnatal brain,[18] this is the first demonstration of this effect in the setting of ischemic brain damage. Cell sources and mechanisms involved in such effect have not been completely elucidated. In this context, we have studied the effect of WIN55212-2 on newly generated OLPs in the EC 7 days after HI. Because BrdU injections were limited to days 5 to 7 after HI (P12 to P14) and WIN55212-2 treatment was administered between days 1 and 7 after HI (P7 to P14), we quantified not only BrdU+/NG2+, but also BrdU−/NG2+ cells to avoid excluding effects of WIN55212-2 on NG2+ cell proliferation before BrdU injections. Thus, we found that WIN55212-2 increases the number of both NG2+ types of cells. Because BrdU was injected in the last 2 days before euthanasia at P14, BrdU+ cells observed in the EC at P14 could be only 1 to 2 days old, a time that appears to be insufficient for their migration from the SVZ to their final location. However, the total increase of BrdU−/NG2+ early OLPs in the WIN55212-2-treated animals suggests that we cannot exclude an effect of WIN55212-2 on the migration of new early OLPs generated in the SVZ during the earliest stages after HI, before proliferating cells were BrdU-labeled.

Interestingly, untreated neonatal brain showed a slower spontaneous recovery of MBP density, which was complete 2 weeks after the onset of the ischemic injury, pointing to a possible involvement of endogenous cannabinoids in this effect that remains to be studied.

Of note, WIN55212-2 is known to increase CBR-dependent gliogenesis in the neonatal SVZ[18] involving the induction of the transcriptional factor Olig2 that actively drives neural stem cells toward an OL phenotype. On the other hand, signaling through CBRs promotes OLPs survival in vitro,[3] an effect that would result in a higher long-term permanence of early OLPs in the EC after HI. Consistently, we have found a transient increase of CB2R mRNA expression together with an induction of SVZ cell proliferation during the treatment with WIN55212-2. These observations account for a SVZ origin of the new early OLPs generated during the treatment. In either case, the treatment with WIN55212-2 results in a higher presence of mature OLs in the ipsilateral boundary of the EC and the injured dorsal striatum 14 and 28 days after HI, indicating that WIN55212-2 not only promotes the generation of new OLPs, but also their survival and differentiation later after HI. This fact results of special interest in relation to previous studies showing that OLP differentiation after neonatal HI is considerably arrested in the injured white matter.[23] Our observations of the stimulating effect of WIN55212-2 on SVZ cell proliferation and OL replacement reinforce the well-known modulatory role of the endocannabinoid system in the control of neural stem cell proliferation and survival,[2–6] which has been shown to involve different intracellular effectors, including extracellular signal-regulated kinase, phosphatidylinositol 3-kinase/Akt, and neurotrophic factors like brain-derived neurotrophic factor and basic fibroblast growth factor.[4,24,25]

Accordingly, we have also found an increased SVZ protein expression of the neuroblast marker Dcx after WIN55212-2 administration, concomitant to the presence of a higher number of newly generated neuroblasts (BrdU+/Dcx+) in the injured striatum 1 week after. However, the long-term survival and differentiation of these cells into a mature neuronal phenotype (BrdU+/NeuN+) is very limited regardless the treatment with WIN55212-2. In this context, the known activating role of CB1R after postinjury neurogenesis[26] suggests that a more prolonged administration of WIN55212-2 could contribute to an increased survival and differentiation of the newly generated neuroblasts into mature neurons in the injured striatum after neonatal HI.

In conclusion, we have demonstrated that the synthetic cannabinoid WIN55212-2 enhances SVZ cell proliferation, oligodendrogenesis, white matter remyelination, and neuroblast generation after neonatal HI. These findings, summed to the previously described neuroprotective properties of cannabinoids after acute brain damage, may possess therapeutic repercussions in the long-term management of neonatal HI encephalopathy, a prevalent and devastating condition for which no pharmacological treatments are yet available. Further experiments using selective CBR1 and CBR2 antagonists and/or CBRs knockout mice would help to elucidate the contribution of each type of CBR on the effects of WIN55212-2 on neural repair after neonatal HI.

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Disclosures

None.

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The Cannabinoid *WIN55212-2* Promotes Neural Repair After Neonatal Hypoxia–Ischemia

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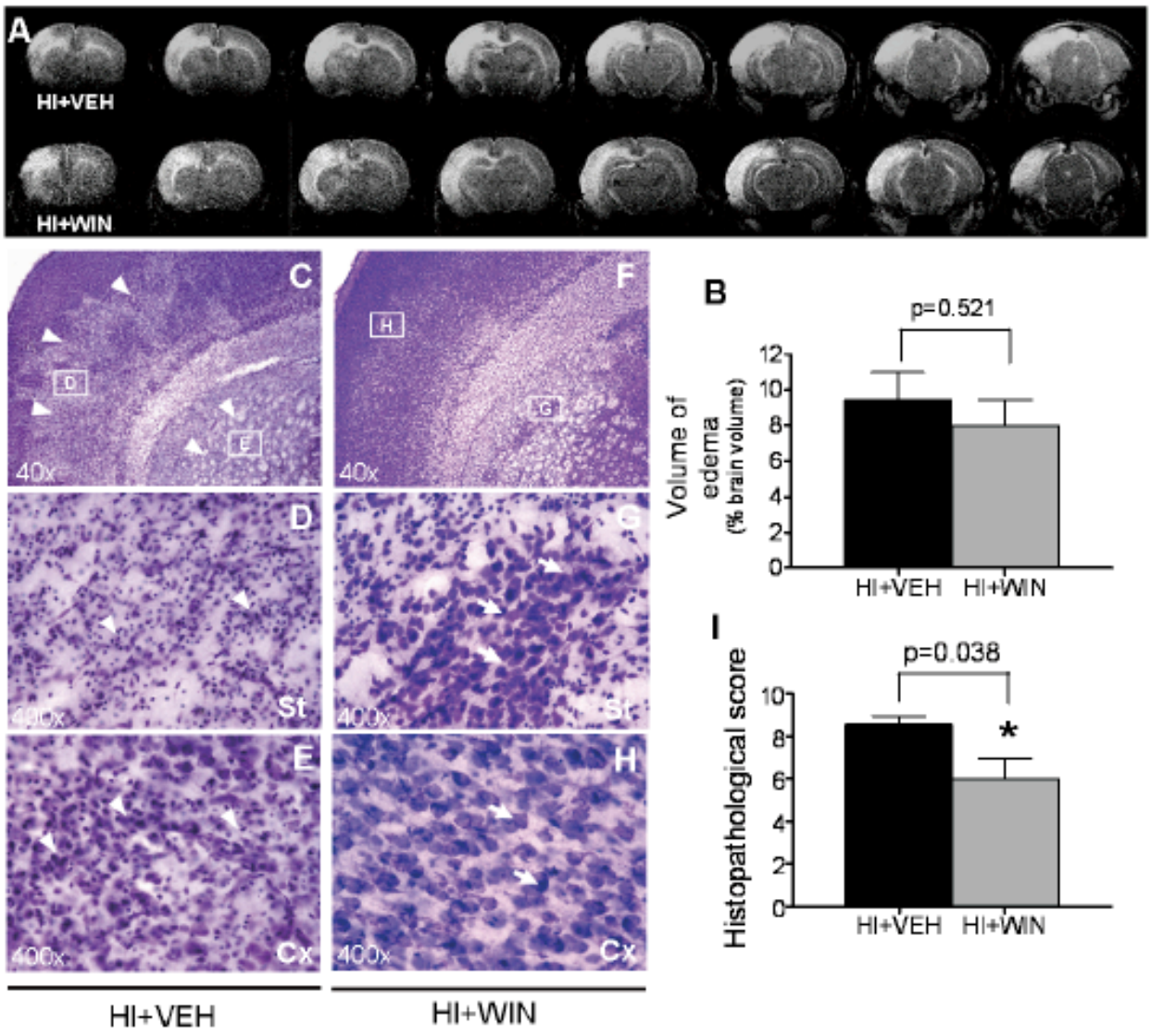
"SUPPLEMENTAL MATERIAL"

Supplementary table 1. Specific primers designed for quantitative RT-PCR analysis

Transcript	*Forward primer*	*Reverse primer*
CB1R	GCTGCAATCTGTTTGCTCGGACAT	CACAATGAACAGCAGCAGCACACT
CB2R	AAAGCACACCAACATGTAGCCAGC	ACCAGCATATGAGCAGAACAGCCA
β-actin	TGAGCGCAAGTACTCTGTGTGGAT	TAGAAGCATTTGCGGTGCACGATG

Supplementary table 2. Parameters used for the stereological quantification of BrdU+ cells in the SVZ.

Parameter	***P14 Brains***	***P21 Brains***	***P35 Brains***
Counting frame area (μm^2)	100 (10x10)	625 (25x25)	100 (10x10)
Sampling grid (X-step) (μm)	84.3	94	94
Sampling grid (Y-step) (μm)	277.8	127.3	127.3
Sampling sites per brain (mean±SD)	66±21	101±24	92±21
Total BrdU+ cells counted (mean±SD)	128±41	138±45	106±34
CE (Gundersen, m=0) (mean±SD)	0.124±0.036	0.116±0.016	0.114±0.016



Supplementary Figure 1. ***Effect of the treatment with WIN on the progression of hypoxic ischemic brain injury in P7 rats. A, B,*** T2WI sequences obtained 24h after neonatal HI (P8) showing a similar extension of brain oedema in animals treated with vehicle (HI+VEH) or WIN (HI+WIN) (n=7) ***C, D, E***, Nissl-stained brain sections showing the presence of numerous pyknotic cells (white arrowheads) in the ipsilateral striatum (St) (***D***) and neocortex (Cx) (***E***) in vehicle-treated rats 7 days after HI (P14). ***F, G, H,*** WIN reduced the extention and intensity of brain injury in the ipsilateral striatum (St) (***G)*** and neocortex (Cx) (***H***) (white arrows). ***I,*** WIN improved the histopathological score of neonatal brains 7 days after HI (n=7).