ABCA1/ApoE/HDL Pathway Mediates GW3965-Induced Neurorestoration After Stroke

Xu Cui, MD, PhD; Michael Chopp, PhD; Zhenggang Zhang, MD, PhD; Rongwen Li, PhD; Alex Zacharek, MS; Julie Landschoot-Ward, BS; Poornima Venkat, PhD; Jieli Chen, MD

Background and Purpose—ATP-binding cassette transporter A1 (ABCA1) is a major reverse cholesterol transporter and plays a critical role in the formation of brain high-density lipoprotein (HDL) cholesterol. Apolipoprotein E (ApoE) is the most abundant apolipoprotein and transports cholesterol into cells in brain. ABCA1 and ApoE are upregulated by liver-X receptors. Activation of liver-X receptors has neurorestorative benefit for stroke. The current study investigates whether ABCA1/ApoE/HDL pathway mediates GW3965, a synthetic dual liver-X receptor agonist, induced neurorestoration after stroke.

Methods—Middle-aged male specific brain ABCA1−/− (ABCA1−B/−B) and floxed-control (ABCA1fl/fl) mice were subjected to distal middle-cerebral artery occlusion (dMCAo) and gavaged with saline or GW3965 (10 mg/kg) or intracerebroinfusion of artificial cerebrospinal fluid or human plasma HDL3 in ABCA1−B/−B stroke mice, starting 24 hours after dMCAo and daily until euthanization 14 days after dMCAo.

Results—No differences in the blood level of total cholesterol and triglyceride and lesion volume were found among the groups. Compared with ABCA1fl/fl ischemic mice, ABCA1−B/−B ischemic mice exhibited impairment functional outcome and decreased ABCA1/ApoE expression and decreased gray/white matter densities in the ischemic boundary zone 14 days after dMCAo. GW3965 treatment of ABCA1fl/fl ischemic mice led to increased brain ABCA1/ApoE expression, concomitantly to increased blood HDL, gray/white matter densities and oligodendrocyte progenitor cell numbers in the ischemic boundary zone, as well as improved functional outcome 14 days after dMCAo. GW3965 treatment had negligible beneficial effects in ABCA1−B/−B ischemic mice. However, intracerebral infusion of human plasma HDL3 significantly attenuated ABCA1−B/−B-induced deficits. In vitro, GW3965 treatment (5 μM) increased ABCA1/synaptophysin level and neurite/axonal outgrowth in primary cortical neurons derived from ABCA1fl/fl embryos, but not in neurons derived from ABCA1−B/−B embryos. HDL treatment (80 μg/mL) attenuated the reduction of neurite/axonal outgrowth in neurons derived from ABCA1−B/−B embryos.

Conclusions—ABCA1/ApoE/HDL pathway, at least partially, contributes to GW3965-induced neurorestoration after stroke. (Stroke. 2017;48:459-467. DOI: 10.1161/STROKEAHA.116.015592.)

Key Words: apolipoprotein E □ ATP-binding cassette transporter A1 □ high-density lipoprotein □ liver-X receptors □ stroke □ white matter

Stroke is one of the most frequent causes of death and disability worldwide. Although the pathogenesis and clinical significance of stroke recovery are clearly related to the functional cerebral lesions both in gray matter and white matter (WM),1 the molecular mechanisms of gray matter and WM remodeling after stroke are not fully understood. New strategies to promote neurorestoration and to improve long-term neurological deficits after stroke are necessary beyond the hyperacute phase of ischemia.

Cholesterol plays major structural and functional roles in both the gray matter and WM. The ATP-binding cassette transporter member A1 (ABCA1), a major cholesterol transporter, regulates efflux of intracellular cholesterol and phospholipids onto lipid-poor apolipoprotein E (ApoE) and plays a critical role in mediating high-density lipoprotein (HDL) cholesterol and ApoE production in the brain.2,3 ApoE is the most abundant apolipoprotein in the brain. It solubilizes phospholipid and transports cholesterol into cells, while stabilizing HDL particles, and enables these proteins to be partners with ABCA1.3–6 Both ABCA1 and ApoE expression are upregulated by nucleic transcription factor liver-X receptors (LXRs).5–8 Treatment of stroke with GW3965, a synthetic dual LXR (including LXRα and LXRβ) agonist, promotes neuroprotection and reduces brain inflammation,9 increases vascularization and WM remodeling in the ischemic brain, and elevates blood HDL level, as well as improves neurological functional outcome in experimental stroke.10 However, the molecular mechanisms of GW3965-induced neurorestorative effects after stroke are not fully elucidated. In this study, using specific conditional brain ABCA1 knockout mice, we tested...
whether the ABCA1/ApoE/HDL signaling pathway mediates GW3965 treatment–induced neurorestoration after stroke.

Materials and Methods
For all in vivo studies, the use of animals and procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Health System. All animal groups, treatment, and their identity were blinded to the surgeon and the investigators who performed behavior tests, lesion volume and biochemical measurements, and immunostaining analysis.

Animals and Stroke Model
Specific brain ABCA1 knockout (ABCA1<sup>−/−</sup>) and ABCA1 floxed control (ABCA1<sup>fl/fl</sup>) mice were used. The parent pairs of animals were generously provided by Dr Michael Hayden (University of British Columbia, Canada). Since female mice have an estrogen protective effect, in this study, we subjected male mice (middle-aged, 13–14 months) to permanent right distal middle cerebral artery occlusion (dMCAo) induced by transcranial ligation with a 10-0 nylon filament. The number of animals was calculated a priori by power calculation. Because dMCAo leads to a consistent lesion volume and low mortality, 9 animals per group were considered to reach 80% power at a significance level of <0.05, assuming 20% difference in both of mean identity, 9 animals per group were considered to reach 80% power at a significance level of <0.05, assuming 20% difference in both of mean and SD at the 95% confidence level and a 2-sided test.

Experimental Groups
To investigate whether ABCA1/ApoE mediates GW3965-induced neurorestoration after stroke, ABCA1<sup>fl/fl</sup> and ABCA1<sup>−/−</sup> stroke mice were randomly divided into 4 groups by a nonteam member using the method of drawing different colored balls. Mice were gavaged starting 24 hours after dMCAo, with saline as vehicle control or GW3965 10 mg/kg (Sigma) daily for 14 days based on previous dose-dependent study. After 14 days of treatment, animals were randomly separated into 2 sets: one set of animals (total 24 mice, n=6/group) were used for Western blot (WB) and reverse transcriptase polymerase chain reaction assay; the other set of animals (total 36 mice, n=9/group) were used for behavioral testing, blood biochemistry, and lesion volume and immunostaining measurements.

To investigate the effect of HDL on GW3965-induced neurorestoration, ABCA1<sup>−/−</sup> stroke mice were randomly assigned to 2 groups (total 18 mice, n=9/group) and intraventricularly infused with artificial cerebrospinal fluid (CSF; Torix Bioscience) 100 μL as vehicle control or human plasma HDL3 (hHDL3; Cell Biolabs Inc) 25 μg in 100 μL artificial CSF by transplanting a micro-osmotic pump (D1002; Alzet) into the right lateral ventricle initiated at 24 hours after dMCAo for 14 days. All mice were euthanized 14 days after dMCAo.

Functional Test
The adhesive removal test, the most sensitive functional test for dMCAo, was performed prior to dMCAo and at 1, 3, 7, and 14 days after dMCAo.

HDL, Total Cholesterol, and Triglyceride Measurement
Blood was collected from the tail vein 14 days after dMCAo, and the blood levels of HDL, total cholesterol, and triglyceride were measured using a CardioChek P-A analyzer and test strips (Polymer 285 Technology System).

Lesion Volume, Histochemical and Immunohistostaining Assessment, Reverse Transcriptase Polymerase Chain Reaction /WB Assay, and Neurite/ Axonal Outgrowth Measurement
Please see methods in the online-only Data Supplement.

Data and Statistical Analysis
Data are presented as mean±standard error. Two-way analysis of variance followed by Tukey post hoc test were performed for analysis: (1) gene/protein expression, lesion volume/blood biochemistry/functional outcome measurement, and in vitro study for comparison of gene-deficient (ABCA1<sup>−/−</sup> versus ABCA1<sup>fl/fl</sup>) and treatment effect (with versus without GW3965 or HDL, respectively); (2) immunostaining measurement for gene-deficient (ABCA1<sup>−/−</sup> versus ABCA1<sup>fl/fl</sup> and ischemic effect (contralateral versus ipsilateral). Independent t test was made between ABCA1<sup>−/−</sup>-stroke mice intracerebral infusion of artificial CSF and ABCA1<sup>−/−</sup>-stroke mice intracerebral infusion of hHDL3 groups. A value of P<0.05 was taken as significant.

Results
GW3965 Treatment Increases Brain ABCA1/ApoE Expression in ABCA1<sup>fl/fl</sup> Stroke Mice, but Not in ABCA1<sup>−/−</sup> Stroke Mice
ABCA1/ApoE protein and mRNA levels significantly decreased measured by WB and reverse transcriptase polymerase chain reaction assays in both the contralateral and the ipsilateral ischemic brain tissues isolated from ABCA1<sup>−/−</sup> saline control stroke mice compared with tissues isolated from ABCA1<sup>fl/fl</sup> saline control stroke mice, respectively; GW3965 treatment of ABCA1<sup>−/−</sup> stroke mice significantly increased ABCA1/ApoE levels in both the contralateral and the ipsilateral ischemic brain tissue compared with ABCA1<sup>fl/fl</sup> saline control stroke mice, respectively. However, GW3965 had a negligible effect on ABCA1 expression, but increases ApoE expression in ABCA1<sup>−/−</sup> stroke mice (Figure 1B through 1D; P<0.05, n=6/group).

GW3965 Treatment Increases Blood HDL Level and Improves Functional Outcome in ABCA1<sup>fl/fl</sup> Stroke Mice, but Not in ABCA1<sup>−/−</sup> Stroke Mice; Intracerebral Infusion of hHDL3 Improves Functional Outcome in ABCA1<sup>−/−</sup> Stroke Mice
There were no significant differences in the indirect lesion volume (Figure 1A in the online-only Data Supplement) and the blood level of total cholesterol and triglyceride among ABCA1<sup>fl/fl</sup> and ABCA1<sup>−/−</sup> stroke mice treated with saline or GW3965. However, GW3965 treatment of ABCA1<sup>fl/fl</sup> stroke mice, but not ABCA1<sup>−/−</sup>-stroke mice, significantly increased blood HDL level compared with ABCA1<sup>fl/fl</sup> saline control stroke mice 14 days after dMCAo (Figure 1B in the online-only Data Supplement; P<0.05, n=9/group). Compared with ABCA1<sup>fl/fl</sup> saline control stroke mice, ABCA1<sup>−/−</sup> saline control stroke mice exhibited more severe neurological deficits 3, 7, and 14 days after dMCAo. Treatment of stroke with GW3965 significantly improved functional outcome 7 and 14 days after dMCAo in ABCA1<sup>fl/fl</sup> stroke mice, but not in ABCA1<sup>−/−</sup>-stroke mice (Figure 1C in the online-only Data Supplement; P<0.05, n=9/group). Compared with intraventricular infusion of artificial CSF in ABCA1<sup>−/−</sup>-stroke mice, no significant differences in the lesion volume (Figure 1A in the online-only Data Supplement) and blood levels of HDL/triglyceride/total cholesterol (Figure 1B in the online-only Data Supplement) were found in ABCA1<sup>−/−</sup>-stroke mice intracerebrally infused with hHDL3. However, these mice exhibited significantly improved functional outcome 14 days after dMCAo (Figure 1C in the online-only Data Supplement; P<0.05, n=9/group).
GW3965 Treatment Increases Syn/MBP Expression in the Ischemic Brain in ABCA1<sup>−/−</sup> Stroke Mice, but Not in ABCA1<sup>−/−−</sup> Stroke Mice; Intracerebral Infusion of hHDL3 Increases Syn/MBP Expression in the Ischemic Brain in ABCA1<sup>−/−−</sup> Stroke Mice

ABCA1<sup>−/−−</sup> saline control stroke mice exhibit significantly decreased Syn protein level measured by both immunostaining (Figure 2A) and WB assay (Figure 2B) and MBP (myelin basic protein) protein/mRNA (Figure 2B) level in both the contralateral and ipsilateral ischemic brain compared with ABCA1<sup>fl/fl</sup> saline control stroke mice, respectively (*P*<0.05, n=9/group). GW3965 treatment of ABCA1<sup>fl/fl</sup> stroke mice, but not ABCA1<sup>−/−−</sup> stroke mice, significantly increased Syn protein and MBP protein/mRNA level in both the contralateral and ipsilateral ischemic brain tissue compared with saline control stroke mice (*P*<0.05, n=6/group). ABCA1 indicates ATP-binding cassette transporter A1; ApoE, apolipoprotein E; BS, Bielschowsky–Silver; LFB, Luxiol–Fast–Blue; PDGFR<sub>α</sub>, platelet-derived growth factor receptor alpha; and RT-PCR, reverse transcriptase polymerase chain reaction.
and ipsilateral ischemic brain compared with ABCA1-/- stroke control stroke mice (Figure 2A and 2B; *P <0.05, n=9/group).

Intraventricular infusion of hHDL3 in ABCA1-/- stroke mice significantly increased Syn protein level measured by immunostaining (Figure 2C; *P <0.05, n=9/group) and Syn/MBP protein/mRNA level (Figure 2D; *P <0.05, n=6/group) compared with intraventricular infusion of artificial CSF in ABCA1-/- stroke mice.

GW3965 Treatment of Stroke Increases WM Densities and OPC Numbers in the IBZ of ABCA1fl/fl Stroke Mice, but Not in ABCA1-/- Stroke Mice; Intracerebral Infusion of hHDL3 Increases WM Densities and OPC Numbers in the IBZ of ABCA1-/- Stroke Mice

Compared with contralateral corpus collasum (CC), the densities of Luxiol–Fast–Blue (LFB+) myelin, Bielschowsky–Silver (BS+) axons, and SMI31+ phosphorlated neurofilament in the ischemic-boundary zone (IBZ) of ipsilateral CC significantly decreased both in ABCA1fl/fl saline control stroke mice and ABCA1-/- saline control stroke mice, respectively. The number of platelet-derived growth factor receptor alpha (PDGFRα+) oligodendrocyte progenitor cells (OPCs) in the IBZ of ipsilateral CC significantly increased in ABCA1fl/fl saline control stroke mice, but not in ABCA1-/- saline control stroke mice. The densities of LFB+/BS+/SMI31+ WM and the numbers of PDGFRα+ OPCs significantly decreased in both the contralateral CC and the IBZ of the ipsilateral CC in ABCA1-/- saline control stroke mice compared with ABCA1fl/fl saline control stroke mice. However, GW3965 treatment of ABCA1fl/fl stroke mice, but not ABCA1-/- stroke mice, exhibited increased densities of LFB+/BS+/SMI31+ in the contralateral CC and increased the densities of LFB+/BS+/SMI31+ and the number of PDGFRα+ OPC in the IBZ of the ipsilateral CC compared with ABCA1fl/fl stroke saline control mice (Figure 3A; *P <0.05, n=9/group).

Compared with intraventricular infusion of artificial CSF in ABCA1-/- stroke mice, intraventricular infusion of hHDL3 increased Syn/MBP level in the contralateral and the ischemic brain in ABCA1-/- stroke mice 14 days after stroke. A and B, Syn immunostaining and quantitative data (A; *P <0.05, n=9/group) and Syn/MBP Western blot (WB)/reverse transcriptase polymerase chain reaction (RT-PCR) assay and quantitative data (B; *P <0.05, n=6/group) in ABCA1fl/fl and ABCA1-/- stroke mice treated with saline or GW3965; C and D, Syn immunostaining and quantitative data (C; *P <0.05, n=9/group) and Syn/MBP WB/RT-PCR assay and quantitative data (D; *P <0.05, n=6/group) in ABCA1-/- stroke mice with intracerebral infusion of artificial cerebral spinal fluid (CSF) or hHDL3. Scale bar=40 μm. ABCA1 indicates ATP-binding cassette transporter A1; and MBP, myelin basic protein.
Figure 3. ABCA1−/− decreases white matter (WM) density/oligodendrocyte progenitor cell (OPC) numbers, GW3965 treatment of ABCA1+/+ mice, but not ABCA1−/− mice, increases WM density/OPC numbers in the ischemic-boundary zone (IBZ) of ipsilateral corpus collasum (CC); intraventricular infusion of human plasma HDL3 (hHDL3) significantly increased WM density/OPC numbers in both of contralateral CC and IBZ of ipsilateral CC 14 days after stroke. A, Immunostainings of LFB+ myelin/BS+ axon/SMI31+ phosphorylated neurofilament/PDGFRα+ OPCs and quantitative data in ABCA1+/+ and ABCA1−/− stroke mice treated with saline or GW3965, (Continued)
in ABCA1−B/−B stroke mice significantly increased the densities of LFB+/BS+/SMI31+ and the number of PDGFRα+ OPCs and quantitative data in ABCA1−B/−B stroke mice intraventricularly infused with artificial cerebrospinal fluid (CSF) or hDHLD, respectively. Scale bar=20 μm in LFB+ myelin and BS+ axon images, 40 μm in SMI31+ neurofilament and PDGFRα+ OPC images. *P<0.05, n=9/group. ABCA1 indicates ATP-binding cassette transporter A1; BS, Bielschowsky–Silver; LFB, Luxol–Fast–Blue; and PDGFRα, platelet-derived growth factor receptor alpha.

GW3965 Treatment Increases ABCA1/Syn Level and Neurite/Axon Outgrowth in ABCA1−B/−B PCNs, but Not in ABCA1−B/−B PCNs; HDL Attenuates the Reduction of Neurite/Axon Outgrowth in ABCA1−B/−B PCNs

The WB and reverse transcriptase polymerase chain reaction assay and quantitative data show that both the Syn protein and ABCA1 protein/mRNA levels were significantly decreased in ABCA1−B/−B primary cortical neurons (PCNs) compared with ABCA1−B/−B PCNs after oxygen and glucose deprivation (OGD). GW3965 treatment significantly increased ABCA1 protein/mRNA and Syn protein levels in ABCA1−B/−B PCNs, but not in ABCA1−B/−B PCNs, after OGD (Figure 4A; *P<0.05, n=6/group).

The neurite outgrowth measurement revealed that ABCA1−B/−B PCNs had significantly decreased neurite outgrowth after OGD compared with ABCA1−B/−B PCNs. GW3965 treatment significantly increased neurite outgrowth in ABCA1−B/−B PCNs, but not in ABCA1−B/−B PCNs, after OGD. HDL treatment not only increased neurite outgrowth in ABCA1−B/−B PCNs, but also attenuated the reduction of neurite outgrowth in ABCA1−B/−B PCNs after OGD (Figure 4B; *P<0.05, n=6/group).

ABCA1−B/−B PCNs exhibited significantly decreased axonal outgrowth compared with ABCA1−B/−B PCNs. GW3965 treatment increased the axonal outgrowth in ABCA1−B/−B PCNs, but did not do so in ABCA1−B/−B PCNs. However, HDL treatment significantly increased axonal outgrowth in both ABCA1−B/−B PCNs and ABCA1−B/−B PCNs (Figure 4C; *P<0.05, n=6/group).

Discussion

WM is composed of bundles of myelinated axons. WM remodeling (including axonal regeneration and remyelination) in the IBZ of cerebral infarcts is essential for long-term stroke recovery, and the process of remyelination is mediated by abundant OPCs located throughout the adult brain.12,13 Cholesterol is a major component of myelin, and axonal regeneration is in part dependent on local cholesterol utilization in regenerating neurons.14,15 Cholesterol is also an important component of neuronal membranes and participates in neuronal survival16 and neuronal function, such as membrane trafficking, signal transduction, and neurotransmitter release.17,18 Synaptic protein is formed from neuronal synapses, and cholesterol is essential lipid substrates for massive synaptogenesis in neurons.19,20 Synaptogenesis parallels functional recovery after cortical injury, including stroke.20

HDL cholesterol plays an important role in WM remodeling and neurological functional recovery after brain injury. Almost all of HDL cholesterol in the central nervous system is derived from in situ biosynthesis mainly by ABCA1.21,22 Mutations in the human ABCA1 gene cause HDL deficiency syndrome, that is, Tangier disease, characterized by little or virtual absence of plasma HDL and prominent cholesterol deposition in tissues, cells, and prevalent atherosclerosis.23,24 In contrast, ABCA1 transgenic mice have a significant increase in cholesterol efflux in different tissues and marked elevation in HDL cholesterol levels.25 Deficiency of ABCA1 also decreases ApoE level and impairs ApoE metabolism in brain.26,27 Both ABCA1 and ApoE play a vital role in cholesterol homeostasis, neuronal repair, and synaptic plasticity.28,29 ABCA1−B/−B mice are generated by crossing loxP-flanked (floxed) ABCA1 mice with transgenic mice expressing Cre recombinant under the control of the neuronal and glial-specific nestin promoter.30 This leads to an absence of ABCA1 in all nestin lineage cells (neral stem cells), including neurons and glia. These mice exhibit selective loss of brain ABCA1 and low level of HDL and ApoE in both brain tissue and CSF and reduced synapse and synaptic vesicle numbers in the brain.25–27 Our previous study showed that there is no significant difference in both the WM density and the number of oligodendrocytes or OPCs in the brain of young (2–3 months) nonstroke ABCA1−B/−B mice; however, ABCA1−B/−B stroke mice exhibited more severe WM damage in the ischemic brain and worse functional outcome compared with ABCA1−B/−B stroke mice 7 days after stroke.30 In the present study, we found that the middle-aged ABCA1−B/−B stroke mice exhibited decreased brain ABCA1/ApoE level, decreased WM remodeling and Syn-protein in both the contralateral and the ipsilateral ischemic brain, as well as decreased functional outcome compared with ABCA1−B/−B stroke mice 14 days after dMCAo. Using PCN cultures, both ABCA1/Syn level and the neurite/axon outgrowth significantly decreased in the ABCA1−B/−B PCNs with or without OGD condition. These data indicate that ABCA1/ApoE play an important role in both the gray/WM remodeling after stroke.

Increase of HDL functionality may have important implications for treatment and prevention of cerebral WM damage after stroke.31 The regulation of ABCA1/ApoE expression by LXRs is physiologically relevant in vivo because both ABCA1/ApoE and LXRs are expressed in neurons and glia in the brain.32–34 Activation of LXRs, which heterodimerizes with retinoid X receptors, induces the transcription of both ABCA1 and ApoE in vivo and in vitro.35,36 In addition, upregulation of ApoE via the LXR activation plays an important role in stroke recovery.37 GW3965 crosses the blood–brain barrier and stimulates expression of many target genes, including ABCA1/ApoE.6 GW3965 treatment raises HDL level in plasma, liver, and macrophages10,32,36,37 and increases expression of ABCA1/ApoE in the hippocampus and cerebral cortex and rescues hippocampus long-term synaptic plasticity in an Alzheimer’s disease mouse model.38 In the present study, we found that GW3965 treatment of ABCA1−B/−B stroke mice significantly increased the levels of brain ABCA1/ApoE and blood HDL, increased gray matter/
Figure 4. ABCA1−B/−B decreased ABCA1/Syn level and neurite/axonal outgrowth in primary cortical neurons (PCNs). GW3965 treatment increased ABCA1/Syn levels and neurite/axonal outgrowth in ABCA1fl/fl PCNs, but not in ABCA1−B/−B PCNs after oxygen and glucose deprivation (OGD); HDL attenuated ABCA1−B/−B–induced neurite/axonal outgrowth with or without hypoxic condition. A, Western blot (WB) image and quantitative data of WB and reverse transcriptase polymerase chain reaction (RT-PCR). B, Neurite outgrowth after OGD and quantitative data. C, Axonal outgrowth and quantitative data. *P<0.05, n=6/group. ABCA1 indicates ATP-binding cassette transporter A1.
WM remodeling, as well as improved functional outcome. In vitro, GW3965 treatment significantly increased ABCA1/Syn level and increased neurite/axonal outgrowth in ABCA1−/− PCNs with or without hypoxic condition. Although GW3965 treatment elevated brain ApoE level in ABCA1−/− stroke mice, however, ABCA1 deficiency abolishes GW3965 treatment–induced benefits in vivo and in vitro. In contrast, HDL supplemental treatment attenuated ABCA1−/−-induced deficits both in vivo and in vitro. These data indicate that substrate (HDL) availability is a predominant factor for neurorestoration through ABCA1/ApoE cholesterol transporters after stroke.

**Limitations**

The risk of hepatic steatosis on pharmaceutical targeting of LXR may be a particularly serious consequence in humans. However, our present study shows that GW3965 treatment did not change blood triglyceride and total cholesterol. Consistent with our study, GW3965 raises HDL without inducing hepatic steatosis and hypertriglyceridemia and do not affect hepatic insulin sensitivity in rodents. Therefore, we should carefully choose LXRs for clinical use. In the present study, we are unable to provide evidence on which cell types, neurons, astrocytes, or oligodendrocytes play a primary role in mediating ABCA1 deficient–induced WM impairment or GW3965 treatment–induced WM remodeling, which is warranted to further studies.

**Conclusions**

We demonstrated, to our knowledge for the first time, that GW3965 treatment increases ABCA1/ApoE/HDL level, promotes gray matter/WM remodeling, and improves neurological functional outcome in ABCA1−/− stroke mice 14 days after dMCAo. These beneficial responses were absent in ABCA1−/−/− stroke mice; however, intracerebral infusion of hHDL3 attenuated ABCA1 deficient–induced deficits. Our data indicate that ABCA1/ApoE/HDL signaling pathway, at least partially, con- contributed to GW3965 induced neurorestoration after stroke.

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**Disclosures**

None.

**References**


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Supplementary Methods

Lesion-volume measurement

The animal brains were fixed, paraffin-embedded and 7 sections (1mm thick) were cut. Briefly, 7 HE-sections representing the entire distal of MCA territory were subjected to a phase-contrast microscope to visualize infarcted area. Infarct volume was determined with Micro Computer Imaging Device (MCID) imaging analysis system (Imaging Research). At 14 days after ischemia, non-injured, scar, and contralateral tissue volumes were measured. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated \(^1\). A representative serial sections (B, C, D and E sections) and the actual indirect lesion volumes were presented to exclude the possible anatomical difference between genotypes \(^2\).

Histochemical and immunohisto-staining measurement

Every 10\(^{th}\) coronal 6-μm section was cut from the center of the lesion (bregma -1 mm to +1 mm), and a total 5 of sections were used for immunostaining. Histochemical-staining for Bielschowsky-silver (BS, an axon marker) and Luxol-Fast-Blue (LFB, a myelin marker), immunostaining was obtained for SMI31 (a marker of phosphorylated-neurofilament, 1:1000, Covance), platelet-derived growth factor receptor alpha (PDGFRα, a marker of oligodendrocyte progenitor cell-OPCs, 1:100, Chemicon) and Synaptophysin (Syn, a plenty of presynaptic protein, herein, as a marker of gray-matter, 1:1000, Chemicon).
Images were acquired at 40× magnification from a total five slides from each brain, with each slide containing 4 fields of view from the ischemic-boundary-zone (IBZ) of the cortex for Syn measurement or from the IBZ of corpus-callosum (CC) for BS/LFB/SMI31/PDGFRα measurements (Figure 1A). The of Syn+/BS+/LFB+/SMI31+ -densities and the PDGFRα+ -OPC numbers in the IBZ were analyzed using MCID.

**RT-PCR assay**

The ipsilateral-ischemic brain tissue and the homologous contralateral-brain tissue (Figure 1A) were isolated, and PCN-cultures derived from ABCA1fl/fl and ABCA1−/−B−B were harvested. Total RNA was isolated and quantitative-PCR was performed in the ABI Prism 7000 sequence detection system, using the Quantitec SYBY Green PCR kit (Qiagen). The following primers were designed using Primer Express software (ABI). **GAPDH**: Fwd, AGAACATCATCCCTGCATCC; Rev: CACATTGGGGGTAGGAACAC. **ABCA1**: Fwd, GCTACCCACCCTACGAACAA; Rev, GGAGTTGGATAACGGGAAC. **ApoE**: Fwd, GAGGAACAGACCCAGCAAATAC; Rev: CAGAGGCCTGTATCTTCTCCAT. **Myelin basic protein (MBP)**: Fwd, ATGGCATCACAGAAGAGACCCTCA; Rev: TAA AGAAGCGCCCGATGGAGTCAA.

**WB assay**

Equal amounts of tissue and cell lysate were used for WB and the following primary antibodies were used: anti-ABCA1 (1:1000, NB400-105, Novus), anti-ApoE (1:1000, ab20874, Abcam), anti-MBP (1:500, MAB386, Millipore), anti-Syn (1:5000, clone SY38, Millipore), and anti-β-actin (1:10000; ab6276, Abcam).
Primary cortical neuron (PCN) culture, ABCA1/Syn level and neurite/axonal outgrowth measurements

Pregnant ABCA1^{fl/fl}-mice were euthanized and E15 embryos were harvested. Genotyping was performed to identify ABCA1^{-B/-B} or ABCA1^{fl/fl}. The PCNs were isolated from both ABCA1^{fl/fl} and ABCA1^{-B/-B} embryos and were cultured separately.

First, we tested whether ABCA1^{-B/-B} decreases ABCA1/Syn level, and whether GW3965-treatment increases ABCA1/Syn expression in PCNs after stroke. To mimic ischemia in vivo, on day-in-vitro (DIV) 3, the PCN-cultures were subjected to 2 hours of oxygen and glucose deprivation (OGD) followed by 24 hours of reperfusion. The OGD-PCNs were randomly divided into (n=6 well/group): 1) non-treatment for control; 2) GW3965-treatment (5µM) for 24 hours. The PCN cultures were collected for WB and RT-PCR assay.

Second, we tested whether ABCA1^{-B/-B} decreases neurite-outgrowth and whether ABCA1/HDL mediates GW3965-treatment induced neurite-outgrowth after stroke, the OGD-PCNs (DIV4) were divided (n=6 well/group): 1) non-treatment; 2) GW3965 5µM; 3) HDL 80µg/ml (human plasma HDL, Calbiochem) for 24 hours. The PCN cultures were then stained with TUJ1 (a phenotypic marker of neural cells, 1:1000, Covance) with Cy3 and photographed using a 10× objective fluorescent microscope (Zeiss). The average length of the 20 longest neurites in each well was calculated.

Third, to further elucidate whether ABCA1^{-B/-B} decreases axonal-outgrowth and whether ABCA1/HDL mediates GW3965-treatment induced axonal-outgrowth, the axonal-outgrowth in the ABCA1^{fl/fl}-PCN and ABCA1^{-B/-B}-PCN cultures were measured by using a microfluidic axonal-outgrowth model (Standard Neuron Device, Xona...
Microfluidics)⁴. On DIV3, the PCN-cultures were divided (n=6 wells/group): 1) non-treatment; 2) GW3965 5µM; 3) HDL 80µg/ml. The PCNs were allowed to grow for DIV5, and the axonal-outgrowth was measured as above.
Supplemental References


Supplemental Figure I. No changes in the lesion volume and the blood level of triglyceride and total cholesterol among the four groups. ABCA1^{B/B} decreases functional outcome. GW3965 treatment increases blood level of HDL and improves functional outcome in ABCA1^{fl/fl}-stroke mice, but not in ABCA1^{B/B}-stroke mice, 7 and 14 days after dMCAo. A: Representative serial sections (from top to bottom: ABCA1^{fl/fl}+saline, ABCA1
-B/-B + saline ABCA1^{fl/fl}+GW3965 and ABCA1^{-B/-B}+GW3965) and the quantitative data of actual indirect lesion volumes; **B**: Blood levels of HDL, triglyceride and total cholesterol; **C**: functional outcome. *p<0.05, n=9/group.
Supplemental Figure II. Intraventricular-infusion of hHDL3 significantly increased functional-outcome in ABCA1^{−/−}/B-stroke mice compared with ABCA1^{−/−}/B-stroke mice intraventricularly-infused with artificial-CSF 14 days after dMCAo. A: Representative B to E sections (top line: ABCA1^{−/−}/B+CSF; bottom line: ABCA1^{−/−}/B+hHDL3) and the
quantitative data of actual indirect lesion volumes; **B:** Blood levels of HDL, triglyceride and total cholesterol; **C:** functional outcome. *p<0.05, n=9/group.
# Stroke Online Supplement

## Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
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<tbody>
<tr>
<td>Experimental groups and study timeline</td>
<td>The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. The account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated. An overall study timeline is provided.</td>
</tr>
<tr>
<td>Inclusion and exclusion criteria</td>
<td>A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article.</td>
</tr>
<tr>
<td>Randomization</td>
<td>Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided. Types and methods of randomization have been described. Methods used for allocation concealment have been reported.</td>
</tr>
<tr>
<td>Blinding</td>
<td>Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible. Blinding procedures have been described with regard to masking of group assignment during outcome assessment.</td>
</tr>
<tr>
<td>Sample size and power calculations</td>
<td>Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided.</td>
</tr>
<tr>
<td>Data reporting and statistical methods</td>
<td>Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. Baseline data on assessed outcome(s) for all experimental groups have been reported. Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. Statistical methods used have been reported. Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures.</td>
</tr>
<tr>
<td>Experimental details, ethics, and funding statements</td>
<td>Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesics, temperature control during experimentation, and postprocedural monitoring have been described. Different sex animals have been used. If not, the reason/justification is provided. Statements on approval by ethics boards and ethical conduct of studies have been provided. Statements on funding and conflicts of interests have been provided.</td>
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