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Stroke. Author manuscript; available in PMC 2014 February 25.

Published in final edited form as:
Stroke. 2013 August ; 44(8): 2284–2291. doi:10.1161/STROKEAHA.113.001320.

Lipoprotein Receptor–Related Protein-6 Protects the Brain From Ischemic Injury

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Abstract

Background and Purpose—Loss-of-function mutations of the lipoprotein receptor–related protein-6 (LRP6), a coreceptor in the Wingless-related integration site-β–catenin prosurvival pathway, have been implicated in myocardial ischemia and neurodegeneration. However, it remains to be established whether LRP6 is also involved in ischemic brain injury. We used $LRP6^{+/-}$ mice to examine the role of this receptor in the mechanisms of focal cerebral ischemia.

Methods—Focal cerebral ischemia was induced by transient occlusion of the middle cerebral artery. Motor deficits and infarct volume were assessed 3 days later. Glycogen-synthase-kinase-3β (GSK-3β) phosphorylation was examined by Western blotting with phosphospecific antibodies, and the mitochondrial membrane potential changes induced by Ca^{2+} were also assessed.

Results—$LRP6^{+/-}$ mice have larger stroke and more severe motor deficits, effects that were independent of intraischemic cerebral blood flow, vascular factors, or cytosolic β-catenin levels. Rather, LRP6 haploinsufficiency increased the activating phosphorylation and decreased the inhibitory phosphorylation of GSK-3β, a kinase involved in proinflammatory signaling and mitochondrial dysfunction. Accordingly, postischemic inflammatory gene expression was enhanced in $LRP6^{+/-}$ mice. Furthermore, the association of mitochondria with activated GSK-3β was increased in $LRP6^{+/-}$ mice, resulting in a reduction in the Ca^{2+} handling ability of mitochondria. The mitochondrial dysfunction was reversed by pharmacological inhibition of GSK-3β.

Conclusions—LRP6 activates an endogenous neuroprotective pathway that acts independently of β-catenin by controlling GSK-3β activity and preventing its deleterious mitochondrial and proinflammatory effects. The findings raise the possibility that emerging treatment strategies for diseases attributable to LRP6 loss-of-function mutations could also lead to new therapeutic avenues for ischemic stroke.

Keywords

cerebral ischemia; glycogen-synthase-kinase-3; mitochondria; stroke; Wnt signaling pathway

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Disclosures None.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.113.001320/-/DC1.

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The Wnt pathway plays a central role in the development of the central nervous system during embryogenesis. Binding of wingless-related integration site (Wnt), a family of cysteine-rich proteins, to the membrane receptor Frizzled and low-density lipoprotein receptor–related protein (LRP) coreceptors initiates a signaling cascade that leads to nuclear β-catenin expression and induction of specific transcriptional programs (canonical Wnt pathway), release of intracellular Ca^{2+} stores (Ca^{2+} pathway), and cytoskeletal reorganization (planar cell polarity pathway).1 In addition to its role in development, Wnt signaling has been implicated in a variety of pathological conditions, including cancer, myocardial ischemia, and neurodegenerative diseases.1 Binding of Wnt to Frizzled induces the formation of a ternary complex with LRP5/6 and tethering of casein kinase 1 to the complex, resulting in phosphorylation and activation of Dishevelled and subsequent inhibition of glycogen-synthase-kinase-3β (GSK-3β). Reduced GSK-3β activity results in stabilization of β-catenin and activation of the canonical Wnt pathway by allowing β-catenin to escape proteasomal degradation and initiate expression of protective genes after binding to T-cell factor/lymphoid enhancer factor transcription factors.1 Besides β-catenin, GSK-3β has been reported to phosphorylate >40 proteins.2 It seems that in the context of Wnt signaling, ≥2 pools of GSK-3β exist. One pool is associated with the β-catenin destruction complex, whereas the other is independent of β-catenin and regulates mammalian target of rapamycin and transforming growth factor-β/Smad1 signaling,3,4 as well as the mitochondrial permeability transition.5

Maintaining mitochondrial function during ischemic stress is essential for neuronal survival. 6 Mitochondrial function is tied to the preservation of the mitochondrial transmembrane potential ($\Delta\Psi_m$), which is essential for oxidative phosphorylation to occur. The collapse of $\Delta\Psi_m$ through the opening of the mitochondrial permeability transition pore (mPTP) constitutes a central step in ischemic cell injury and the subsequent induction of apoptosis.7 The mPTP is a multiprotein complex whose activity is controlled by several molecular signals and upstream protein kinases, including GSK-3β. Augmented GSK-3β activity results in enhanced mPTP permeability, potentially leading to the collapse of $\Delta\Psi_m$.

The Wnt pathway has recently been linked to neuronal cell death after cerebral ischemia.8,9 Although modulation of Frizzled has been implicated in cell survival through β-catenin signaling,10 the role of the Wnt coreceptor LRP6, previously implicated in Alzheimer disease11 and in a hereditary form of coronary artery disease,12 remains to be established. Here, we set out to investigate the role of LRP6 in cerebral ischemic injury in mice. We found that LRP6 haploinsufficiency resulted in increased ischemic injury through GSK-3β–mediated impairment of mitochondrial function and upregulation of postischemic inflammatory gene expression. The data identify LRP6 as a critical component of a potent endogenous neuroprotective pathway that acts by suppressing the deleterious effects of GSK-3β, and could be the presumptive target of preventive or therapeutic interventions in stroke.

Material and Methods

A detailed description of the methods is provided in the online-only Data Supplement.

Mice

All procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Experiments were performed in 2- to 3-month-old male $LRP6^{+/-}$ mice13 (weight, 20–24 g), obtained from in-house colonies. The mice were kept on a C3H/HeJ background. We used heterozygous mice because LRP6−/− mice are not viable.13 Wild-type siblings were used as wild-type controls.

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Middle Cerebral Artery Occlusion

Procedures for transient middle cerebral artery (MCA) occlusion were identical to those previously described.14–16 Under isoflurane anesthesia with cerebral blood flow (CBF) monitoring, the MCA was occluded for 40 minutes. Only animals that exhibited a 85% reduction in CBF during MCA occlusion (MCAo) and in which CBF recovered by 80% after 10 minutes of reperfusion were included in the study.15–17 The number of excluded animals was not significantly different in $LRP6^{+/+}$ and $LRP6^{+/-}$ groups (12% versus 17%; $P>0.05$), and the survival rate after MCAo was comparable (83% in $LRP6^{+/+}$ versus 87% in $LRP6^{+/-}$; $P>0.05$).

Assessment of Motor Function and Measurement of Infarct Volume

Motor function and edema-corrected infarct volume were assessed by an investigator blinded to the experimental groups on day 3 after reperfusion. Motor function was assessed by the hanging wire test, as described in detail elsewhere.15 Infarct volumes were determined in cresyl violet–stained brain sections as described.15–17

Cerebrovascular Regulation

Procedures for testing cerebrovascular regulation in mice have been described previously.18 After cannulation of the femoral artery, tracheal intubation, and artificial ventilation under isoflurane, anesthesia was maintained with urethane and chloralose. The somatosensory cortex was exposed through a small craniotomy (2×2 mm). The dura was removed, and the site was superfused with a modified Ringer solution. CBF was continuously monitored with a laser-Doppler probe, positioned stereotaxically on the somatosensory cortex, a region supplied by the MCA. CBF changes were expressed as percentage increase relative to the resting level. To study the increase in CBF produced by neural activity (functional hyperemia), the somatosensory cortex was activated by gently stroking the contralateral whiskers with a cotton-tipped applicator. Endothelium-dependent vasodilation was tested by topical superfusion of acetylcholine (10 μmol/L) or bradykinin (50 μmol/L) for 3 to 5 minutes, and the CBF increase was recorded. The CBF response to adenosine (400 μmol/L), an agent that produces vasodilation by acting directly on vascular smooth muscles, was also tested.

Real-Time Polymerase Chain Reaction

The mRNA for proinflammatory genes was examined after ischemia using quantitative real-time polymerase chain reaction as previously described.16,19 A group of genes that have been previously shown to be regulated after MCAo and have been implicated in neuronal cell death and postischemic inflammation were included in the mRNA expression screen. Mice were euthanized 0, 2, 6, 24, and 72 hours after ischemia. Primer details are presented in Table 1 in the online-only Data Supplement.

Double-Label Immunohistochemistry

For identification of the cell types expressing LRP6, paraformaldehyde-fixed brain sections were first processed for LRP6 immunocytochemistry, followed by incubation with antibodies against the neuronal marker NeuN, the endothelial cell marker CD31, the astrocytic marker glial fibrillary acidic protein or the microglial marker ionized calcium–binding adaptor molecule 1. Images of double-labeled neocortex were sequentially acquired using confocal laser-scanning microscope.

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Western Blot Analysis

In GSK-3β phosphorylation studies, mice were euthanized 0, 1, 3, 6 hours after ischemia-reperfusion. The cortex of the ischemic hemisphere was sampled, and equal amount of proteins were gel-separated and transferred to polyvinylidene fluoride membranes. The blots were probed with phospho-GSK-3β-Ser9 or phospho-GSK-3β-Y216–specific antibodies and reprobed with pan-GSK-3β antibody or β-actin–specific antibody to verify equal loading. For β-catenin detection, cytosolic protein lysates were prepared from the cortex of right hemisphere at each time point and analyzed by Western blotting with antiβ-catenin antibodies.

Mitochondrial Transmembrane Potential

Crude mitochondria from mouse brain were isolated as previously described.6 The mitochondria were used for experiments within 4 hours. $\Delta\Psi_m$ was measured according to the method of Liu et al20 using safranine O as a probe as previously described.6 Changes in $\Delta\Psi_m$ were induced by adding 50 nmol aliquots of $CaCl_2$. In some experiments, LRP6$^{+/+}$ and +/− mice were treated with the GSK-3β inhibitor SB216763 (1 mg/kg; IP)[21] for 16 hours. The effectiveness and specificity of the dose of SB216763 has been previously documented. 21

Statistical Analysis

Data are presented as mean±SEM. Comparisons between 2 groups were statistically evaluated by the Student *t* test. Multiple comparisons were evaluated by ANOVA followed by the Newman–Keuls test. Differences were considered significant for $P<0.05$.

Results

LRP6 Is Localized to Neurons in the Mouse Neocortex

First, we sought to determine the cellular localization of LRP6 in the adult mouse neocortex. LRP6 immunoreactivity was observed throughout the neocortex clearly demarcating the plasma membrane outlining the somata of large cells consistent with a predominantly plasmalemmal localization of the receptor (Figure 1A). We found that LRP6 was colocalized with the neuronal marker NeuN, but not with the astroglial marker glial fibrillary acidic protein or the microglial marker ionized calcium–binding adaptor molecule 1 (Figure 1). Similarly, LRP6 was not observed in CD31-positive endothelial cells (Figure 1). Therefore, in the mouse neocortex, LRP6 is localized to the plasma membrane of neurons.

LRP6$^{+/-}$ Mice Have Larger Infarcts and More Severe Motor Deficits After MCAo

Next, we examined the role of LRP6 in the brain injury 72 hours after transient MCAo. The infarct was larger in LRP6$^{+/-}$ mice, the expansion involving both the neocortex and the basal ganglia (Figure 2A, 2C, and 2D). The motor deficits induced by MCAo, as reflected by the latency to fall at the hanging wire test,22 were more severe in LRP6$^{+/-}$ than in LRP6$^{+/+}$ mice (Figure 2B). The increase in infarct volume could not be attributed to differences in the degree of ischemia because the reduction in CBF was comparable in the center and periphery of the ischemic territory in LRP6$^{+/+}$ and LRP6$^{+/-}$ mice (Figure 3A, and 3B). Furthermore, no differences in arterial pressure were observed between LRP6$^{+/+}$ and LRP6$^{+/-}$ mice during and after MCAo (Figure 3C). Similarly, plasma glucose did not differ between LRP6$^{+/-}$ (204±14 mg/dL) and LRP6$^{+/+}$ mice (190±35 mg/dL; $P>0.05$; n=10/group). Next, we examined whether defects in neurovascular regulation could play a role in the increased ischemic injury observed in LRP6$^{+/-}$ mice. In anesthetized mice, the increase in CBF produced by whisker stimulation, by the endothelium-dependent vasodilators

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acetylcholine and bradykinin or the smooth muscle relaxant adenosine was comparable between LRP6$^{+/+}$ and LRP6$^{+/-}$ mice (Figure 3D). Therefore, the increased stroke volume in LRP6$^{+/-}$ cannot be attributed to alterations in cerebrovascular regulation.

GSK-3β Phosphorylation Is Altered in LRP6$^{+/-}$ Mice

GSK-3β, a kinase regulated by activating (Y216) and inhibitory (Ser9) phosphorylation,23 is a critical component of the LRP6-Wnt signaling pathway. Because GSK-3β activation is deleterious to the brain24,25 and LRP6 inhibits GSK-3β activity,26,27 we examined the phosphorylation state of GSK-3β in LRP6$^{+/-}$ mice. After MCAo in LRP6$^{+/+}$ mice, GSK-3β activating phosphorylation (pGSK3-Y216) in the ischemic lesion remained stable, whereas the inhibitory phosphorylation (pGSK3-Ser9) increased at 1 hour and returned to normal by 3 hours (Figure 4A and 4B). In sham-operated LRP6$^{+/-}$ mice, pGSK3-Ser9 was reduced, whereas pGSK3-Y216 was increased (Figure 4A and 4B). After MCAo in LRP6$^{+/-}$ mice, the pGSK3-Y216 was not different from LRP6$^{+/+}$ mice (Figure 4A), whereas pGSK3-Ser9 was suppressed compared with LRP6$^{+/+}$ mice (Figure 4B). These observations are consistent with the hypothesis that GSK-3β activity is increased in LRP6$^{+/-}$ mice.

Mitochondrial translocation of GSK-3β is associated with mitochondrial dysfunction and injury.5,28 Therefore, we examined the association of GSK-3β with mitochondria and its phosphorylation state. In sham-operated mice, pGSK3-Y216 associated with mitochondria tended to be more abundant in LRP6$^{+/-}$ mice than in LRP6$^{+/+}$ mice (Figure 4C). An increase was also observed 6 hours after MCAo, but no differences were seen at 24 hours (Figure 4C).

β-Catenin Levels Are Not Reduced in LRP6$^{+/-}$ Mice

β-catenin is a key signaling molecule in the LRP6-Wnt pathway, which has a protective role in cerebral ischemic injury.9 Phosphorylation of β-catenin by GSK-3β leads to its degradation and loss of its prosurvival effects.23 Therefore, we examined whether the increased injury in LRP6$^{+/-}$ mice is related to suppression of β-catenin expression. β-Catenin protein levels were not significantly reduced in LRP6$^{+/-}$ mice, either before or after ischemia (Figure 4D), indicating that increased degradation of this protein is unlikely to play a role in the increased injury in LRP6$^{+/-}$ mice.

Postischemic Expression of Inflammatory Genes Is Enhanced in LRP6$^{+/-}$ Mice

GSK-3β activation is well known to promote inflammatory signaling.2 Therefore, we examined whether the exacerbation of the ischemic injury in LRP6$^{+/-}$ mice was related to enhancement of postischemic inflammation (Figure 5). Cyclooxygenase-2 and prostaglandin E synthase, which are mainly localized to neurons,29,30 were markedly upregulated in LRP6$^{+/-}$ mice. The upregulation of the chemokine monocyte chemotactic protein-1, and of Rac2, a small guanosine triphosphatase enriched in neutrophils, was increased in LRP6$^{+/-}$ mice (Figure 5). Endothelial-leukocyte adhesion molecule 1 expression was significantly higher in LRP6$^{+/-}$ mice, while intercellular adhesion molecule 1 showed the same trend without reaching significance. In contrast, the upregulation of the cytokine interleukin-1β and tumor necrosis factor, the chemokine IP-10, and of the NOX2 subunit of the superoxide-producing enzyme NADPH oxidase did not differ between LRP6$^{+/+}$ and LRP6$^{+/-}$ mice (Figure 5). As anticipated, LRP6 mRNA expression was reduced in sham-operated LRP6$^{+/-}$ mice (Figure 5).

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Mitochondria of LRP6$^{+/-}$ Mice Are More Susceptible to Ca^{2+}-Induced Depolarization, an Effect Mediated by GSK-3β

Mitochondrial localization of GSK-3β may increase their susceptibility to injury.31 Therefore, we examined whether the mitochondria of LRP6$^{+/-}$ mice are more vulnerable to the loss of $\Delta\Psi_m$ induced by Ca.$^{2+}$ In isolated mitochondrial preparations of LRP6$^{+/+}$ mice, administrations of Ca^{2+} aliquots ($CaCl_2$; 50 nmol) produced transient mitochondrial depolarizations, which fully recovered up to the third or fourth aliquot administered, at which point $\Delta\Psi_m$ remained elevated after the Ca^{2+} challenge (Figure 6A). In contrast, LRP6$^{+/-}$ mitochondria were unable to maintain $\Delta\Psi_m$ and, subsequent to the first Ca^{2+} challenge, they progressively became more depolarized (Figure 6A). Administration of the GSK-3β inhibitor SB216763, before mitochondria isolation, did not affect mitochondrial depolarization in LRP6$^{+/+}$ mice, but it reversed the increased Ca^{2+}-induced depolarization in LRP6$^{+/-}$ mice (Figure 6B).

Discussion

We have demonstrated that LRP6 haploinsufficiency exacerbates the brain injury and motor deficits produced by MCAo. The effect was associated with increased phosphorylation of GSK-3β at the activating site (Y206) and reduced phosphorylation at the inhibitory site (Ser9), suggesting increased GSK-3β activity. β-Catenin levels were not reduced in LRP6$^{+/-}$ mice, but MCAo results in an enhancement of the expression of inflammatory genes, well known to contribute to ischemic brain injury.32 In addition, consistent with an increase in p-GSK-3β targeted to mitochondria, resistance to Ca^{2+}-induced depolarization was reduced in LRP6$^{+/-}$ mitochondria, an effect reversed by the GSK-3β inhibitor SB216763. Collectively, the data indicate that LRP6 haploinsufficiency leads to activation of GSK-3β, which exacerbates ischemic injury by promoting postischemic inflammation and reducing mitochondrial resistance to stress. Therefore, neuronal LRP6 may act as an endogenous neuroprotective receptor that seeks to maintain the homeostasis of the postischemic brain by suppressing GSK-3β activity.

The enhancement of injury observed in LRP6$^{+/-}$ mice could not be attributed to an increase in the intensity of the ischemic insult because the reduction in CBF produced by MCAo in the center and periphery of the ischemic territory was comparable in LRP6$^{+/+}$ and LRP6$^{+/-}$ mice. Similarly, alterations in the ability of cerebral blood vessels to increase CBF in response to vasoactive stimuli are unlikely to play a role in the increased injury because the reduction in CBF produced by MCAo and the increase in CBF produced by functional hyperemia and endothelium-dependent relaxing agents did not differ between LRP6$^{+/+}$ and LRP6$^{+/-}$ mice.

Given its predominant neuronal localization, we hypothesized that LRP6 haploinsufficiency might contribute to ischemic neuronal injury by decreasing canonical Wnt signaling, resulting in reduced β-catenin activity. However, cytosolic β-catenin levels were not different in LRP6$^{+/-}$ mice before or after cerebral ischemia, although activating GSK-3β-Y216 phosphorylation was increased at the same time points. We, therefore, investigated whether other downstream targets of the GSK-3β pathway could potentially account for the increased ischemic brain injury observed in LRP6$^{+/-}$ mice.

In addition to its role in the Wnt signaling pathway, GSK-3β has been linked with enhanced proinflammatory gene transcription and impaired mitochondrial function.2,5 Analysis of a panel of proinflammatory genes shown to be involved in neuronal injury and postischemic inflammation revealed a gene-specific effect of LRP6 expression. Genes associated with neuronal injury, such as cyclooxygenase-2 and Ptges,29,30 were upregulated in LRP6$^{+/-}$ mice undergoing MCAo. Similarly, we observed increased expression of the chemokine

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monocyte chemotactic protein-1 and increased presence of Rac2 transcripts, possibly indicating greater leukocyte infiltration and larger infarcts in $LRP6^{+/-}$ mice 72 hours after ischemia-reperfusion. Expression of endothelial adhesion molecules, endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1, was moderately increased, whereas tumor necrosis factor and interleukin-1β mRNA levels were not different in $LRP6^{+/+}$ and $LRP6^{+/-}$ animals. The apparent selectivity of LRP6-dependent postischemic inflammatory gene expression could be indicative of a divergent role of GSK-3β in regulating gene expression in different inflammatory models, having suppressive or enhancing activities depending on the inflammatory cascades involved.33 To more definitively establish the involvement, the role of neuronal GSK-3β in postischemic inflammation mice with conditional deletion of neuronal GSK-3β would be needed.

Elevated GSK-3β activity has been shown to decrease mitochondrial $\Delta\Psi_m$ stability by reducing the threshold for mPTP opening.5 The fact that mitochondria isolated from the cerebral cortex of $LRP6^{+/-}$ mice showed less tolerance for Ca^{2+}-induced $\Delta\Psi_m$ alterations is consistent with a decreased mPTP threshold, an effect that was dependent on increased GSK-3β activity as shown by its reversal after treatment with the GSK-3β inhibitor SB216763. Therefore, loss of mitochondrial integrity is also likely to contribute to the enhanced cerebral ischemic injury in $LRP6^{+/-}$ mice. Mitochondria dysfunction could lead to brain damage injury through different mechanisms, including, for example, release of proapoptotic factors, oxidative stress, and energy failure.34 The specific mechanisms through which mitochondrial dysfunction increases the susceptibility to ischemic injury in $LRP6^{+/-}$ mice remain to be established.

LRP6 mutations are involved in several human diseases, including osteoarthritis, premature osteoporosis, coronary artery disease, and Alzheimer disease.35 The present results raise the possibility that LRP6 loss-of-function mutations may also increase the susceptibility to ischemic stroke. Although the clinical association with stroke remains to be established, several approaches to modulate LRP5 and 6 signaling are under development, especially for bone diseases,35 and could also benefit patients at risk for ischemic stroke. A limitation of this approach is that excessive or dysregulated activation of Wnt signaling could also lead to pathology.36 Therefore, approaches to provide tight control of the activation would be needed.37

In summary, LRP6 haploinsufficiency leads to exacerbation of ischemic brain injury. The effect is independent of vascular factors or suppression of β-catenin levels, but is related to increased GSK-3β activating phosphorylation and mitochondrial association of GSK-3β, leading to increased inflammatory gene expression and mitochondrial dysfunction, respectively. The data unveil a novel role of LRP6 in neuronal resistance to injury and raise the possibility that loss-of-function mutations of LRP6, in addition to promoting myocardial ischemia and Alzheimer diseases, may also increase the propensity to ischemic stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health grants NS34179, NS73666, and HD067244.

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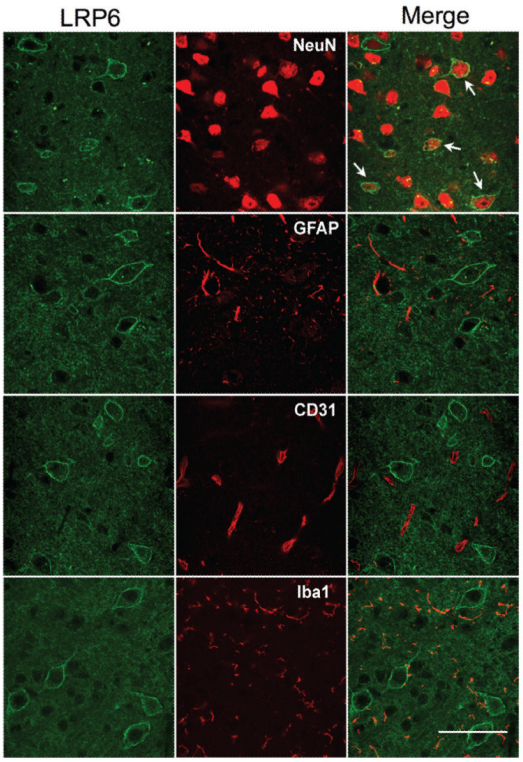

Figure 1.

Lipoprotein receptor–related protein-6 (LRP6) is localized to neurons in the adult mouse cerebral cortex. Representative micrographs (n=4) of mouse cortex stained with antibodies specific for LRP6, NeuN (neurons), glial fibrillary acidic protein (GFAP; astrocytes), CD31 (endothelium), and ionized calcium–binding adaptor molecule 1 (Iba1; microglia). Scale bar=50 μm.

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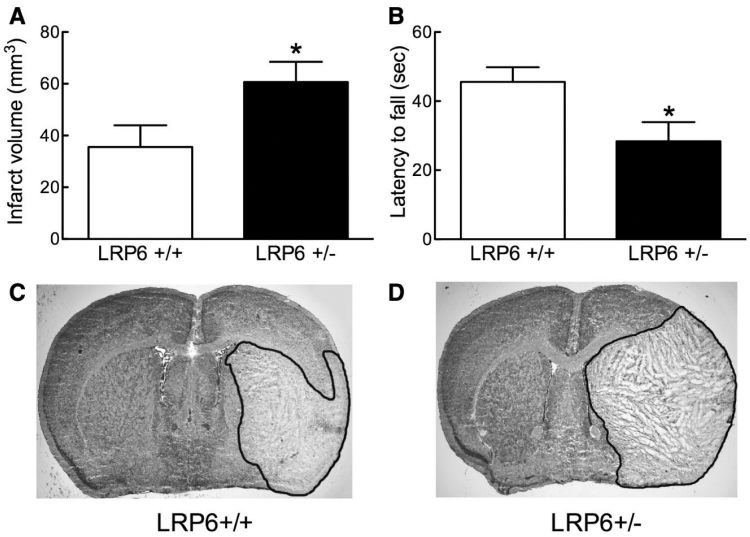

Figure 2.

Increased infarct volumes and impaired motor function in lipoprotein receptor–related protein-6 (LRP6) $^{+/-}$ mice. **A**, Edema-corrected infarct volume was determined 72 hours after MCAo (n=8/group); **P*<0.05 Student *t* test. **B**, The Hanging Wire Test was applied 72 hours after tMCAo (n=9/group); **P*<0.05 Student *t* test. **C** and **D**, Representative Nissl-stained brain sections derived from animals analyzed in **A**.

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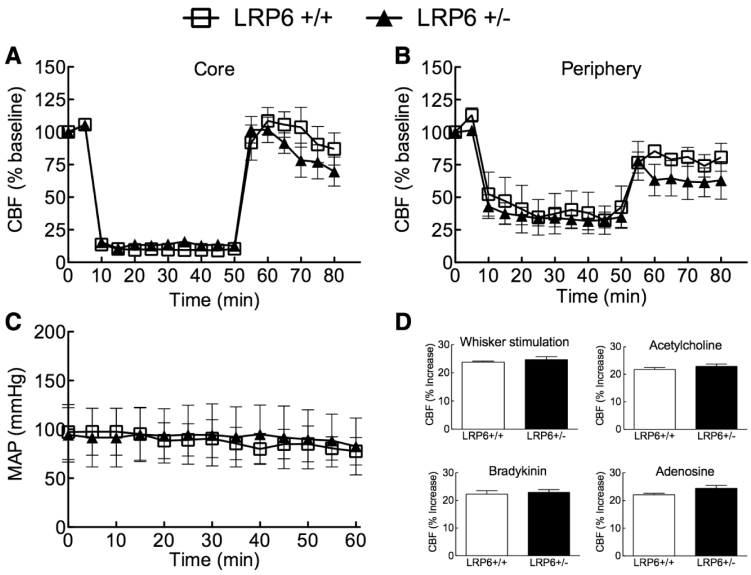

Figure 3.

Cerebral perfusion and cerebrovascular regulation are not compromised in lipoprotein receptor–related protein-6 (LRP6)$^{+/-}$ mice. Core (**A**) and penumbral (**B**) blood flow during and after middle cerebral artery occlusion (MCAo; n=5/group). **C**, Intraarterial blood pressure recordings in mice undergoing MCAo (n=5/group). **D**, Cerebral blood flow (CBF) responses in LRP6$^{+/+}$ or LRP6$^{+/-}$ mice undergoing whisker stimulation or topical application of acetylcholine, bradykinin, or adenosine. Significance was tested by ANOVA followed by the Newman–Keuls test (**A**–**C**) or Student *t* test (**D**). MAP indicates mean arterial pressure.

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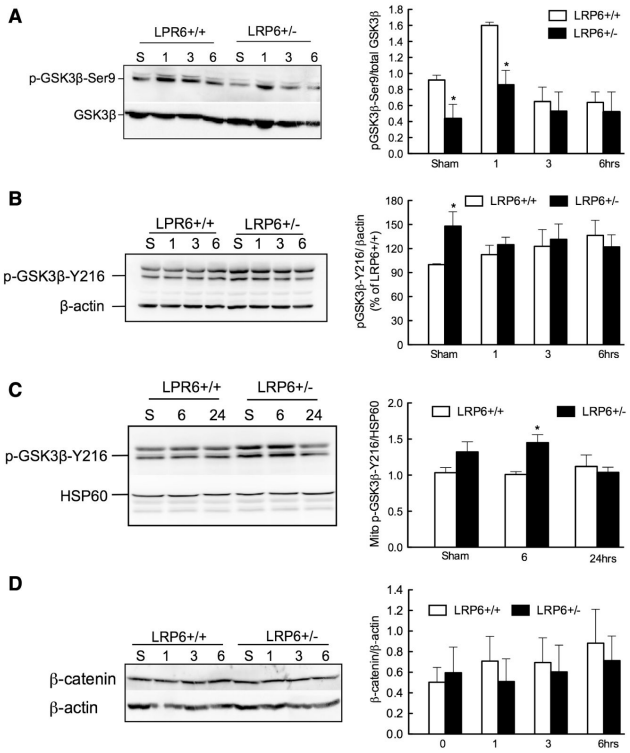

Figure 4.

Increased cerebral glycogensynthase-kinase (GSK)-3β activity in lipoprotein receptor–related protein-6 (LRP6)$^{+/-}$ mice. **A**, Western blot analysis of inhibitory GSK-3β-Ser9 phosphorylation; quantification of p-GSK-3β-Ser9 signals after normalization by total-GSK-3β signals of the same sample (**right**, n=4). **B**, Western blot analysis of activating GSK-3β-Y216 phosphorylation; quantification of p-GSK-3β-Y216 signals after normalization by β-actin signals of the same sample (**right**, n=4). **C**, Western blot analysis of mitochondrial GSK-3β-Y216 phosphorylation; quantification of p-GSK-3β-Y216 signals after normalization by heat shock protein 60 (HSP60) signals of the same sample (**right**, n=4). **D**, Western blot analysis of cytoplasmic β-catenin levels; quantification of β-catenin signals after normalization by β-actin signals of the same sample (**right**, n=4). *P<0.05 (ANOVA and Newman–Keuls test).

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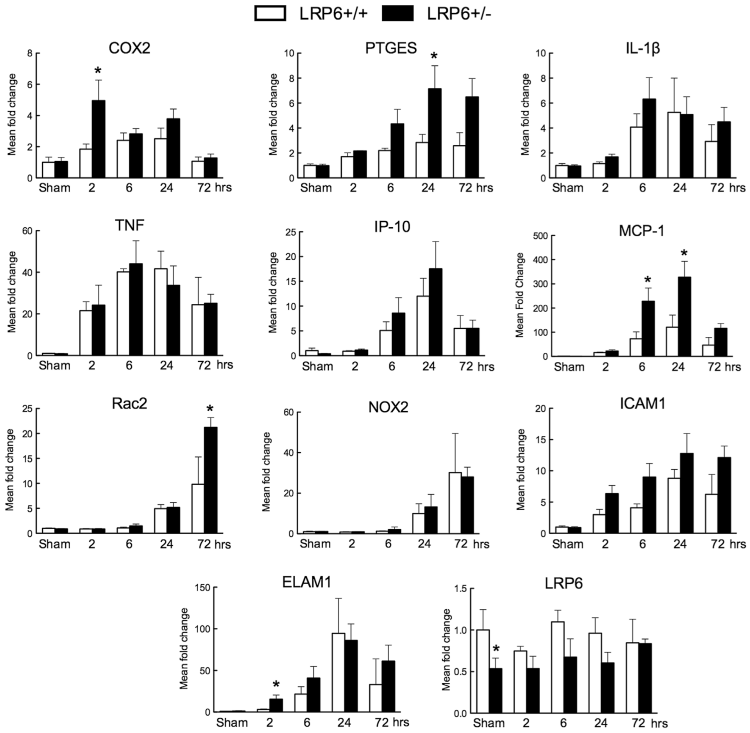

Figure 5.

Inflammatory marker genes are increased in the cerebral cortex of lipoprotein receptor–related protein-6 (LRP6)$^{+/-}$ mice after middle cerebral artery occlusion (MCAo). mRNA expression of cyclooxygenase-2 (COX-2), prostaglandin E synthase (PTGES) interleukin (IL)-1β, tumor necrosis factor (TNF), IP-10, monocyte chemotactic protein-1 (MCP-1), Rac2, NOX2, intercellular adhesion molecule 1 (ICAM1), endothelial-leukocyte adhesion molecule 1 (ELAM1), and LRP6 were analyzed by quantitative real-time polymerase chain reaction 2, 6, 24, and 72 hours after reperfusion. Mean fold increase was calculated relative to sham-operated LRP6$^{+/+}$ animals (n=4–7/group). *P<0.05 from LRP6$^{+/+}$ mice at the same time point (Student t test).

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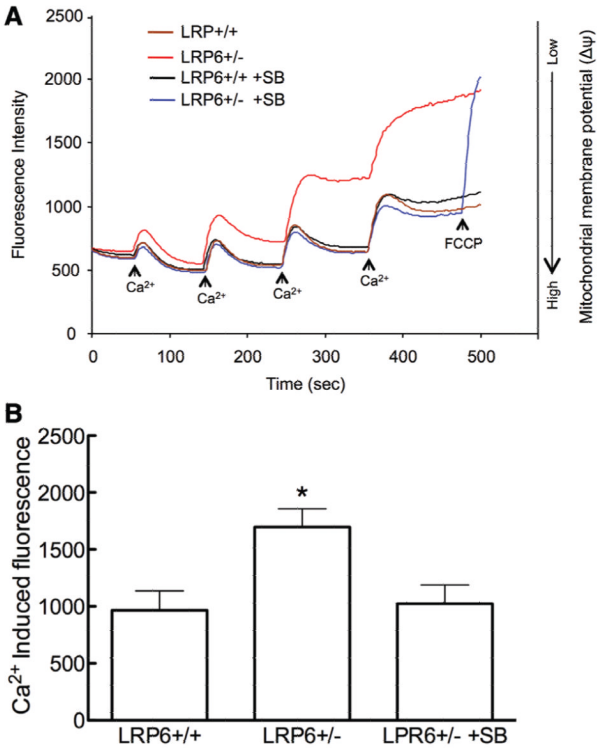

Figure 6.

Increased glycogen-synthase-kinase (GSK)-3β activity impairs mitochondrial transmembrane potential in lipoprotein receptor–related protein-6 (LRP6)$^{+/-}$ mice. **A**, Representative traces of Ca^{2+}-induced mitochondrial membrane depolarization. Mitochondria isolated from the cerebral cortex of LRP6$^{+/+}$ or LRP6$^{+/-}$ mice treated with vehicle or the GSK-3β inhibitor SB216763 were exposed to increasing Ca^{2+} concentrations, and safranine O fluorescence was recorded. Addition of the uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 0.15 μm) to mitochondria from LRP6$^{+/-}$ mice treated with SB216763 led to complete depolarization attesting to the integrity of the preparation. Traces shown are representative of 3 independent experiments with pooled mitochondria from 3 mice in each group. **B**, Mean fluorescence was recorded during the time period of 500 sec (n=3); *P<0.05 ANOVA followed by the Newman–Keuls test.