

Seladin-1/DHCR24 Is Neuroprotective by Associating EAAT2 Glutamate Transporter to Lipid Rafts in Experimental Stroke

Macarena Hernández-Jiménez, PhD; Diego Martínez-López, MSc; Enrique Gabandé-Rodríguez, PhD; Adrian Martín-Segura, MSc; Ignacio Lizasoain, PhD, MD; María D. Ledesma, PhD; Carlos G. Dotti, PhD, MD; María A. Moro, PhD

Background and Purpose— 3β -Hydroxysteroid- Δ 24 reductase (DHCR24) or selective alzheimer disease indicator 1 (seladin-1), an enzyme of cholesterol biosynthetic pathway, has been implicated in neuroprotection, oxidative stress, and inflammation. However, its role in ischemic stroke remains unexplored. The aim of this study was to characterize the effect of seladin-1/DHCR24 using an experimental stroke model in mice.

Methods—*Dhcr24*^{+/-} and wild-type (WT) mice were subjected to permanent middle cerebral artery occlusion. In another set of experiments, WT mice were treated intraperitoneally either with vehicle or U18666A (seladin-1/DHCR24 inhibitor, 10 mg/kg) 30 minutes after middle cerebral artery occlusion. Brains were removed 48 h after middle cerebral artery occlusion for infarct volume determination. For protein expression determination, peri-infarct region was obtained 24 h after ischemia, and Western blot or cytometric bead array was performed.

Results—*Dhcr24*^{+/-} mice displayed larger infarct volumes after middle cerebral artery occlusion than their WT littermates. Treatment of WT mice with the seladin-1/DHCR24 inhibitor U18666A also increased ischemic lesion. Inflammation-related mediators were increased after ischemia in *Dhcr24*^{+/-} mice compared with WT counterparts. Consistent with a role of cholesterol in proper function of glutamate transporter EAAT2 in membrane lipid rafts, we found a decreased association of EAAT2 with lipid rafts after ischemia when DHCR24 is genetically deleted or pharmacologically inhibited. Accordingly, treatment with U18666A decreases [³H]-glutamate uptake in cultured astrocytes.

Conclusions—These results support the idea that lipid raft integrity, ensured by seladin-1/DHCR24, plays a crucial protective role in the ischemic brain by guaranteeing EAAT2-mediated uptake of glutamate excess. (*Stroke*. 2016;47:206-213. DOI: 10.1161/STROKEAHA.115.010810.)

Key Words: DHCR24 ■ EAAT2 ■ lipid raft ■ neuroprotection ■ seladin-1 ■ stroke

Stroke is one of the main causes of death and disability in developed countries (the third leading cause of death in the United States). In recent years, several studies have focused on the development of new drugs with brain-protective effects in experimental models.

Selective alzheimer disease indicator 1 (seladin-1)/ 3β -hydroxysteroid- Δ 24 reductase (DHCR24), encoded by *Dhcr24* gene, was identified as a human homolog of the DIMINUTO/DWARF1 gene described previously in plants.¹ Seladin-1/DHCR24 is highly conserved in mammals; specifically, in central nervous system, seladin-1/DHCR24 is highly expressed in neurons where it is located in endoplasmic reticulum under basal conditions.¹ Many

functions have been described for this protein: seladin-1/DHCR24 catalyzes one of the last steps, via the Bloch pathway, in cholesterol biosynthesis.² Consistently, seladin-1/DHCR24 deficiency decreases cholesterol levels in plasma membrane and, subsequently, in the formation and stability of lipid rafts,³ important platforms for cell function mediators,⁴ thus affecting downstream cellular signaling.¹ In addition to the role in cholesterol metabolism, seladin-1/DHCR24 has been reported to play a role in cellular responses against oxidative and oncogenic stress, apoptotic signaling, and inflammation.^{1,5-9} From these series of evidences, it seems reasonable to consider that situations accompanied by a reduction in seladin-1/DHCR24, similarly to that reported in certain Alzheimer's disease brains,¹

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From the Unidad de Investigación Neurovascular, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense and Instituto de Investigación Hospital 12 de Octubre (i+12), Madrid, Spain (M.H.-J., D.M.-L., I.L., M.A.M.); and Centro de Biología Molecular Severo Ochoa, CSIC, Madrid, Spain (E.G.-R., A.M.-S., M.D.L., C.G.D.).

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Correspondence to Macarena Hernández-Jiménez, PhD, Unidad de Investigación Neurovascular, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, Avenida Complutense s/n, 28040 Madrid, Spain, E-mail macarenh@ucm.es or María A. Moro, PhD, Unidad de Investigación Neurovascular, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, Avenida Complutense s/n, 28040 Madrid, Spain, E-mail neurona@ucm.es

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will have important consequences in the response of brain tissue to ischemia. Thus, paucity in seladin-1/DHCR24 could affect size and time of recovery to ischemic damage, as this is highly dependent on the level of stress of the affected area. Alternatively, a reduction in seladin-1/DHCR24 can alter the distribution of EAAT2, a glutamate transporter primarily localized on astrocytic processes. This transporter, which is responsible for $\leq 90\%$ of all glutamate transport in adult tissue,¹⁰ requires to be associated to lipid rafts to carry out an adequate glutamate uptake,^{11,12} thereby decreasing excitotoxicity after ischemia. All in all, a deficit in seladin-1/DHCR24 may affect the response to ischemia through the stress or cholesterol functions of seladin-1/DHCR24. Importantly, stroke affects mainly the aged population. In this context, it has been suggested that loss of brain membrane cholesterol underlies deficits of cognition in aging rodents.¹³ Therefore, the decay in brain cholesterol found in seladin-1/DHCR24-deficient mice makes these animals a useful tool to investigate the effects of stroke in the aged population.

To assess the veracity of the above prediction, we have used both *in vivo* and *in vitro* models of experimental ischemia to characterize the effect of seladin-1/DHCR24 after stroke.

Materials and Methods

Animals

Heterozygous breeding pairs with target depletion of one *Dhcr24* allele were provided by E Feinstein (Quark Biotech Inc). *Dhcr24*-deficient

mice were bred and genotyped as previously described.¹⁴ We obtained gene-deficient (*Dhcr24*^{-/-}) and wild-type (WT) male animals, 2 months old, from the same littermates. The genetic background of all animals used was C57BL/6 (albino). Mice were housed in ventilated cages on a 12-h light/dark cycle at 22°C and 35% humidity with *ad libitum* access to food and water. All testing was performed during the light phase of the cycle.

All procedures were performed in accordance with the European Communities Council Directive (86/609/EEC) and reviewed by the Ethics Committees on Animal Welfare of University Complutense. A special effort was made to reduce the number of animals used in the study and to provide them with the most comfortable conditions possible.

Permanent Cerebral Ischemia by Middle Cerebral Artery Occlusion

All experiments have been performed and quantified in a randomized manner by investigators blinded to treatment groups for the prevention of bias. Mice were anesthetized with 3% isoflurane (in 70% N₂O, 30% O₂) for induction and with 1.5% isoflurane for maintenance. Rectal temperature was maintained at 36.5°C with a heating pad. Left common carotid and middle cerebral artery were exposed and occluded permanently by ligation as previously described.¹⁵ Complete interruption of the blood flow was confirmed under an operating microscope. Sham-operated animals were subjected to anesthesia and the surgical procedure, but the occlusion of the arteries was omitted. After surgery, subjects were returned to their cages and allowed free access to water and food. The survival rate of the animals until the end of the experiment was 97.5%.

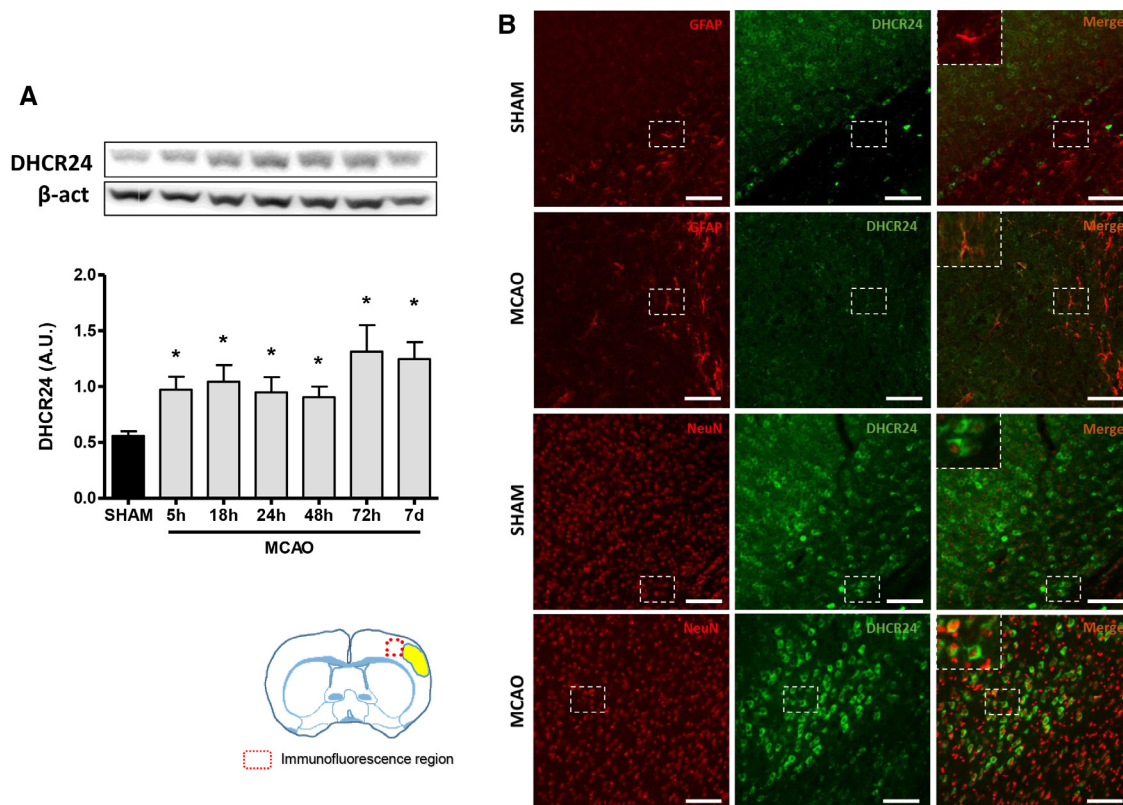


Figure 1. Seladin-1/DHCR24 levels increase after ischemia in mouse brain. **A**, Quantification of seladin-1/DHCR24 protein levels 5, 18, 24, 48, and 72 h and 7 days after ischemia in peri-infarct region of wild-type (WT) mice. **B**, Representative DHCR24-GFAP or DHCR24-NeuN immunofluorescence staining in brains from SHAM or middle cerebral artery occlusion (MCAO)-exposed mice 24 h after ischemia. Data are mean \pm SD ($n=6$; * $P < 0.05$). DHCR indicates 3 β -hydroxysteroid- Δ 24 reductase; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; and seladin-1, selective alzheimer disease indicator 1.

Middle Cerebral Artery Occlusion Experimental Groups

All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. The set of experiments aimed to study the effect of the pharmacological modulation of seladin-1/DHCR24 was performed in 2-month-old WT mice (n=6–10/group) using the seladin-1/DHCR24 inhibitor U18666A¹⁶ (10 mg/kg; Calbiochem), administered intraperitoneally 30 minutes, 6 h, and 18 h after ischemia. U18666A is a cell-permeable, amphiphilic amino-steroid, high-affinity-binding inhibitor of the Δ 24-reductase activity of seladin-1/DHCR24¹⁷ (half maximal inhibitory concentration [IC₅₀]=0.15 μ mol/L; inhibitory constant [K_i]=0.000157 mmol/L). A second set of experiments was performed in 2-month-old WT and *Dhcr24*^{-/-} mice.

Determination of Physiological Parameters

Physiological parameters (mean arterial blood pressure, blood P_{O₂}, P_{CO₂}, and pH, hematocrit and hemoglobin, and rectal temperature) were measured as described.¹⁸

Motor-Deficit Evaluation

Two days after surgical procedure, motor deficit was assessed by 2 independent researchers blinded to experimental conditions. Motor assessment was performed by using the walking test that is graded on a scale of 0 to 3, with a higher score indicating more severe motor deficits, where 0=normal walk, 1=inability to walk straight, 2=circling toward the paretic side, and 3=fall down to the paretic side.

Determination of Infarct Size

Two days after middle cerebral artery occlusion (MCAO), animals were killed by cervical dislocation to assess infarct outcome. Brain was removed and cut into 1-mm-thick coronal slices and stained with 2,3,5-triphenyltetrazolium chloride (1% 2,3,5-triphenyltetrazolium chloride in 0.1 mol/L phosphate buffer). Infarct size was determined as described.¹⁸

Primary Culture of Mouse Cortical Astrocytes

Cortical primary astrocyte cultures were prepared from postnatal day 1 pups of C57BL/6 mice, as previously described.¹⁹ After the cells became confluent, nonastrocytes, such as microglia, were removed by shaking during 12 h followed by a change of medium, and astrocytes were detached by trypsin and reseeded on 6-well plates. After 24 h, astrocytes were washed with serum-free medium and incubated in a serum-free medium supplemented with dibutyl-cAMP 100 μ mol/L for 72 hours as described.²⁰ Cells present in the culture were shown to be 90% astrocytes by immunostaining with the astrocytic marker glial fibrillary acidic protein using a primary specific anti-glial fibrillary acidic protein antibody (Chemicon, Temecula, CA; 1:100 dilution).

Cells were treated for 2 days with the seladin-1/DHCR24 inhibitor U18666A (Calbiochem; 2.5, 5, and 10 μ g/mL, once a day).

[³H]-Glutamate Uptake by Astrocytes

[³H]-Glutamate uptake by astrocytic cultures was determined as described.²¹ Briefly, cultures were washed in control solution and incubated in control solution containing 3 μ mol/L glutamate and 8 μ Ci/mL of [³H]-glutamate for 90 s, a time in which glutamate uptake was found to proceed linearly with time. In a parallel set of experiments, cultures were incubated during the same time in a solution of the same composition but in which Na⁺ was equiosmotically substituted by choline. At the end of the incubation, solution was collected, and cells were lysed by addition of perchloric acid (0.3 mol/L). [³H]-Glutamate uptake was calculated by subtracting the uptake in the absence of Na⁺ from the uptake in its presence and expressed as percentage of total [³H]-glutamate.

Western Blot

Brain tissue was collected from peri-infarct area of mice killed 5, 18, 24, 48, and 72 h and 7 days after MCAO for seladin-1/DHCR24 determination. For other proteins, animals were killed 24 h after MCAO. Protein concentration was determined in peri-infarct tissue homogenates with detergent compatible protein assay (Bio-Rad). Equal amounts of total protein (20 μ g) were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Immunodetection

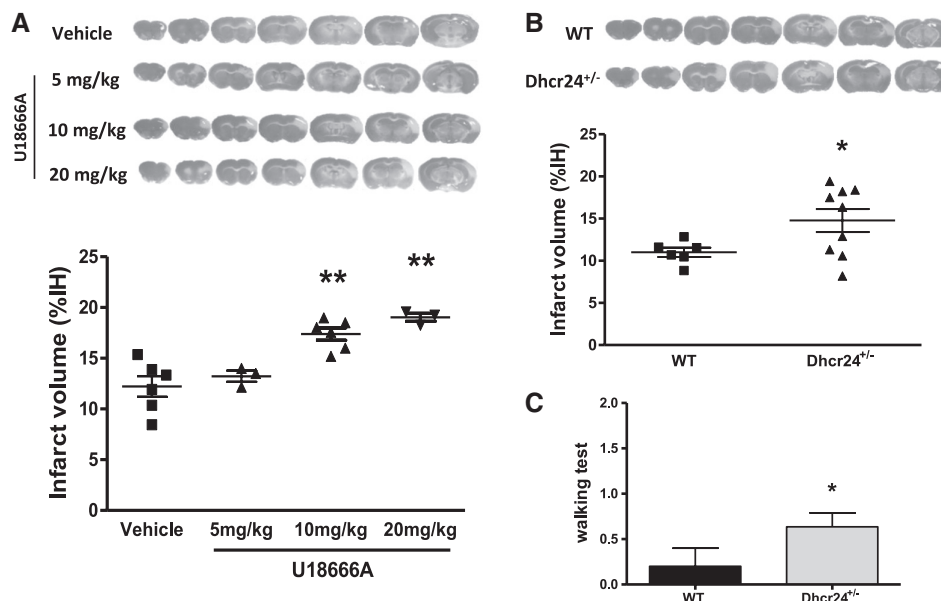


Figure 2. 3 β -Hydroxysteroid- Δ 24 reductase (DHCR24) pharmacological inhibition or partial genetic deletion worsen stroke outcome. Quantification of 2,3,5-triphenyltetrazolium chloride-stained brain slices and neurological outcome. **A**, Effect of DHCR24 inhibitor U18666A at different concentrations: 5 mg/kg, 10 mg/kg, and 20 mg/kg, intraperitoneally, 30 minutes after middle cerebral artery occlusion (MCAO) on infarct volume determined 48 h after ischemia in wild-type (WT) mice. **B**, Effect of partial genetic deletion of *Dhcr24* in *Dhcr24*^{-/-} vs WT mice 48 h after ischemia. **C**, Effect of partial genetic deletion of *Dhcr24* in *Dhcr24*^{-/-} and WT mice on functional deficits, monitored 48 h after MCAO by using the walking test. Data are mean \pm SD (n=6–9; **P*<0.05).

was performed by standard procedures. The membranes were blocked with 5% nonfat milk in TBS-T (0.05% Tween 20 in Tris-buffered saline [TBS]) and probed with specific primary antibodies against seladin-1/DHCR24 (Cell Signaling; 1:1000 dilution), cyclooxygenase-2 (COX-2, Santa Cruz; 1:1000), inducible nitric oxide synthase (iNOS, Santa Cruz; 1:1000), EAAT2 (Cell Signaling; 1:1000), and flotillin-1 (Cell Signaling; 1:1000), and mouse anti- β -actin (Sigma; 1:5000) was included to ensure equal protein loading. Specific signals were quantified using densitometry analysis software (ImageJ).

Double Immunofluorescence Staining

To examine the cellular location of seladin-1/DHCR24, free-floating coronal brain slices (30 μ m) were processed 24 h after MCAO (n=3 in each group) as described previously.²² In brief, brain sections were blocked with 5% goat serum and incubated with mouse anti-neuronal nuclei and anti-glial fibrillary acidic protein and rabbit anti-DHCR24 (Cell Signaling) 1:100 at 4°C overnight followed by the appropriate rabbit secondary antibody Alexa 488 (Invitrogen A-11008) and anti-mouse Cy3 (Jackson Immuno Research; 715-165-151) 2 h at room temperature. All immunofluorescence images were obtained in a blinded manner from 7 correlative slices of each brain. Image acquisition was performed with a laser-scanning confocal imaging system (Zeiss LSM710), and image analysis was performed with the ZEN 2009 software (Zeiss). All colocalization images shown were confirmed by orthogonal projection of z-stack files.

Cytometric Bead Array

Protein homogenates from brain peri-infarct tissue obtained 24 h after MCAO were used to measure the protein levels of tumor necrosis factor- α and interleukin-10 by a cytometric bead array (Beckton Dickinson).

Lipid Rafts Isolation

Mouse brains were obtained from peri-infarct area 24 h after ischemia and homogenized in phosphate buffer saline containing 9% sucrose and protease inhibitors (pepstatin, antipain, chymostatin, each at a final concentration of 25 mg/mL) using 20 strokes in a dounce homogenizer on ice. The samples were centrifuged for 10 minutes at 4°C and 700 g, and the supernatants considered as total extracts. A further centrifugation of the supernatant was performed at 100 000 g for 1 h at 4°C to pellet the membrane fraction. Protein concentration was quantified by the detergent compatible method (Bio-Rad).

The homogenates were then incubated with Tris-NaCl-EDTA (TNE)-Triton X-100 (Tris-HCl 50 mmol/L, pH 7.4, NaCl 150 mmol/L, EDTA 5 mmol/L, and protease inhibitors) for 40 minutes at 4°C in rotation and centrifuged at 11 752 g for 3 minutes. The supernatant was then placed in a centrifuge tube mixed with 80% sucrose prepared in TNE buffer (final concentration 60%). The samples were overlaid with 35% and 5% sucrose, respectively. The gradient was centrifuged for 19 h at 100 000 g at 4°C. Nine fractions, 1 mL each sample, were collected from the top of each tube, and lipid raft fractions were identified by the presence of the lipid raft marker, flotillin-1.

Statistical Analysis

Results are expressed as mean \pm SD for the indicated number of experiments. Prism5 (GraphPad Software, Inc, La Jolla, CA) was used for statistical analysis. Unpaired Student's *t* test was used to compare 2 groups. One- or 2-way ANOVA was used to compare >2 groups or parameters with the Tukey and Bonferroni post hoc tests, respectively. Values of *P*<0.05 were considered statistically significant.

Results

Seladin-1/DHCR24 Expression Increases After Experimental Stroke

Exposure of 2-month-old WT mice to MCAO increased seladin-1/DHCR24 protein levels, as shown by Western blot in

homogenates from peri-infarct area of ipsilesional cortices at all the time points of the study (n=6, *P*<0.05; Figure 1A).

The expression of seladin-1/DHCR24 was also examined by immunofluorescence and confocal microscopy. To analyze its cellular location, double immunofluorescence staining was used in equivalent sections. Our data show that seladin-1/DHCR24 is mainly located in neurons (neuronal nuclei-positive) of both sham and MCAO-exposed animals. Interestingly, we detected seladin-1/DHCR24 immunoreactivity in astrocytes (glial fibrillary acidic protein-positive) after MCAO but not in SHAM-operated animals (Figure 1B).

Infarct Volume Is Increased After Seladin-1/DHCR24 Pharmacological Inhibition

The increased expression of seladin-1/DHCR24 observed after experimental stroke moved us to test whether this enzyme influences infarct volume in vivo. To this aim, we studied the effect of the high-affinity-binding seladin-1/DHCR24 inhibitor¹⁷ U18666A (5, 10, and 20 mg/kg), administered intraperitoneally 30 minutes after MCAO, on infarct volume. Infarct lesion was determined 48 h after MCAO in vehicle- and U18666A-treated mice. The treatment with 10 and 20 mg/kg U18666A but not with 5 mg/kg caused a significant increase in the infarct volume when compared with the vehicle-treated group (n=6, *P*<0.05; Figure 2A). However, the effect of U18666A (10 mg/kg) was no longer observed

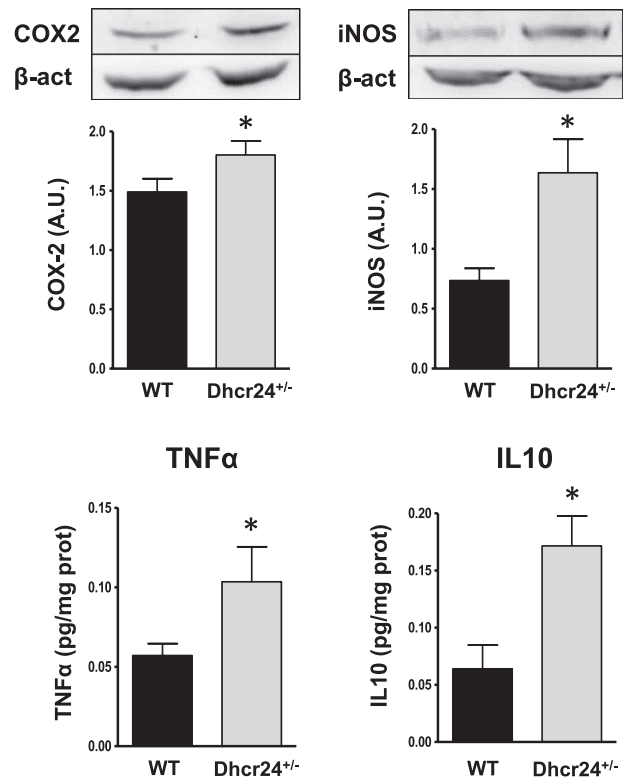


Figure 3. Dhcr24-deficient mice show an increase in inflammation-related mediators after stroke. Quantification of COX2, iNOS, IL-10, and TNF α in wild-type (WT) and Dhcr24^{-/-} mice 24 h after ischemia in peri-infarct region. Data are mean \pm SD (n=7; **P*<0.05). DHCR24 indicates 3 β -hydroxysteroid- Δ 24 reductase; IL, interleukin; and TNF, tumor necrosis factor.

when this compound was administered 6 or 18 h after MCAO (Figure I in the online-only Data Supplement).

Haploinsufficiency of Seladin-1/DHCR24 Worsens Stroke Outcome and Brain Inflammation

To confirm the role of endogenous seladin-1/DHCR24 in cerebral ischemia, 2-month-old *Dhcr24*^{+/-} and WT mice were subjected to MCAO and euthanized 48 h after. In agreement with the data obtained with the DHCR24 inhibitor, our data show that the size of the ischemic lesion was significantly higher in *Dhcr24*^{+/-} than in WT mice (n=9–6 respectively, $P<0.05$, Figure 2B) suggesting that endogenous seladin-1/DHCR24 plays a protective role during cerebral ischemia.

To assess whether seladin-1/DHCR24 deficiency had an effect not only in infarct volume but also in functional deficits, we monitored motor abilities by using the walking test (normal=0, maximum=3) in WT and *Dhcr24*^{+/-} mice before killing, 48 h after MCAO. The score of WT mice was significantly lower than that in *Dhcr24*^{+/-} animals, indicating a better performance in mice with a normal expression of seladin-1/DHCR24 (Figure 2C).

As an additional parameter of brain damage, we also analyzed the expression of inflammatory mediators in brain homogenates from peri-infarct tissue of MCAO-injured mice 24 h after the surgery. Both, proinflammatory mediators, such as iNOS, COX-2, and tumor necrosis factor- α and anti-inflammatory cytokines like interleukin-10 showed higher levels (n=7, $P<0.05$; Figure 3) in *Dhcr24*^{+/-} mice when compared with their WT littermates. This indicates exacerbated

stress response, resulting in pro and anti-inflammatory activities after experimental ischemia in *Dhcr24*^{+/-} animals. Physiological parameters measured in arterial blood 10 minutes before and 30 minutes after MCAO were not affected by the partial deletion of seladin-1/DHCR24 in *Dhcr24*^{+/-} mice (n=6; Table I in the online-only Data Supplement).

Seladin-1/DHCR24 Deficiency Decreases Cellular EAAT2 Levels and Lipid Rafts Location in Peri-Infarct Region

Excitotoxicity because of glutamate increase in brain after cerebral ischemia contributes to infarct lesion. A key molecule in this event is the glutamate transporter EAAT2, whose association to lipid rafts where it is more active increases after ischemic injury. It has been shown that seladin-1/DHCR24 alters raft partitioning of certain molecules. To ascertain whether seladin-1/DHCR24 deficiency affects EAAT2 association to lipid rafts in our model, homogenates from peri-infarct area of ipsilesional cortices from WT and *Dhcr24*^{+/-} mice were subjected to a discontinuous sucrose gradient in the presence of detergent, and 9 fractions were collected from the top of the gradient (fractions 1–9) according to their density. Flotillin-1, a canonical marker of lipid rafts,²³ was found mainly in fractions 3 to 5 from MCAO-WT mice (n=5, $P<0.05$; Figure 4A); consistent with the association of EAAT2 to these membrane domains, most of the protein (70%) colocalized with flotillin-1 in these fractions. In contrast, in *Dhcr24*^{+/-} mice, both flotillin-1 and EAAT2 were displaced to non-raft fractions 7 to 9 ($\approx 50\%$ flotillin-1 and 85% EAAT2; Figure 4A).

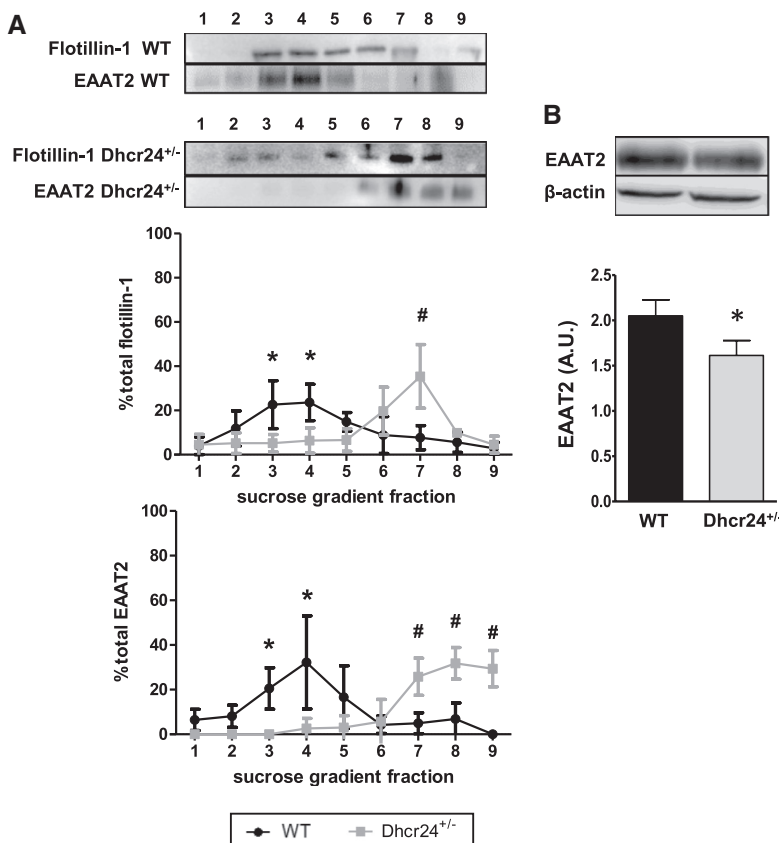


Figure 4. Seladin-1/DHCR24 haploinsufficiency leads to decreased cellular EAAT2 levels and lipid rafts association in the peri-infarct region. **A**, Effect of partial deletion of *Dhcr24* on brain EAAT2 association to lipid rafts in mouse brain. The figure shows flotillin-1 and EAAT2 expression in each fraction in peri-infarct region 24 h after ischemia. Data are mean \pm SD (n=5; * $P<0.05$ comparing wild-type (WT) fractions; # $P<0.05$ comparing fractions from *Dhcr24*^{+/-} mice). **B**, Levels of total EAAT2 in peri-infarct region 24 h after ischemia in WT and *Dhcr24*^{+/-} mice. Data are mean \pm SD (n=8; * $P<0.05$). DHCR24 indicates 3 β -hydroxysteroid- Δ 24 reductase; and seladin-1, selective alzheimer disease indicator 1.

In addition, our results also show a significant decrease ($\approx 25\%$) in total EAAT2 protein levels in *Dhcr24*^{+/-} animals 24 h after MCAO (n=8, $P < 0.05$; Figure 4B).

Pharmacological inhibition of seladin-1/DHCR24 induces a decrease in glutamate uptake in astrocytes and in EAAT2 association with lipid rafts in peri-infarct area

To determine whether the loss of EAAT2 association to lipid rafts found in *Dhcr24*^{+/-} mice has functional consequences on this transporter, we monitored first the effect of seladin-1/DHCR24 inhibition on the uptake of ³H-glutamate in cultured astrocytes. To this aim, we used mouse astrocytic cultures after treatment with U18666A at different concentrations (2.5, 5, and 10 $\mu\text{g/mL}$) or with vehicle during 48 h. Our results show a decrease in glutamate uptake after treatment with U18666A at all the concentrations studied (n=5, $P < 0.05$; Figure 5A), confirming that DHCR24 inhibition affects EAAT2 function. To determine whether this correlates with an altered membrane distribution of this glutamate transporter, we assessed the presence of EAAT2 in lipid rafts after pharmacological inhibition of seladin-1/DHCR24 in vivo. Homogenates from peri-infarct area from vehicle- and U18666A (10 mg/kg)-treated mice were subjected to a discontinuous sucrose gradient in the presence of detergent as described previously. Although flotillin-1 and EAAT2 mainly localize in raft fractions 3-4-5 in vehicle-treated animals, the pharmacological inhibition of seladin-1/DHCR24 resulted in the displacement of both flotillin-1 and EAAT2 to non-raft fractions (7-9;

$\approx 70\%$ both flotillin-1 and EAAT2; n=5, $P < 0.05$; Figure 5B). This behavior is similar to that observed in *Dhcr24*^{+/-} mice (see Figure 4A).

Discussion

The presence of seladin-1/DHCR24 in central nervous system has been reported in many studies where seladin-1/DHCR24 has been shown to play an important role in neuroprotection.²⁴ In brain and other tissues, several authors have described the implication of seladin-1/DHCR24 in different processes like apoptosis, inflammation, oxidative stress, and neurodegeneration.^{1,3,5,8,25} We hereby demonstrate for the first time the protective role of seladin-1/DHCR24 against experimental stroke induced by MCAO in mice.

First, we have found that exposure to MCAO induces an overexpression of seladin-1/DHCR24 protein in mouse brain early after the ischemic occlusion that was maintained even 7 days thereafter, suggesting that seladin-1/DHCR24 might play a role in ischemic stroke. To ascertain this issue, we first performed pharmacological loss-of-function experiments using U18666A, an inhibitor of seladin-1/DHCR24 enzymatic activity,¹⁶ showing that DHCR24 inhibition is associated to an increase in the infarct volume and strongly supporting a protective role of endogenous seladin-1/DHCR24 in cerebral ischemia. To confirm these data, we explored the effect of DHCR24 haploinsufficiency using *Dhcr24*^{+/-} mice. Importantly, *Dhcr24*^{+/-} animals presented larger infarct

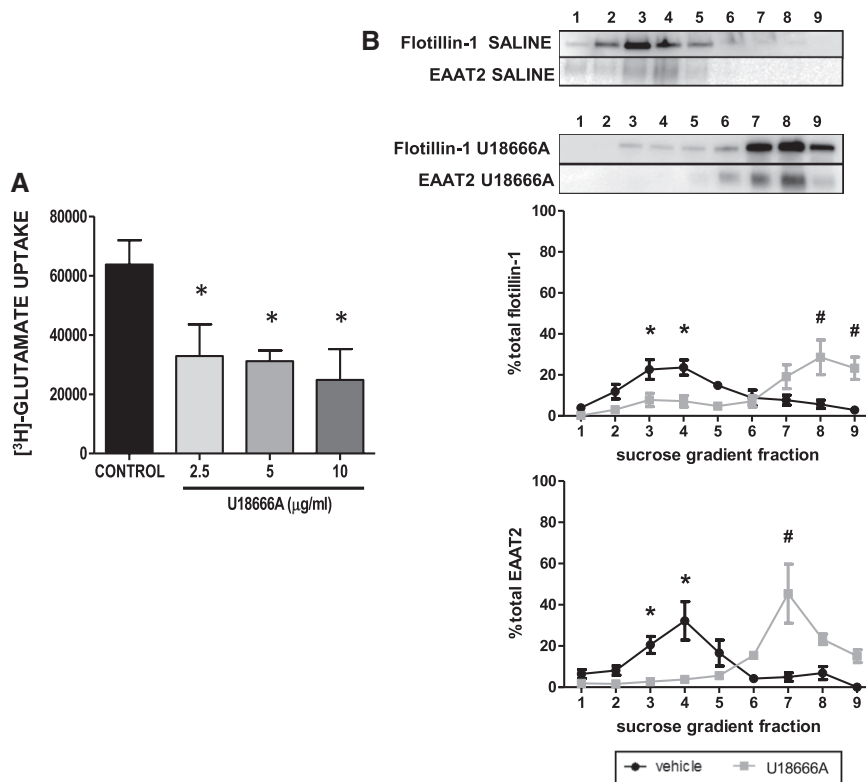


Figure 5. Inhibition of 3β -hydroxysteroid- $\Delta 24$ reductase (DHCR24) leads to a decreased association of EAAT2 to lipid rafts and astrocytic glutamate uptake. **A**, [³H]-glutamate uptake after DHCR24 inhibition in mouse astrocytic cultures. DHCR24 inhibitor (U18666A) was added 48 h before glutamate determination at different concentrations (2.5, 5, and 10 $\mu\text{g/mL}$). Data are mean \pm SD (n=5; * $P < 0.05$). **B**, Association of mice brain EAAT2 to lipid rafts and effect of partial deletion of *Dhcr24*. The figure shows flotillin-1 and EAAT2 expression in each fraction. Data are mean \pm SD (n=5; * $P < 0.05$ comparing vehicle-treated mice fractions; # $P < 0.05$ comparing fractions from U18666A-treated mice).

volumes and worse motor outcome than their WT littermates. In addition, our results show a significant increase in inflammatory response after ischemia in *Dhcr24*^{+/-} compared with their WT littermates, as demonstrated by increased levels of the inflammation-related markers COX-2, iNOS, tumor necrosis factor- α , and interleukin-10, which participate in the ischemic inflammatory cascade and in the subsequent ischemic brain damage.²⁶ Altogether, these results confirm the neuroprotective role of seladin-1/DHCR24 in cerebral ischemia.

Ischemia initiates with severe focal hypoperfusion that leads to excitotoxicity and oxidative damage which, in turn, initiate postischemic inflammation that contribute to brain injury and to the expansion of the ischemic lesion.²⁷ We and others have previously shown that glutamate increases in brain after cerebral ischemia in this permanent MCAO model^{28–30} and that strategies that diminish extracellular glutamate levels could ameliorate the symptoms in rats.^{31–33} In this context, glutamate uptake by glutamate transporters play a major role; specifically, EAAT2 or GLT1, that is predominantly located in astrocytes, has been reported to account for $\approx 95\%$ of glutamate uptake in the adult central nervous system.³⁴ Interestingly, this transporter needs to be associated to lipid rafts for a proper cellular function,¹¹ an association that is increased after ischemic injury.³² Our results show an increase in seladin-1/DHCR24 expression in astrocytes after MCAO that could constitute a stress-response aimed to increase EAAT2 association to lipid rafts. Further studies are necessary to discriminate the role of astrocytes and neurons in this setting. Because seladin-1/DHCR24, by regulating cholesterol biosynthesis, has a crucial role in the correct function of these microdomains,³ we reasoned that the absence or inhibition of this enzyme might have a major impact on glutamate levels and subsequent excitotoxicity after brain ischemia. Indeed, our results show that the association of EAAT2 to lipid rafts was lower in *Dhcr24*^{+/-} animals in comparison with WT mice after ischemia. The same effect was observed when seladin-1/DHCR24 was inhibited pharmacologically with the inhibitor U18666A. These results support the idea that the decrease in seladin-1/DHCR24 alter lipid rafts and thus impede a correct association of EAAT2 to these platforms that may lead to a decrease in glutamate uptake and subsequent excitotoxicity in *Dhcr24*^{+/-}. Supporting this idea, our results demonstrate that glutamate uptake is markedly decreased when DHCR24 activity is inhibited in astrocytic cultures, confirming the involvement of seladin-1/DHCR24 at this level. We also found that the expression of EAAT2 is significantly lower in heterozygous mice, suggesting that the altered association of this protein to lipid rafts may be indirectly affecting its synthesis or degradation. Finally, our data show that the action of seladin-1/DHCR24 takes place in the first hours after the ischemic onset, supporting excitotoxicity as the target of the beneficial actions of this protein after stroke. However, because several important cellular functions are exerted by other proteins associated to lipid rafts, we cannot discard the involvement of other mechanisms in the effect of seladin-1/DHCR24 in the ischemic scenario.

Summary

In summary, this is the first report showing that seladin-1/DHCR24 has a neuroprotective role against cerebral ischemia in mice. Decreased levels of this protein exacerbate the inflammatory response and cell death after ischemia. Our results suggest that these effects are caused by excitotoxicity mediated by a failed glutamate uptake caused by a decrease in the association of EAAT2 to lipid rafts. Importantly, because seladin-1/DHCR24-deficient mice recapitulate brain lipidic changes typical of aging, our findings also suggest important mechanisms that explain increased vulnerability of the aged brain to stroke. Our results open a new line of investigation aimed to design therapeutic strategies that increase seladin-1/DHCR24 expression or activity for a correct functioning of EAAT2 and other important proteins associated to lipid rafts. These strategies may be beneficial to improve the outcome of stroke and also of other pathologies in which seladin-1/DHCR24 deficiency has been reported like Alzheimer's disease.

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Disclosures

None.

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Macarena Hernández-Jiménez, Diego Martínez-López, Enrique Gabandé-Rodríguez, Adrian Martín-Segura, Ignacio Lizasoain, María D. Ledesma, Carlos G. Dotti and María A. Moro

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