Title: TREATMENT OF NEURAL INJURY AND NEURODEGENERATIVE DISEASE WITH STATINS

Abstract: A method of promoting neural restoration and enhancing neural function by administering a therapeutic amount of a statin compound to a patient in need of neurogenesis promotion is disclosed. A composition for promoting neural restoration and enhancing neural function having an effective amount of a statin compound sufficient to promote neurogenesis is disclosed. A method of augmenting the production of brain cells and facilitating cellular structural and receptor changes by administering an effective amount of a statin compound to a site in need of augmentation is disclosed. A method of increasing both neurological and cognitive function by administering an effective amount of a statin compound to a patient is disclosed.
TREATMENT OF NEURAL INJURY AND NEURODEGENERATIVE DISEASE WITH STATINS

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present application relates to a method and compound for promoting recovery after neural injury and for promoting neuroregeneration. More specifically, the present invention relates to a method and composition for promoting plasticity in the nervous system.

2. DESCRIPTION OF RELATED ART

Stroke occurs when a section of the brain becomes infarcted, resulting in death of brain tissue from interruption of cerebral blood supply. Cerebral infarcts associated with acute stroke cause sudden and dramatic neurological impairment. Stroke is the third most common cause of death in the adult population of the United States, and is a major cause of disability.

Pharmacological interventions have attempted to maximize the blood flow to stroke affected brain areas that might be able to survive, but clinical effectiveness has proven elusive. As stated in Harrison's Principles of Internal Medicine (9th Ed., 1980, p. 1926), "despite experimental evidence that...[cerebral vasodilators] increase the cerebral blood flow, as measured by the nitrous oxide method, they have not proved beneficial in careful studies in human stroke cases at the stage of transient ischemic attacks, thrombosis-in-evolution, or in the established stroke. This is true of nicotinic acid, Priscoline, alcohol, papaverine, and inhalation of 5% carbon dioxide. . .In opposition to the use of these methods is the suggestion that vasodilators are harmful rather than beneficial, since by lowering the systemic blood pressure they reduce the intracranial anastomotic flow, or by dilating blood vessels in the normal parts of the brain they steal blood from the infarct."

It would therefore be useful to develop a compound and method for
lessening the disabling effects of stroke by enabling functional recovery and for promoting neuroregeneration.

SUMMARY OF THE INVENTION

According to the present invention, there is provided a method of promoting neurogenesis by administering a therapeutic amount of a statin compound to a patient in need of neurogenesis promotion. Also provided is a composition for providing neurogenesis having an effective amount of a statin compound sufficient to promote neurogenesis. Further, a method of augmenting the production of brain cells and blood vessels, reducing glial scaring, enhancing cell survival, promoting neural connections and facilitating cellular structural and receptor changes by administering an effective amount of a statin compound to a site in need of augmentation is provided. There is provided a method of increasing both neurological and cognitive function by administering an effective amount of a statin compound to a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1A shows the suture model of MCAo and Figure 1B shows 2,3,5-triphenyltetrazolium chloride (TTC) stained coronal section of retired breeder rat brain at 2 days after MCAo;

Figures 2A and B are graphs showing that after MCAo, functional scores prior to the treatment were balanced among different doses of the atorvastatin treatment group and the control MCAo group;

Figures 3A and B are graphs showing significant improvement in functional recovery in atorvastatin treatment animals compared with control animal;

Figures 4A and B are graphs showing significant neurological functional recovery in the simvastatin treatment group compared with
control animals;

Figures 5 A-F are photographs that show how vessels containing BrdU reactive endothelial cells were affected when treated with atorvastatin;

Figures 6 A-F are photographs that show reconstructed 3D cerebral microvessels from the striatum (Figures 6A-C) in a normal adult rat, and from MCAo rat (Figures 6D-F);

Figures 7 A and B are photographs that show that atorvastatin significantly augmented corneal neovascularization (Figure 7B) compared with control animals (Figure 7A);

Figures 8 A-D are photographs and graphs that show how atorvastatin induced mouse brain-derived endothelial cells to form a network of capillary-like structures with numerous intercellular contacts;

Figures 9 A and B are graphs that show that simvastatin-induced tube formation was dose dependent (Figure 9A) and inhibitors of VEGFR2, NOS and p-PI3K, significantly inhibited simvastatin-induced tube formation (Figure 9B);

Figures 10 A-G are photographs and graphs that show BrdU positive cells significantly increased in the ipsilateral SVZ (Figure 10B, C) and DG (Figure 10D) in low dose atorvastatin (1 mg/kg and 3 mg/kg), but not high dose (8 mg/kg) treated groups compared to the control treatment group (Figure 10A-C for SVZ, Figure 10D for DG);

Figures 11 A-C are photographs and a graph that show synaptophysin expression significantly increased in the low dose (Figure 11B, 1mg/kg Atorvastatin) but not in high dose atorvastatin treatment rats (Figure 11C) compared with control rats (Figure 11A, C);

Figures 12 A-C are photographs and a graph showing that simvastatin significantly increases neuronal migration at concentrations 0.01 µM (Figure 12B,C) compared with control (Figure 12A,C), but not at 0.005 µM and 0.1 µM (Figure 12C);

Figures 13 A-C are photographs and a graph showing that LY294002, a specific PI-3K inhibitor, dose dependently inhibits SVZ cell
migration;

Figures 14 A-C are photographs and a graph showing that simvastatin (Figures 14B, C) led to significant dendrite elongation compared with control (Figures 14A, C);

Figures 15 A and B are photographs showing that incubation of atorvastatin (0.1 \(\mu\text{M}\)) in cultured primary cortical neurons markedly increased p-AKT (265%, Figure 15A) and p-ERK (140%, Figure 15B) compared with control;

Figure 16 is a schematic showing how the compound of the present invention function;

Figure 17 is a photograph that shows the eight brain regions of the ipsilateral, contralateral homologous hemispheres and BrdU in SVZ, DG in standard vibratome sections;

Figures 18 A-H are photographs showing that atorvastatin induces angiogenesis, as indicated by increased BrdU immunoreactive endothelial cells, vascular density and perimeter;

Figures 19 A-G are photographs that show capillary-like tube formation;

Figures 20 A-H are photographs that show that atorvastatin induces neurogenesis;

Figures 21 A-C are photographs showing synaptophysin expression increased in the low dose (1mg/kg) atorvastatin treatment rats (Figure 21B) compared with control rats (Figure 21A) and Figure 21C shows quantitation of synaptophysin expression in the ischemic boundary area; and

Figures 22 A and B are photographs showing atorvastatin induces phosphorylation of Akt and Erk.

Figure 23 shows significant improvement in functional recovery using the mNSS and the Corner test in atorvastatin treated rats subjected to cortical contusion injury compared with saline treated animals.

Figure 23 and 24 show that atorvastatin increases the survival of neurons in the boundary zone of the injured area and the CA3 region of the hippocampus, respectively, in rats subjected to cortical contusion injury;
Figure 25 shows that atorvastatin augments synaptic density defined by synaptophysin expression in the boundary zone of the injured cortex;

Figure 26 shows that atorvastatin augments synaptic density defined by synaptophysin expression in the CA3 region of the hippocampus;

Figure 27 shows that atorvastatin induces angiogenesis in the boundary zone of the lesion and in the CA3 hippocampus after cortical contusion injury; and

Figures 28A-J show that atorvastatin augments angiogenesis in the injured brain after TBI.

DESCRIPTION OF THE INVENTION

Generally, the present invention provides a method and compound for promoting brain plasticity, encompassing neurogenesis, angiogenesis, synaptogenesis, enhancing neural communication, and promoting selective neuronal survival after neural injury. More specifically, the compound of the present invention can be used for promoting brain repair and remodeling after neural injury. More specifically, the present invention provides a method for promoting brain repair utilizing compounds containing statins.

By “statins” it is meant a compound from the family of HMG-CoA reductase inhibitors. Statin compounds are drugs that are capable of lowering blood cholesterol levels by inhibiting hydroxymethylglutaryl CoA (HMG-CoA) reductase. Examples of the statin compounds include, but are not limited to compounds such as LIPICTOR™, pravastatin and its sodium salt, simvastatin, lovastatin, atorvastatin, fluvastatin, and cerivastatin.

By “promoting brain repair and remodeling” as used herein, it is meant that neurogenesis, angiogenesis, dendritic arborization, and synaptogenesis are enhanced and promoted and structural changes in brain tissue that facilitate neurite extension, reduce glial scaring, and promote neuronal survival are also included. The term “neurogenesis"
indicates that neural generation is promoted or enhanced. This can include, but is not limited to, new neuronal generation or enhanced growth of existing neurons, as well as growth and proliferation of parenchymal cells and cells that promote tissue plasticity. Neurogenesis also encompasses, but is not limited to, neurite and dendritic extension and synaptogenesis. The term “angiogenesis” refers to an increase in the number of blood vessels. The term “synaptogenesis” refers to neuronal circuit formation and strengthening.

By “augmentation” as used herein, it is meant that growth is either enhanced or suppressed as required in the specific situation. Therefore, if additional neuron growth is required, the addition of a statin increases this growth. Alternatively, if neuron growth is to be retarded, statins and natural equivalents thereof can be suppressed. Statins prime cerebral tissue to compensate for damage brought on by injury, neurodegeneration, or aging. This is accomplished by enhancing receptor activation and promoting cellular morphological change and cellular proliferation.

By “neurological” or “cognitive” function as used herein, it is meant that the new neural growth (i.e. neurogenesis), neural survival and synaptogenesis in the brain enhances the patient’s ability to think, function, etc. Humans treated with statins have increased production of brain blood vessels, brain cells, and enhanced cell connectivity that facilitate improved cognitive, memory, and motor function. Further, patients suffering from neurological disease or injury, when treated with statins, have improved cognitive, memory, and motor function.

Angiogenesis is associated with improved neurological recovery from stroke, and the stimulation of neovascularization and angiogenesis are important therapeutic targets for recovery. Stroke patients with a higher cerebral blood vessel density appear to make better progress and survive longer than patients with lower vascular density. The preliminary data indicate that statins induce angiogenesis in ischemic brain. Growth factors such as VEGF and basic fibroblast growth factor (bFGF) are potent angiogenic molecules and are hypoxia-inducible secreted proteins. Statins promote production of growth factors and neurotrophic factors (e.g. VEGF)
in injured and ischemic brain. VEGF or bFGF treatment after stroke induces angiogenesis and promotes functional recovery after stroke. Statin therapy also promotes endothelial progenitor cell (EPCs) and neuronal progenitor and stem cell migration and differentiation \textit{in vivo} and \textit{in vitro}.

Neurogenesis and synaptic reorganization are important for functional improvement after stroke. Neurogenesis, the process through which precursor cells proliferate, migrate, and differentiate into a mature neuronal phenotype, persists in discrete regions of adult brain, including the rostral subventricular zone (SVZ) and the subgranular zone (SGZ) of hippocampal dentate gyrus (DG). Focal stroke induces forebrain and hippocampal neurogenesis and striatal neuron replacement in rats. Stroke promotes cell proliferation in the SVZ and SGZ, and the proliferating cells migrate into the severely damaged area of the striatum, where they express markers of developing and mature neurons. However, most neurons generated after stroke fail to survive and integrate into cerebral tissue. The low survival of newly formed neurons can be related to lack of trophic support and connections, and exposure to the detrimental environment of severely damaged tissue. VEGF, an angiogenic factor, not only induces angiogenesis, but also induces neurogenesis and stimulates axonal outgrowth and improves the survival of neurons. The data show that statin promotes VEGF secretion in the ischemic boundary area. Statins also reduce mRNA levels of plasminogen activator inhibitor-1 (PAI-1) and endothelin-1, and increase the mRNA levels of thrombomodulin, t-PA and u-PA. t-PA and u-PA are not only anticoagulation agents, statins also promote neurogenesis, neuronal migration and synaptogenesis. The data demonstrate that statins enhance proliferation of SVZ cells in the ischemic brain, and promote postnatal SVZ migration.

Synaptic plasticity is related to behavioral change and functional recovery after brain injury. Increases in dendritic arborization and spine structure and density are potential morphological strategies that enable the brain to reorganize its neuronal circuits. Moreover, recent evidence demonstrates that functional alterations in motor cortex organization are accompanied by changes in dendritic and synaptic structure, as well as
alterations in the regulation of cortical neurotransmitter systems. Statins regulate small Rho GTPase proteins. The small GTPase, RhoG is a key regulator for neurite outgrowth. PI3K promotes synaptic plasticity in hippocampus. Activated ERK1/2 supports axonal outgrowth. The data included herein show that statins increase expression of (phosphorylated-p) p-PI3K and p-ERK, which can promote synaptogenesis and synaptic plasticity in the ischemic boundary zone and induce growth cone guidance and neuritic outgrowth in primary cortical neurons in vitro.

Neurotrophic and growth factors, such as VEGF and bFGF administered to animals one or more days after stroke, improve neurological function and evoke neurogenesis and angiogenesis. The data included herein show that statin treatment after stroke enhances VEGF expression in the ischemic boundary area. VEGF is a potent angiogenic factor, which plays a key role in endothelial cell differentiation, vasculogenesis, and in the sprouting of new blood vessels from preexisting ones (angiogenesis). Intravenous injection of VEGF two days after stroke in rats significantly improves functional outcome without reducing the volume of cerebral infarction. VEGF appears to have a direct neurotrophic effect, as it stimulates axonal outgrowth and increases the survival of mouse superior cervical and dorsal root ganglion neurons. Trophic factors can promote neuronal sprouting, both from the ends of damaged axons (regenerative sprouting) and from intact axons into denervated synapses (collateral sprouting). Such sprouting appears to be associated with functional recovery in some model systems. VEGF exerts biological functions via two related receptor tyrosine kinases VEGF receptor 1 (VEGFR1, flt-1) and VEGF receptor 2 (VEGFR2, flk-1). Binding of VEGFR2 leads to receptor phosphorylation and subsequent activation of p-PI3K, and other signaling proteins. Moreover, downstream activation of AKT by PI3K is responsible for phosphorylation and activation of eNOS by VEGF. VEGF promotes cell migration through a novel pathway regulating p-PI3K/AKT/eNOS. VEGF also exerts cell proliferation partly through activation of mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (MAPK/ERK1/2). Cells activated by VEGF showed
increased ERK1/2 levels. Activated ERK1/2 supports axonal outgrowth, enhances cell survival and regulates the turnover of the NO/cGMP-pathway. Inhibition of NO synthase or overexpression of a dominant negative AKT abrogates VEGF-induced cell migration. The Sandwich ELISA data indicate that VEGF is increased at 14 days after MCAo with statin treatment, compared with rats subjected to MCAo without treatment.

The Rho-family of GTPases regulate a wide range of biological functions including neuronal development, neuronal migration, neurogenesis, axonal guidance, growth cone behavior and dendritic growth in both neuronal cell lines and primary neurons. Rho family GTPases also play a key role in growth inhibition, since a RhoA activator, lysophosphatidic acid (LPA) induces neurite retraction and growth cone collapse. Inactivation of RhoA with C3 transferase and a Rho kinase inhibitor Y-27632, promote neurite growth on inhibitory substrate. The pleiotropic effects of statins can, at least in part be ascribed to the regulation of the small Rho GTPase family (Rac, RhoA and Cdc42). Statins inhibit Rho isoprenylation and promote Rac activity. Rac is a key member of the Rho family of small GTPases and acts as a molecular switch. When GTP-bound, Rac binds specific effectors to induce downstream signaling events, including actin cytoskeletal rearrangements. Rac is involved in the morphogenesis of dendritic spines. Rac activity is regulated by guanine nucleotide exchange factors, which can be further regulated by extracellular factors. Thus, the Rac signal transduction pathway can provide links between extracellular ligands or synaptic activity and the regulation of the actin cytoskeleton in spine morphogenesis. In addition, Rho GTPases are important mediators of vascular function. For example, RhoA negatively regulates eNOS mRNA stability and the release of t-PA. Statins increase eNOS, t-PA and u-PA by inhibiting RhoA function. t-PA and u-PA play important roles in fibrinolysis, cell migration, tissue destruction, angiogenesis and tissue remodeling. t-PA and u-PA promote angiogenesis. Statins promote p-PI3K expression in cultured endothelial cells. Blocking of PI3K (LY294002) inhibits angiogenesis in vitro.

Nogo-A is a potent neuritic growth inhibitor in vitro and suppresses
axonal regeneration and structural plasticity in the adult mammalian CNS in vivo. Inhibition of RhoA improves neurite outgrowth response of cerebellar granule cells. Nogo receptor (NGR) is necessary for Nogo-A-induced RhoA activation. Enhancing RhoA and suppressing Rac1 activities in cerebellar granule cells increase Nogo-66 expression and inhibit neurite growth.

Functional improvement cannot only be associated with cellular survival and angiogenesis, but also with structural changes in neurons, including increase in synaptic density, following treatment with statin. GAP-43 is thought to play an essential role in axon outgrowth and synaptogenesis in the developing and regenerating nervous system. MAP2, is a marker of dendrites; and synaptophysin, a marker of synapse formation. Thus, GAP43, MAP2 and synaptophysin are measured as an index of the neuronal structural changes after statin treatment of stroke.

Neurotrophins are key regulators of the shape and fate of neuronal cells and act as guidance cues for growth cones by remodeling the actin cytoskeleton. Actin dynamics are controlled by Rho GTPases. The Rho family of small GTPases has been shown to be involved in the regulation of neuronal morphology, and RhoA exerts antagonistic actions on neurite formation. Statins block the synthesis of isoprenoids. Isoprenoids permit the subsequent membrane translocation and activation of RhoA. Thus, isoprenylation can play an important role in mediating the direct cellular effects of statin. Simvastatin reduces the binding of the small GTPase RhoA to cellular membranes. Statins can inhibit RhoA and therefore activate Rac-1, PI3K/AKT/eNOS and Ras/ERK, which promote cell survival and neuronal plasticity. Nogo-A, an integral membrane protein predominantly expressed by oligodendrocytes, has been demonstrated to impair neurite growth in vitro and in vivo. The Nogo-A protein contains at least two active domains, NiG and Nogo-66, with diverse effects on neurite outgrowth and cell spreading. Nogo-A is inhibited by an antagonistic regulation of the small GTPases RhoA. By inactivating RhoA, the inhibitory effects of Nogo-A on neurite outgrowth and oligodendrocyte-mediated growth cone collapse were abolished.
The purpose of the present invention is to promote an improved outcome from ischemic cerebral injury, or other neuronal injury, or deficits resulting from neurodegenerative disease and aging by inducing neurogenesis and cellular changes that promote functional improvement. Patients suffer neurological and functional deficits after stroke, CNS injury, and neurodegenerative disease. The composition of the present invention provides a means to enhance brain compensatory mechanism to improve function after CNS damage degeneration and normal conditions. The induction of neurons, blood vessels and cellular changes induced by statin administration promotes functional improvement after stroke, injury, aging, degenerative disease and normal conditions. This approach can also provide benefit to patients suffering from other neurological disease including, but not limited to, ALS, and Huntington's disease. Statins administered at propitious times after CNS injury promote cell proliferation and enhanced synaptic function in brain.

The experimental data included herein show that the compound of the present invention, namely a statin (e.g. Lipitor) administered to rats 24 hours after stroke onset or traumatic brain injury, significantly reduces neurological deficits and improves functional outcome after stroke and traumatic brain injury. Developing a way to promote brain remodeling, i.e. brain cell production, proliferation and synaptogenesis opens up the opportunity to treat a wide variety of neurological disease, CNS injury and neurodegeneration. Using the compound of the present invention it is possible to augment the production of neurons and other cells and enhance synaptic function in non-damaged brain, so as to increase function.

Additionally, the experimental data show that administration of statin to rats subjected to stroke and traumatic brain injury one or more days after stroke significantly improves functional recovery, without necessarily reducing the volume of cerebral infarction.

Functional benefit induced by statin treatment of stroke derives from enhancement of: a) angiogenic factors, which promote angiogenesis within the 'penumbral-like' regions of brain and b) cell proliferation within the
subventricular zone (SVZ) and hippocampal dentate gyrus (DG), neuronal migration from the SVZ, and synaptic protein expression. Further, statins promote activation of Rac, and activates Ras/ERK, PI3K/AKT/eNOS signaling pathways. These pathways promote angiogenesis, neurogenesis, neuronal migration and neurite outgrowth. Stains reduce gliosis and facilitate neuronal connections. Statins enhance the expression of VEGF and its receptors VEGFR1 (flt-1) and VEGFR2 (flk-1), which evoke angiogenesis, neurogenesis and neurite growth. Enhancement of eNOS expression by statins contributes to angiogenesis, neurogenesis, and neurite outgrowth.

The composition of the present invention, when administered one or more days after stroke and traumatic brain injury, enhances brain plasticity and thereby significantly reduces functional deficits after ischemic stroke and traumatic brain injury in the rat. The following shows the restorative therapy in young and old rats, and also shows the molecular sequelae that activate statin mediated alterations of ischemic and injured brain in a preclinical rodent model of middle cerebral artery occlusion and in a preclinical model of traumatic brain injury. Additionally, the administration of statins provides selective neuroprotection post injury. In other words, the administration of statins protects and thereby reduces the progression of cell damage in an area of injury.

Treatment of stroke and trauma with statins, initiated at one day after stroke or traumatic brain injury onset improves neurological functional recovery in old and young adult rats. The data included shows the mechanisms that promote therapeutic benefit of statin in vivo. In other words, the effects of statin treatment on, angiogenesis, neurogenesis, neuronal migration and synaptogenesis, were measured in old and young rats after stroke. Additionally, to elucidate molecular mechanisms that promote therapeutic benefit of statin in vitro, the effects of statin on, the regulation of small G-proteins, activation of phosphatidylinositol 3-kinase (PI3K)/AKT/endothelial nitric oxide synthase (eNOS) and the Ras/extracellular signal-regulated kinase (ERK) pathways, and expression of vascular endothelial growth factor (VEGF) and associated receptors...
were measured in cultured mouse brain-derived endothelial cells, primary cortical neurons and adult SVZ explant culture.

The data show that administration of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) reduce neurological deficits after induction of middle cerebral artery occlusion (MCAo) or traumatic brain injury (TBI) in rat without reducing volume of cerebral infarction. These data indicate that statins promote neurorestoration.

The present invention demonstrates that the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor atorvastatin and simvastatin enhance functional outcome and induces brain plasticity when administered after stroke and trauma to rats. With atorvastatin treatment initiated one day after stroke, animals exhibited significant increases in vascular endothelial growth factor (VEGF), cyclic guanosine monophosphate (cGMP), angiogenesis, endogenous cell proliferation and neurogenesis, as well as an increase in the synaptic protein, synaptophysin. Atorvastatin induced angiogenesis in a tube formation assay was reduced by an antibody against the VEGF receptor 2 (FLK-1). Atorvastatin also induced phosphorylation of Akt and Erk in cultured primary cortical neurons. These data indicate that atorvastatin induced brain plasticity and has neurorestorative activity after experimental stroke and trauma.

The present invention demonstrates that widely used statins (atorvastatin and simvastatin) when administered at one day after stroke or trauma is highly effective in reducing neurological deficits in a rat model of middle cerebral artery occlusion (MCAo) and traumatic brain injury (TBI). These benefits are mediated by statin induced amplification of angiogenesis, neurogenesis, and synaptogenesis.

The market for a class of drugs that promotes functional recovery after injury or disease and improvement of function under normal conditions is vast. Statins, of which Lipitor is but one example, promote cellular proliferation, production and synaptogenesis and enhance synaptic
function. An increase in neurogenesis, angiogenesis, and synaptogenesis translates into a method to increase and improve neurological, behavioral, and cognitive function, injured because of age, injury, or disease.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for the purpose of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

**EXAMPLES**

**Example 1:**

**MCAo model:** The suture model of MCAo was employed in both young and old rats (Figure 1A). The MCAo model was successfully employed in adult Wistar young (2-3 months), retired breeder (10-12 months) and old (18-20 months) rats. Figure 1B shows 2,3,5-triphenyltetrazolium chloride (TTC) stained coronal section of retired breeder rat brain at 2 days after MCAo. Two hours of MCAo gives rise to a highly reproducible lesion volume in the striatum and cortex in adult male Wistar rats.

**Functional outcome of young adult rats subjected to MCAo and treated with or without atorvastatin:** Adult male Wistar rats (2-3 months) were subjected to 2 hours of MCAo. One day after stroke, animals were fed atorvastatin or saline daily at doses of 1, 3 or 8 mg/kg for 7 days. All rats were sacrificed 14 days after stroke. The effect of atorvastatin treatment at these doses on a battery of functional outcome tests was measured. Figure 2 shows that after MCAo, functional scores prior to the treatment were balanced among different doses of the atorvastatin treatment group and the control MCAo group (Figures 2A, B). Compared to the control treated group, rats treated with 1 and 3 mg/kg atorvastatin had significantly (P<0.05 n=8/group) improved functional recovery. No
functional benefit was detected with 8mg/kg atorvastatin (Figures 2A, B). No significant differences of ischemic lesion volumes in atorvastatin treated groups (29±7.0% for 1 mg/kg atorvastatin; 28.1±4.9% for 3 mg/kg atorvastatin; 42.3±6.9% for 8 mg/kg atorvastatin) were detected compared with control treated rats (34.7±5.6 %), respectively.

**Functional outcome after MCAo treated with or without atorvastatin in retired breeder rats:** Retired breeder male rats (10-12 months) were subjected to permanent MCAo and treated with or without atorvastatin (3 mg/kg) starting at 24 hours after stroke. The animals were sacrificed at 28 days after stroke. Functional tests were performed. Figure 3 shows significant improvement in functional recovery in atorvastatin treatment animals compared with control animal (Figure 3A, B; n=9/group).

**Functional outcome after MCAo in adult young rats treated with or without simvastatin:** Adult young rats (2-3 months) were treated with or without simvastatin 1 mg/kg at 24 hours after stroke. Functional tests were performed. The animals were sacrificed at 14 days after stroke. Figure 4 shows significant neurological functional recovery in the simvastatin treatment group compared with control animals (Figure 4A, B; n=8/group).

**Endothelial cell proliferation and angiogenesis.** Since angiogenesis contributes to functional improvement after stroke, microvascular structure and density in atorvastatin treated and control groups in adult young rats were measured at 14 days after stroke. Enlarged and thin walled vessels, termed “mother” vessels, are formed under conditions of cerebral ischemic angiogenesis. Figure 5 shows that enlarged vessels containing BrdU reactive endothelial cells significantly increased in the ipsilateral hemisphere of the low dose atorvastatin (1 and 3 mg/kg) treatment group (Figures 5C, D), but not in the high dose atorvastatin (8 mg/kg) compared with the ipsilateral hemisphere of MCAo control animals (Figures 5B, D) or the contralateral hemisphere of treated animals (Figure 5A). Figures 5 E-F shows that treatment with low dose (1 and 3 mg/kg) significantly (P<0.05) increased vascular perimeter and
density in the ipsilateral hemisphere compared with the control rats but not in high dose (8 mg/kg) atorvastatin. These data indicate that low dose atorvastatin enhances angiogenesis in ischemic brain.

Three-dimensional measurement of cerebral microvessels: To quantify cerebral microvessels in three dimensions, a computerized system was developed to analyze cerebral microvessels perfused in vivo by FITC-dextran. The program quantifies vascular structures in three-dimensional images obtained from LSCM, such as number of branch points, segment length and diameters of vessels. Figure 6 shows reconstructed 3D cerebral microvessels from the striatum (Figures 6A-C) in a normal adult rat, and from MCAo rat (Figures 6D-F). Different colors in Figures 6B and 6E represent individual vessels which are not connected to each other, and green and red colors in Figure 6C and 6F code for diameters of blood vessels less than 7.5 μm and larger than 7.5 μm, respectively. These reconstructed 3D images reflect the original images obtained from LSCM (Figures 6A, D). These data demonstrate that the software program can analyze in three dimensions cerebral microvascular structures.

Atorvastatin promotes corneal neovascularization. As a complementary measurement of atorvastatin mediated angiogenesis, a corneal assay for angiogenesis was performed (n=6). Mixed atorvastatin (2 μl, 10μg) with 12% hydron (2 μl) was inserted into the cornea of the right eye. 12% hydron (4 μl) alone was inserted into left eye for control. Corneas were digitized for measurement of vessel length extension and density at day 7. Atorvastatin significantly augmented corneal neovascularization (Figure 7B) compared with control animals (Figure 7A). The total vascular lengths significantly increased in the atorvastatin treatment group (Figure 7B, 29.9±7.9 mm/cornea) compared with control animals (Figure 7A, 1.5±1.0 mm/cornea).

Atorvastatin promotes VEGF secretion in the ischemic boundary zone (young adult rats). Since atorvastatin promotes angiogenesis, it was tested whether atorvastatin evokes production of VEGF, a principal angiogenic factor in ischemic brain. Adult young rats
were subjected to 2 hours of MCAo and treated daily with or without atorvastatin (1 mg/kg) beginning at 24 hours after stroke for 7 days. The rats were sacrificed at 14 days after treatment. Brain extract from control MCAo and atrovasatatin treated animals were obtained from the ischemic boundary zone (bregma -2~2 mm, penumbral region encompassing the ischemic core). Using ELISA, VEGF production significantly increased in the atorvastatin treatment group (25.7±2.7 ng/ml) compared with the MCAo control group (10.5±1.7 ng/ml) and normal brain (13.4±1.5 ng/ml, P<0.05, n=6/group).

**Atorvastatin induces angiogenesis in vitro.** The contribution of VEGF to statin induced angiogenesis was tested using an in vitro endothelial cell tube formation assay. Mouse brain-derived endothelial cell capillary-like cell tube formation was measured. Cells were incubated in: (1) DMEM for control; (2) 0.01 μM atorvastatin; (3) 0.1 μM atorvastatin; (4) 0.5 μM atorvastatin; (5) 1.0 μM atorvastatin; (6) 10 μM atorvastatin; (7) 0.5 μM atorvastatin with 10 μg/ml neutralizing antibody to VEGFR2 (DC101, Imclone System). (8) 0.5 μM atorvastatin with 1mM L-NAME (Sigma). (9) 0.5 μM atorvastatin with 10 μg/ml DC101 and 1mM L-NAME. All assays were performed in triplicate and quantitated. Atorvastatin induced mouse brain-derived endothelial cells to form a network of capillary-like structures with numerous intercellular contacts (Figure 8B, Atorvastatin 0.1μM). Low concentrations of atorvastatin (0.01-1 μM), but not a high concentration (10 μM) significantly induced tube formation compared with control medium (Figure 8C). Total capillary tube length significantly increased in medium containing atorvastatin (0.5 μM, Figure 8B, C) compared with the control medium (Figure 8A, C). Both anti-VEGFR2 (DC101) and NOS inhibitor L-NAME significantly decreased atorvastatin induced capillary tube formation compared with atorvastatin alone (P<0.05, Figure 8D). The combination of DC101 with L-NAME significantly inhibited atorvastatin induced capillary tube formation (Figure 8D) compared to atorvastatin alone (P<0.05) and tube formation was significantly reduced compared to atorvastatin with L-NAME (P<0.05) (Figure 8D). These data indicate that statins have a
biphasic effect on angiogenesis, with promotion of angiogenesis at low concentration, but not at high concentration.

**Simvastatin induces angiogenesis in vitro.** Simvastatin induces mouse brain-derived endothelial cells to form a network of capillary-like structures with numerous intercellular contacts. Cells were incubated in: (1) DMEM for control; (2) 0.01 μM simvastatin; (3) 0.1 μM simvastatin; (4) 0.5 μM simvastatin; (5) 1.0 μM simvastatin; (6) 10 μM simvastatin; and 0.5 μM simvastatin with (7) 10 μg/ml neutralizing antibody to VEGFR1 (MF1). (8) with 10 μg/ml anti-VEGFR2 (DC101). (9) with 1 mM L-NAME. (10) with 10 μM LY294002 (a p-PI3K inhibitor); (11) with 10 μM U0126 (a MEK inhibitor). All assays were performed in n=5/group. Simvastatin-induced tube formation was dose dependent (Figure 9A), as was atorvastatin. In addition, inhibitors of VEGFR2, NOS and p-PI3K, significantly inhibited simvastatin-induced tube formation (Figure 9B). There were no significant differences in tube formation in brain endothelial cells treated with U0126, an inhibitor of MEK compared to simvastatin treated alone (0.5 μM simvastatin). These data show that VEGF, NO and PI3K but not ERK facilitate statin induced angiogenesis.

**Cell proliferation in the SVZ and dentate gyrus (DG) in vivo.**

Since endogenous cerebral cell proliferation and neurogenesis furthers functional benefit, numbers of newly formed cells were measured at 14 days after stroke within two neurogenic regions of brain, the DG and the SVZ in atorvastatin treated and control animals. Adult young rats were treated with or without atorvastatin at 24 hours after MCAo. BrdU was injected daily beginning at 24 hours after treatment for 14 days. Rats were sacrificed at 14 days after treatment. Double immunostaining was performed on coronal sections for BrdU with tubulin 3 (TUJ1), a marker for new neurons. BrdU positive cells significantly increased in the ipsilateral SVZ (Figure 10B, C) and DG (Figure 10D) in low dose atorvastatin (1 mg/kg and 3 mg/kg), but not high dose (8 mg/kg) treated groups compared to the control treatment group (Figures 10A-C for SVZ, Figure 10D for DG). Using confocal imaging, double staining immunohistochemistry shows that
some BrdU positive cells (Figure 10E and G) colocalized with the developmental neuronal marker TUJ1 (Figure 10F and G) in the low dose (1 and 3 mg/kg) atorvastatin treatment group. The percentage of BrdU reactive cells which express TUJ1 in the ipsilateral SVZ, increased in low dose atorvastatin (1 and 3 mg/kg) treated animals (25.4±4.5%, 21.4±2.1%, P<0.05), but not in high dose treated animals (8 mg/kg, 15±5.8%) compared with the control group (14.4±6.2%). This shows that atorvastatin induces neurogenesis after stroke.

**Synaptophysin.** New neuronal sprouting and synapse formation parallels functional recovery after cortical injury, including stroke. Functional benefit derived from treatment of stroke with atorvastatin suggests an effect of atorvastatin on synaptic plasticity. Synaptophysin is a marker for presynaptic plasticity and synaptogenesis. Rats were subjected to 2 hours MCAo and treated daily for 7 days with or without atorvastatin starting one day after stroke. Animals were sacrificed at 14 days after stroke. Synaptophysin immunostaining was performed on coronal sections. For semi-quantification of synaptophysin immunoreactivity, five immunostained coronal sections and eight fields of view from the ischemic penumbra (cortex and striatum) and contralateral hemisphere in each section were digitized under a 20x objective (Olympus BX40) via the MCID computer image analysis system. The optical density was measured. All values of density are presented as a percentage compared with the contralateral homologous region on the same section. Figure 11 shows synaptophysin expression significantly increased in the low dose (Figure 11B, 1mg/kg Atorvastatin) but not in high dose atorvastatin treatment rats (Figure 11C) compared with control rats (Figures 11A, C). This shows that low dose atorvastatin treatment promotes synaptic plasticity.

**Effect of simvastatin on cell migration in postnatal SVZ explant cultures.** Newly generated neurons derived from the SVZ migrate long distances, invade the mature neuronal tissue and establish new functional circuits with existing neurons. SVZ explant cultures were prepared from 14 day-old (p14) post-natal Wistar rat pups. The SVZ explant cultures were
treated with or without simvastatin at different concentrations (0.005, 0.01, 0.1 \( \mu M \)). After incubation, dishes were observed at 4 days with a phase contrast microscope and photographed at 10X magnification. Neuronal migration was measured and averaged using the MCID analysis system in 4 microscopic fields per well (n=6/group). The dishes were fixed at 7 days for immunostaining. GFAP (FITC, green) and TUJ1 (Cy5, red) double immunostaining were performed. Figure 12 shows that simvastatin significantly increases neuronal migration at concentrations 0.01 \( \mu M \) (Figures 12B,C) compared with control (Figure 12A,C), but not at 0.005 \( \mu M \) and 0.1 \( \mu M \) (Figure 12C). These data show that statin promotes neuronal migration in a dose dependent manner.

**PI3K inhibitor (LY294002) inhibits cell migration on adult brain SVZ explant culture.** The adult brain SVZ explant cultures were prepared and treated with or without a PI3K inhibitor, LY294002, at different concentrations (2 \( \mu M \), 10 \( \mu M \), 30 \( \mu M \)). After incubation, dishes were observed at 4 days with a phase contrast microscope and photographed at 10X magnification (n=6/group). Cell migration was measured and averaged using the MCID analysis system (Figure 13C). Figure 13 (A-C) shows that LY294002 dose dependently inhibits SVZ cell migration.

**Effect of simvastatin on dendritic structure in cultured primary cortical neurons.** Primary cortical neurons were treated with or without simvastatin 0.01 \( \mu M \) for 24 hours. Axon and dendritic outgrowth were measured. To trace the axonal arbors of fluorescently labeled neurons, photomicrographs were captured at 10X magnification with a digital camera. The total dendritic of 20 neurons were measured using the MCID analysis system. Figure 14 shows that simvastatin (Figures 14B, C) led to significant dendrite elongation compared with control (Figure 14A, C).

**Effect of atorvastatin on activation of AKT, ERK in cultured primary cortical neurons.** Statins activate PI3K-AKT and ERK pathways. PI3K/AKT and ERK transduce cell survival signals and promote synaptic plasticity. p-AKT and p-ERK were measured in primary cortical neurons. Incubation of atorvastatin (0.1 \( \mu M \)) in cultured primary cortical neurons
markedly increased p-AKT (265%, Figure 15A) and p-ERK (140%, Figure 15B) compared with control. Pretreatment with LY294002, a stable PI3K inhibitor significantly reduced atorvastatin induced p-AKT and p-ERK 75% and 2.7%, respectively (Figures 15 A, B). These data indicate that atorvastatin activates the PI3K/AKT and ERK in cultured cortical neurons.

**Serum total cholesterol, blood pressure (BP) and CBF.** Serum total cholesterol was not significantly decreased in any group of rats subjected to MCAo and treated with or without atorvastatin (1 mg/kg, 66.3±4.9 mg/dl; 3 mg/kg, 71±11.5 mg/dl; 8 mg/kg, 64±10.1 mg/dl; and control group, 76.4±9.4 mg/dl). This suggests that atorvastatin induced brain plasticity is not dependent on lowering of cholesterol. High dose (8 mg/kg) treatment with atorvastatin caused a significant reduction (P<0.05) of systemic arterial BP levels at 1 hour after treatment (51±10.6 mmHg) compared with levels obtained before treatment (85.0±3.3 mmHg). BP was not decreased at 1 hour after low dose (1 and 3 mg/kg) atorvastatin administration (78.3±4.7 mmHg and 81.6±12.1 mmHg, respectively) compared with BP measured before treatment (84.3±4.0 mmHg and 76.3±6.3 mmHg, respectively). Local cortical rCBF continuously measured using laser doppler flowmetry (LDF), significantly increased in the ischemic hemisphere in low dose atorvastatin treatment (1 mg/kg) from 40 minutes to 120 minutes after atorvastatin administration (range-165±22% - 130±3.9%, P<0.05), but not in high dose treatment (8 mg/kg, range-86.2±9.4%-115.3±15.1%) compared with pretreatment levels. No significant differences in rCBF were detected 2 hours after atorvastatin treatment among all groups.

**GENERAL METHODS:**

**The MCAo model and statin administration:** Adult male Wistar rats (age 2 months or 18 months) are purchased from Charles River Breeding Company (Wilmington, MA). Rats were anesthetized with halothane. The right femoral artery and vein was cannulated for measuring blood gases (pH, pO₂, pCO₂) and BP as basic physiological parameters.
MCAo was induced by advancing a 4-0 (for young rats) or 3-0 (for old rats) surgical nylon suture (18.5-19.5 mm for young rats and 20-22 mm for old rats determined by body weight) with an expanded (heated) tip from the external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA, and reperfusion was performed by withdrawal of the suture. Twenty-four hours after MCAo, rats were fed atorvastatin or simvastatin, dissolved in saline. In order to identify newly formed DNA in endogenous ischemic brains, rats received by injections of BrdU (as a tracer, 100 mg/kg in 0.007N NaOH physiological saline) intraperitoneally daily for 14 consecutive days before sacrifice.

**Cholesterol, BP and rCBF measurement.** Serum total cholesterol was quantified using a Sigma cholesterol measurement kit before treatment with simvastatin and before sacrifice (14 day, 28 days and 3 months). The systemic BP was measured using BP detection system (Protocol Systems. INS. Beaverton Orgeon USA). Regional CBF (rCBF) was measured during the initial treatment using laser-Doppler flowmetry (LDF) 30 minutes before treatment to 3 hours after treatment. The data of BP and rCBF was presented as a percentage of before treatment baseline values.

**Behavioral tests:** A battery of behavioral tests was performed to assess the somatosensory, motor and balance functions. **Adhesive-removal somatosensory test:** Somatosensory deficit was measured both pre- and postoperatively. All rats were familiarized with the testing environment. In the initial test, two small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The time to remove each stimulus from forelimbs was recorded on 5 trials per day. Before surgery, the animals were trained for 3 days. If the rats were able to remove the dots within 10 seconds, they are subjected to MCAo. The time to remove the left dot was recorded. **Modified Neurological Severity Score (mNSS):** Neurological function was graded on a scale of 0 to 18 (normal score 0; maximal deficit score 18. mNSS is a composite of motor, sensory,
reflex and balance tests. In the severity scores of injury, one score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher score, the more severe is the injury.

**Light, fluorescent and laser scanning confocal microscopy (LSCM):** Animals were anesthetized with ketamine (44-80 mg/kg, i.p.) and xylazine (13 mg/kg, i.p.) and the vascular system transcardially perfused with heparinized PBS followed by ice-cold 15% sucrose in PBS. Brains were immersed in 4% paraformaldehyde in PBS. Using a rat brain matrix (Activational Systems Inc., Warren, MI), each forebrain was cut into 2 mm thick coronal blocks for a total 7 blocks from bregma 5.2 mm to bregma -8.8 mm per animal. The tissues were processed and 20 or 100 μm thick free-floating vibratome coronal sections from each block were cut. Volume of cerebral tissue infarction was measured by light microscope using a Global Lab Image analysis program (Data Translation, Marlboro, MA). To reduce errors associated with processing of tissue for histological analysis, an indirect volume calculation was measured.

**Immunohistochemistry:** For vibratome sections, free-floating sections were sequentially incubated in 3% hydrogen peroxide, 0.1% Triton X-100 and 1.5% normal serum from the animal source of the secondary antibody. Sections were incubated with a primary antibody. Antibodies for growth factors, receptors and cell type specific proteins) at 4°C and then incubated with secondary antibody conjugated to chromogens. Double immunofluorescence labeling protocol: free-floating sections were incubated with the first primary antibody and sections were then incubated with the secondary antibody conjugated to FITC. Sections were incubated with second primary antibody and then the secondary antibody conjugated to Cy5.

**Quantification:** In each 20th coronal section from reference (from bregma 2 mm to bregma -2 mm per animal) vibratome coronal sections (20 μm) were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad, Cambridge, MA). A 10x objective with a numerical aperture of 0.3 and
a 40x oil immersion objective with a numerical aperture of 1.3 were used for data acquisition. Since the size of the fluorescent spots in a two-dimensional image depends on the laser power, iris, gain, and duration of sampling time, these parameters were fixed within the same section during the acquisition of data. For immunofluorescence single, double or triple labeled sections, green FITC and far red Cy5 or red Cy3 fluorochromes on the sections were excited by the laser beam at 488 nm (FITC), 568 nm (Cy3), and 647 nm (Cy5), and emissions were acquired sequentially with a photomultiplier tube through 522 nm, 605 nm, and 670 nm emission filters, respectively. **a**) For semi-quantification: TUJ1, DCX, VEGF, VEGFR1, VEGFR2, synaptophysin, MAP2, GAP43, p-PI3K and NGR positive cells were measured in the brain regions of the ipsilateral (Figure 17), contralateral homologous hemispheres and BrdU in SVZ, DG in standard vibratome sections were scanned in 512 x 512 pixel (279 x 279 μm²) format in the x-y direction using a 4x frame-scan average and twenty thin optical sections along the z-axis with a 0.5 μm step-size under a 40x objective was acquired for cell identification. The tissue volume or image size will be 279 x 279 x 20 μm³. To avoid working with a single composite or compressed image, the MCID system (Imaging Research, St. Catharines, Canada) was used. All z-axis LSCM images were imported into the system as individual 256-gray-scale, TIF format images using software of Confocal Assistant (Bio-Rad). The total volume of staining present in the rendered cube of tissue was calculated in μm³ and divided by the total tissue volume to determine the percentage of tissue volume that was fluorescently marked. **b**) The percentage of cell identification *in vivo*: Using double or triple staining in each section (20 μm thick), a total of 500 BrdU-positive cells per rat was counted to obtain the percentage of each cell type specific marker, i.e., BrdU-TUJ1, BrdU-DCX, BrdU-MAP2, BrdU-GFAP, BrdU-t-PA, BrdU-p-PI3K, BrdU-VEGF, BrdU-VEGFR1, BrdU-VEGFR2. For tissue samples with double or triple (FITC, Cy3 and Cy5) staining, two or three sets of three-dimensional images was constructed, each one representing the distribution of a different fluorescent marker. A fixed gray-
scale display cutoff was applied to the model to ensure that the three-dimensional reconstruction was an accurate rendering of the original tissue-staining pattern. LSCM is employed for analysis of cell phenotype.

To measure in three dimensions morphological changes of vessels: Seven 100 μm thick vibratome coronal sections were screened at 2 mm intervals from bregma 5.2 mm to bregma -8.8 mm (from each animal injected with FITC-dextran. Green fluorochrome (FITC-dextran perfused microvessels) on the sections was excited by a laser beam at 488 nm and emissions were acquired with a photomultiplier tube through 522 nm emission filters. Eight brain regions (Figure 17), three fields view within each region in the ipsilateral and the contralateral hemispheres for rats subjected to stroke within a reference coronal section (interaural 8.8 mm, bregma 0.8 mm) were scanned in 512x512 pixel (279 x 279 μm2) format in the x-y direction using a 4X frame-scan average and forty thin optical sections along the z-axis with an 1 μm step-size were acquired under a 40X objective. All z-axis LSCM images of cerebral microvessels perfused by FITC-dextran were converted into a TIF format using software of Confocal Assistant (Bio-Rad). Vascular branch points, segment length and diameter were measured in three dimensions using software developed in the laboratory. The methodology involves voxels coding, branch labeling and quantitative techniques.

To measure in two dimensions vascular density and morphological changes of vessels: Eight brain regions (Figure 17), three field views (40x objective) within each region were acquired and numbers of vWF immunoreactive vessels were counted throughout the field view by the MCID system. The total numbers of immunoreactive vessels were divided by the total tissue areas to determine vascular density. Vessel perimeter was measured and numbers of vessels with sprouting and intussusception was counted in each of eight brain regions using the MCID system.

For quantification of endothelial cell proliferation: BrdU immunostained sections were digitized using a 40X objective (Olympus
BX40) via the MCID computer imaging analysis system (Imaging Research, St. Catharine's, Canada). Total endothelial cells and BrdU positive cells within a total of 20 enlarged and thin walled vessels located in the boundary area of the ischemic lesion and in homologous contralateral tissue (Figure 17) were counted in each section. In each 20th coronal section from the reference section eight regions in each section were counted per rat with the number of BrdU reactive cells averaged. Data was presented as the percentage of the number of the BrdU immunoreactive cells within vessel/total endothelial cell number.

**Capillary-like tube formation assay:** In vitro angiogenic activity of conditioned medium was determined by testing its ability to induce microvascular endothelial cells to form of capillary-like tubes as described by Haralabopoulos, et al. Briefly, 0.8 ml of 4°C growth factor reduced Matrigel (Becton Dickinson) was added to pre-chilled 35 mm culture dishes and allowed to polymerize at 37°C for 2 to 5 hours. Microvessel endothelial cells (2x10⁴ cells) (above) in 1.5ml culture medium with or without simvastatin were seeded in each dish. Dishes were incubated at 37°C for 24 hours in a humidified atmosphere of 5% CO₂ in air. All assays were performed in triplicate. After incubation, dishes were observed with a phase contrast microscope and photographed at 10x magnification. Tracks of endothelial cells organized into networks of cellular cords (tubes) in randomly selected 5 microscopic fields were counted and averaged.

**SVZ explant cultures:** The SVZ in old and young rats were dissected from a frontal slice (2 mm thick) extending between the crossing of the interior commissure and the rostral opening of the third ventricle. A strip of tissue (100 µm wide; 2-4 mm long) was cut along the lateral wall of the lateral ventricle under the corpus callosum down to the ventral tip of the lateral ventricle. Tissue was minced with scalpels into pieces of =0.15 mm. Explant was cultured within Matrigel (BD Biosciences) in wells containing 500 µl of culture medium. The medium composition was as follows: Neuralbasal-A Medium (GIBCO) containing 2% B27 medium-supplement (GIBCO). The cultures were fixed after 7 days in vitro (DIV).
Quantification of neuronal migration *in vitro*: SVZ explant culture was performed with a phase contrast microscope and photographed at 10x magnification with a digital camera. Several photomicrographs were joined in Adobe Photoshop to reproduce the complete migration of SVZ explant culture. The average linear distance of cell migration from the SVZ explant culture edge was captured and measured at day 2, 4 and 7 using the MCID software. This average distance was assessed in each explant culture.

**Primary cortical neuronal cell culture**: Cultured cortical neurons were prepared from embryonic day 17 (ED 17) Wistar rats. Cells are mechanically dissociated from tissue pieces by titration following enzymatic incubation for 20 minutes in calcium- and magnesium-free Hank’s balanced saline buffer (HBSS) containing 2 mg/ml of trypsin. Dissociated cells are plated in poly-D-lysine-coated dishes and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin overnight at 37°C with 5% CO₂. The medium was removed the next day and replaced with Neurobasal medium supplemented with 2% B27 supplement and 100 µg/ml streptomycin and 100 units/ml penicillin (Invitrogen) for 10-12 days *in vitro* before the start of experiments.

**Neurite outgrowth assay (image acquisition and morphometry)**: To trace the axonal arbors of fluorescently labeled neurons, photomicrographs were captured at 50X magnification with a digital camera. Where necessary, several photomicrographs were joined in Adobe Photoshop to reproduce the complete axonal arbor. The axons were then traced in adobe photoshop and again measured using MCID analysis system. Twenty neurons per condition were documented. The tracings were measured using the perimeter/length function. Axon branching was quantified from the tracings using a custom-written branchpoint counting program (P.Pomorski, UNC). To determine axon caliber and soma size, pictures of 20 stained neurons per condition were taken at 150X magnification. The width of axons was measured in adobe Photoshop at 20 µm and 50 µm from cell body. In case of cells bearing
more than one axon, the thickest axon was measured. For soma size, the circumference of the cell bodies was traced and the footprint area measured using MCID system. To determine growth cone morphology, the primary cortical neurons was double-labeled with an antityrosinated a-tubulin antibody and FITC-phalloidin. The mean growth cone area within 10 random fields from four independent cultures was measured.

**Immunocytochemistry:** Cells grown on chamber slides were fixed in 4% paraformaldehyde and then incubated in 20% normal serum for 30 minutes, depending upon the specific primary antibody to be used. Cells were treated with a predetermined dilution of each of the primary antibody against p-AKT, p-ERK, p-PI3K, VEGF, VEGFR1, VEGFR2, t-PA, u-PA, synaptophysin, MAP2, NGR for 1 hour at 4°C. Following incubation with primary antibody, cells were washed 3 to 4 times in cold PBS and then reacted with fluorescein or Texas red-labeled secondary antibody. The visualization of neural markers was accomplished by immunofluorescence technique and LSCM.

**Enzyme-linked immunosorbent assay (ELISA).** Brain extract from control MCAo and simvastatin treated animals were obtained from the ischemic boundary zone, at 14, after MCAo. The infarct rims in the parietal cortex, at 2 mm posterior to the bregma within the region, is considered to be the penumbral zone. Tissue blocks were dissected on ice and wet weight is rapidly measured. 150 mg/ml of tissue was homogenized in DMEM and centrifuged for 10 minutes at 10,000x g at 4°C. The brain extracts were then divided into 200 μl triplicate samples. To measure the release of factors such as VEGF, VEGFR1, VEGFR2, p-AKT or p-PI3K, p-ERK, NGR from cultured cortical neurons and endothelial cells in vitro or in vivo ischemic brain tissue under experimental conditions, commercially available factor specific ELISA kits from (R&D system inc., MN and CALBIOCHEM) was used according to manufacturer’s instructions.

**Western blot analysis:** Enhanced chemiluminescence (ECL) Western blot is a light emitting nonradioactive method for detection of specific antigens, conjugated directly or indirectly with horseradish
peroxidase-labeled antibodies. Animals were perfused transcardially with heparinized isotonic saline solution. The samples, brain tissue, were homogenized and measured for protein content. Proteins were resolved by 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. The ABC (Avidin-Biotin Complex) method was used to detect specific proteins, e.g., p-PI3K, p-AKT, p-ERK, t-PA, u-PA, VEGF, VEGFR1 and VEGFR2, and synaptophysin, GAP43, NGR. The ECL Western blotting detection system (Amersham. IL) and exposure to Hyperfilm was used to detect proteins. Proteins are quantified by a scanning densitometer using digital imaging and analysis systems (Alpha Innotech Co. IL).

**Rac1 and RhoA activity assays**: The samples were lysed in RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail set I from Calbiochem). RhoA and Rac1 activity assay kits from Upstate Biotechnology were used according to the manufacturer's instructions. Equal amount of lysates were incubated with GST-PBD (p21-binding domain of human PAK-1) or GST-RBD (Rho binding domain of rhotekin) to precipitate GTP-bound Rac1 and GTP-bound RhoA, respectively. Precipitated GTP-bound Rac1 or RhoA was resolved on a 14% SDS-PAGE and immunoblotted using monoclonal antibodies specific for Rac1 and RhoA (Upstate Biotechnology). The total amount of Rac1 or RhoA was also measured by immunoblotting using the same set of lysates and antibodies.

**STATISTICAL ANALYSIS**

The primary purpose of this experiment was to test statin (atorvastatin, simvastatin) treatment efficacy on neurological functional recovery in rats after stroke, to provide insight into the underlying mechanisms of therapeutic benefit of angiogenesis, synaptogenesis, neurogenesis and neuronal migration, and to elucidate the molecular pathways mediating simvastatin induced therapeutic benefit.

**Dose-finding study for atorvastatin efficacy**: Rats were
randomized into groups: saline, and various dose levels of atorvastatin at administrations 1 day after stroke stratified by age (young and old). Neurological functional deficit was measured the day before and after simvastatin therapy at 7 and 14 days with two functional tests (mNSS, Adhesive-removal test). The outcome measurement for testing efficacy is neurological functional recovery at 14 days after treatment that is compatible to clinical stroke study.

Study of the effectiveness of statin therapy was taken death, deterioration/worsening into account. If a rat dies before 14 days after treatment, the worst functional scores were assigned as the 14-day functional status. If a rat is deteriorating at 14 days, status was measured by functional tests, or the worst scores were assigned if the rat is not capable of performing the tests. The analysis of variance (ANOVA) test was used to assess simvastatin efficacy on mNSS at 14 days. Analysis began by testing for interaction between treatment and age, followed by the main effect at the 0.05 level, if no treatment interaction is detected at 0.10. Pair-wise comparison was further tested at the 0.05 level, if there is a significant interaction or main effect at the 0.05 level. The same analysis approach was used to study statin efficacy on Adhesive-removal test at 14 days.

The dose is effective, if there is a significant improvement on neurological functional recovery at 14 days after the treatment with p-value <0.05 compared to the placebo treated group (saline), adjusting for age. The treatment effects were tested at 14-day functional recovery between different doses.

Repeated measure analysis of covariance was used to test each function score changes over time, with independent factors of the simvastatin treatment, rat age and dependent factor of time. PROC MIXED in SAS was used to analyze the data, if data is normal. Otherwise, PROC GEMODE was used to handle data with weak assumption on data distribution. If an interaction is detected at level 0.05, the subgroup analysis was conducted to at 0.05 level.

Functional deficits were evaluated prior to the treatment and at 14
days and 28 days after stroke before sacrificed for the measurements of angiogenesis, synaptogenesis, neurogenesis and neuronal migration. Analysis of variance and covariance was performed to test the statin effect on functional recovery, angiogenesis, synaptogenesis, neurogenesis and neuronal migration respectively, adjusting for sacrifice time in 3 levels, age of levels, and the multiple data collections per subject (rat), if there are any. Analysis started with testing for the interaction between the treatment and the other covariate, followed by the subgroup analysis.

In general, a complete 2 X 2 factorial design was used to study the statin effect on outcome (e.g., angiogenesis) with a total four groups; DMEM as control, statin alone, VEGFR2 inhibitor alone and simvastatin+VEGFR2 inhibitor. Analysis began with testing for interaction of statin by VEGFR2 inhibitor, followed by testing for the main effect of statin or VEGFR2 inhibitor, if there was no interaction at the 0.05 level. A significant interaction indicates the effect of statin on angiogenesis was dependent on the condition of VEGF. Subgroup analysis was further tested between statin alone and statin+ VEGFR2 inhibitor or between s statin+VEGFR2 inhibitor and control groups. No interaction detected at the 0.05 level, indicates that the effect of statin on the angiogenesis expression is not dependent on the VEGF condition and therefore VEGF is not an important pathway factor.

**EXAMPLE 2**

**METHODS:**

**Animal model of traumatic brain injury (TBI)** A controlled cortical impact model of TBI in rat was utilized. Male Wistar rats (300-400 g) were anesthetized with chloral hydrate, 350 mg/kg/body weight, intraperitoneally. Rectal temperature was controlled at 37°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in a stereotactic frame. Two 10-mm diameter craniotomies were performed adjacent to the central suture, midway between lambda and bregma. The second craniotomy allowed for movement of cortical tissue laterally. The dura was kept intact over the
cortex. Injury was induced by impacting the left cortex (ipsilateral cortex) with a pneumatic piston containing a 6-mm diameter tip at a rate of 4 m/s and 2.5 mm of compression. Velocity was measured with a linear velocity displacement transducer. Brain injury in this model is characterized by cystic cavity formation in cortex and selective cell damage in hippocampal formation. In cortex, the injured area incorporates the frontal cortex areas 1-3, which serve as the primary motor cortex. It also includes three other areas: forelimb area, hindlimb area and parietal cortex area 1. These three areas are considered as the primary somatosensory cortex. Therefore unilateral damage to these areas causes somatomotor and somatosensory disorders which are evident on asymmetry deficiency tests, such as Beam Balance Test and Corner Test.

**Experimental Groups:** Twenty male Wistar rats were randomly divided into two groups with 10 animals per group. As experimental control, one group of rats was exposed to TBI and orally given saline. Rats in another group were subjected to TBI, and one day later, atorvastatin was orally administered at a dose of 1 mg/kg/day for seven consecutive days. All rats were sacrificed 14 days after TBI.

**Neurological Functional Evaluation:** Neurological functional measurement was performed using a modified Neurological Severity Score (mNSS) and a Corner Test. The tests were carried out on all rats preinjury and on days 1, 4, 7, and 14 after TBI. The mNSS is a composite of the motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive) and reflex tests. Motor tests of the mNSS include seven items with a maximum of 6 points, which mainly reflect the function of the motor representation area in the contralateral cortex. Damage to this area causes contralateral limb paralysis, leading to high scores on the mNSS motor tests. Sensory tests include two items with a maximum score of 2, reflecting a combination of visual, tactile and deep sensation. Unilateral lesion of the sensory and motor representations of the forelimb in the somatosensory cortex can produce contralateral asymmetry. Indeed, the placing test included in sensory tests of the mNSS also reflects an aspect
of the motor function, because the corticospinal pathway mediates the
execution of the placing reaction (DETA-6) and their lesions produce an
enduring forelimb placing deficit. Beam Balance Tests, belonging to the
asymmetry test, have seven items with a maximum score of 6, mainly
reflecting hindlimb placing performance, which is controlled by the
contralateral cortical representation of motor function. Damage to this area
causes dragging of the contralateral hindlimb (the hindlimb is not placed on
the beam), or the hindlimb is placed on the vertical surface of the beam to
help support the animal's weight and to aid in maintaining balance, which
reflects a high score on Beam Balance Tests. The last part of the mNSS
includes reflexes, such as pinna, corneal and startle reflexes, and
abnormal movements. In this model, injury in the left hemisphere cortex of
rats causes sensory and motor functional deficiency with elevated scores
on motor, sensory, and Beam Balance Tests in the early phase after injury
(day 1 after injury). Absent reflexes and abnormal movements can be
measured on rats with severe injury. The Corner Test was developed for
measurement of long-term functional recovery for the rat. The test is more
sensitive to unilateral cortical injury because it reflects multiple
asymmetries, including postural, vibrissae sensory, and forelimb and
hindlimb use asymmetries, which all combine at the same time to bias
turning. The non-injured rat randomly turns either left or right, but the
injured rat preferentially turns toward the non-impaired, ipsilateral (left)
side. The number of right turns was recorded from ten trials for each test
and used for statistical analysis.

**Tissue Preparation:** Rats were anesthetized intraperitoneally with
ketamine and xylazine, and perfused transcardially first with saline solution
containing heparin, followed by 4% paraformaldehyde in 0.1 M phosphate
buffered saline (PBS), pH 7.4. Their brains were removed, post-fixed in
10% formalin for 1-2 seconds day at room temperature, and then
processed for paraffin sectioning. A series of 6-μm thick sections were cut
with a microtome through each seven standard sections.
**Immunoperoxidase Staining:** To identify the vascular structure, brain sections, after being deparaffinized, were incubated in 2% bovine serum albumin (BSA)-phosphate buffered saline (PBS) at room temperature for 30 min, subsequently treated with mouse anti-Von Willebrand Factor (vWF, Dako, Carpinteria, CA) antibody diluted at 1:200 in PBS at 4°C overnight. Following sequential incubation with biotin-conjugated anti-mouse IgG (dilution 1:100; Dakopatts, CA), the sections were treated with an avidin-biotin-peroxidase system (ABC kit, Vector Laboratories, INC.). DAB was then used as a sensitive chromogen for light microscopy.

**Immunofluorescence:** After dehydration, sections were boiled in 1% citric acid buffer (pH 6.0) in a microwave oven for 10 minutes, cooled down to room temperature, and incubated in 1% sapomlin for 3 hours. Subsequently, the sections were incubated in 1% BSA to block the non-specific signals. Using the same buffer solution, the sections were incubated overnight at 4°C in primary antibodies [monoclonal mouse anti-MAP-2 and monoclonal mouse anti-synatophysin (Chemicon Temecula, CA) followed by 2 hours at room temperature in corresponding fluorochrome-conjugated goat secondary antibodies (anti-mouse FITC, Jackson ImmunoResearch, West Grove, PA). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Dako, Carpinteria, CA). Each of the above steps was followed by four 5 minute rinses, three times in PBS. The sections were mounted with ProLong antifade medium (Molecular Probes, Eugene, OR). Double immunofluorescent staining was performed as described for the identification of the newly generated vessels that contain BrdU-labeled cells. Briefly, BrdU labeling was performed after staining for vWF of the endothelial cell marker. After dehydration, sections were incubated for 1 hour in 0.1 M PBS containing 1% goat serum and 0.3% Triton X-100. Using the same buffer solution, the sections were incubated overnight at 4°C in primary antibodies [monoclonal mouse anti-vWF (1:200, Dako, Carpinteria,
CA), and followed by 2 hours at room temperature in corresponding fluorochrome-conjugated goat secondary antibodies (anti-mouse FITC). The sections were incubated with monoclonal mouse anti-BrdU (Dako, Carpinteria, CA) at room temperature for 2 hours and subsequently with anti-mouse Cy5 at room temperature for 2 hours. All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Each of the above steps was followed by four 5 minute rinses, three times in PBS. The sections were mounted with ProLong antifade medium (Molecular Probes, Eugene, OR). Sections were observed under a fluorescent microscope.

**Cell Counting:** To evaluate whether orally administered atorvastatin reduces the damage of TBI to the neurons, cell counts were performed by observers blinded to the individual treatment status of the animals. MAP-2/DAPI-positive cells were defined as the survival neurons and counted in the CA3 region of hippocampal formation and the boundary zone the injured cortex. **CA3 of hippocampus:** Cell counting in this region was performed as described previously. Five sections with 50-μm interval through the dorsal DG were analyzed with a fluorescent microscope at 400 × magnification (at the interaural 5.20-mm levels). The number of MAP-2/DAPI positive cells was counted in the CA3 region both in the ipsilateral and contralateral hippocampus by using an MCID image analyzer (MCID, St. Catherine's, Ontario). The percentage of MAP-2/DAPI positive cells of CA3 regions in the ipsilateral hippocampus compared to those in the contralateral hippocampus was estimated and used as a parameter to evaluate histological changes, and subsequently employed for statistical analysis also. **Boundary zone of the injured cortex:** MAP-2/DAPI positive cells in the boundary zone of the injury were counted on the same sections as used for the hippocampus. The boundary zone was defined as the area surrounding the lesion cavity, which morphologically differs from the surrounding normal tissue. Surviving neuronal cells were calculated as a density (cell number/mm²). The density was used for statistical analysis. Using cell density to compare the cell proliferation avoids bias due to
sample size and unevenly distributed cells.

**Volumetric Analysis** To estimate the volume of the hippocampus, sections were stained with hematoxylin and eosin (H&E staining) and analyzed using a 10× objective and a computer image analysis system. The volume of hippocampus at the dorsal DG level was calculated by measuring the hippocampus areas from each section and multiplying by the section thickness and the sampling intervals. The lesion volumes of the cortex were estimated using the same image analysis system, as previously described.

**Density Measurement of Synaptophysin:** To investigate whether atorvastatin administration protects synapses and/or promotes synaptogenesis, an additional five sections with 50-μm intervals through the dorsal DG were stained for synaptophysin an index of synaptic activity and the images were digitized with a fluorescent microscope at 400× magnification (at the interaural 5.20-mm levels). Synaptophysin density (pixels/mm²) was calculated by dividing the pixels measured in the CA3 region by the square area (mm²) both in the ipsilateral and contralateral hippocampus by using an MCID image analyzer (MCID, St. Catherine's, Ontario). The density (pixel/mm²) of synaptophysin in the boundary zone was only measured in the ipsilateral hemisphere. The percentage of the synaptophysin density in the ipsilateral and the contralateral CA3 were further calculated and used as a parameter to evaluate the synaptic changes. Density measurements were performed by an investigator who was blind to the treatment of the animals.

**Measurement of Vascular Density and Perimeters:** Five sections with 50-μm interval through the dorsal DG were stained for vWF and the images were digitized with a light microscope at 400× magnification (at the interaural 5.20-mm levels). The vWF positive vessels were counted in the boundary zone of the lesion and the CA3 region of the hippocampus, using the MCID system. The vascular density in both two regions were determined by dividing the immunoreactive vessels by the corresponding area and used as a parameter of angiogenesis. Because angiogenesis
comprises two different mechanisms: endothelial sprouting and intussusceptive microvascular growth, the endothelial cell proliferation, measured by counting both vWF and BrdU vessels, and intussusceptive microvascular growth were also investigated morphologically to evaluate angiogenesis.

Results

Atovastatin Improves Neurological Functional Outcome. Injury in the left hemisphere cortex of rats causes neurological functional deficits as measured by mNSS (Figure 23a). These rats present with high scores on motor, sensory and Beam Balance Tests in the early phase after injury (day 1 after injury). Absent reflexes and abnormal movements are evident in rats with severe injury. By day 4 after injury, recovery began, and this recovery persisted at all subsequent evaluation time points in both saline-treated and atorvastatin-treated groups. Motor function tested by the mNSS recovered faster than sensory and beam balance functions. On day 14 after injury, the residual deficit scores were mainly present on the Beam Balance Tests and sensory test (placing test) of the mNSS. The mNSS scores for the atorvastatin-treated group were significantly decreased at day 14 (5.2 ± 0.6) after TBI when compared with the saline-treated groups (6.9 ± 0.8, p < 0.05) (Figure 23a). These results were also supported by data from the Corner Test, a sensitive test for asymmetry deficiency (Figure 24b). Damage to the left hemisphere caused the rats to make fewer right turns. The Corner Test scores from the atorvastatin-treated group were significantly improved at days 4, 7 and 14 (1.4 ± 0.7, 2.4 ± 0.9 and 3.3 ± 1.0, respectively) after TBI when compared with the saline-treated groups (0.7 ± 0.4, 1.3 ± 0.4 and 2.1 ± 0.7, p < 0.05). There were no significant differences at other time points between two groups for these tests. These data demonstrate that atorvastatin reduces the asymmetry deficiencies caused by TBI in rats.

Lesion Volume and Hippocampal Volume. There was no significant difference in the cortical lesion volume between the atovastatin-
treated (6.1±3.7 mm³) and the control (5.1±3.5 mm³) groups. Likewise, no difference was detected in the volume of hippocampus at the dorsal DG level between the atovastatin-treated (3.3 ± 1.2 mm³) and the control (3.1 ± 0.9 mm³) groups. These data demonstrate that atorvastatin does not reduce the lesion volume.

**Atorvastatin Increases the Survival of Neurons in the Boundary Zone of the Injured Area and the CA3 of the Hippocampus.** In the cortex of the contralateral hemisphere, the pyramidal neurons show intense signals for MAP-2 and distributed within different layers of the cortex, with some neurons exhibiting a long axon and some processes (Figure 24a). MAP-2 positive. MAP-2 positive days after TBI, the density of synaptophysin signals in the boundary zone was less than that of the intact cortical area (Figure 26a). After treatment with atovastatin, the density significantly increased in this area compared to the control rats (Figure 26b and c). These data suggest that atovastatin protects synapses from the impact and/or induces synaptogenesis in the boundary zone.

**Hippocampal CA3:** Synaptophysin signals were evident, and contained fewer processes than homologous neurons of the morphologically intact area adjacent to the boundary zone (Figure 24b). After treatment with atovastatin, the density of MAP-2/DAPI positive cells (neurons) in boundary zone of the injured area was significantly higher in the atovastatin treated group (222.5±68 neurons/mm³) than in the control group (145±32 neurons/mm³) (Figure 24c and d). This suggests that atovastatin can protect the damaged neurons in the boundary zone of the lesion area from death induced by TBI.

In the CA3 region of the contralateral hippocampus, the neurons in the pyramidal cell layer (PCL) had large cell body with long processes projecting to the stratum lucidum (SL) (Figure 25a). Fourteen days after the TBI, the number of MAP-2 positive cells in the CA3 region compared with the contralateral hemisphere significantly declined and with survival neurons showing a loss of processes, resulting in the disruption of the
pyramidal layer (Figure 25b). Many more MAP-2 negative and DAPI-labeled cells in the SL region were found compared to the contralateral SL (Figure 25b). After treatment with atorvastatin, the number of the surviving neurons in the pyramidal cell layer significantly increased with augmentation of processes of the pyramidal cells compared to the control (Figure 25c). The survival rate of the pyramidal cells was 21 ± 3% after TBI and 52 ± 7% after TBI and atorvastatin treatment (p<0.05) (Figure 25d). These data suggest that atorvastatin can rescue the damaged pyramidal cells in CA3 region due to TBI.

Atorvastatin Augments Synaptic Density Defined by Synaptophysin. Boundary Zone of the Injured Cortex: Almost no positive signals were found in the body of the pyramidal cells (Figure 27a). Fourteen days after TBI, almost no synaptophysin positive signals were detected in the stratum lucidum and some weak signals were observed in the pyramidal cell layer (Figure 27b). After the treatment of atorvastatin, intense synaptophysin immunoreactive signals were found in the pyramidal cell layer as well as in the stratum lucidum (Figure 27c). Synaptophysin staining density increased twofold in the treated group over the control (Figure 27d). These data indicate that atorvastatin can protect synapses from impact and induce synaptophysin expression.

Atorvastatin Induces Angiogenesis. Fourteen days after TBI, the capillary vessels in the contralateral cortex had very clear margins and tight connections with the adjacent parenchymal tissue, and narrow clefts between the vessels and the parenchyma were observed (Figure 28a and b). In the boundary zone of the lesion area of the ipsilateral hemisphere, disruption of capillaries and the enlarged vascular clefts were observed and enlarged or intussusception vessels were found (Figure 28c, d and e). Many large and thin-wall-vessels (“mother” vessels) and/or intussusception vessels were, however, found in the boundary zone after atorvastatin treatment (Figure 28f and g). The capillary density in the boundary zone of the lesion and the CA3 region of the hippocampus from the atorvastatin
treated group significantly increased when compared with the control by counting the vWF positive vessels (Figure 28h). This evidence of angiogenesis was further supported by the presence of newly generated vessels marked by Brdu-labeled endothelial cells in the boundary zone and the CA3 region after atorvastatin treatment (Figure 28h and j). These data suggests that atorvastatin augments angiogenesis in the injured brain after TBI.

Discussion

This study shows that treatment with atorvastatin reduces neurological functional deficits in rat after controlled cortical impact. Based on the immunohistostaining and morphological observation, the functional improvement is likely partially mediated by atorvastatin increasing neuronal survival in the boundary zone of the lesion area and the CA3 region of the hippocampus after injury. The functional improvement also results from synaptic protection and/or the induction of synaptogenesis, and the increased angiogenesis by atorvastatin in the boundary zone and the CA3 region.

In controlled cortical impact model of TBI, direct impact of the piston rod on brain tears the vessels and disrupts axonal integrity, causing diffuse axonal injury (DAI), diffuse vascular injury (DVI), intracerebral hemorrhage and concussion. After these primary injuries, subsequent secondary insults occur and result in ischemic brain injury. This is the leading cause of brain death from TBI. Beside the primary neuronal necrosis after injury, delayed neuronal cell death occurs in the boundary zone of the injured cortical area and the CA3 regions of hippocampus. Both patterns of the neuronal death cause certain neuronal loss in these areas and subsequently result in the neurological functional deficits in animals. These areas are potentially salvageable. Rescuing the damaged neurons and maintaining or enhancing synaptic activity in these areas promotes the functional outcome after brain injury. Significant functional improvements in the atorvastatin treated group were detected at days 4, 7 and 14 after TBI.
on the Corner Test, and at day 14 on mNSS when compared with the control. Since both functional tests are composed of sensory, motor and reflex aspects, atorvastatin treatment appears to augment the overall improvement of the neurological function. Significant functional improvement occurring at day 4 after TBI (atorvastatin treatment for 3 days) strongly suggests that atorvastatin may rescue and/or promote the survival of the damaged neurons or synaptic connections. This is supported by the increased survival number of neurons and the synaptophysin density in the boundary zone and the CA3 region in the ipsilateral hemisphere after atorvastatin treatment in TBI rats.

The data also demonstrate that atorvastatin induces angiogenesis in the injured brain after TBI.

In summary, the data show a significant therapeutic benefit of atorvastatin treatment on TB, which can be mediated by induction of angiogenesis, enhancement of synaptic activity, neurogenesis and neuronal survival.

Throughout this application, author and year and patents by number reference various publications, including United States patents. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology that has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the described invention, the invention may be practiced otherwise than as specifically described.
What is claimed is:

1. A method of promoting neural restoration and enhancing neural function comprising the step of:
   administering a therapeutic amount of a statin compound to a patient in need of such promotion.

2. The method according to claim 1, wherein said administering step includes administering a statin compound selected from the group consisting essentially of pravastatin and its sodium salt, simvastatin, lovastatin, atorvastatin, fluvastatin, and cervistatin.

3. The method according to claim 1, wherein said administering step includes administering therapeutic amount of a statin compound to a patient to promote a result selected from the group consisting essentially of promote brain plasticity and functional benefit and recovery from neural injury, and neurodegeneration.

4. The method according to claim 3, wherein said administering step includes administering therapeutic amount of a statin compound to a patient to promote a result that is accomplished by a function selected from the group consisting essentially of induction of neurogenesis, angiogenesis, synaptogenesis, selective neuronal survival, reduced gliosis, dendritic arborization, enhanced progenitor and stem cell migration, generation of growth and neurotrophic factors and activation of g-proteins, and upregulation of cGMP.

5. A composition for promoting neural restoration and enhancing neural function comprising an effective amount of a statin compound sufficient to promote neurogenesis.

6. The composition according to claim 5, wherein said statin compound is selected from the group consisting essentially of pravastatin and its sodium salt, simvastatin, lovastatin, atorvastatin, fluvastatin, and cervistatin.

7. The composition according to claim 5, wherein said statin
compound is capable of a function selected from the group consisting essentially of induction of neurogenesis, angiogenesis, synaptogenesis, selective neuronal survival, reduced gliosis, dendritic arborization, enhanced progenitor and stem cell migration, generation of growth and neurotrophic factors and activation of g-proteins, and upregulation of cGMP.

8. A neural restoration and neural function promoter comprising a statin compound in a pharmaceutically acceptable carrier.

9. The neurogenesis promoter according to claim 8, wherein said statin compound has a function selected from the group consisting essentially of augmenting neural growth in a tissue, promoting brain plasticity and functional benefit and recovery from neural injury, and neurodegeneration.

10. The neurogenesis promoter according to claim 8, wherein said statin compound is selected from the group consisting essentially of pravastatin and its sodium salt, simvastatin, lovastatin, atorvastatin, fluvastatin, and cervistatin.

11. A method of augmenting the production of brain cells by administering an effective amount of a statin compound to a patient that can benefit from augmentation.

12. The method according to claim 11, wherein said administering step includes administering a statin compound selected from the group consisting essentially of pravastatin and its sodium salt, simvastatin, lovastatin, atorvastatin, fluvastatin, and cervistatin.

13. A method of augmenting the production of brain cells and facilitating cellular structural and receptor changes by administering an effective amount of a statin compound to a site in need of augmentation.
FIG - 5a  FIG - 5b  FIG - 5c

FIG - 5d  FIG - 5e  FIG - 5f

SUBSTITUTE SHEET (RULE 26)
**FIG - 9a**

**FIG - 9b**

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**FIG - 11a**

Synaptophysin

MCAo

**FIG - 11b**

MCAo + Atorvastatin

**FIG - 11c**

![Graph showing synaptophysin levels](image)

* P < 0.05

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**FIG - 12a**

Control

**FIG - 12b**

+Simvastatin

**FIG - 12c**

Migration distance (µm)

- Control
- 0.005µM
- 0.01µM
- 0.1µM

*p<0.05

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**FIG - 13a**

**FIG - 13b**

Control  
+LY10μM

**FIG - 13c**

Migration distance (pm, mean±SE)

Control  2μM  10μM  30μM

* p<0.05

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Fig. 16
FIG - 21

![Figure 21](image)

**FIG - 21**

![Figure 21](image)

**FIG - 21c**

![Figure 21c](image)

**FIG - 22a**

![Figure 22a](image)

**FIG - 22b**

![Figure 22b](image)

SUBSTITUTE SHEET (RULE 26)
**FIG - 23a**

NSS Scores (Mean±SD)

- Lipitor
- Control

**FIG - 23b**

Corner Test Scores

- Lipitor
- Control

SUBSTITUTE SHEET (RULE 26)
FIG - 26a

FIG - 26b

FIG - 26c

SYNAPTOPLYSIN DENSITY (PIXEL/MM²)

lipitor control

SUBSTITUTE SHEET (RULE 26)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/21, 31/225
US CL : 514/510, 548
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/510, 548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 6,274,603 B1 (FOIRIER) 14 August 2001 (14.08.2001), column 4, lines 26-34; column 5, lines 46, 66; column 6, lines 6-24, 65-67; column 8, lines 3-5.</td>
<td>1-13</td>
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<td>P,X</td>
<td>US 6,472,421 B1 (WOLOZIN) 29 October 2002 (29.10.2002), column 3, lines 8-12; column 5, lines 35-67; column 6, lines 1-4.</td>
<td>5-10</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

See patent family annex.

Date of the actual completion of the international search 05 July 2003 (05.07.2003)

Date of mailing of the international search report 08 AUG 2003

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Form PCT/ISA/210 (second sheet) (July 1998)
Continuation of B. FIELDS SEARCHED Item 3:
WEST, STN (file medicine)
search terms: statin and (neural restoration or neural function or brain plasticity or neural injury or neurodegeneration or neural growth)