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Simvastatin Treatment Enhances NMDAR-Mediated Synaptic Transmission by Upregulating the Surface Distribution of the GluN2B Subunit

Marc-Alexander L. T. Parent,

Department of Experimental and Clinical Pharmacology, University of Minnesota, McGuire Translational Research Facility, 2001 6th St SE, Minneapolis, MN 55455, USA

David A. Hottman,

Department of Experimental and Clinical Pharmacology, University of Minnesota, McGuire Translational Research Facility, 2001 6th St SE, Minneapolis, MN 55455, USA

Shaowu Cheng,

Department of Experimental and Clinical Pharmacology, University of Minnesota, McGuire Translational Research Facility, 2001 6th St SE, Minneapolis, MN 55455, USA

Wei Zhang,

Department of Experimental and Clinical Pharmacology, University of Minnesota, McGuire Translational Research Facility, 2001 6th St SE, Minneapolis, MN 55455, USA

Lori L. McMahon,

Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, USA

Li-Lian Yuan, and

Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA

Ling Li

Department of Experimental and Clinical Pharmacology, University of Minnesota, McGuire Translational Research Facility, 2001 6th St SE, Minneapolis, MN 55455, USA

Li-Lian Yuan: lilian.yuan@dmu.edu; Ling Li: lil@umn.edu

Abstract

The ramifications of statins on plasma cholesterol and coronary heart disease have been well documented. However, there is increasing evidence that inhibition of the mevalonate pathway may provide independent neuroprotective and procognitive pleiotropic effects, most likely via inhibition of isoprenoids, mainly farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate

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Correspondence to: Li-Lian Yuan, lilian.yuan@dmu.edu; Ling Li, lil@umn.edu.

Present Address: M.-A. L. T. Parent, Department of Neurobiology, John B. Pierce Laboratory, Yale University, 290 Congress Ave, New Haven, CT 06519, USA

Present Address: L.-L. Yuan, Department of Physiology and Pharmacology, Des Moines University, 3200 Grand Ave, Des Moines, IA 50312, USA

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(GGPP). FPP and GGPP are the major donors of prenyl groups for protein prenylation. Modulation of isoprenoid availability impacts a slew of cellular processes including synaptic plasticity in the hippocampus. Our previous work has demonstrated that simvastatin (SV) administration improves hippocampus-dependent spatial memory, rescuing memory deficits in a mouse model of Alzheimer's disease. Treatment of hippocampal slices with SV enhances long-term potentiation (LTP), and this effect is dependent on the activation of Akt (protein kinase B). Further studies showed that SV-induced enhancement of hippocampal LTP is driven by depletion of FPP and inhibition of farnesylation. In the present study, we report the functional consequences of exposure to SV at cellular/synaptic and molecular levels. While application of SV has no effect on intrinsic membrane properties of CA1 pyramidal neurons, including hyperpolarization-activated cyclic-nucleotide channel-mediated sag potentials, the afterhyperpolarization (AHP), and excitability, SV application potentiates the *N*-methyl *D*-aspartate receptor (NMDAR)-mediated contribution to synaptic transmission. In mouse hippocampal slices and human neuronal cells, SV treatment increases the surface distribution of the GluN2B subunit of the NMDAR without affecting cellular cholesterol content. We conclude that SV-induced enhancement of synaptic plasticity in the hippocampus is likely mediated by augmentation of synaptic NMDAR components that are largely responsible for driving synaptic plasticity in the CA1 region.

Keywords

Simvastatin; Pyramidal neurons; Intrinsic membrane properties; Glutamatergic transmission; *N*-methyl *D*-aspartate receptor; GluN2B subunit

Introduction

The most common use of statins in the general population is to reduce plasma levels of cholesterol, specifically low-density lipoprotein (LDL)-associated cholesterol, the primary causal factor for coronary heart disease (Kannel et al. 1971; Prospective Studies et al. 2007). Statins reduce plasma LDL-cholesterol by competing with the endogenous substrate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) for the binding site on HMG-CoA reductase (Endo 2004), the rate-limiting step of the mevalonate synthesis pathway responsible for production of cholesterol (Brown and Goldstein 1986). Due to statins' greater affinity for the binding site, these drugs effectively inhibit cellular production of cholesterol, yielding a homeostatic increase in LDL receptors responsible for cellular uptake of LDL-cholesterol from plasma, and ultimately a reduction in plasma LDL-cholesterol levels. However, while the effects of statins on plasma cholesterol and coronary heart disease have been well documented, there is increasing evidence that inhibition of the mevalonate pathway may provide positive pleiotropic effects that are independent of cholesterol levels and that statins may serve as neuroprotective agents against various forms of nervous system disorders (Liao 2002; Vaughan 2003).

While inhibition of HMG-CoA reductase by statins reduces cholesterol synthesis, a concomitant effect of this functional blockade is decreased availability of isoprenoids, the major donors of prenyl groups for protein prenylation. A branch point downstream of mevalonate synthesis draws substrates away from cholesterol synthesis and yields the

production of non-sterol isoprenoids such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Houten et al. 2003). Many of the pleiotropic effects of statins are mediated through inhibition of isoprenoid synthesis and subsequent protein prenylation (Liao 2002; Vaughan 2003).

Treatment with statins has also been reported to decrease susceptibility of individuals to cognitive dysfunction such as dementia and Alzheimer's disease (AD). Patients prescribed statins for hypercholesterolemia were less likely to develop dementia in retrospective studies (Jick et al. 2000; Wolozin et al. 2000). Normocholesterolemic patients with probable AD given statins demonstrated a more gradual cognitive decline relative to patients given placebo. These patients also had a significantly correlated decrease in amyloid- β protein (Ab) with decreased levels of a cholesterol precursor in the mevalonate pathway (Simons et al. 2002). Further, another study on statins in probable AD patients demonstrated the possible utility of cholesterol-lowering drugs in the treatment of cognitive decline (Sparks et al. 2005). However, these studies reporting a neuroprotective role of statins are tentative as prospective studies have come forth showing that statins provide no protection from the development of AD in a group of patients at risk of cardiovascular disease (Shepherd et al. 2002; Collins et al. 2002). Recent clinical trials using statins in patients with AD also have not shown consistent benefits (reviewed by (Shepardson et al. 2010)). Possible reasons for such discrepancies may include differences in the blood-brain barrier permeability and the dose of statins, the population of subjects, and the stage of the disease at which statins are administered.

While the therapeutic potential of statins for AD remains to be determined, neuroprotective effects of statins through both cholesterol-dependent and cholesterol-independent mechanisms have been demonstrated in multiple model systems. For example, by cholesterol-dependent mechanisms, several statins were shown to protect cultured cortical neurons from excitotoxicity after exposure to *N*-methyl *D*-aspartate (NMDA) (Zacco et al. 2003). The protective effects were mimicked by acute treatment with the cholesterol-extracting agent β -cyclodextrin and attenuated by co-treatment with mevalonate or cholesterol (Zacco et al. 2003). In another study, a cholesterol-mediated effect in neuroprotection against NMDA was also observed in primary neuronal cultures treated with simvastatin (Ponce et al. 2008). The study showed that simvastatin treatment lowered the neuronal cholesterol level, resulting in reduced association of an NMDA receptor (NMDAR) subunit with lipid rafts although no change was found in the total level of this NMDAR subunit (Ponce et al. 2008). In addition, cholesterol extraction by β -cyclodextrins protected cortical neuronal cultures against ischemic and excitotoxic insults by affecting NMDAR subunit distribution and signal transduction (Abulrob et al. 2005).

However, a large body of evidence indicates that neuroprotective effects of statins are mediated by cholesterol-independent (pleiotropic) effects. For instance, the protection of cultured cortical neurons from exposure to monosodium glutamate by a statin treatment was not affected by re-establishing cholesterol biosynthesis pathway (Bosel et al. 2005). Chronic statin treatment in rats stimulates production of brain-derived neurotrophic factor (BDNF) in the hippocampus after traumatic brain injury (TBI) by activating Akt-mediated signaling pathway (Wu et al. 2008); increases levels of NMDA receptors (Wang et al. 2009); and

promotes neurogenesis and increases cerebral blood flow by upregulating vascular growth factor, synaptophysin, and activation of Akt and ERK (Chen et al. 2003). In a rabbit ischemic stroke model, simvastatin (SV) exhibits neuroprotective effects by inhibiting Rho-associated kinase (ROCK) (Lapchak and Han 2010). In a mouse model for Parkinson's disease, SV prevents dopaminergic neuronal loss by inhibiting the activation of Ras (Ghosh et al. 2009). Interestingly, among all commercially available statins, SV was found to be the most effective at protecting against kainate-induced excitotoxicity and memory impairment (Ramirez et al. 2011). Previous studies in our laboratory also show that chronic SV treatment improves learning and memory performance in both transgenic AD mice and aged wild-type mice without affecting total brain cholesterol levels (Li et al. 2006). More recently, we found that acute in vitro SV treatment of hippocampal slices from young adult mice enhances long-term potentiation (LTP) in the CA1 region in a phospho-Akt-dependent manner and that this effect is mediated by modulating prenylation-dependent molecular pathways downstream of farnesyltransferase (Mans et al. 2010; Mans et al. 2012).

Employing both whole-cell patch clamp and extracellular dendritic field potential recordings in hippocampal slices, in the present study, we investigated potential effects of SV on the intrinsic (passive and active) membrane properties of CA1 pyramidal neurons and on pharmacologically isolated NMDA receptor (NMDAR) currents that could contribute to the enhancing effects of SV on LTP. In addition, using biochemical methods, we studied the effect of SV on the cell surface distribution of different NMDAR subunits in mouse hippocampal slices and human neuronal cells.

Experimental Procedures

Animals

All procedures used in this study were carried out with the approval of the University of Minnesota Institutional Animal Care and Use Committee. The mice used in this series of experiments were 3–4 months old, male wild-type C57B6/J (Jackson Labs, Bar Harbor, ME). Animals were housed in a specific-pathogen-free facility and kept on a 12 h/12 h light/dark cycle. Animals were provided rodent chow and water ad libitum. All experiments were performed using in vitro transverse slices of hippocampus. Whole-cell current clamp recordings were performed to explore intrinsic membrane properties of CA1 pyramidal neurons exposed to SV. Extracellular dendritic field potential (fEPSP) recordings were used to probe the actions of SV on pharmacologically isolated NMDAR-mediated synaptic potentials at Schaffer collateral CA1 pyramidal cell synapses. Hippocampal slices treated with vehicle were used as controls.

Drugs

SV solution was prepared by procedures described previously (Mans et al. 2010). In brief, simvastatin powder (TCI America, Portland, OR) was dissolved in ethanol and converted into its active form via alkaline hydrolysis in 1 N NaOH. Aliquots of the activated SV stock solution were kept frozen and used within 1 month. On the day of use, the stock solution is diluted in cell culture grade PBS and neutralized with 1 N HCl to pH 7.4 to produce a working stock solution (10 mM SV), which was further diluted 10009 in artificial

cerebrospinal fluid (aCSF) to a final concentration of 10 μ M SV and bath applied to hippocampal slices. Vehicle (Veh) solution was prepared by the same procedure without SV. Selective antagonists for AMPA and NMDA receptors, respectively, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate) and APV (D(-)-2-Amino-5-phosphonopentanoic acid), were purchased from Sigma (St. Louis, MO, USA).

Preparation of Mouse Transverse Hippocampal Slices

Mice were anesthetized using isoflurane followed by decapitation. Brains were removed and placed into icy cutting solution containing (mM): 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 0.5 CaCl_2 , 7 MgCl_2 , 7 Dextrose, and 240 sucrose (Sigma). Transverse slices through the hippocampus were cut with a vibratome (Microm or Leica) using cutting solution and then placed into a holding chamber with aCSF containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 2 CaCl_2 , 1 MgCl_2 , and 25 dextrose (pH 7.4) with constant bubbling of carbogen (95 % O_2 –5 % CO_2). Slices were allowed to recover for at least 1 h prior to recording.

Whole-Cell Recordings

Following recovery, the hippocampal slices were incubated in either 10 μ M SV/aCSF or aCSF containing Veh for 2 h at 27–29 °C before being transferred into the recording chamber for whole-cell patch clamp experiments. The internal pipette solution contained (mM): 120 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl_2 , 4 Na_2 -ATP, 0.3 Tris-GTP, and 14 phosphocreatine. Intrinsic membrane properties of CA1 pyramidal cells were determined experimentally from 3–5 neurons per experimental group per animal over a two-hour period following incubation in either Veh or SV. SV and Veh treatments were counterbalanced to control for the length of time since slicing before experiments commenced.

Resting Membrane Potential (V_{rest})—The V_{rest} was monitored for half of the cells recorded in these experiments immediately following “break in” to achieve a whole-cell recording configuration.

Input Resistance (R_{in})—The steady-state input resistance (R_{in}) was estimated via a –10 pA hyperpolarizing current injection, 800-ms duration at –70 mV. The change in membrane potential (ΔV_{m}) due to the current injection was the difference between the steady-state membrane potential ($V_{\text{m,ss}}$) at the end of the current injection and the pre-injection baseline holding potential (V_{hold}). R_{in} was computed as $R_{\text{in}} = \Delta V_{\text{m}} / -10 \text{ pA} = (V_{\text{m,ss}} - V_{\text{hold}}) / -10 \text{ pA}$.

Rheobase and Action-Potential Threshold (V_{thresh})—Cells were injected with a 100 pA/second current ramp for 2 s. The action potential (AP) threshold was determined as the membrane potential where the rate of membrane potential change exceeds 2 mV/msec at the first AP during the current ramp. The time of V_{thresh} during the current injection indicates the amount of current required to elicit an AP. To compare between groups, all neurons were recorded at $V_{\text{hold}} = -70 \text{ mV}$.

HCN-Current-Mediated Sag Potential (V_{sag})— V_{sag} was generated by a -150 pA, 800-ms duration current injection and computed as the difference between the peak hyperpolarization at the start of the current injection ($V_{\text{m, peak}}$) and the steady-state voltage during the last 50 ms of the current injection. Thus, $V_{\text{sag}} = V_{\text{m,ss}} - V_{\text{m,peak}}$. All neurons were held at $V_{\text{hold}} = -70$ mV at baseline.

Input-Frequency Curves—To explore how SV may affect supra-threshold translation of current to AP firing, a series of supra-threshold currents were injected into neurons, and the numbers of APs generated over 800 ms, at each current step, were plotted. All neurons were held at $V_{\text{hold}} = -70$ mV at baseline. The average number of APs at each level of injected current was compared between SV- and Veh-treated groups.

After-Hyperpolarization Potentials—After-hyperpolarization potentials (AHPs) in the two treatment groups were generated with a burst of ten 700 pA, 3-ms duration, depolarizing current injections to induce 10 APs. Current injections were done at 77 Hz (13 ms between injections), and the protocol was repeated 10 times with 10 s between induced bursts of APs. The 10 traces recorded from a single cell were then averaged, and the average AHP waveform was compared between groups. All neurons were held at $V_{\text{hold}} = -70$ mV at baseline.

Field Recordings

During recording, slices were placed into a two-well, submerged-style recording chamber (Automate Scientific, Berkeley, CA) permitting the recording of two slices in parallel. Each well within the recording chamber is served via an independent solution reservoir and plumbing system with aCSF flowing through each well at a rate of roughly 1.5 ml/min at 29–31 °C.

Evoked, field excitatory post-synaptic potentials (fEPSPs) were recorded with pulled borosilicate glass micropipettes filled with aCSF using a two channel 700B multiclamp amplifier, 1440A Digidata DA/AD converter, and pClamp data acquisition software (Molecular Devices, Sunnyvale, CA). Recording electrodes were placed in the stratum radiatum within CA1 and presynaptic stimulation was given at the Schaffer collaterals within, or directly adjacent to, the CA3/CA1 boundary. Noise was reduced via a faraday cage surrounding the recording setup and Humbug 50/60 Hz noise removing hardware (Quest Scientific, North Vancouver, BC, Canada). A 100-lsec presynaptic stimulation pulse was evoked using 1 MX, bipolar, tungsten-stimulating electrodes (FHC, Bowdoin, ME) driven by A365R constant-current stimulus isolators (World Precision Instruments, Sarasota, FL). fEPSPs mediated by NMDA receptors were isolated pharmacologically via application of CNQX and transition to a modified aCSF containing low magnesium content (0.1 mM Mg^{2+}). Stimulation intensity was adjusted to yield NMDAR-mediated responses of 0.4–0.6 mV and 20-min stable baselines were established prior to application of SV.

Surface Biotinylation Assay and Immunoblot Analysis

Mouse hippocampal slices were prepared and treated with SV or Veh for 2 h as in electrophysiological experiments and subjected to the surface biotinylation assay as

described previously (Snyder et al. 2005). In brief, the slices were rinsed in ice-cold aCSF, and incubated in aCSF containing 0.5 mg/ml sulfo-NHS-LC-biotin (21335; Pierce/Thermo Scientific, Rockford, IL) for 45 min at 4 °C in dark. After rinsing twice in aCSF, the hippocampal area was dissected under microscope and lysed in 100 µl tissue lysis buffer (PBS with 0.1 % SDS, 1 % Triton X-100, and complete protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO)). The tissue lysate was obtained after centrifugation at 14,000 rpm for 10 min at 4 °C. For measuring the total level of proteins by immunoblot analysis, 10 % of the tissue lysate was removed. The remaining 90 % of the tissue lysate was used to isolate biotinylated proteins by incubating with 30 µl NeutrAvidin agarose (PI29200; Pierce/Thermo Scientific). The bound proteins were subjected to SDS-PAGE and immunoblot analysis with specific antibodies (Millipore, Billerica, MA) to GluN2B (06–600), GluN2A (07–632), GluN1 (05–432), and GluA1 (AB1504), followed by HRP-conjugated secondary antibodies. Signal was detected by the ECL plus Western Blotting System (GE Healthcare, Pittsburgh, PA) and quantified by the image J software. The ratio of biotinylated (surface) to total protein for a given culture were calculated and normalized to Veh-treated cultures. For a loading control, the blots were stripped and reprobed with a goat polyclonal antibody against a cytosolic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-20357; Santa Cruz Biotechnology, Dallas, TX). We also studied the effect of SV treatment on surface distribution of GluN2B in human neuroblastoma SH-SY5Y cells (ATCC® CRL-2266; Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum at 37 °C with 5 % of CO₂ in 10 cm culture dishes. When the cells reached approximately 90 % confluency, SV or Veh was added to the medium, and the culture was continued for 1–2 h, followed by the surface biotinylation assay as described above for mouse hippocampal slices.

Cellular Cholesterol Assay

Mouse hippocampal slices were prepared and treated with SV or Veh for 2 and 4 h. The hippocampal area was dissected and homogenized in a tissue lysis buffer (PBS with 5 mM sodium deoxycholate, 0.1 % Triton X-100, and complete protease and phosphatase inhibitor cocktails). The cholesterol level in the tissue lysate was measured by the Infinity™ Cholesterol Liquid Stable Reagent (Thermo Scientific, Middletown, VA). The cellular cholesterol content was normalized by the cellular protein concentration measured by the BioRad Protein Assay (BioRad, Hercules, CA).

Statistics

Analysis of input resistance, input/frequency curves, sag potentials, rheobase, and AP threshold were done using custom scripts written by Marc Parent in MATLAB. Analysis of the AHP experiment was done manually using the AxographX data acquisition software's in house analysis suite (Sidney, Australia). Finally, all field recordings were analyzed using Clampfit (Molecular Devices, Sunnydale, CA). Statistics and graphing were done in Origin-Pro (Northampton, MA). Comparison of data from different treatment groups was performed by Student's *t* test, and *P* < 0.05 was considered statistically significant.

Results

Enhancement of LTP at hippocampal CA3-CA1 synapses following treatment with SV *in vitro* has been linked to suppression of prenylation via the inhibition of isoprenoids (Mans et al. 2012). However, over 300 proteins can be modified posttranslationally via prenylation, and thus the downstream effects of varying prenylation levels may affect various aspects of synaptic and cellular processing. We hypothesized that the LTP-enhancing effects of SV and isoprenoid modulation on LTP could be a consequence of modulation of intrinsic active and passive membrane properties as well as NMDAR transmission which is required for LTP induction.

Intrinsic Properties

Resting Membrane Potential (V_{rest})—From each mouse, half of the slices were treated with SV and half with Veh. The effects of SV treatment on intrinsic membrane properties of CA1 pyramidal neurons were measured on the same neurons.

Initially, the network-independent membrane properties of CA1 pyramidal neurons were compared via analysis of the resting membrane potential, excitability, and AP threshold. If SV treatment depolarizes the resting membrane potential, then this could facilitate NMDAR activation and explain the enhanced LTP magnitude relative to untreated slices as previously reported (Mans et al. 2010). However, there was no difference in the resting membrane potential recorded from neurons in the SV-treated group compared to Veh-treated group (SV: -60.1 ± 1.1 mV, $n = 23$ neurons/5 mice vs Veh: -60.9 ± 1.0 mV, $n = 23$ neurons/5 mice) (Fig. 1a).

Similarly, if SV-treated hippocampal neurons show an enhancement of excitability, and are generally driven to more depolarized voltages than untreated neurons when given a similar amount of current, then these enhanced depolarizations may yield a greater activation of those mechanisms responsible for plasticity at the Schaffer collateral-to-CA1 synaptic network. General levels of excitability in CA1 pyramidal neurons were compared via calculation of input resistance and rheobase within SV- and Veh-treated groups. Pyramidal neurons were held at -70 mV using current clamp and given a series of current injections from -150 to 100 pA (Fig. 1b). The steady-state input resistance was computed using the potential difference between the holding potential and the final 50 ms of the -10 pA current injection. Neurons in the SV-treated group had an input resistance of 199.4 ± 10.8 MX ($n = 45$ neurons/10 mice), while neurons treated with Veh demonstrated an input resistance of 209.6 ± 7.2 MX ($n = 45$ neurons/10 mice). These differences were not statistically significant (Fig. 1c).

Consistent with no difference in the input resistance between SV- and Veh-treated neurons, we found no significant difference in the rheobase of these same neurons. The rheobase of a neuron is the current at which it fires an AP when injected with ramping positive current from a standardized holding potential (Fig. 1d). Neurons recorded in these experiments were driven to fire APs with a depolarizing current ramp of 100 pA per second for two seconds. SV-treated neurons fired APs when injected with a current of 100.7 ± 6.2 pA on average, which is not significantly different from 90.3 ± 3.3 pA for Veh-treated neurons (Fig. 1e).

This is in agreement with the similar levels of excitability in these neurons, as characterized by their similar levels of input resistance (Fig. 1c). Consistently, there was no significant difference in the firing threshold between SV- and Veh-treated groups (Fig. 1f). Neurons treated with SV fired APs during the ramping positive current injection protocol at -49.5 ± 0.3 mV, while Veh-treated neurons fired APs at a threshold of -50.6 ± 0.6 mV.

Another way of examining neuronal excitability is to characterize the firing pattern induced by a series of steady current injections. Stepwise increases in depolarizing current injection generate progressively larger numbers of APs (Fig. 1g). Plotting the number of APs as the function of current amplitude results in an input–output curve. Within the range of 100–325 pA currents, curves obtained from Veh- and SV-treated groups showed no significant difference (Veh: $n = 45$ neurons/10 animals; SV: $n = 44$ neurons/10 animals), further supporting the conclusion from current ramp experiments described above.

HCN-Current-Mediated Sag Potential (Vsag)—HCN channels are active channels incorporated into the processing of synaptic integration at hyperpolarized membrane potentials and are modulated by cyclic nucleotides within the brain. Up- or down-regulation of these conductances will change resting membrane potential and membrane excitability, thus representing an important candidate mechanism for SV-induced modulation of synaptic plasticity. Injection of a large negative current will hyperpolarize the membrane potential enough for HCN channel activation that then depolarizes the membrane potential until a new stable membrane potential is reached. This hyperpolarization-induced membrane depolarization exhibits a characteristic sag and the sag amplitude is an indirect measure of HCN channel activity (Fig. 2a). We found that treatment of slices with SV had no effect on the magnitude of sag potentials (Fig. 2b). In response to negative current injection (-150 pA, 800-ms duration), neurons from slices treated with SV had a sag potential of 5.8 ± 0.3 mV ($n = 45$ neurons/10 animals), while Veh-treated neurons had an average sag potential of 6.3 ± 0.1 mV ($n = 45$ neurons/10 animals).

After-Hyperpolarization Potentials—Successful LTP induction requires supra-threshold depolarizations that yield APs in the postsynaptic neurons. The kinetics of individual APs and the firing pattern of a neuron play an important role in synapse recruitment. After-hyperpolarization potentials (AHPs), mediated by specific ion channels, contribute to the firing pattern of hippo-campal CA1 neurons, thus may be ultimately involved in LTP induction process. Specifically, presence of AHPs (and the underlying ion channels) limits the ability of an induction protocol to drive plasticity in the hippocampus. Thus, we next investigated whether SV incubation resulted in changes in AHPs, and whether these changes may be part of the mechanisms by which SV exerts its effects on synaptic plasticity.

On the basis of their kinetics, AHPs can be divided into two groups, fast AHPs (fAHP), occurring within 500 ms following the last AP of a burst, and slow AHPs (sAHP), activated at time scales greater than 500 ms (Fig. 2c). sAHPs may remain present for many seconds following an AP burst. Following a current injection that elicited a burst of 10 APs, fAHP amplitude and sAHP area were measured and compared between conditions. SV-treated neurons demonstrated fAHP amplitudes of -3.1 ± 0.1 mV and sAHP area of -782.3 ± 149.5

mV*ms ($n = 41$ neurons/ 10 animals), while Veh-treated neurons had fAHP amplitudes and sAHP areas of -3.0 ± 0.2 mV and -725.4 ± 118.7 mV*ms ($n = 39$ neurons/10 animals), respectively (Fig. 2d, e). The differences between SV and Veh groups are not statistically significant. Thus, there was no effect of SV on the fAHP or sAHP.

NMDAR-Mediated Synaptic Transmission

Results from above experiments indicate that SV's effect on synaptic plasticity is not mediated through significant changes in intrinsic membrane properties of CA1 neurons. Previous work has shown that SV treatment exerts no effect on basal AMPA receptor (AMPA)-mediated synaptic transmission or on the paired-pulse ratio suggesting that SV does not alter baseline presynaptic release probability; however, following high-frequency stimulation, LTP in SV-treated slices was enhanced (Mans et al. 2010). This work was done using extracellular dendritic field potential recordings in hippocampal slices, and thus synaptic responses are measured from neuron populations generally maintained at their resting membrane potential. As such, the contribution of NMDA receptors (NMDARs) to basal evoked synaptic transmission is present but minimal (Sah et al. 1989) and does not begin to make significant contributions to synaptic communication until strong postsynaptic depolarizations are generated, as occurs during high-frequency stimulation used to induce LTP. Thus we considered the possibility that SV increases NMDAR-mediated transmission, which could explain the greater LTP in SV-treated slices. To test this, we isolated NMDAR-mediated dendritic field potentials by applying an AMPAR antagonist CNQX (20 μ M) and decreasing Mg^{2+} to 0.1 mM in the aCSF to reduce Mg^{2+} blockade of NMDAR. fEPSP under this condition was further proven to be solely mediated by NMDAR by addition of an NMDAR antagonist APV (50 μ M) (Fig. 3a). After establishing a baseline of NMDAR-mediated fEPSPs, SV or Veh was washed into the bath and the fEPSP was monitored for one hour following application (Fig. 3b). Because of the slow kinetics of NMDAR-mediated fEPSP, total charge transfer, as quantified by area covered by fEPSP, is more sensitive than fEPSP amplitude (Welch et al. 2007). SV treatment significantly increased the area of the NMDAR-mediated fEPSP response by 20.6 % ($n = 15$ slices/14 mice; $p < 0.05$), while treatment of slices with Veh did not significantly change the response from baseline (-4 %, $n = 11$ slices/6 mice; $p > 0.3$) (Fig. 3c).

SV Treatment Increases the Cell Surface Distribution of the GluN2B Subunit of NMDAR

To explore the molecular basis for the enhancement of NMDAR-mediated synaptic transmission, we employed biochemical approaches. Mouse hippocampal slices were treated with 10 μ M SV or Veh for 2 h, followed by the isolation and measurement of cell surface and total level of different receptor subunits by the surface biotinylation assay and immunoblot analysis. The results show that SV treatment significantly increased the surface distribution of the GluN2B subunit of NMDAR but had no significant effect on the surface distribution of the GluN2A and GluN1 subunits of NMDAR or the GluA1 subunit of AMPAR in the hippocampus (Fig. 4). The acute SV treatment also did not affect the total level of different receptor subunits. In addition, cellular cholesterol levels were measured in hippocampal slices treated with SV or Veh for 2 and 4 h. The results show that the acute SV treatment did not affect the cellular cholesterol content in the hippocampus (Fig. 5),

suggesting that the effect of SV on GluN2B is mediated by cholesterol-independent mechanisms.

To test if the effect of simvastatin on GluN2B extends to human neuronal cells, human neuroblastoma SH-SY5Y cells were treated with different concentrations of SV for 1–2 h, followed by the isolation and measurement of surface and total GluN2B subunit of NMDAR by the surface biotinylation assay and immunoblot analysis. The results show that SV treatment led to an increase of surface GluN2B in a dose-dependent manner (Fig. 6). As seen in hippocampal slices, SV at 10 μ M, the concentration used in electrophysiological studies, significantly increased the level of surface GluN2B in SH-SY5Y cells.

Discussion

While the current understanding of the mechanisms underlying the pleiotropic actions of SV on brain function remains incomplete, previous studies have focused attention onto meta-plasticity within the hippocampus. Chronic treatment with SV in mice enhances hippocampus-dependent spatial memory in aged wild-type mice and rescues spatial memory deficits in AD mouse models, which was associated with an upregulation of phosphorylated, and thus activated, Akt in brain (Li et al. 2006). Prolonged incubation of acute hippocampal slices in vitro with SV enhances LTP in a PI3 K/p-Akt-dependent manner (Mans et al. 2010). Further studies demonstrate that this effect is mediated by modulating prenylation-dependent molecular pathways downstream of farnesyltransferase (Mans et al. 2012). In the present study, we employed both whole-cell patch clamp and extracellular dendritic field potential recordings from acute in vitro hippocampal slices to investigate the functional consequences of exposure to SV on intrinsic membrane properties and NMDAR transmission. Here we show, for the first time, that, while application of SV has no effect on hyperpolarization-activated cyclic-nucleotide channel-mediated sag potentials, after-hyperpolarization potentials, and excitability in hippocampal neurons, SV treatment leads to an increase in the NMDAR-mediated synaptic transmission, which may contribute to SV-induced enhancement of synaptic plasticity and hippocampal-dependent cognitive function (Lapchak and Han 2010; Ghosh et al. 2009; Ramirez et al. 2011; Mans et al. 2010; Mans et al. 2012; Li et al. 2006). Previous studies have also shown that localization of GluN2B subunit-containing NMDARs to synapses is crucial for synaptic function (Akashi et al. 2009), and that neurons overexpressing GluN2B exhibit enhanced LTP (Tang et al. 1999). In addition, pharmacologically induced increase in GluN2B-mediated synaptic current underlies the enhancement of hippocampal-dependent learning (Vedder et al. 2013). In the present study, using mouse hippocampal slices and a human neuronal cell culture, we demonstrated that SV treatment increases the surface distribution of the GluN2B subunit of the NMDAR, consistent with an increase in function of NMDAR at synapses. Importantly, the acute SV treatment did not change cellular cholesterol content in the hippocampus. Our data extend a previous report, in which chronic SV treatment upregulates the expression of NMDAR in the hippocampus of rats (Wang et al. 2009).

However, it is worth noting that statin treatments that lead to a significant reduction of cellular cholesterol levels may have different effects on NMDAR distribution and function. Ponce et al. (2008) demonstrated that a decrease in cholesterol by simvastatin treatment

reduced the association of an NMDAR subunit to lipid rafts in primary neuronal cultures, which protected the neurons from NMDA toxicity (Ponce et al. 2008). Similarly, Abulrob et al. (2005) also showed that cholesterol extraction from detergent-resistant microdomains by β -cyclodextrins affected NMDAR subunit distribution and signal transduction, resulting in neuroprotection of cortical neuronal cultures against ischemic and excitotoxic insults (Abulrob et al. 2005). In addition, Borisova et al. (2010) and Krisanova et al. (2012) showed that a decrease in the level of membrane cholesterol by methyl- β -cyclodextrin in nerve terminals reduced transporter-mediated glutamate release, providing another mechanism for neuroprotective effects of cholesterol reduction under the pathological conditions of stroke, ischemia, and traumatic brain injury (Borisova et al. 2010; Krisanova et al. 2012). However, the normal level of cholesterol in neuronal membrane is crucial for proper synaptic function. Besides the aforementioned pathological conditions, drastic cholesterol reduction in the neuronal membrane may pose negative effects on synaptic function because it impairs glutamate transport (Borisova et al. 2010; Krisanova et al. 2012). Of note, depending on the treatment regimens and animal models used, statins may or may not affect the cholesterol level in the brain in vivo (Kirsch et al. 2003; Johnson-Anuna et al. 2005; Fassbender et al. 2001; Li et al. 2006). In the present study, we used a SV treatment protocol that does not affect cellular cholesterol content and avoids those potential negative consequences. Under our experimental conditions, SV treatment increased cell surface distribution of GluN2B. This finding may have important implications for Alzheimer's disease as it has been shown that β -amyloid down-regulates the cell surface distribution of GluN2B in neurons in a mouse model of Alzheimer's disease (Snyder et al. 2005).

The impact of SV treatment on NMDAR function/trafficking is most likely related to the effects of SV on protein prenylation, in particular protein farnesylation. SV acts as a HMG-CoA inhibitor and decreases the production of downstream intermediates of the mevalonate pathway, including non-sterol isoprenoids: FPP and GGPP. FPP and GGPP serve as lipid substrates for protein farnesylation and geranylgeranylation, collectively called protein prenylation, a post-translational modification of proteins (McTaggart 2006). The hydrophobic lipid moiety facilitates the anchoring of proteins in the cell membrane. The largest group of proteins undergoing protein prenylation is the small GTPase superfamily. Prenylation status of small GTPases affects their trafficking and localization within cells, regulates their interactions with other intracellular signaling molecules, and ultimately affects cellular functioning (McTaggart 2006). In support of the hypothesis that SV-mediated inhibition of isoprenoid production and protein prenylation modulates LTP, we have demonstrated previously that supplement of FPP abolishes the enhancement of LTP by SV and that inhibition of farnesyltransferase mimics the effects of SV treatment (Mans et al. 2012). Recently, we found that genetic reduction of farnesyltransferase rescues cognitive function in a mouse model of Alzheimer's disease (Cheng et al. 2013). Others have also shown that SV exerts neuroprotective effects by inhibiting activation of small GTPases (Lapchak and Han 2010; Ghosh et al. 2009). These data strongly implicate SV-induced changes in neuronal function via isoprenoid-mediated modulation of small GTPases. Indeed, activation of small GTPase H-Ras, which depends on farnesylation for function, decreases the surface distribution of the NR2 subunits of NMDAR (Suvana et al. 2005). Accordingly, deletion of H-Ras increases NMDAR-dependent synaptic conductance and LTP magnitude

(Manabe et al. 2000). In addition, inhibition of hyperactive Ras by a farnesyltransferase inhibitor (FTI) or a statin rescues synaptic and cognitive deficits in mouse models of neurofibromatosis type 1 (Costa et al. 2002; Li et al. 2005). Certainly, further studies are needed to identify particular small GTPases and related signaling pathways that mediate the neuronal effects of SV.

Conclusions

The present study employed both whole-cell patch clamp and extracellular dendritic field potential recordings in acute hippocampal slices *in vitro* to isolate the major location of SV's effects on plasticity at Schaffer collateral-CA1 synapses to intrinsic membrane or synaptic components. The data demonstrate that, while SV treatment does not have significant effects on intrinsic membrane properties of CA1 pyramidal neurons, it increases NMDAR-mediated synaptic transmission. Biochemical studies in mouse hippocampal slices and human neuronal cell cultures further show that SV treatment increases the cell surface distribution of the GluN2B subunit of the NMDAR without affecting cellular cholesterol content, suggesting that SV-induced enhancement of synaptic plasticity in the hippocampus is likely mediated by augmentation of synaptic NMDAR components through cholesterol-independent mechanisms. These findings provide new insights into the cellular and molecular mechanisms for SV-mediated enhancement of synaptic and cognitive function.

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Abbreviations

aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AHPs	Afterhyperpolarization potentials
Akt	Protein kinase B
AMPA and AMPAR	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid and AMPA receptor
fEPSPs	Field excitatory postsynaptic potentials
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GluA1	A subunit of AMPA receptor
GluN1, GluN2A and GluN2B	Subunits of NMDA receptor

HCN	Hyperpolarization/cyclic-nucleotide
HFS	High-frequency stimulation
HMG-CoA	3-Hydroxy-3-methylglutaryl Coenzyme A
LTP	Long-term potentiation
NMDA and NMDAR	<i>N</i> -methyl <i>D</i> -aspartate and NMDA receptor
SV	Simvastatin
Veh	Vehicle

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Fig. 1.

Passive neuronal properties and action potential generation are not affected by simvastatin treatment. **a** The resting membrane potential (V_{rest}) of neurons was recorded after breakthrough of the cell membrane to achieve a whole-cell configuration (vehicle: $n = 23$ neurons/5 mice; simvastatin: $n = 23$ neurons/5 mice). **b** A series of current injections, ranging from -150 to 100 pA, were performed to explore any possible differences in the input resistance of neurons in these two treatment conditions. **c** Input resistance (R_n) of neurons treated with vehicle ($n = 45$ neurons/10 mice) and simvastatin ($n = 45$ neurons/10

mice). Input resistance was computed from a -10 pA current injection. **d** Neurons were held at -70 mV, and a current ramp was applied at 100 pA per second to determine rheobase, the minimal current required to yield an action potential. **e** The rheobase of neurons in slices incubated in simvastatin ($n = 44$ neurons/10 mice) and vehicle ($n = 45$ neurons/10 mice). **f** The voltage threshold of action potentials in simvastatin- and vehicle-treated neurons. **g** A series of currents from 100 to 325 pA were injected into neurons to induce action potential firing. Number of action potentials was plotted against current injection in order to construct an input–output curve

Fig. 2.

Active cellular properties are unchanged following incubation with simvastatin. **a** Negative 150 pA of current was injected into neurons held at -70 mV to hyperpolarize the membrane potential and open HCN channels. Sag potential was measured as the voltage difference between the most negative potential and the steady-state potential, as indicated by the double-headed arrow). **b** The sag potentials between vehicle ($n = 45$ neurons/10 mice) and simvastatin ($n = 45$ neurons/10 mice) incubated slices were similar in amplitude. **c** To measure after-hyperpolarization potentials (AHPs), neurons were held at -70 mV and

subsequently injected with 10 current pulses at 77 Hz to induce a train of APs. Following the last AP in the train, fast (fAHP) and slow (sAHP) AHPs are distinct on the basis of their time course. **d, e** SV treatment ($n = 41$ neurons/10 mice) resulted in no significant change in either fAHPs or sAHP, compared with the vehicle group ($n = 39$ neurons/10 mice)

Fig. 3.

Evoked NMDAR-mediated field potentials are augmented following application of simvastatin. **a** Field EPSPs were first evoked in normal aCSF. NMDAR-mediated responses were then isolated by AMPA receptors antagonist CNQX (20 μ M) in the presence of 0.1 Mg-containing aCSF. Subsequent application of 50 μ M APV (a selective antagonist of NMDAR) to the aCSF blocks the remaining field response, confirming the success in isolating the NMDAR component from the total synaptic response. **b** Representative traces of evoked NMDAR field responses before and one hour after application of vehicle or 10 μ M simvastatin. The waveforms are depiction of the baseline NMDAR-mediated response (dotted line, pre-) and the response following incubation with vehicle or simvastatin (solid line, post-). **c** Comparison of the NMDAR-mediated response area after application of vehicle or simvastatin. There was no significant difference from baseline following application of vehicle ($n = 11$ slices/6 mice); however, the NMDAR-mediated response was significantly increased (20.6 %) relative to baseline following application of simvastatin ($n = 15$ slices/14 mice; * $p < 0.05$)

Fig. 4.

Simvastatin treatment increases the surface distribution of GluN2B in the hippocampus. Mouse hippocampal slices were treated with 10 μ M simvastatin (SV) or vehicle (Veh) for 2 h, followed by the isolation and measurement of surface and total level of different receptor subunits by the surface biotinylation assay and immunoblot analysis. **a** Representative immunoblot images of total and cell surface distribution of different receptor subunits with a cytosolic protein, GAPDH, as a loading control. As expected, the amount of GAPDH in the cell surface fraction is negligible. **b** The relative level of different receptor subunits on the

cell surface with the levels in the Veh-treated slices set as 100 %. The ratio of surface to total level was determined by densitometric analysis of immunoblots and normalized by the ratio in Veh-treated slices ($n = 5-6$ per treatment; $*p < 0.05$). **c** The relative total level of different receptor subunits normalized by the amount of GAPDH with the levels in the Veh-treated slices set as 100 % ($n = 5-6$ per treatment)

Fig. 5.

Simvastatin treatment does not affect cellular cholesterol content in the hippocampus.

Mouse hippocampal slices were treated with 10 µM simvastatin (SV) or vehicle (Veh) for 2 and 4 h, followed by measurements of cellular cholesterol and protein concentrations ($n = 6$ slices per treatment)

Fig. 6.

Simvastatin treatment increases the surface distribution of the GluN2B subunit of the NMDAR in human neuronal cells. Human neuroblastoma SH-SY5Y cells were treated with different concentrations of SV for 1–2 h, followed by the isolation and measurement of surface and total GluN2B subunit of NMDAR by the surface biotinylation assay and immunoblot analysis. **a** Representative immunoblot images of total and cell surface GluN2B with a cytosolic protein, GAPDH, as a loading control. As expected, GAPDH is not present in the cell surface fraction; the irregular spot on the blot is a non-specific stain. Con, cells not treated with Veh or SV. **b** The relative level of surface GluN2B with the levels in the Veh-treated cells set as 100 %. The ratio of surface to total GluN2B was determined by

densitometric analysis of immunoblots and normalized by the ratio in Veh-treated cells ($n = 6$ per treatment; $*p < 0.05$)