THE EFFECTS OF SIMVASTATIN ON LEARNING AND MEMORY MECHANISMS IN MICE

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ABSTRACT

Statins, a widely prescribed class of cholesterol-lowering drug, inhibit HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway. Due to the identification of cholesterol as a risk factor for developing Alzheimer’s disease (AD), a number of studies have examined whether statins are neuroprotective against developing AD or dementia. While some epidemiological studies do indicate a lower prevalence of AD in people taking statins, other reports are contradictory. A consensus has therefore not been reached regarding the neuroprotective effects of statin treatment.

Aside from the well-characterized reduction in circulating cholesterol resulting from statin therapy, numerous cholesterol-independent, pleiotropic effects have been observed in the central nervous system (CNS). These include enhanced neurogenesis in the dentate gyrus, improved cerebral blood flow, augmented functional recovery from cerebral ischemia and enhanced neurite outgrowth. The mechanisms underlying many pleiotropic effects are derived from altered isoprenylation of signaling molecules, including small GTPases. In light of data showing dramatic improvements in learning and memory in wild-type mice after statin treatment, the current study was conducted to
elucidate how a lipophilic statin, simvastatin (SV), affects long-term potentiation (LTP) in the hippocampus of young adult mice. It is shown that *in vitro* SV treatment for several hours increases the magnitude of LTP at CA3-CA1 synapses in a manner independent of changes in basal synaptic transmission, as neither paired-pulse ratios, nor input/output curves were affected by SV. Also, it is determined that SV-mediated LTP enhancement is dependent upon PI3-K activation during the induction phase. Furthermore, data are presented suggesting SV increases LTP by decreasing farnesylation of isoprenylated proteins, and that inhibiting farnesyl transferase promotes recruitment of PI3-K to the induction of LTP. Overall, the present study provides a framework for understanding how SV affects the mechanisms of synaptic plasticity in the adult hippocampus and highlights the need for further investigation into how isoprenylation might affect cognitive function in statin-prescribed populations.
DEDICATION

Respect your elders and yourself.

Work hard.

Be honest.

Be humble.

Always do your best.

Treat others how you wish to be treated.

I attribute my successful completion of graduate school to remembering these maxims during the triumphs, tribulations and grueling stretches that encompass biomedical research. My incredible parents, Glenn and Judy Mans, have by example fostered these principles in me for as long as I can remember. For this blessing and their limitless devotion to my success and happiness, I dedicate my dissertation to them.
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INTRODUCTION

Long-term Potentiation

Since its discovery by Lomo and Bliss (1973) in rabbit dentate gyrus, long-term potentiation (LTP) has undergone extensive characterization and dissection due to its promise as a cellular correlate of learning and memory (Bliss and Collinridge, 1993). Hippocampal LTP in CA1, defined as an enduring change in the efficacy of a synapse resulting from prior activity at the synapse (Bliss and Lomo, 1973), is orchestrated by several signaling cascades, resulting in both pre-synaptic and post-synaptic modifications (Lisman, 2003). The post-synaptic component of LTP, for which activation of NMDA receptors (NMDARs) is essential (Collinridge, 1983), ultimately results in the integration of AMPA-type glutamate receptors into the post-synaptic density and structural modifications of dendritic spines (Lisman, 2003). In some instances, pre-synaptic changes are known to drive LTP (Zakharenko, 2003), in addition to those occurring in dendrites. However, the predominant focus of research into NMDAR-dependent LTP resides in area CA1 and the post-synaptic density.

LTP is grossly divided into two phases: (1) protein-synthesis-independent induction, expression and early maintenance phase; (2) the protein-synthesis-dependent consolidation phase (Malenka and Bear, 2004). Early LTP can be induced by several means. These include high-frequency stimulation (HFS) protocols, consisting of single or repeated trains of stimuli delivered commonly at 100 Hz in a number of patterns,
including the theta-burst rhythm known to occur naturally in the hippocampus (Vertes, 2005). Pairing protocols, which employ coincident low-frequency pre-synaptic stimulation and post-synaptic depolarization also induce LTP. These protocols are meant to mimic coincident activation of two or more inputs onto a single neuron, such as might occur during learning when two independent stimuli converge onto a common target within a narrow time span.

The NMDAR, now established as critical for hippocampal-dependent learning, is uniquely equipped to serve as a coincidence detector in hippocampal circuitry due to a magnesium block impeding conductance through its central pore. The initial depolarization in the protocols described above expels the magnesium ion (Mayer et al., 1984; Nowak, 1984), and subsequent depolarization(s) of close temporal proximity drives extracellular calcium through NMDARs and into dendritic spines. Calcium influx then initiates a host of signaling cascades that mediate augmentation of glutamatergic synaptic transmission (Lynch et al., 1983; Malenka, 1988), the primary mechanism of which being lateral movement of AMPARs from non-synaptic reserve pools to the synaptic pool (Makino and Malinow, 2009). It is interesting to note that non-NMDA voltage-gated calcium channels (VGCCs) may also be recruited for LTP induction if a 200 Hz stimulation is employed (Lisman, 2003). Also, an activity-independent form of LTP, induced by BDNF application, has also been observed. The most well characterized LTP inducing enzymes are CaMKII, MEK and phosphotidyl-inositol-3 kinase (PI3-K), and they are discussed below.
CaMKII

CaMKII, “the memory molecule”, is necessary and sufficient for LTP induction in CA1 (Giese et al., 1998; Lledo et al., 1995; Otmakhov et al., 1997). It relies upon calcium and calmodulin for its activation characterized by autophosphorylation (Fukunaga, et al., 1993). Calcium influx through NMDARs or VGCCs (depending on the induction protocol) triggers activation of CAMKII localized to NMDARs at the postsynaptic density (Gardoni et al., 1998). Activated CamKII subsequently phosphorylates GluR1 subunits of AMPA receptors (AMPARs), which induces both, increased single-channel AMPAR conductance (Barria et al., 1997; Benke et al., 1998) and insertion of GluR1-containing AMPA channels into the synaptic membrane (Chen et al., 2000, Hayashi, et al., 2000). CamKII thus induces an increase in glutamatergic synaptic transmission in response to coincident synaptic activity. Another calcium sensor, Ca/calmodulin-activated adenylate cyclase (cAMP) (Chetkovitch et al., 1993; Wong, et al., 1999) facilitates phosphorylation of GluR1 by inhibiting protein phosphatase 1, an enzyme which inactivates CAMKII (Brown et al., 2000; Genoux et al., 2002). In addition to phosphorylating GluR1, CamKII triggers insertion of empty synaptic “slots” (Lisman, 2003) into the synaptic membrane, and activates the transcription factor cyclic-AMP-responsive element-binding protein (CREB), an important mediator of structural modification in late LTP (Thomas et al., 2004).
**MAPK/ERK Kinase**

MAPK/ERK kinase (MEK) activation is known to contribute to LTP induction (Thomas et al., 2004), as early LTP can be inhibited in the presence of specific MEK inhibitors (English and Sweatt, 1997; Selcher et al., 2003). Activation of MAPK during LTP also depends upon calcium influx. Specifically, calcium-mediated activation of the small GTPase Ras induces phosphorylation and activation of Raf, which then phosphorylates and activates MAPK/ERK kinase (MEK). MEK phosphorylates ERK1 (p44) and ERK2 (p42 MAPK), which leads to phosphorylation and synaptic trafficking of GluR2L-containing AMPA receptors (Lisman, 2003, Qin et al., 2000). It has also been proposed that MEK activation changes K⁺ channel conductance to facilitate theta-burst LTP (reviewed in Sweatt, 2004). It is important to note that several groups have demonstrated HFS-induced LTP in CA1 of adult mice to be independent of Erk activation (Winder et al., 1999; Watabe et al., 2000; Liu et al., 1999). It is becoming increasingly apparent that the pattern and degree of postsynaptic depolarization can affect the coupling of NMDARs to intracellular signaling pathways (Jin and Feig, 2010).

**PI3-K/Akt Pathway**

The PI3K-Akt signaling pathway underlies multiple cellular events. These include cell differentiation, proliferation, survival and inflammation. In neurons, another role for the pathway has been revealed: mediator of synaptic plasticity and memory consolidation (Mizuno et al., 2003; reviewed in Sweatt, 2001).

The role of PI3-K in early LTP is less characterized than those of CAMKII and MEK and somewhat controversial. While it is unclear if PI3-K is necessary for LTP
induction or expression of hippocampal LTP, there is evidence that PI3-K mediates events preceding maintenance (Opazo et al., 2004; Qin et al., 2000). Blockade of PI3-K with LY294002 or Wortmannin severely inhibits early LTP in cultured neurons (Man et al., 2003), and in acutely prepared hippocampal slices, application of LY294002 (20 μM) for at least 40 min prior to HFS and for the duration of the experiment reduces LTP by approximately 50% (Opazo et al., 2004). Also, phosphorylation of Akt at residue Ser473, a common indirect measure of PI3-K activity, is elevated in region CA1 five minutes after LTP induction, and this augmented activation state is maintained for as long as thirty minutes (Racaniello et al., 2009). It is known that other kinases, such as ERK, act in conjunction with PI3-K during LTP (Thomas and Huganir, 2004). It has been suggested that the Ras-PI3K-Akt-GluR1 and Ras-ERK-GluR2 signaling pathways each contribute approximately 50% to early LTP expression (Qin et al., 2005). However, the relative contributions of PI3-K and ERK likely depend upon the LTP induction protocol (Opazo et al., 2003, Jin and Feig, 2010).

The exact mechanistic role for PI3-K in the process of LTP is currently under investigation. Initial studies have shown that PI3-K colocalizes with AMPARs in dendrites (Man et al., 2003), suggesting a role for PI3-K in the regulation of glutamate receptor (GluR) phosphorylation. In addition, PI3-K activity has been linked to trafficking of AMPAR subunits (Qin et al., 2005) and PSD-95 (Yoshii et al., 2007), which is known to anchor AMPARs to the synaptic membrane in the proximity of NMDARs (Ehrlich et al., 2004). Thus, it likely that PI3-K mediates several events necessary for insertion and anchoring of AMPARs at the synapse. It is also quite possible that PI3-K plays a modulatory role in the magnitude of LTP expressed under given
conditions. The fact that incomplete (Opazo et al., 2003) or selective (Mans et al., 2010) blockade of murine hippocampal LTP in the presence of a PI3-K inhibitor has been observed supports this possibility.

In the process of memory consolidation, described further below, it is likely that PI3-K mediates the neuronal expression of immediate early genes (IEG’s), such as Arc/Arg3.1, after behavioral or electrical stimulation. The NMDAR-PI3K-Akt-mTOR cascade, which can be initiated by BDNF application or electrical stimulation \textit{in vitro} (Chen et al., 2009; Kelly et al., 2000), is upstream of Arc expression, and shown to be required for BDNF-induced upregulation of Arc (Chen et al., 2009). Furthermore, dendritic size and complexity, which are modified during late-LTP, have been shown to be mediated by PI3-K/Akt signaling (Kumar et al., 2005).

\textit{Late LTP}

Memory consolidation occurs during the protein-synthesis-dependent phase of LTP, or late LTP (Frey et al., 1996; Nguyen and Kandel, 1996; reviewed in Bramham et al., 2008 and Lisman et al., 2003). Late LTP is characterized by the synthesis and delivery of proteins to synapses which have undergone stimulation (Bliss and Collinridge, 1993). The presence of ribosomes within dendritic spines supports the notion that new proteins can be synthesized specifically at synapses which have been “tagged” for potentiation (Frey et al., 2008). Synaptic tagging has been proposed as a mechanism by which previous synaptic events, such as early LTP, may be integrated with temporally distinct occurrences in the same cell (reviewed in Redondo and Morris, 2011). Perhaps the most vital characteristic of late LTP entails protein-synthesis dependent structural
reorganization of dendritic spines. This structural plasticity allows for changes in synaptic efficacy to persist despite rapid turnover of kinase activity.

Potentiated synapses are characterized by enlargement of the dendritic spine on which they reside. The spine typically transforms from a small spine into large, mushroom-shaped spine (Bramham, 2008). This enlargement can be triggered using several LTP-inducing protocols, including application of exogenous BDNF or pairing postsynaptic spikes with theta-burst stimulation or glutamate uncaging (Redondo and Morris, 2011). Interestingly, early, protein-synthesis-independent spine enlargement occurs immediately following LTP inducing stimuli, but sustained spine-head enlargement requires synthesis of new proteins. Reorganization of the actin cytoskeleton is thought to promote both, the anchoring of new AMPARs to the PSD and the maintenance of molecular scaffolds at the expanded PSD (Bramham, 2008; Redondo and Morris, 2011).

Structural plasticity, including spine enlargement, is dependent upon cytoskeletal reorganization, specifically the polymerization of filamentous actin (F-actin) (Kitanishi et al., 2009; Kuijk, 2008; Matsuzaki et al., 2004). A pool of rapidly turned over F-actin, known as the dynamic pool, is maintained in the spine head, and a more persistent, “stable” pool exists in the dendritic shaft. A third pool, called the enlargement pool, is thought to be the site of cytoskeletal expansion or retraction via actin polymerization or cleavage, respectively. A key regulator of cytoskeletal dynamics is cofilin, an actin-binding protein that severs actin monomers from the ends of cytoskeletal filaments. Inhibition of cofilin via cofilin kinases, such as LIM kinase-1, facilitates actin polymerization (Pak et al., 2008). In accord with this mechanism, an increase in the
number of phospho-cofilin-positive spines has been observed following theta-burst LTP in hippocampal region CA1 (Chen, 2007).

In addition to cofilin, Arc is thought to play a critical role in synaptic remodeling during LTP (Bramham, 2008). Arc mRNA is rapidly transcribed and transported to the dendritic spines of stimulated neurons in response to HFS or behavioral stimulation (Dickey et al., 2004; Guzowski et al., 2000, Guzowski et al., 1999, Link et al., 1995, Palop et al., 2005). There it is known to reside in the post-synaptic density and interact with F-actin. Application of antisense Arc after LTP induction blocks late LTP, inactivates actin-remodeling enzymes and leads to the disappearance of new F-actin at synapses (Bramham, 2008). A variety of learning tasks, including taste aversion, novel object recognition, contextual fear conditioning and Morris water maze have been shown to elicit or require Arc function (Dickey et al., 2004; Guzowski et al., 1999; Inoue et al., 2005, Palop et al., 2005, Plath et al., 2006). Accordingly, mice lacking the Arc/Arg3.1 gene perform poorly in several of the aforementioned behavioral tests (Plath et al., 2006). Also, two transgenic mouse models of Alzheimer’s disease, APP + PS1 and J20 have been shown to have reduced hippocampal Arc expression, and fail to upregulate Arc after exposure to a novel environment (Palop et al., 2005; Dickey et al., 2004). The deficit appears to be specific to granule cells of DG, and may be caused by amyloid-beta mediated disruption of the PI3K-Akt-mTOR pathway which induces Arc (Chen et al., 2009; Wang et al., 2006).

In addition to the signaling cascades associated with glutamate and NMDAR-dependent LTP described above, non NMDA receptors, such as those for serotonin, acetylcholine, adenosine and BDNF, further influence synaptic plasticity, as do genetic
changes and epigenetic modifications. Elucidating the context in which each of these factors modulate synaptic plasticity, and how their respective signaling cascades interact, will be of great importance to understanding the cellular mechanisms of learning.
**Small GTPases**

The small GTPases, 20-40 kDa guanine-nucleotide-binding molecules, act as molecular switches to regulate signaling cascades. This responsibility includes the coupling of extracellular stimuli to intracellular effectors as well as managing cross-talk between cascades. All small GTPases fall within the Ras superfamily, which is further divided into five subfamilies (Ras, Rho, Rab, Ran and Sar1/Arf). Given that small GTPases participate in a myriad of cellular processes, including the cell cycle and apoptosis, glycogen metabolism, cytoskeletal organization, visual signal transduction (Lane and Beese, 2006) and synaptic plasticity, it is important to understand the mechanics of small GTPase activity (reviewed in McTaggart, 2006), as well as factors modulating their function directly and indirectly. Guanine nucleotide exchange factors (GEFs), guanine dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs) directly affect GTPase activation, while isoprenylation exerts indirect effects that are often unpredictable.

_Guanine nucleotide exchange factors (GEFs)_

Activation of a small GTPase entails exchange of bound guanine diphosphate (GDP) for guanine tri-phosphate (GTP), a process catalyzed by guanine nucleotide exchange factors (GEFs). The presence of GTP induces a conformational change
promoting interaction between GTPases and their effector proteins. Nucleotide exchange is facilitated by a specific domain within GEFs, though the exact amino acid sequence within this domain varies depending on the GEF of interest. A well conserved GEF motif is the Dbl homology (DH) domain, first identified within oncogenic Rho GEF, the first mammalian GEF identified, in diffuse B cell lymphoma (Dbl) cells (Erickson and Cerione, et al., 2004). The DH domain has since been identified in a number of other human GEFs (Rossman et al., 2005), and it facilitates nucleotide exchange activity by interacting with switch regions of the Rho GTPases (Erickson and Cerione et al., 2004; Rossman et al., 2005) and Ras GTPases (Vetter et al., 2001). Non-conserved residues within DH domains are known as specificity patches (Erickson and Cerione et al., 2004), and they confer selectivity to the interaction of GEFs with specific GTPases.

Working in tandem with the DH domain is an adjacent C-terminal pleckstrin homology (PH) domain that binds to lipid products of PI3-K. The PH domain localizes Dbl-containing GEFs to the plasma membrane, and appears to augment the nucleotide exchange activity of the enzyme (Liu et al., 1998; Rossman et al., 2005). It has been proposed that PH domains affect GEF activity through allosteric regulation or by influencing membrane orientation (Rossman et al., 2005). In another form of positive regulation, the catalytic activity of Ras GEF Son of sevenless (Sos) is enhanced by interaction with GTP-bound Ras (Margarit et al., 2003). Positive cooperativity thereby adds an additional level of GTPase modulation.

In the absence of the DH-PH tandem motif, the dock homology region 2 (DHR-2), or ‘Docker’ (Cote et al., 2002) domain, found in members of the DOCK 180 family of Rho GEFs, may facilitate GEF-mediated nucleotide exchange. Whether a DHR-2 domain
is sufficient to catalyze nucleotide exchange is subject to debate, as some studies have shown the need for an accessory protein known as ELMO (Erickson et al., 2004; Brugnera et al., 2002), while others demonstrate that DHR-2 may act independently (Kiyokawa, et al., 1998; Meller et al., 2002). While a number of GEFs and their key structural components have been identified, continued efforts are needed to understand the complexities of their activity.

**Guanine Dissociation Inhibitors (GDIs)**

GDIs implement three mechanisms to regulate small GTPases (reviewed in McTaggart, 2006). The first entails prolonging the GDP-bound (inactive) state by stabilizing GDP to the switch region, inhibiting its dissociation. This tight binding impairs GEF-mediated GTP exchange and prevents activation. A second method of GTPase inhibition is to directly interfere with interactions between GTPases and their effectors by binding and segregating GTP-bound (active) GTPases. However, it is important to consider that this action from GDI also prevents interaction between activated GTPases and deactivating enzymes termed GAPs (described below). This may result in a prolonged state of activation. The third regulatory mechanism is indirect, and entails enveloping the C-terminal isoprenoid attachment. By hiding this hydrophobic moiety, GDIs prevent membrane association and induce cytosolic accumulation (A more detailed review of the effects of isoprenylation on GTPase activity can be found below). Intriguingly, GDIs are themselves regulated by a class of proteins termed GDI displacement factors (GDFs), providing yet another level of control for regulating GTPase-mediated signaling.
**GTPase Activating Proteins (GAPs)**

Small GTPases employ their intrinsic GTP hydrolysis capability to convert bound GTP to GDP, though this deactivating step occurs quite slowly if unassisted. GAPs facilitate GTP hydrolysis by stabilizing the existing catalytic machinery of GTPases, and thereby augment GTPase deactivation. Similar to GEFs, GAPs contain a PH domain, but also incorporate a Src homology domain into their structure. Two structures, an arginine finger (Scheffzek et al., 1998) and an asparagine thumb (Li et al., 2004), have been identified as critical to their catalytic activity. As in the case of GDIs, GAPs themselves are susceptible to regulation by additional factors. These include subcellular translocation, phosphorylation, targeted degradation and protein-protein interactions (reviewed Bernards et al., 2004). There are at least 160 human genes predicted to encode GAP-like proteins (Bernards et al., 2003; Bernards et al., 2004), and given the influence of secondary regulation described above, the level of complexity in the regulation of GAPs and the GTPases they interact with is potentially quite staggering.

**Isoprenylation**

Isoprenylation, the covalent linkage of small, non-sterol lipids at or near the carboxy terminus of proteins, has been recognized as a key regulator of intracellular protein-protein interactions and membrane-associated protein trafficking. Two major types of prenylation, farnesylation and geranylgeranylation have been identified, and they are catalyzed by farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) type I/II, respectively. The substrates for FTase and GGTase, farnesyl pyrophosphate
(FPP) and geranylgeranyl pyrophosphate (GGPP), are non-sterol intermediates in the biosynthetic pathway for cholesterol, also known as the mevalonate pathway (Figure 1). FPP, a 15-carbon moiety, is generated by FPP-synthase from the precursor geranyl diphosphate. FPP serves as a branchpoint in the mevalonate pathway, as it may be converted to either the 20-carbon GGPP moiety via GGPP synthase, or squalene, a precursor to cholesterol.

The isoprenylation target sequence is a C-terminal CaaX motif, where “C” represents cysteine, “a” represents generally aliphatic residues, and “X” represents a residue that confers specificity to the isoprenylation reaction. In general, FTase prefers methionine, serine, glutamine or alanine in the X position, while GGTase-I prefers leucine or phenylalanine. However, the substrate specificity rules of these prenylation enzymes are not always strictly adhered to. For instance, K-Ras, which contains a FTase CaaX sequence, can be geranylgeranylated in the presence of FTase inhibition (Gibbs et al., 2001; Yokoyama et al., 2001), and RhoB, a known target for GGTase, may also be farnesylated (Armstrong et al., 1995). Also, there is evidence that GGTase-I can mistransfer FPP, rather than GGPP, onto a host (Yokoyama et al., 1995). A third type of prenylation is conducted by GGTase Type II enzymes (reviewed in Stenmark and Olkkonen, 2001), also called Rab transferases due to the distinction that they exclusively target the Rab subfamily of GTPases. Type II transferases target a highly variable sequence at the carboxyl terminal (CXXX, CC, CCXX, CCXXX) where X is any amino acid. Unique to type-II transferases is the ability to transfer multiple GGPPs to a single host. Two additional processing steps occur at the endoplasmic reticulum following
prenylation in the cytoplasm. These entail (1) proteolytic removal of the aaX sequence, and (2) cleavage of the pyrophosphate group from the prenylcysteine residue.

Isoprenylation dramatically increases the lipophilicity of isoprenylated proteins. It thereby facilitates anchoring of proteins into cell membranes, including the inner leaflet of the plasma membrane and membranes of the endoplasmic reticulum and golgi apparatus (reviewed in McTaggart, 2006). The major consequences of this post-translational modification on small GTPase function are three-fold: (1) They may interact with regulatory enzymes, such as GEFs, GAPs and GDIs, that reside at the plasma membrane; (2) they may engage effector kinases that reside at the plasma membrane; and (3) they may be embedded into the outer leaflet of vesicles and be trafficked between the ER and the plasma membrane. However, the exact functional outcome from reduced prenylation is not always easy to predict and is the subject of active investigation.

A well-established effect of inhibiting prenyl transferases is the exclusion of small GTPases from membranes and subsequent accumulation in the nucleus or cytosol (Samuel and Hynds, 2010) This outcome hampers GTPase-mediated processes on two fronts: (1) by preventing interaction with membrane-bound GTP-loading GEFs, and (2) inhibiting GTPase-effector protein interaction. However, mounting evidence indicates that cytosolic localization does not preclude GTPase activation. For instance, there are reports of unprenylated forms of Ras and Rho proteins retaining partial function (Allal, et al., 2000; Lebowitz et al., 1997). Furthermore, a pool of GTP-loaded Rho GTPases have been observed in the cytosol in an unpublished study by Samuel and colleagues (reviews in Samuel and Hynds, 2010). They propose that pools of GDP- and GTP-bound Rho GTPases reside at the plasma membrane and in the cytosol. These emerging findings
highlight the need for conservative conclusions regarding the effects of isoprenoid modulation on small GTPase activity.

**Isoprenylation, GTPases and LTP**

Several mechanistic components of LTP, including cytoskeletal dynamics and AMPAR trafficking have been shown to involve small GTPases. Due to the role of isoprenylation in the regulation of their activity, some groups have tested the effects of inhibiting isoprenylation via prenyl transferase inhibitors or by reducing isoprenoid production in the mevalonate pathway using statins.

A key component of synaptic plasticity entails cytoskeletal reorganization (Bramham, 2008), and Rho GTPases, which include RhoA, RhoB and Rac1, have been observed participating in cytoskeletal dynamics at dendritic spines (Narumiya et al., 1997). Several groups have therefore tested if Rho GTPases are necessary for persistent changes in synaptic strength. Surprisingly, whether Rho GTPases facilitate or restrict LTP is not entirely clear. It is known that HFS triggers activation of hippocampal RhoA, RhoB and Rac1 (O’Kane et al. 2004). Moreover, theta-burst-induced LTP in rat CA1 is impaired if either, Rac1 or an effector kinase for RhoA, ROCK, is inhibited (Rex et al. 2009). In direct conflict with these results, O’Kane et al. (2003) show that inhibiting ROCK or GGtase type-1 prenylation actually augments HFS-induced LTP. It is not clear why these studies obtain conflicting results, but at a minimum, it is apparent that Rho GTPase can contribute to LTP in some regard.

The roles of farnesylated proteins, including Ras and RhoB, in LTP are similarly complex. Three principal actions of Ras in the context of LTP are most well
characterized: (1) coupling of adrenergic receptor activity to ERK phosphorylation, (2) calcium-dependent coupling of NMDAR activity to ERK phosphorylation, and (3) NMDAR-dependent activation of the PI3-K/Akt pathway (reviewed in Sweatt, 2002; Huganir 2004). The exact conditions under which Erk and PI3-K are recruited and/or necessary for LTP in CA1 is highly controversial (Opazo et al., 2003; Qin et al., 2009; Jin and Feig 2010), though it is well established that Ras can initiate all three cascades. In accord with this assertion, FTase inhibition, which alters the intracellular localization of Ras and its ability to interact with effector kinases (Omerovic and Prior), can reduce the magnitude of LTP (O’Kane et al, 2004). However, these results are in conflict with several findings. First, in genetically modified mice lacking the neuron-specific Ras-guanine-nucleotide-releasing factor (GRF), an activator of Ras GTPase, hippocampal learning and LTP magnitude are normal (Brambilla et al., 1997). Second, there are several lines of evidence that Ras negatively regulates NMDAR transmission. Specifically, it has been shown that an H-Ras deficient mouse exhibits both enhanced NMDAR conductance and greater LTP in CA1 compared to wild-type mice (Manabe et al. 2000). Also, there is evidence that inhibiting a Ras effector protein, RACK1, increases NMDAR currents in hippocampal neurons, suggesting that Ras inhibition can augment NMDAR-dependent plasticity. More studies are clearly needed to truly understand how prenylation states modulate the small GTPases and their participation in synaptic plasticity.
Statins

Pharmacology

Statins inhibit hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the cholesterol biosynthetic pathway, and they are widely prescribed to reduce circulating cholesterol levels in patients with hypercholesteremia. While the primary action of statins is to inhibit cholesterol production, the goal of statin treatment is achieved via a secondary, compensatory mechanism, in which hepatic low-density lipoprotein (LDL) receptors are increased and subsequent clearance of LDL from the bloodstream is augmented (Kovanen et al., 1981, Bilheimer et al., 1983 in Gerson et al.). Interestingly, unlike humans, rats do not upregulate LDL receptors, but circulating cholesterol levels are still reduced.

The first statin approved for clinical use, lovastatin, was initially isolated from the mold Aspergilus terreus in 1987 (Jones, 2003). Chemical modifications of lovastatin have resulted in several statin variants, including pravastatin and simvastatin (SV). Fully synthetic statins, such as fluvastatin, cerivastatin and atorvastatin (ATV), are also available. The naturally derived statins share two inhibitory structural elements. The first is comprised of a hydropnaphthalene ring that interacts with the coenzyme-A recognition site of HMG-CoA reductase (HMG-CoAR), and the second is a hydroxyl acid side chain that mimics mevalonate. Though structurally distinct, synthetic statins also inhibit HMG-
CoAR by interacting with the binding sites of coenzyme-A and mevalonate (Blum, 1994).

Some statins, such as SV and lovastatin, are administered in an inactive form, while others, like pravastatin, are delivered in the active conformation. The inactive state consists of a lactone that must be converted to a beta-hydroxy acid in the liver (Figure 2). The inactive lactone form is favored due its greater hepatic extraction efficiency, resulting in more direct targeting to the liver, the primary site of cholesterol biosynthesis.

Slight structural differences between statins confer unique properties regarding lipid-lowering effectiveness, absorption efficiencies, metabolic bi-products, half-life and lipophilicity. The inhibition potency ranks in the order of rosuvastatin > ATV > SV > lovastatin = pravastatin > fluvastatin (Jones, 2003), while the ability to cross the blood-brain barrier is highest in SV. The absorption efficiency varies quite widely, with SV absorbed with high efficiency (≈ 80%), but pravastatin absorbed at only 46% efficiency (Blum, 1999). As for the metabolic by-products released into plasma, the metabolites of fluvastatin do not inhibit HMG-CoAR, but active metabolites have been detected from pravastatin, SV and lovastatin with varying inhibitory efficiencies ranging from 2% to 80% (Blum, 1999).

**Effects of Statins on the Central Nervous System: Vascular Health and Traumatic Brain Injury**

A key aim of statin treatment is to improve cardiovascular health, and reductions in vascular inflammation (Maeda et al., 2003), platelet aggregation and thrombosis (Laufs et al., 2003) are evident after statin use. These benefits extend to the CNS, as cerebral
vascular diseases (Essig et al 1998 from Lu 2007) and risk of ischemic stroke are also decreased by statin treatment. Attempts have been made to harness these cholesterol-dependent effects and other, cholesterol-independent properties of statins to improve outcomes from traumatic brain injury (TBI). In in vivo studies, chronic administration of ATV or SV following cerebral ischemia have been shown to improve rehabilitation of spatial memory (Lu et al., 2007a), reduce inflammatory cytokine production (Balduini et al., 2003), and improve cerebral blood flow to the injury site (Chen et al., 2003). Also, angiogenesis and neurogenesis are enhanced in the dentate gyrus (Chen et al., 2003, Lu et al., 2007a), while neuronal loss in hippocampal region CA3 is diminished. In vitro, statins promote neurite outgrowth in terms of length and branching (Holmberg et al., 2006; Pooler et al., 2006), suggesting statins may enhance regeneration after neuronal trauma. Additionally, the excitotoxic effects triggered by excessive glutamate release after TBI appear to be ameliorated by statins (Zacco et al., 2003; Bosel et al., 2005). In the context of TBI, the cocktail of beneficial effects induced by statins point to a promising future for HMG-CoAR inhibitors in TBI therapy. However, another consideration, the impact of statins on cognitive function, complicates the issue of whether statins are ultimately beneficial or detrimental to brain function.

Effects of Statins on the Central Nervous System: Alzheimer’s Disease and Cognition

The identification of hypercholesterolemia and cardiovascular disease (Kivipelto et al., 2002; Skoog et al., 1996; Soneira and Scott, 1996; Sparks et al., 1990) and several genes related to cholesterol metabolism (Jarvik et al., 1995; Kolsch et al., 2003; Papassotiropoulos et al., 2003) as risk factors for sporadic AD raised the question of
whether HMG-CoAR inhibitors may prevent the onset of AD or dementia. Indeed, some studies indicate a lower prevalence of AD in statin-prescribed populations (Jick et al., 2000, Wolozin et al., 2000), and statin treatment has been associated with a decreased rate of cognitive decline (Szwast, 2011). Several mechanisms have been proposed to explain the apparent cognitive benefits of statins as they pertain to AD. These include improved cerebro-vascular health, reduced Aβ production and augmented synaptic plasticity.

Imaging studies show widespread alterations in brain hemodynamics in AD brains (Gonzalez et al., 1995; Harris et al., 2000) characterized by hypoperfusion of frontal, temporal, parietal and cingulate regions (Alsop et al., 2000). Statin-induced improvements in cerebral blood flow could therefore contribute to reductions in AD incidence among statin-prescribed populations. Cholesterol-mediated reduction of amyloid-beta (Aβ) peptide production, a hallmark of AD, could also be a factor. In support of this hypothesis, animals maintained on cholesterol-rich diets demonstrated an increase in Aβ load that was reversed when animals were returned to a regular chow diet (Sparks, 1996; Sparks et al., 1994). Furthermore, in vitro studies have shown that increased cellular cholesterol levels facilitate amyloidogenic processing of amyloid precursor protein, while lowering cellular cholesterol results in a decrease in Aβ secretion (Bodovitz and Klein, 1996; Ehehalt et al., 2003; Fassbender et al., 2001; Galbete et al., 2000; Kojro et al., 2001; Simons et al., 1998).

The ability of some statins to cross the blood-brain barrier has spurred investigations into whether neuroprotective effects of statins occur by a mechanism within the CNS or through a peripherally mediated effect (reviewed in Cole and Vassar,
2006). Given that hydrophilic statins, which cannot cross the blood-brain barrier, may lower AD risk by up to 70% (Wolozin et al., 2000), it seems likely that a peripherally-mediated mechanism is at work. Furthermore, concentrations of statins found in the cerebro-spinal fluid of statin-prescribed patients have been found to be quite low (< 0.5 ng/ml) (Triscari et al., 1993), reducing the likelihood of profound direct action in the brain. Still, evidence does exist suggesting CNS-localized protective effects. For instance, pro-inflammatory responses of microglia after amyloid-β peptide exposure are ameliorated after statin treatment in vitro (Cordle and Landreth, 2005) and in vivo (Clarke et al., 2008), and glutamate-receptor mediated excitotoxicity is reduced in the presence of statins (Zacco et al., 2003; Bosel et al., 2005).

While the retrospective epidemiological and basic-science studies described above demonstrate great promise for statin-mediated neuroprotection in AD or for even slowing cognitive decline in normocholesteromic patients (Simons et al., 2002), there is mounting contradictory evidence. For instance, two studies (Fassbender et al., 2002; Haglund et al., 2004) report no change in levels of Aβ-42 in plasma or cerebrospinal fluid following administration of low-dose statin, and other prospective epidemiological reports show no association between statin use and neuroprotection (Collins et al., 2002; Shepherd et al., 2002). A small population of non-AD statin users have even reported memory loss as a side effect (Wagstaff 2003).

Unfortunately, contradictions similar to those complicating the human literature have arisen from experiments in laboratory animals. For instance, a SV-supplemented diet has been shown to rescue learning and memory in a transgenic mouse model of AD independent of changes in amyloid beta pathology, and these improvements strikingly
extended to wild-type mice (Li et al., 2006). A similar effect has been observed in adult rats administered SV for twenty-five days prior to testing in passive avoidance or object-in-place tasks (Douma et al. 2011). However, these results are directly contradicted by a study in which adult rats administered SV for six weeks showed impaired performance in the Barnes Maze (Baytan, et al., 2008).

A monumental confound to clearly understanding statin-mediated neurological effects is the fact that statins induce a myriad of cholesterol-independent, pleiotropic effects, due to the reduction of isoprenoid intermediates in the cholesterol biosynthetic pathway that regulate enzymatic activity. To better understand how these secondary effects impact learning and memory, several studies have tested how statins modulate hippocampal LTP. In keeping with the theme of statin research, a consensus is far from being reached. In favor of benefiting cognition, ATV increases LTP in the hippocampus of rats following administration of Aβ, (Clarke et al., 2007). Also, a four-week treatment with SV increases NMDAR abundance in the prefrontal cortex, primary motor cortex, cingulate cortex, hippocampus, caudate putamen, nucleus accumbens and amygdala (Wang et al., 2008). Furthermore, in vitro inhibition of farnesyl transferase or geranylgeranyl transferase type-I, major prenylation enzymes, have been shown to increase LTP in slices from the hippocampal CA1 region in BL-6 mice compared to treatments with vehicle. However, the potentially beneficial effects of statins/reduced isoprenylation are countered by findings that isoprenoid production is necessary for normal LTP in CA1 (Kotti et al., 2006; Kotti et al., 2008). Also, the small GTPases modulated by prenylation have been implicated in LTP induction, and there is evidence
that inhibiting them is detrimental to LTP, though opposing results have also been obtained.

The present study was undertaken to ascertain whether the pleiotropic effects of statins extend to the cellular mechanisms of synaptic plasticity in the brains of wild-type C57/BL6 mice. To this end, several questions were addressed: (1) Can SV treatment for several hours in vitro enhance the magnitude of LTP at CA3-CA1 synapses in C57/BL6 mice; (2) Do the isoprenoids produced in the mevalonate pathway play a role in SV-mediated effects on synaptic plasticity; (3) Does PI3-K activation, which is known to increase in neurons following statin treatments in vivo and in vitro, play a role in SV-induced changes in synaptic plasticity?
**Fig. 1.** The biosynthetic pathway for cholesterol (also known as the mevalonate pathway). Farnesyl-PP and geranylgeranyl-PP, non-sterol isoprenoid intermediates, serve as substrates for isoprenylation via farnesyl transferase and geranylgeranyl transferase, respectively. A vast number of proteins, including all of the small GTPases, are prenylated, altering their intracellular localization and protein-protein interaction potentials. Statins inhibit HMG-CoA reductase, and thereby exert pleiotropic effects stemming from reduced prenylation of small GTPases and other regulatory proteins.
Fig. 2. Structures of inactive and active simvastatin. Some statins are administered as an inactive lactone, then converted *in vivo* into a pharmacologically active hydroxy acid. (Used with permission from Vickers et al., 1999).
SIMVASTATIN ENHANCES HIPPOCAMPAL LONG-TERM POTENTIATION
IN C57BL/6 MICE

by

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Abstract

Statins inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA), the rate-limiting enzyme in the cholesterol biosynthetic pathway, and they are widely used to control plasma cholesterol levels and prevent cardiovascular disease. However, emerging evidence indicates that the beneficial effects of statins extend to the central nervous system. Statins have been shown to improve the outcome of stroke and traumatic brain injury, and statin use has been associated with a reduced prevalence of Alzheimer’s disease (AD) and dementia. However, prospective studies with statins in AD have produced mixed results. Recently, we reported that simvastatin, a widely used statin in humans, enhances learning and memory in non-transgenic mice as well as in transgenic mice with AD-like pathology on a mixed genetic background. However, the cellular and molecular mechanisms underlying the beneficial effects of simvastatin on learning and memory remain elusive. The present study was undertaken to investigate the effect of acute simvastatin treatment on hippocampal long-term potentiation (LTP), a cellular model of learning and memory, in brain slices from C57BL/6 mice. Our results demonstrate that a prolonged in vitro simvastatin treatment for 2-4 hrs, but not a short-term 20-min exposure, significantly increases the magnitude of LTP at CA3-CA1 synapses without altering basal synaptic transmission or the paired-pulse facilitation ratio in hippocampal slices. Furthermore, we show that phosphorylation of Akt (protein kinase B) is increased significantly in the CA1 region following 2-hour treatment with
simvastatin, and that inhibition of Akt phosphorylation suppresses the simvastatin-induced enhancement of LTP. These findings suggest activation of Akt as a molecular pathway for augmented hippocampal LTP by simvastatin treatment, and implicate enhancement of hippocampal LTP as a potential cellular mechanism underlying the beneficial effects of simvastatin on cognitive function.

**Introduction**

Statins inhibit the rate-limiting reaction in the mevalonate biosynthetic pathway for cholesterol, which entails conversion of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) to mevalonate via HMG-CoA reductase (Brown and Goldstein, 1986). Inhibiting this reaction not only reduces *de novo* cholesterol synthesis, but also prevents the formation of isoprenoid intermediates, such as farnesyl-pyrophosphate (PP) and geranylgeranyl-PP, which serve as lipid attachments for various intracellular signaling molecules like the small GTP-binding proteins Rho, Ras and Rac (Liao, 2002, Vaughan, 2003). The subcellular localization, intracellular trafficking, and function of these GTPases depend on their isoprenylation state. Consequently, inhibition of isoprenoid production by statins induces pleiotropic effects that go beyond the reduction of cholesterol. A growing body of literature supports the notion that these pleiotropic effects extend to brain.

A diverse characterization of the cognitive effects of statins has been published, with many studies focusing on the potential therapeutic benefits of statins for the treatment of stroke or traumatic brain injury (Balduini et al., 2003, Chen et al., 2003, Lu et al., 2007a, Wu et al., 2008). In rat models, chronic administration of atorvastatin or
simvastatin following traumatic brain injury have been shown to improve rehabilitation of spatial memory (Lu et al., 2007a), reduce inflammatory cytokine production (Balduini et al., 2003), and improve cerebral blood flow to the injury site (Chen et al., 2003). Furthermore, neurogenesis in the dentate gyrus (Chen et al., 2003, Lu et al., 2007a), angiogenesis and neurite outgrowth (Pooler et al., 2006) are stimulated by chronic (seven days) statin treatment.

Statins have received much attention in the field of Alzheimer’s disease (AD), as well, due in large part to findings from epidemiological studies indicating a lower prevalence of AD/dementia in statin-prescribed populations (Jick et al., 2000, Wolozin et al., 2000), suggesting a neuroprotective role for statins. However, prospective studies with statins have produced mixed results. While some studies showed no protection of statins in preventing AD in a group of patients at risk for cardiovascular disease (Collins et al., 2002, Shepherd et al., 2002), others reported that statin (simvastatin or atorvastatin) treatment improved cognitive function in normocholesterolemic patients (Simons et al., 2002) or slowed the decline in cognitive function of AD patients (Sparks et al., 2005). Also, some studies showed a decrease of amyloid-β peptide (Aβ) after statin treatments (Buxbaum et al., 2002, Sjogren et al., 2003), whereas others found no effects of statin treatments on Aβ levels (Fassbender et al., 2002, Hoglund et al., 2004, Hoglund et al., 2005). Subsequent work has examined the contribution of the anti-inflammatory properties of statins. Namely, pro-inflammatory responses of microglia after Aβ exposure are ameliorated after statin treatment in vitro (Cordle and Landreth, 2005) and in vivo (Clarke et al., 2008). Also, statins protect cultured cortical neurons from excitotoxicity after exposure to N-methyl D-aspartate (NMDA) (Zacco et al., 2003) and monosodium
glutamate (Bosel et al., 2005). In addition, atorvastatin increases long-term potentiation (LTP) in the hippocampus of rats following acute in vivo administration of amyloid-β peptide (Clarke et al., 2007). However, these potentially beneficial effects of statins are countered by a report that acute in vitro treatment of mouse hippocampal slices with mevastatin (also known as compactin; a statin not approved for human use) inhibits LTP at CA3-CA1 synapses (Kotti et al., 2006). While statins share a common mechanism for inhibiting cholesterol synthesis, differences exist regarding their potency (rosuvastatin > atorvastatin > simvastatin > lovastatin = pravastatin > fluvastatin) and their ability to cross the blood-brain barrier (simvastatin > atorvastatin and rosuvastatin) (Jones, 2003). Recently, we reported that chronic simvastatin treatment in vivo enhances hippocampal-dependent learning and memory in non-transgenic mice as well as in a transgenic mouse model of AD (Li et al., 2006).

The goal of the present study was to ascertain whether the pleiotropic effects of statins extend to the cellular mechanisms of synaptic plasticity that contribute to hippocampal-dependent learning. To this end, we sought to determine whether acute in vitro exposure of hippocampal slices to simvastatin increases the magnitude of NMDA receptor-dependant LTP at CA3-CA1 synapses in slices from C57BL/6 mice. Our results demonstrate that a prolonged in vitro simvastatin treatment for 2-4 hr, but not a short-term 20-min exposure, increases the magnitude of LTP at CA3-CA1 synapses without changing basal synaptic transmission or the paired-pulse facilitation (PPF) ratio in hippocampal slices. Furthermore, we show that simvastatin treatment for 2 hr, but not 20 min, significantly increases the phosphorylation of protein kinase B (Akt). These findings suggest that modulation of hippocampal LTP and related signaling pathways could be
one of the underlying cellular and molecular mechanisms by which simvastatin treatment enhances learning and memory.

**Experimental Procedures**

*Animals*

Three- to four-month-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME; stock no. 000664) were used in this study. The mice were housed in a specific-pathogen-free facility under veterinary supervision at an ambient temperature of 22-23 °C and under a 12:12-hr light/dark cycle. The mice were allowed *ad libitum* access to food and water. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

*Preparation of simvastatin solution for electrophysiology experiments*

Simvastatin was kindly provided by Merck & Co., Inc., West Point, PA. Prior to its use in experiments, simvastatin was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by alkaline hydrolysis (first dissolving 50 mg of the compound in 1 ml of ethanol (100%) and then adding 0.813 ml of 1 N NaOH). This stock solution was stored in aliquots at -20°C (for up to 1 month). On the day of use, the simvastatin stock solution was neutralized with 1 N HCl to pH of 7.4 and diluted in artificial cerebral spinal fluid (aCSF). The final concentration of simvastatin used in the aCSF was 10 µM. In addition, a vehicle solution without simvastatin was prepared and added to the aCSF to serve as controls.
Slice preparation and electrophysiology

Hippocampal slices (400 μM) were prepared from male C57BL/6 mice using methods described previously (Smith and McMahon, 2006) with modifications. Briefly, mice were anesthetized with isoflurane and decapitated. Their brains were removed and immersed in ice-cold “high-sucrose” aCSF composed of (in mM): NaCl 85; KCl 2.5; MgSO₄ 4; CaCl₂ 0.5; NaH₂PO₄ 1.25; NaHCO₃ 25; glucose 25; sucrose 75; 290-300 mOsm. This sucrose-based aCSF, which contains less Na⁺ and Ca²⁺ and higher Mg²⁺ than the regular sodium-based aCSF promotes neuronal survival during the slicing procedure by reducing excitotoxicity (Kuenzi et al., 2000). Coronal slices of the dorsal hippocampus were cut on a vibratome (Vibratome, St. Louis, MO) and incubated in sucrose-based aCSF for ten min, then for ≥ one hr in regular aCSF containing (in mM): NaCl 119; KCl 2.5; CaCl₂ 2.5; MgSO₄ 1.3; NaH₂PO₄ 1; NaHCO₃ 26; and glucose 10 saturated with 95% O₂-5% CO₂ (pH 7.4). To record field excitatory postsynaptic potentials (fEPSPs), slices were placed in a submersion recording chamber and continuously perfused at 3-5 ml/min with aCSF warmed to 26-28°C. CA1 extracellular dendritic fEPSPs were recorded (Axopatch 200B, Molecular Devices, Sunnyvale, CA) using standard methods (Smith and McMahon, 2005). Stimulus frequency was 0.1 Hz (100-μs duration), and stimulus intensity was adjusted to yield fEPSPs with amplitudes of 0.5-0.8 mV. Schaffer collaterals were stimulated with a bipolar tungsten stimulating electrode placed in CA1 stratum (s.) radiatum, and fEPSPs were recorded using a glass microelectrode filled with aCSF, also placed in CA1 s. radiatum. If stable fEPSPs were
maintained for at least 20 minutes, NMDA receptor-dependent LTP was induced with a high-frequency stimulation (HFS) protocol (four 0.5-s 100 Hz trains applied at a 20-s interval) (Smith and McMahon, 2005). The stimulus intensity was increased to 1.5 times the baseline intensity during the HFS to ensure strong postsynaptic depolarization and NMDA receptor activation, and was returned to the baseline intensity immediately after HFS.

**Simvastatin treatment**

Two simvastatin treatment protocols were used: 1) a 20-minute bath application of simvastatin during collection of baseline transmission and 2) a 2-4 hr simvastatin incubation prior to HFS induction of LTP; simvastatin was bath applied throughout the recording period.

**Twenty-minute treatment**: First, stable baseline transmission was recorded for at least 10 min in regular aCSF, after which time bath application of either simvastatin (10 μM) or vehicle commenced and continued for 20 min. Once a stable 20 min baseline in simvastatin was acquired, HFS was delivered to induce LTP. Simvastatin (or vehicle) perfusion continued throughout the experiment.

**Two-to four-hour incubation**: Following slicing and 10-min incubation period in sucrose-based aCSF, half of the slices were incubated at room temperature in regular aCSF supplemented with simvastatin (10 μM), and half were incubated in regular aCSF supplemented with vehicle. The experimenter was blinded to the identity of the solutions. The slices were incubated in these solutions for a period of one hour. Following this, recordings were performed, alternating between simvastatin- and vehicle-treated slices in
an interleaved fashion. Importantly, the order of recordings was reversed between mice, to ensure that the results are not biased towards longer incubation times in simvastatin or vehicle. Because experiments were interleaved between the two conditions, and the order of the experiments was reversed between animals, the simvastatin incubation times (time from onset of drug exposure to HFS) varied between 2 to 4 hours. Simvastatin (or vehicle) was perfused continuously throughout the recording period. For experiments with a phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Sigma, St. Louis, MO), LY294002 stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and added to the aCSF in a final concentration of 20 µM containing 0.1% DMSO. In these experiments, the same concentration of DMSO (0.1%) was added to vehicle as a control.

In a separate set of experiments, slices were prepared in regular aCSF, rather than in the sucrose-based aCSF. In these experiments, slices also underwent the 2-4 hour simvastatin incubation period as described above.

**Immunoblot analysis**

Hippocampal slices were prepared as described above for LTP experiments. For each mouse, 6 hippocampal slices were obtained: 3 slices were incubated at room temperature in regular aCSF supplemented with simvastatin (10 µM), and 3 slices were incubated in regular aCSF supplemented with vehicle. After 20 min or 2 hr, the CA1 region was dissected and pooled from 3 slices followed by an immediate homogenization in SDS sample buffer (Invitrogen, Carlsbad, CA) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitor
cocktail (Sigma, St. Louis, MO). For some slices, the entire hippocampal area was dissected out and homogenized in SDS sample buffer. After a brief sonication, protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Twenty-five µg of proteins for each sample were separated by SDS-PAGE, and blotted to PVDF membranes. The membranes were incubated with primary antibodies followed by biotinylated or HRP-conjugated secondary antibodies. Signal was detected by the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometric scanning using the LabWorks image acquisition and analysis software (UVP Inc., Upland, CA). For a loading control, the blots were stripped and re-probed with a mouse anti-tubulin monoclonal antibody (Sigma, St. Louis, MO). Primary antibodies used for immunoblot analysis are rabbit polyclonal antibodies against Akt (protein kinase B) (Santa Cruz) and phospho-Akt (p-Akt, Ser473) (Cell Signaling, Danvers, MA).

Data analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparison of data from different treatment groups was performed by two-tailed Student's t test (for normally distributed data) or Mann-Whitney rank sum test (for non-normally distributed data). P < 0.05 was considered statistically significant. Data from electrophysiology experiments were filtered at 3 kHz, digitized at 10 kHz, and acquired using LabVIEW data acquisition software (Richard Mooney, Duke University). The slope of the rising phase of the fEPSP was measured and plotted versus time. Each point represents the average of five raw data points. To determine the magnitude of LTP, the slopes of the
rising phase of the fEPSPs were normalized to baseline, and the fEPSPs between thirty-five and forty minutes post-HFS were averaged. For the 20-min treatment and incubation experiments with slices prepared in sucrose-based aCSF, data were analyzed in pairs such that experiments were not included in the analyzed data set unless a pair of simvastatin- and vehicle-treated experiments were successfully completed from the same mouse brain. This requirement controlled for inter-animal variability as well as potential differences in slice health between batches of slices. For incubation experiments from slices prepared in regular aCSF, pairs of data were not always achieved, so LTP values from all animals were included in the final data set.

Results

Twenty-minute simvastatin treatment is not sufficient to affect the magnitude of hippocampal LTP

We tested the effects of acute simvastatin exposure in vitro on synaptic plasticity in mouse hippocampus. LTP experiments were performed at CA3-CA1 synapses in coronal slices from C57BL/6 mice. Upon completion of the resting period (one hr), slices were moved to the recording chamber, and a stable (>20 min) baseline was obtained in regular aCSF. Simvastatin or vehicle was then bath applied and synaptic transmission was acquired for an additional 20 min. There was no effect of simvastatin on basal synaptic transmission, as neither the paired-pulse facilitation (PPF) ratio (Fig. 1A) nor the fEPSP slope (Fig. 1B) changed significantly during simvastatin perfusion. Following acquisition of a stable baseline, high frequency stimulation (HFS; 4 trains, 100 Hz, 0.5 s in duration, 20 s interval) was applied to induce LTP. As shown in Fig 1B, there
was no significant effect of the 20-min simvastatin treatment on the magnitude of LTP expressed (Fig. 1B) (147 ± 11% and 137 ± 12% of baseline fEPSP slope in simvastatin-treated slices (n = 7 slices/6 mice) and in vehicle-treated slices (n = 7 slices/6 mice), respectively; P > 0.05).

Two-to-four-hour simvastatin treatment significantly increases the magnitude of hippocampal LTP

To determine whether a longer simvastatin exposure would affect the magnitude of LTP, hippocampal slices from C57BL/6 mice were incubated in regular aCSF supplemented with simvastatin or vehicle. Incubation began immediately after the 10-min post-slicing resting period in sucrose-based aCSF and continued until slices were transferred to the recording chamber. The recording chamber was continuously perfused with simvastatin- or vehicle-containing aCSF. Because experiments from simvastatin-treated slices were interleaved with experiments from vehicle-treated slices from the same animal using the same electrophysiology recording setup, the simvastatin incubation times varied from 2-4 hours before inducing LTP with HFS. Consistent with the results from the 20-min treatment experiments, basal transmission was unaffected by longer incubation in simvastatin, as there was no difference in the input/output relationship (Fig. 2A). Furthermore, there was no difference in the PPF ratio (Fig. 2B). However, in contrast to the 20-min treatment, 2-4-hr simvastatin incubation produced a significant increase in the mean LTP magnitude recorded at CA3-CA1 synapses in hippocampal slices (Fig. 2C) (149 ± 10% and 128 ± 7% of baseline fEPSP slope in
simvastatin-treated slices (n = 9 slices/9 animals) and vehicle-treated slices (n = 9 slices/9 animals), respectively; P = 0.007).

Sucrose-based aCSF was used to facilitate the health of brain slices. However, preparing slices in sucrose-based aCSF can limit the magnitude of LTP due to increased synaptic inhibition that occurs as a consequence of the sucrose-mediated neuroprotection of GABAergic interneurons (Kuenzi et al. 2000). Therefore, to rule out the possibility that the increase in LTP magnitude stimulated by simvastatin could be underestimated because of stronger GABAergic inhibition, we repeated the simvastatin incubation experiments in slices prepared using regular aCSF, rather than sucrose-based aCSF. As shown in Fig. 3A and consistent with results obtained in sucrose-prepared slices, simvastatin incubation did not affect the input/output relationship (Fig. 3A) or the PPF ratio (Fig. 3B), but it significantly increased the magnitude of LTP (Fig. 3C) (148 ± 7% and 127 ± 5% of baseline fEPSP slope in simvastatin-treated slices (n = 11 slices/8 animals) and vehicle-treated slices (n = 10 slices/9 animals), respectively; P = 0.025). These experiments demonstrate that simvastatin treatment over a period of 2-4 hours enhances the magnitude of LTP at CA3-CA1 synapses, regardless of slicing conditions.

**Acute simvastatin treatment increases the phosphorylation of Akt**

To explore the potential molecular mechanisms by which simvastatin modulates synaptic plasticity, we measured the levels of phospho-Akt (p-Akt) and total Akt, which are increased by chronic simvastatin treatment *in vivo* (Li et al., 2006), by immunoblot analyses. The results showed that the levels of p-Akt in the CA1 region were increased significantly (about 2.5 fold, P < 0.01) in the hippocampal slices treated with simvastatin.
(n=15 slices/5 mice) for 2 hr, but not for 20 min, compared to slices treated with vehicle (n=15 slices/5 mice) (Fig. 4A and 4B). To confirm the upregulation of Akt phosphorylation by simvastatin in the same slices used for LTP recording, we also measured the level of p-Akt in the homogenate of individual hippocampi that had undergone LTP induction (Fig. S1). Consistent with results obtained from non-stimulated slices, the level of p-Akt in simvastatin-treated slices increased by about 2.8 fold compared to vehicle-treated slices (n = 7 slices/animals, \( P = 0.026 \)), corresponding with enhanced magnitude of LTP in simvastatin-treated slices (140 ± 12% ) compared to that (120 ± 11%) in vehicle-treated slices (n = 7 slices/animals, \( P = 0.047 \)).

**PI3K inhibitor suppresses simvastatin-induced enhancement of LTP**

To determine if upregulation of Akt phosphorylation is responsible for the enhancement of LTP by simvastatin treatment, we used a PI3K inhibitor LY294002 that inhibits the phosphorylation of Akt (Sanna et al., 2002, Opazo et al., 2003). We used 20 µM LY294002 in our experiments, as it has been shown that high concentrations of LY294002 affect basal synaptic transmission (Opazo et al., 2003). Before conducting LTP experiments, immunoblot analysis was performed to confirm that a 40 min incubation in LY294002 at 20µM decreases the levels of p-Akt in hippocampal slices. As the results show (Fig. S2), LY294002 partially but significantly decreased the level of p-Akt in simvastatin- and vehicle-treated slices.

Next, we studied the effect of LY294002 on the magnitude of LTP from simvastatin and vehicle-treated slices. In the literature, there are inconsistent results regarding the role of the PI3K/Akt pathway in the induction and expression of LTP at
hippocampal CA3-CA1 synapses (Sanna et al., 2002, Opazo et al., 2003). To study the effect of LY294002 on different phases of LTP, we first conducted experiments investigating the effect of LY294002 on LTP expression (already-induced LTP). Hippocampal slices were treated with simvastatin or vehicle as described above for LTP experiments. Twenty minutes after LTP was induced, LY294002 bath application began and continued for the remainder of the experiment. As shown in Fig. 5A, LY294002 application had no differential effect on established LTP in simvastatin or vehicle slices. Although there was a decrement of the LTP magnitude in both simvastatin- and vehicle-treated slices coinciding with the addition of LY294002, the decrement also occurred in vehicle-treated slices in the absence of LY294002, indicating that the decrementing LTP magnitude was not caused by the presence of LY294002. The reason for the decrement in the LTP magnitude is not clear but it might be caused by the presence of dimethyl sulfoxide (DMSO), the solvent for LY294002, which was added in the vehicle as a control. The side effects of DMSO on synaptic activity have been reported (Sawada and Sato, 1975, Lu and Mattson, 2001, Tsvyetlynska et al., 2005). Thus, when DMSO is used as a solvent in an experiment, proper control experiments are required to control for potential effects of DMSO. While the exact mechanism responsible for the effects of DMSO is beyond the scope of this study, our results show that LY294002 (inhibition of p-Akt) had no significant effect on the expression of LTP.

Next, we investigated the effect of LY294002 on the induction of LTP. Hippocampal slices were subjected to 4 different treatments: vehicle, simvastatin, vehicle+LY294002, and simvastatin+LY294002. LY294002 was bath applied at least 40 min prior to LTP induction and was present throughout the experiment. As shown in Fig.
5B, the LTP magnitude from simvastatin-treated slices was increased significantly compared to that from vehicle-treated slices (145 ± 9% vs. 129 ± 6%, simvastatin vs. vehicle, n = 10 slices/animals, \( P = 0.025 \)), consistent with data in Fig. 2C and 3C. For slices treated with simvastatin+LY294002, the LTP magnitude was significantly decreased to 119 ± 7% (n = 7 slices/animals, \( P = 0.045 \) compared with the LTP magnitude from simvastatin slices), indicating that inhibiting phosphorylation of Akt suppressed the enhancement of LTP by simvastatin treatment. Interestingly, for slices treated with vehicle+LY294002 (n = 10 slices/animals), the LTP magnitude was unchanged (129 ± 7%) compared to slices treated with vehicle alone (129 ± 6%).

**Discussion**

We have shown recently that chronically administered simvastatin in vivo enhances spatial learning and memory in non-transgenic mice as well as in a transgenic mouse model of AD (Li et al., 2006). Our findings corroborate evidence in human epidemiological studies (Jick et al., 2000, Wolozin et al., 2000, Wolozin et al., 2007) and rodent models of cerebral ischemia and/or traumatic brain injury which argue for a positive effect of statins on cognition (Balduini et al., 2003, Chen et al., 2003, Lu et al., 2007a, Wu et al., 2008). However, the cellular and molecular mechanisms underlying the beneficial effects of simvastatin on learning and memory remain elusive. The present study was carried out to investigate the effect of acute in vitro simvastatin treatment on LTP at CA3-CA1 synapses, a cellular model of learning and memory, recorded in brain slices from wild type C57BL/6 mice. Our results demonstrate that: 1) Acute simvastatin treatment for up to 4 hrs does not alter basal synaptic transmission or the PPF ratio in
hippocampal slices; 2) While a 20-min treatment with simvastatin is not sufficient to affect the magnitude of hippocampal LTP, a prolonged incubation with simvastatin for 2-4 hr significantly enhances the magnitude of LTP in hippocampal slices; 3) Consistent with the results from LTP experiments, simvastatin treatment for 2 hr, but not 20 min, significantly increases the phosphorylation (activation) of Akt in the CA1 region of the hippocampus; and 4) A PI3K inhibitor LY294002, which inhibits the phosphorylation of Akt, suppresses the enhancement of LTP in SV-treated slices.

In a previous study, we have shown that enhancement of learning and memory by chronic simvastatin treatment is associated with an increased level of activated (phosphorylated) Akt (p-Akt) in the brain of wild type mice (Li et al., 2006). In a rat model of TBI, simvastatin treatment activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in hippocampus (Wu et al. 2008), and is responsible for mediating neurorestorative effects of simvastatin. In addition, simvastatin rapidly triggers translocation of Akt to the plasma membrane of cultured endothelial cells (Skaletz-Rorowski et al., 2003). The activation of Akt (i.e. the level of p-Akt) is enhanced as early as 15 minutes after exposure to simvastatin (Kureishi et al., 2000, Skaletz-Rorowski et al., 2003). Another statin, pravastatin, was also found to activate Akt in endothelial cells (Nakao et al., 2007).

Our current understanding of NMDA receptor-dependant LTP divides the phenomenon into “early”, protein-synthesis independent, and “late”, protein-synthesis dependent, phases (Malenka and Bear, 2004). The present study examines early LTP, for which the underlying intracellular signaling cascades have been extensively investigated. The role of the PI3K/Akt pathway in the regulation of LTP is not well understood. While
one study using rat hippocampal slices showed that PI3K/Akt activation is required for the expression but not the induction of LTP at CA3-CA1 synapses (Sanna et al., 2002), another study using mouse hippocampal slices demonstrated that PI3K/Akt activation contributes to the induction of LTP rather than the expression or maintenance of LTP at CA3-CA1 synapses (Opazo et al., 2003). The reasons for the discrepancy were not clear but might be attributable to the differences in the concentration of PI3K inhibitors and in the animal species (rat vs. mouse) used in these studies. In the present study, we found that inhibition of Akt phosphorylation by LY294002 had no effect on the expression of hippocampal LTP in either simvastatin or vehicle-treated slices but it significantly suppressed the induction of LTP in simvastatin-treated slices such that the LTP magnitude was similar to that from vehicle-treated slices (Fig. 5). Interestingly, LY294002 did not affect the induction of LTP in vehicle-treated slices under our experimental conditions. It is plausible that, in vehicle-treated slices, the inhibition of basal level of p-Akt by LY294002 was not sufficient to affect the magnitude of LTP, as other LTP-inducing mechanisms exist. However, in simvastatin-treated slices, the level of p-Akt is upregulated, causing a greater contribution of p-Akt to the induction process and enhancing LTP. Thus, inhibition of Akt phosphorylation by LY294002 would produce a significant suppression of LTP in simvastatin-treated slices. Taken together, our data indicate that enhanced Akt phosphorylation is a molecular mechanism contributing to the simvastatin-induced increase in the magnitude of early LTP.

If p-Akt levels can be enhanced in cultured endothelial cells after only 15 minutes of exposure to simvastatin (Kureishi, 2000, 1004), why did the 20-minute exposure in our study not significantly affect the level of p-Akt? We may not be able to compare a
monolayer endothelial cell culture with a 400 µm-thick brain slices. It is likely that a continuously perfused hippocampal slice requires a longer exposure to allow full penetration of simvastatin into the slice and/or sufficient upregulation of the PI3K/Akt pathway. This notion is consistent with our finding that incubation of brain slices with simvastatin for 2 hr indeed increases the level of p-Akt significantly and enhances the magnitude of hippocampal LTP. Therefore, we conclude that hippocampal slices require an exposure to simvastatin greater than 20 min before LTP enhancement can occur under the experimental conditions used in the present study.

While our findings that simvastatin increases hippocampal LTP in C57BL/6 mice parallel the behavioral data that simvastatin enhances hippocampal-dependent learning and memory, these results are seemingly in contrast with reports showing that hippocampal LTP is decreased by an acute treatment with a different statin, compactin, in mice (Kotti et al., 2006, Kotti et al., 2008). However, we do not feel that these results are necessarily in conflict. The differences in experimental conditions may explain the discrepancy. Firstly, although all statins block HMG-CoA reductase, they possess subtle differences in their chemistry that endow them with different properties (Jones, 2003). While simvastatin was used in our study, compactin was used in the study of Kotti et al. (2006). Compactin is the first statin isolated, but it is not approved for human use because of toxicity concerns (Endo, 2004). Therefore, it is possible that the impact of compactin on neuronal function could be different from that of simvastatin. Secondly, there is a difference in LTP induction protocols. While we used a high-frequency stimulation protocol, Kotti et al. (2006) utilized a theta-burst protocol. It is now appreciated that different stimulation protocols induce LTP with varying molecular mechanistic
requirements (Lisman, 2003). Other studies have reported inconsistencies between HFS- vs. theta-burst-induced LTP within a single experimental system (Hoffman et al., 2002, Costa and Grybko, 2005). Thirdly, the age of the animals is different between the two studies. While we used mature (3-4 months old) mice, Kotti et al. (2006) used young (4-6 wks old) mice. The molecular mechanisms underlying LTP induction evolve during development (Esteban et al., 2003, Yasuda et al., 2003). C57BL/6 mice younger or older than 2 months have been shown to require different molecular mechanisms for expression of early LTP (Lu et al., 2007b). Therefore, it is possible that modulation of LTP by statin exposure depends upon the developmental state of the animals.

In conclusion, the present study demonstrates that simvastatin can enhance early LTP in hippocampal slices from C57BL/6 mice when administered acutely over 2-4 hours, likely by modulating a signaling pathway involving the activation of Akt. Our findings provide a potential cellular basis for the beneficial effects of simvastatin on cognitive function.

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Fig. 1. Basal synaptic transmission and early LTP are unaffected by a 20-minute exposure to simvastatin. Vehicle was bath applied to hippocampal slices for the first 10 min of baseline stimulation, and simvastatin (10μM) application ensued for the final 20 min of baseline prior to HFS (arrow) and continued for the duration of the experiment. **A:** Paired-pulse facilitation (PPF) ratio was unchanged during simvastatin exposure (1.7 ± 0.05) compared to vehicle (1.7 ± 0.06). **B:** Mean magnitude of LTP from slices treated with simvastatin or vehicle. Mean magnitude of LTP, averaged over the last 5 min of run-out (min 65-70), was unaffected by 20-min simvastatin treatment ($P = 0.583$). LTP from simvastatin-treated slices was 147 ± 11% (n = 7 slices/6 mice), and 137 ± 12% (n=7 slices/6 mice) from vehicle-treated slices. Also, fEPSP slope did not change after onset of drug application, indicating basal synaptic transmission was unaffected by simvastatin. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from a pair of slices treated with vehicle or simvastatin.
Fig. 2. Prolonged incubation in simvastatin significantly enhances early LTP in slices prepared in sucrose-based aCSF.  

A: Stimulus response curves for slices prepared in sucrose-based aCSF and incubated in simvastatin or vehicle for 2-4 hours. Incubation in simvastatin (n = 5 slices) did not alter the input/output curves over the range of baseline stimulation intensities utilized for baseline compared to vehicle-treated slices (n = 4 slices).  

B: Paired-pulse facilitation (PPF) ratio from slices prepared in sucrose-based aCSF and incubated in simvastatin or vehicle. PPF ratio was unchanged during baseline stimulation after incubation in simvastatin (1.6 ± 0.06) compared to vehicle (1.6 ± 0.06).  

C: The mean magnitude of LTP from simvastatin-treated slices (149 ± 10%, n= 9 slices/9 animals) was significantly higher than that from vehicle-treated slices (128 ± 7%, n= 9 slices/9 animals) (**P = 0.007). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from a pair of slices treated with vehicle or simvastatin.
Fig. 3. Slicing in regular aCSF followed by 2-4 hr incubation in simvastatin significantly enhances early LTP. **A:** Stimulus response curves for slices prepared in regular aCSF and incubated in simvastatin or vehicle for 2-4 hours. Incubation in simvastatin (18 slices/7 animals) did not alter basal transmission over the range of stimulus intensities utilized for baseline acquisition compared to slices incubated in vehicle (9 slices/5 animals). **B:** Paired-pulse facilitation (PPF) ratio after incubation in simvastatin or vehicle. PPF ratio was not affected by simvastatin incubation. **C:** Mean magnitude of LTP from slices incubated in simvastatin or vehicle. The magnitude of LTP from simvastatin-treated slices (148 ± 7%, n = 11 slices/8 animals) was significantly higher (*P = 0.025) than that from slices incubated in vehicle (127 ± 5%, n = 10 slices/9 animals). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from slices treated with vehicle or simvastatin.
Fig. 4. Effect of simvastatin treatment on the level of p-Akt and total Akt. **A:** Representative immunoblot images of p-Akt and total Akt in the homogenate of CA1 region of hippocampal slices treated with simvastatin (SV) or vehicle (Veh) for 20 min or 2 hr. **B:** Densitometric analysis of immunoblots (normalized by the amount of tubulin) with the levels in vehicle-treated group set as 100%. Data represent means ± SEM (n=15 slices/5 mice per treatment). The results showed that the levels of p-Akt in the CA1 region were increased significantly (about 2.5 fold) in the hippocampal slices treated with simvastatin for 2 hr, but not for 20 min, compared to slices treated with vehicle. **P = 0.008.**
Fig. 5. PI3K inhibitor LY294002 suppresses simvastatin-induced enhancement of LTP.  
**A.** Mean magnitude of LTP from slices incubated in simvastatin or vehicle with LY294002 (LY) (20 µM) applied 20 min after LTP induction in simvastatin (SV) + LY and vehicle (Veh) + LY slices. The vehicle slices were treated with vehicle (containing 0.1% DMSO) with no application of LY and served as control. The magnitude of LTP from simvastatin + LY slices (141 ± 9%, n = 8 slices/8 animals) was significantly higher (*P = 0.038) than that from vehicle + LY slices (116 ± 4%, n = 8 slices/8 animals). The magnitude of LTP from vehicle + DMSO was 118 ± 6% (n = 6 slices/6 animals) similar to that from vehicle + LY slices. The results show LY does not affect the expression of LTP in either simvastatin- or vehicle-treated slices.  
**B.** Mean magnitude of LTP from slices incubated in simvastatin or vehicle and treated with LY294002 (20 µM) or DMSO for at least 40 min pre-induction and for the rest of the experiment. The LTP magnitude from simvastatin-treated slices was increased significantly compared to that from vehicle-treated slices (145 ± 9% vs. 129 ± 6%, n = 10 slices/animals, *P = 0.025). From the slices treated with simvastatin + LY, the LTP magnitude was significantly decreased to 119 ± 7% (n = 7 slices/animals, *P = 0.045 compared with the LTP magnitude from simvastatin-treated slices). From slices treated with vehicle + LY (n = 10 slices/animals), the LTP magnitude was unchanged (129 ± 7%) compared to slices treated with vehicle + DMSO (129 ± 6%). For clarity purposes, error bars are shown in only one direction. Waveforms show fEPSPs during baseline (dotted) and 40-min post-tetanus (solid) from slices in different treatment groups.
SIMVASTATIN-MEDIATED ENHANCEMENT OF LONG-TERM POTENTIATION IS DRIVEN BY FARNESYL-PYROPHOSPHATE DEPLETION AND INHIBITION OF FARNESYL TRANSFERASE

by

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Abstract

In previously published work, we have demonstrated the ability of simvastatin (SV) to enhance long-term potentiation (LTP) in the CA1 region of the hippocampus. The present study was conducted to better understand the molecular mechanisms underlying SV-induced enhancement of LTP. Specifically, it was found that inhibiting production of isoprenoid intermediates in the biosynthetic pathway for cholesterol triggers the downstream events leading to enhanced LTP. Replenishment of farnesyl pyrophosphate, but not geranylgeranyl pyrophosphate, abolished the LTP-enhancing ability of SV. In parallel to this finding, inhibiting farnesylation, but not geranylgeranylation replicated the enhancement of LTP caused by SV. Finally, inhibiting farnesylation promotes the activation of Akt during the induction phase.

Introduction

Statins directly inhibit the rate-limiting enzyme of the cholesterol biosynthetic pathway, HMG-CoA reductase, which converts HMG-CoA to mevalonate (Endo, 2004). Inhibiting this reaction not only reduces do novo cholesterol biosynthesis, but also reduces production of non-sterol intermediates, known as isoprenoids, downstream of mevalonate and preceding cholesterol. Isoprenoids, such as farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) serve as lipid attachments for all members of the small GTPase superfamilies which include the well-known Ras, Rho and Rac (reviewed in McTaggart, 2006). The isoprenylation state of GTPases alters their intracellular trafficking, subcellular localization and interactions with substrates and, therefore, modifies their activity and the activity of enzymes they regulate (McTaggart,
Consequently, reducing isoprenoid availability or the process of isoprenylation itself can affect a diverse group of intracellular signaling pathways and processes. Some lipophilic statins, such as simvastatin and lovastatin, are capable of crossing the blood-brain barrier (Jones, 2003). The effects of statins are diverse and extend across several disciplines. Consequently, it is imperative to understand how statins affect cognitive function, and a number of experimental and epidemiological studies have been conducted to this end (reviewed in Cole and Vassar, 2005).

Statins have been shown, to some extent, to be therapeutic and neuroprotective in humans, though these results are not without controversy. Some epidemiological studies indicate a reduced prevalence of AD or dementia in statin-prescribed populations (Jick et al., 2000; Wolozin, 2000). However, there are conflicting reports that statins are not neuroprotective (Collins et al., 2002, Shepherd et al., 2002), and some statin users suffer memory loss that is ameliorated by withdrawal from statin treatment (Wagstaff, 2003). In support of a neuroprotective role for statins, it has been observed that statins reduce pro-inflammatory responses of microglia after amyloid-β peptide exposure in vitro (Cordle and Landreth, 2005) and in vivo (Clarke et al., 2008), and protect cultured cortical neurons from excitotoxicity after exposure to N-methyl D-aspartate (NMDA) (Zacco et al., 2003) and monosodium glutamate (Bosel et al., 2005). Also, a recent study found elevated levels of FPP and GGPP in the brains of AD patients suggesting that reducing isoprenoid production may prove therapeutic (Eckert et al., 2009). Indeed, in previously published work from our laboratory, a simvas tatin (SV)-supplemented diet rescued learning and memory in a transgenic mouse model of AD independent of changes in amyloid beta pathology (Li et al., 2006). Interestingly, dramatic memory improvements
are also observed in non-transgenic wild type (WT) littermate controls. A similar effect has been observed in adult rats administered SV for twenty-five days prior to testing in passive avoidance or object-in-place tasks (Douma et al., 2011). It is therefore possible that SV can augment the processes underlying learning and memory in normal, non-diseased brains. Chronic statin treatment stimulates production of brain-derived neurotrophic factor (BDNF) (Wu et al., 2008), increases levels of NMDA receptors (Wang et al., 2009), promotes neurogenesis and increases cerebral blood flow (Chen et al., 2003). Additionally, we recently reported that treatment of hippocampal slices for several hours with SV increases the magnitude of NMDA receptor-dependent long-term potentiation (LTP), a mechanism thought to mediate memory at the cellular level, in the CA1 subregion in the brains of young adult C57BL/6 mice (Mans et al., 2010). Many of the pleiotropic effects described above have been attributed to reduced isoprenoid production or altered small GTPase activity.

In the current study, we investigate the mechanism by which SV augments LTP in the CA1 region of the hippocampus of C57BL/6 mice. We test the hypothesis that SV-induced reduction of isoprenoid production leads to enhanced LTP. We also test the related hypothesis that inhibition of isoprenylation contributes to the SV-induced LTP enhancement we observe. In support of these proposals, evidence is presented demonstrating that replenishing FPP, but not GGPP, abolishes SV-induced LTP enhancement. Furthermore, we find that inhibiting farnesylation, but not geranylgeranylation, mimics the LTP-enhancing property of SV. Lastly, we present data suggesting that inhibiting farnesylation augments the recruitment of PI3-kinase activity during LTP induction.
Experimental Procedures

Animals

Three- to four-month-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME; stock no. 000664) were used in this study. The mice were housed in a specific-pathogen-free facility under veterinary supervision at an ambient temperature of 22-23 °C and under a 12:12-hr light/dark cycle. The mice were allowed *ad libitum* access to food and water. All animal procedures used for this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Slice preparation and electrophysiology

Hippocampal slices (400 μM) were prepared from male C57BL/6 mice using methods described previously (Mans et al., 2010) with modifications. Briefly, mice were anesthetized with isoflurane and decapitated. Their brains were removed and immersed in ice-cold “high-sucrose” aCSF composed of (in mM): NaCl 85; KCl 2.5; MgSO4 4; CaCl2 0.5; NaH2PO4 1.25; NaHCO3 25; glucose 25; sucrose 75; 290-300 mOsm. This solution contains less Na+ and Ca2+ and higher Mg2+ than sodium-based aCSF and promotes neuronal survival during the slicing procedure by reducing excitotoxicity (Kuenzi et al., 2000). Coronal slices of dorsal hippocampi were cut on a vibratome (Leica) and incubated in high-sucrose aCSF for ten min, then for ≥ one hr in regular aCSF containing (in mM): NaCl 119; KCl 2.5; CaCl2 2.5; MgSO4 1.3; NaH2PO4 1; NaHCO3 26; and
glucose 10 saturated with 95% O₂-5% CO₂ (pH 7.4). To record field excitatory postsynaptic potentials (fEPSPs), slices were placed in a submersion recording chamber and continuously perfused at approximately 1.5-2.0 ml/min with aCSF warmed to 26-28 °C and recirculated via peristaltic perfusion pump. CA1 extracellular dendritic fEPSPs were recorded (Axopatch 200B, Molecular Devices, Sunnyvale, CA) using standard methods (Mans et al., 2010). Stimulus frequency was 0.1 Hz (100 μs duration), and stimulus intensity was adjusted to yield fEPSPs with amplitudes of 0.5-0.8 mV. Schaffer collaterals were stimulated with a bipolar tungsten stimulating electrode placed in CA1 stratum (s.) radiatum, and fEPSPs were recorded using a glass microelectrode filled with aCSF, also placed in CA1 s. radiatum. If stable fEPSPs were maintained for at least 15 minutes (min), NMDA receptor-dependent LTP was induced with a high-frequency stimulation (HFS) protocol (four 0.5-s trains of 100 Hz stimulation applied at 20 s intervals) (Mans et al., 2010). The stimulus intensity was increased to 1.5 times the baseline intensity during the HFS to ensure strong postsynaptic depolarization and NMDA receptor activation, and was returned to baseline intensity immediately after HFS.

**Preparation of solutions for electrophysiology experiments**

*Simvastatin (SV):* Simvastatin was purchased from Calbiochem (Cat#567020). Prior to its use in experiments, SV was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by alkaline hydrolysis (first dissolving 50 mg of the compound in 1 ml of ethanol (100%) and then adding 0.813 ml of 1 N NaOH). This stock solution was stored in aliquots at −20 °C (for up to 1 month). On the day of use, the
SV stock solution was neutralized with 1 N HCl to pH of 7.4 and diluted in artificial cerebral spinal fluid (aCSF). The final concentration of SV in the recording solution was 10 µM. In addition, a vehicle solution lacking SV was added to aCSF to serve as control.

Mevalonate: Mevalonate was purchased from Sigma (Cat # M-4667) and prepared according to previously published methods (Wagner et al., 2000; Essig et al., 1998). Briefly, mevalonate was dissolved before undergoing alkaline hydrolysis in 1N NaOH (heated at 50 C for 2 hrs), and 0.2 M stock solutions (pH 7.0) were stored at -20 °C.

Farnesol: Trans, trans-farnesol (96%) was purchased from Sigma (Cat# 27754). To prepare 0.2 mM farnesol, 1.85 uL of farnesol were initially pipetted into 4 uL of ethanol to improve solubility. This solution was then diluted into 40 mL of aCSF to reach a final concentration of 0.2 mM. 0.01% ethanol was used for vehicle control.

Geranylgeraniol (GGOH): Geranylgeraniol (≥ 85%) was purchased from Sigma (Cat# G3278). To prepare 0.2 mM GGOH, 2.42 uL of GGOH were pipetted into 4 uL of ethanol to improve solubility. This solution was then diluted into 40 mL of aCSF to reach a final concentration of 0.2mM. Control solutions contained 0.01% ethanol.

Farnesyl transferase inhibitor (FTI): FTI-277 was purchased from Calbiochem (Cat# 344555). A 1 mM stock solution was prepared by reconstituting 250 ug FTI in 559 uL dimethylsulfoxide. This stock solution was aliquoted and stored at -80 °C for a maximum of one week. On the day of use, 40 uL of stock solution were dissolved in 40 mL of aCSF to reach a final concentration of 1 uM. Vehicle solutions contained 0.1% DMSO and served as controls.
Geranylgeranyl transferase inhibitor type-I (GGTI): GGTI-2133 was purchased from Calbiochem (Cat# 345884). A 1 mM stock solution was prepared by reconstituting 250 μg GGTI in 547 μL dimethylsulfoxide. This stock solution was aliquoted and stored at -80 °C for a maximum of one week. On the day of use, 40 μL of stock solution were dissolved in 40 mL of aCSF to reach a final concentration of 1 μM. Vehicle solutions contained 0.1% DMSO and served as controls.

Simvastatin treatment

Slices were treated with aCSF containing 10 μM simvastatin (SV-aCSF) or vehicle (veh-aCSF) for a minimum of 100 min before HFS. The 100-min treatment entailed two steps: 1) a 60-min incubation at room temperature in a beaker containing oxygenated 10 μM SV- or veh-aCSF; 2) continuous perfusion in the recording chamber with SV- or veh-aCSF warmed to 26-28 °C for at least 40 min. Beaker incubations in SV or vehicle for subsequent experiments began immediately after HFS of the preceding experiment to ensure consistent incubation times between groups. Control experiments were performed in an interleaved fashion, and the order of experiments was reversed over multiple days.

Mevalonate, farnesyl-diphosphate or geranylgeranyl-diphosphate replenishment

An initial 60 min inhibition of the cholesterol biosynthetic pathway with 10 μM oxygenated SV-aCSF occurred in a beaker at room temperature. This was followed by a metabolite replacement period lasting a minimum of 40 min pre-HFS. Metabolite replacement entailed continuous perfusion in the recording chamber with SV- or veh-
aCSF supplemented with the metabolite of interest (or its vehicle) warmed to 26-28 °C. Therefore, the minimum SV treatment was 100 min, and the minimum period of metabolite replacement lasted 40 min. Baseline responses were obtained during treatment in the recording chamber, and the recording solution was unaltered after HFS.

*Immunoblot analysis*

Hippocampal slices were prepared as described above for LTP experiments. For each mouse, six hippocampal slices were obtained. In interleaved trials, slices were moved to the recording chamber after a 40 min preincubation in FTI (1 μM), GGTI (μM) or DMSO (0.1%) at 26 °C. Once moved to the recording chamber, bath application of FTI, GGTI or DMSO continued during approximately 20 min of baseline stimulation. Slices then underwent either HFS or continued baseline stimulation, and region CA1 was harvested 5 min after this point, then immediately homogenized in SDS sample buffer (Invitrogen, Carlsbad, CA) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). The entire sample (15 μL) from each experiment was separated by SDS-PAGE, and blotted to PVDF membranes. The membranes were incubated with primary antibody for p-Akt (Ser473) (Cell Signaling) followed by biotinylated or HRP-conjugated secondary antibodies. Signal was detected by the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and quantified by densitometric scanning using the LabWorks image acquisition and analysis software (UVP Inc., Upland, CA). For a loading control, the blots were stripped and re-probed with a mouse anti-pan Akt antibody (Cell Signaling).
Data analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparison of data from different treatment groups was performed by two-tailed Student’s *t* test (for normally distributed data), and *P* < 0.05 was considered statistically significant. Data from electrophysiology experiments were filtered at 3 kHz, digitized at 10 kHz, and acquired using LabVIEW data acquisition software (Richard Mooney, Duke University). The slope of the rising phase of the fEPSP was measured and plotted versus time. Each point represents the average of five raw data points. To determine the magnitude of LTP, the slopes of the rising phase of the fEPSPs were normalized to baseline, and the fEPSPs between thirty-five and forty minutes post-HFS were averaged. Comparisons between treatment groups were not made unless all treatments were successfully completed from the same mouse brain. This requirement controlled for inter-animal variability as well as potential differences in slice health between batches of slices.

Results

Mevalonate enrichment suppresses SV-mediated LTP enhancement

The pleiotropic effects of statins are often attributed to the reduced production of metabolites downstream of HMG-CoA reductase (HMG-CoAR) and upstream of cholesterol in the cholesterol biosynthetic pathway, also known as the mevalonate pathway. We therefore hypothesized that SV’s ability to enhance LTP in CA1 (Mans et al., 2010) could be suppressed by replenishing products downstream of HMG-CoAR. To
test this hypothesis, we employed a two-step *in vitro* treatment protocol. Initially, hippocampal slices were incubated in SV for one hour. Mevalonate (mev; 0.2 mM) was then bath applied in the recording chamber in the presence of SV for at least 40 min, and a stable baseline fEPSP was evoked. After the treatments with SV (100 min) and mev (40 min) were completed, a high frequency-stimulation protocol induced LTP in CA3-CA1 synapses. To establish the enhancement of LTP caused by SV, interleaved experiments from three animals and a pair of independent experiments from one animal were performed on slices treated with SV or vehicle (veh). Consistent with previous findings (Mans et al., 2010), SV-treated slices achieved significantly more LTP (Fig 1, 166 ± 0.07%), than veh-treated slices (112 ± 0.07%) \((P=0.009)\). To test if mev could reverse the SV-mediated enhancement of LTP, SV + mev-treated slices were interleaved with SV and veh control experiments. SV + mev-treated slices achieved only 120 ± 0.07% potentiation in contrast to 157 ± 0.04% potentiation in slices treated with SV alone (Fig 1) \((P=0.002)\). Also, the LTP magnitude in SV + mev-treated slices was statistically indifferent from veh-treated slices \((P > 0.05)\). To verify that mevalonate was specifically reversing the effect of statin as opposed to non-specifically inhibiting LTP, interleaved control experiments were conducted using veh + mev-treated slices or slices treated with veh alone. The magnitude of LTP was not reduced in veh + mev-treated slices (119 ± 0.04%) compared to those treated with veh alone (117 ± 0.08%) \((P > 0.05)\), indicating that the LTP-suppressing effect of mevalonate was specific to statin-treated slices. These results support our hypothesis that replenishing metabolites in the mevalonate pathway reverses the ability of SV to enhance LTP in CA1.
Farnesyl-pyrophosphate enrichment suppresses SV-mediated LTP enhancement

Given our positive findings in the mevalonate-replenishment experiments, we began testing if restoring isoprenoids with known regulatory effects downstream of mev could also reverse SV-induced increases in LTP. To this end, we coupled the same two-step treatment protocol used for mev with an established method for resupplying isoprenoids (Crick et al., 1997) in which farnesol (FOH) is bath applied, incorporated into the intracellular space, then converted to FPP via endogenous salvage mechanisms. It has been demonstrated that little if any FPP formed from FOH is converted to GGPP when this protocol is implemented (Crick et al. 1997). When LTP was induced after this treatment, it was found that a high dose (0.2 mM) of FOH caused a significant reduction of LTP, even in the absence of statin. As seen in Figure 2A, the amount of LTP in slices treated with FOH-supplemented veh (115 ± 0.08%) was lower than LTP in slices treated with veh + ethanol (FOH’s vehicle), which averaged 134 ± 0.10% (P = 0.046). These data suggest that the amount of intracellular FPP can bidirectionally modulate LTP induction. Namely, reduced FPP, which occurs during SV treatment, can lead to enhanced LTP induction, but high levels of FPP can suppress LTP.

To test this hypothesis further, we lowered the FOH dosage by 100 fold. Our goal was to increase FPP production to a degree that would restore FPP levels in SV-treated slices, but not appreciably affect total FPP in veh-treated slices. In interleaved trials, we found that a 2 uM dose of FOH does not reduce LTP in veh-treated slices. As seen in Fig 2B, the LTP in slices treated with FOH-supplemented veh (135 ± 0.10%) was not different from that achieved in slices treated with veh + ethanol (129 ± 0.08%) (P > 0.05). However, treatment with 2 uM FOH does reduce LTP in statin-incubated slices,
reducing the average magnitude of potentiation from $142 \pm 0.08\%$ in SV + ethanol-treated slices to only $116 \pm 0.03\%$ in slices treated with FOH-supplemented SV (Fig 2C) ($P = 0.005$). These results strongly suggest that FOH specifically reverses SV’s ability to enhance LTP, and that it does so by reversing the limitation on FPP availability caused by statin.

*Statin-induced LTP enhancement is mimicked by inhibiting farnesyl transferase*

Prenylation, an important regulatory mechanism conducted by prenyl transferase enzymes, entails the covalent attachment of prenyl moieties, such as FPP, to a specific CAAX motif (McTaggart, 2006). Our finding that FPP enrichment suppresses LTP in CA1 raises the possibility that prenylation, and specifically farnesylation, might modulate LTP induction. To test this hypothesis, we inhibited farnesylation for at least 60 min pre-HFS via bath application of a farnesyl transferase inhibitor (FTI-277), and asked if FTI-277 can mimic the LTP enhancement we observe in slices treated with statin. Interleaved treatments with FTI (1 uM) or 0.1% DMSO revealed that inhibiting farnesyl transferase for at least 60 min does indeed mimic SV (Fig 2D): the average LTP from FTI-treated slices reached $144 \pm 0.06\%$, but the average LTP from DMSO-treated slices reached only $126 \pm 0.04\%$ ($P = 0.011$). These data suggest that the increase in LTP we observe after statin incubation may arise from reduced farnesylation of downstream proteins.

*Geranylgeranyl-diphosphate replacement does not suppress LTP in SV-treated slices*

Similar to FPP, the amount of geranylgeranyl-pyrophosphate (GGPP) produced via the mevalonate pathway can exert regulatory changes on molecules subject to
geranylgeranylation. To test if GGPP can also suppress LTP, 0.2 mM GGOH was bath-applied in the same two-step treatment protocol used for FPP, and we relied upon endogenous salvage pathways to convert GGOH to GGPP (Crick et al., 1997). In contrast to FOH treatment, GGOH did not reverse the enhancement of LTP caused by SV. As seen in Fig. 3A, the average LTP in SV + GGOH-treated slices (151 ± 0.13%) was significantly enhanced over the average LTP from slices treated with vehicle (130 ± 0.08%) (P = 0.035). Furthermore, the magnitude of LTP in SV + GGOH-treated slices was similar to the LTP from slices treated with SV alone (146 ± 0.08%). From these data we conclude that the LTP enhancement we observe in SV-treated slices does not arise due to reduced production of GGPP, and that the LTP-suppressing effects of FOH treatment are specific to the farnesyl pathway.

**Inhibiting geranylgeranyl transferase type I does not change LTP magnitude**

To confirm the negative result of the GGPP-enrichment experiments, we utilized a geranylgeranyl transferase type-I inhibitor (GGTI-2133) to inhibit geranylgeranylation. It was expected that reducing geranylgeranylation would have no effect on the magnitude of potentiation. GGTI or 0.1% DMSO were bath applied for at least one hour pre-HFS. In agreement with our hypothesis, LTP in GGTI-treated slices (122 ± 0.13%) did not differ from LTP in DMSO-treated slices (123 ± 0.06%) (Fig 3B) (P > 0.05). This negative result, in conjunction with the inability of GGPP to suppress SV’s LTP-enhancing abilities, leads us to conclude that SV enhances potentiation using mechanisms independent of GGPP production and type I geranylgeranylation.
Inhibiting farnesylation promotes recruitment of the PI3-K/Akt signaling cascade during LTP induction

In a previous study, we showed that a PI3-K inhibitor, LY294002 (LY), prevents SV from enhancing LTP in CA1 (Mans et al. 2010) if applied for at least 40 min pre-HFS. Interestingly, we also found that LY did not decrease the magnitude of LTP in vehicle-treated slices. This finding suggests that under our experimental conditions, PI3-K/Akt signaling are only recruited for LTP induction in the presence of SV. Since blocking farnesylation mimics the LTP-enhancing property of SV, we hypothesized that FTI-277 might facilitate the recruitment of PI3-K during LTP induction. To test this hypothesis, we treated hippocampal slices with FTI, GGTI or DMSO for one hour, tetanized the Schaffer-collateral pathway in CA1, then homogenized the CA1 subfield for Western blot analysis five minutes post-HFS. We chose this time-point based on the work of Racaniello et al. (2009) in which tetanus-induced phosphorylation of Akt, an indirect measure of PI3-K activity, peaked at five-minutes post-tetanus. To generate interleaved internal controls from non-tetanized slices from the same animals, CA1 subfields were also harvested from slices treated with FTI, GGTI or DMSO for one hour prior to stimulation at baseline intensity. These slices served as estimators of the basal p-Akt level under each treatment condition per animal. To account for the variability of the raw ratio of p-Akt/total Akt between mice, all data were normalized to the non-tetanized control within each treatment group. We used this experimental design to test the hypothesis that only slices treated with FTI would display an increase in p-Akt after tetanus. Relative to their non-tetanized controls, we found the level of post-HFS p-Akt in DMSO-, FTI- and GGTI-treated slices to be $0.94 \pm 0.08$ (n=13), $1.34 \pm 0.16$ (n=12) and $1.07 \pm 0.13$ (n=12),
respectively (Fig 4B). After a paired, one-tailed t-test, only FTI-treated slices showed a significant increase in p-Akt following tetanus (p= 0.027). We interpret this result as an indication that FTI facilitates the recruitment of PI3-K/Akt during LTP induction.

It is notable that a statistically insignificant (P > 0.05) decrease in basal p-Akt occurred after FTI treatment (Fig 4C). This analysis was conducted by normalizing the p-Akt/total Akt ratio from the non-tetanized slices for each drug treatment to the p-Akt/total Akt ratio in DMSO-treated slices from the same animal. The average p-Akt levels in non-tetanized slices after incubation in FTI or GGTI were 0.86 ± 0.11 and 1.03 ± 0.12, respectively.
Fig 1. Mevalonate (mev) enrichment suppresses SV-mediated LTP enhancement. Hippocampal slices were pre-incubated in SV (10 µM) or veh for one hr, then moved to the recording chamber and treated with SV or veh in the presence or absence of mev (0.2 mM) for at least 40 min prior to HFS. The average magnitude of LTP from SV-treated slices (166 ± 0.07% LTP) was significantly higher (* P=0.009) than that from veh-treated slices from the same animals (112 ± 0.07% LTP, both treatments: n=4 slices, 4 animals). To test if mev enrichment can suppress this enhancement, SV + mev-treated slices were interleaved with SV and veh controls, and an additional independent pair of experiments without a veh control were performed (n=4 slices, 4 animals). Mev reduced the LTP magnitude (P=0.002) from 157 ± 0.04% in SV-treated slices to only 120 ± 0.07% in SV+mev-treated slices.
**Normalized fEPSP Slope**

**Time (min)**

**Vehicle or Vehicle + Farnesol (0.2 mM)**

**A**

**Vehicle or Vehicle + Farnesol (2 mM)**

**B**

**SV or SV + Farnesol (2 mM)**

**C**

**FTI-277 or DMSO**

**D**
Fig 2. SV-mediated LTP enhancement is suppressed by restoring FPP and mimicked by inhibiting farnesylation. (A) Mean LTP from slices pre-incubated in veh for one hour, then treated with veh containing either, 0.2 mM farnesol (FOH) or 0.01% ethanol for at least 40 min in the recording chamber pre-HFS. This high dose of FOH significantly (*P=0.046, both treatments: n=4 slices, 4 animals) lowered LTP in veh-treated slices (115 ± 0.08% LTP) compared to LTP in slices treated with veh alone (134 ± 0.10% LTP). (B) Mean LTP from slices pre-incubated in veh for one hour then treated with veh containing either, 2 µM FOH or 0.01% ethanol for at least 40 min in the recording chamber pre-HFS. The lower dose of FOH did not reduce LTP in veh-treated slices. 129 ± 0.08% LTP was achieved in veh + ethanol-treated slices, and 135 ± 0.10% LTP was achieved in veh + FOH-treated slices (P>0.05, both treatments: n=6 slices/6 animals). (C) Mean LTP from slices pre-incubated in SV (10 µM) for one hour then treated with SV containing either, 2 µM FOH or 0.01% ethanol for at least 40 min in the recording chamber pre-HFS. Unlike in veh-treated slices, 2 µM FOH reduced LTP in slices pre-treated with SV. LTP was reduced (*P=0.005) from 142 ± 0.08% in SV + ethanol-treated slices to only 116 ± 0.03% in SV + FOH-treated slices (both treatments: n=5 slices, 5 animals). (D) Inhibiting farnesyl transferase mimics the LTP-enhancing property of SV. Hippocampal slices were treated in the recording chamber with FTI-277 (1 µM) or 0.1% DMSO for at least one hour-pre HFS. FTI significantly (*P=0.011) enhanced LTP (144 ± 0.06% LTP, n=8 slices, 6 animals) compared to DMSO (126 ± 0.04% LTP, n=6 slices, 6 animals)
Fig 3. SV-mediated LTP enhancement is not affected by GGPP or mimicked by inhibiting geranylgeranylation. (A) Hippocampal slices were pre-incubated in SV (10 µM) or veh for one hr, then moved to the recording chamber and treated with SV or veh in the presence or absence of GGOH (0.2 mM) for at least 40 min prior to HFS. The average magnitude of LTP from SV-treated slices (146 ± 0.08% LTP) was significantly higher (*P=0.009) than that from veh-treated slices from the same animals (130 ± 0.08% LTP, both treatments: n=5 slices, 5 animals). To test if GGPP enrichment can suppress this enhancement, SV + GGOH-treated slices were interleaved with SV and veh controls (5 slices, 5 animals). GGOH did not (P>0.05) reverse the SV-induced LTP enhancement, as LTP in SV + GGOH-treated slices averaged 151 ± 0.13%. (B) Inhibiting geranylgeranyl transferase type 1 does not affect LTP. Hippocampal slices were treated in the recording chamber with GGTL-2133 (1 µM) or 0.1% DMSO for at least one hour-pre HFS. LTP in the presence of GGTL (122 ± 0.13% LTP) was not different (P>0.05) from LTP in DMSO (123 ± 0.06% LTP, both treatments: n= 6 slices, 5 animals).
**Fig 4.** HFS-induced activation in FTI-treated slices. (A) Representative immunoblot images of p-Akt and total Akt in the homogenate of CA1 region of hippocampal slices treated with FTI-277 (FTI), GGTI-2133 (GGTI) or DMSO for one hour, then harvested 5 min after HFS or baseline stimulation. (B) Densitometric analysis of immunoblots from tetanized slices (normalized to total Akt) with non-tetanized slices set as 1. Data represent means±SEM (n=12 slices from 12 animals). The results show the ratio of activated Akt increased after HFS compared to non-tetanized controls in FTI-treated slices only (*P*=0.027). (C) Densitometric analysis of immunoblots from slices that received baseline stimulation (normalized to total Akt) with DMSO-treated slices set as 1. The results show a statistically non-significant (*P* > 0.05) decrease in basal Akt activation in FTI-treated slices.
Discussion

Previously, we showed that chronically administered SV enhances spatial learning and memory in both a transgenic mouse model of AD and non-transgenic C57BL/6 controls (Li et al., 2006). A subsequent study demonstrated that incubation in SV for 2-4 hours enhances LTP in hippocampal slices from young adult C57BL/6 mice. This enhancement of LTP was found to be dependent upon PI3-K activity during the induction phase (Mans et al. 2010). In the present study we dissect the mevalonate pathway to identify the key metabolite(s) contributing to this effect. Specifically, we test the hypothesis that SV’s ability to inhibit isoprenoid production is driving the SV-induced LTP enhancement in CA1. Our results demonstrate that: (1) modulating levels of FPP, but not GGPP, alters the magnitude of early LTP in region CA1 of mature C57BL/6 mice, (2) inhibiting FTase, but not GGTase Type I for one hour preceding HFS mimics the LTP-enhancing property of SV, and (3) FTase inhibition increases the recruitment of PI3-K during LTP induction.

The small GTPases, which include the most-commonly studied RhoA, RhoB, Rac1 and Ras, are regulated by prenylation, and facilitate the coupling of extracellular signals to intracellular kinases while simultaneously regulating the activity of other GTPases (Reviewed in McTaggart 2006 and Hoof et al. 2010). The two major families of small GTPases, Rho and Ras, are differentially regulated by geranylgeranylation and farnesylation, respectively, though some exceptions do occur (reviewed in McTaggart, 2006). Prenylation enzymes and small GTPases have been implicated in regulating LTP, but their exact roles are not clearly understood.
A key component of synaptic plasticity entails cytoskeletal reorganization (Bramham, 2008). The Rho GTPases, which include RhoA, RhoB and Rac1, have known roles in cytoskeletal dynamics (Narumiya et al., 1997). Several groups have therefore tested if Rho GTPases are necessary for persistent changes in synaptic strength. Whether Rho GTPases facilitate or restrict LTP is subject to debate. In support of a role for geranylgeranylated GTPases in LTP, HFS triggers activation of hippocampal RhoA, RhoB and Rac1 (O’Kane et al. 2004). Also, several studies demonstrate a role for Rho GTPases in structural plasticity at dendritic spines (Murakoshi et al., 2011; reviewed in Tolias et al., 2011). Moreover, theta-burst-induced LTP in rat CA1 is impaired if either Rac1 or an effector kinase for RhoA, ROCK, is inhibited (Rex et al. 2009). In direct conflict with these results, O’Kane et al. (2004) show that inhibiting ROCK or GGTase Type-1 prenylation actually augments HFS-induced LTP. It is not clear why these studies obtain conflicting results, but at a minimum it seems likely that Rho GTPases contribute to LTP in some regard. Therefore, it is surprising that our experimental manipulations of geranylgeranylation, either by GGPP enrichment or GGTase Type 1 inhibition, do not affect LTP magnitude. However, it is now well appreciated that experimental conditions, especially the degree and pattern of post-synaptic depolarization, can dramatically alter the recruitment of signaling molecules during the induction phase of LTP (Opazo et al., 2003; Jin and Feig, 2010). Our use of HFS rather than theta-burst is an obvious deviation from the protocol in Rex et al. (2010). As for the study by O’Kane and colleagues, it is notable that their baseline stimulations and tetanus were strong enough to evoke population spikes, but our stimulations during baseline and HFS were of sub-threshold intensity. It also important to consider that the levels of GGPP measured in the brains of
mice are nearly three-fold higher than FPP (Eckert et al., 2009; Tong et al., 2008). Due to the high volume of GGPP present, GGTase inhibition under our conditions may not have induced a change in the percentage of geranylgeranylated proteins of sufficient magnitude to affect LTP. There may be a threshold of prenylation necessary for normal function that was not crossed under our conditions. It is also possible that other GTPases, which may be farnesylated or geranylgeranylated, such as RhoB, have compensated for the affects induced by GGTI-2133. A more detailed biochemical analysis of GTPase localization, activation state and coupling to effector proteins will be necessary to fully understand how our stimulation protocol and pharmacological manipulations of geranylgeranylation affect Rho GTPase activity. Also, small GTPases are known to retain some functionality while unprenylated (Allal et al., 2000; Lebowitz et al., 1997; reviewed in Samuel and Hynds, 2010). How these unprenylated GTPases function in the context of LTP is mostly unexplored. Finally, other post-translational modifications, such as palmitoylation, incur changes in protein activity (Omeric and Prior, 2008), and they may contribute to our observations.

The roles of farnesylated proteins, including RhoB and Ras, in LTP are similarly complex. Three principal actions of Ras in the context of LTP are most well characterized: 1) coupling of adrenergic receptor activity to ERK phosphorylation, 2) calcium-dependent coupling of NMDAR activity to ERK phosphorylation, and 3) the coupling of calcium-dependent NMDAR activity to PI3-K/Akt signaling (reviewed in Sweatt 2004, Thomas and Huganir, 2004). The exact conditions under which Erk and PI3-K are recruited and/or necessary for LTP in CA1 is highly controversial (Opazo et al., 2003; Qin et al., 2005; Jin and Feig 2010). It is now well established that LTP
induction can initiate both the Ras-MEK-Erk and the Ras-PI3-K/Akt signaling cascades, though several groups have shown that HFS-induced LTP in CA1 of adult mice occurs independent of Erk activation (Opazo et al. 2003—Winder et al., 1999; Watabe et al., 2000; Liu et al., 1999). Regardless, both pathways have been shown to facilitate AMPA receptor trafficking and insertion into synapses during long-term changes in synaptic strength (Man et al., 2003; Qin et al., 2005; Hu et al., 2008; Jin and Feig, 2010; Patterson et al., 2010). In accord with this mechanism, FTase inhibition, which alters the intracellular localization of Ras and its ability to interact with effector kinases (Omerovic and Prior, 2008), can reduce the magnitude of LTP (O’Kane et al, 2004). However, these results are in conflict with several findings.

First, in genetically modified mice lacking the neuron-specific Ras-guanine-nucleotide-releasing factor (GRF), an activator of Ras GTPase, hippocampal learning and LTP magnitude are normal (Brambilla et al., 1997). Second, there are several lines of evidence that Ras negatively regulates NMDAR transmission. Specifically, it has been shown that an H-Ras deficient mouse exhibits both enhanced NMDAR conductance and greater LTP in CA1 compared to wild-type mice (Manabe et al. 2000; Thornton et al., 2003). Also, there is evidence that inhibiting a Ras effector protein, RACK1, increases NMDAR currents in hippocampal neurons (Yaka et al., 2002), suggesting that Ras inhibition can augment NMDAR-dependent plasticity. Our findings are in agreement with this hypothesis. Namely, incubation in the FTase inhibitor, FTI-277, which blocks farnesylation of H-ras, enhanced LTP in CA1. Furthermore, increasing FPP levels, and presumably farnesylation of Ras, inhibited LTP. Interestingly, abnormally high levels of activated Ras were found in a genetically-engineered mouse model of mental retardation
(neurofibromatosis type 1), and learning deficits in this model were ameliorated by treatment with statin. Furthermore, elevated levels of FPP and GGPP have been found in the brains of Alzheimer’s disease patients (Eckert et al. 2009). More experiments are required to verify that Ras GTPases modulate NMDAR transmission under our experimental conditions. Also, separate contributions of all Ras isoforms (K-ras, N-ras and H-ras), whose activities are differentially regulated (Omerovic and Prior 2008), and the actions of less-well studied GTPases must be considered. Finally, farnesylated proteins outside of the small GTPase superfamily should not be excluded when evaluating the results described here.

In an attempt to better understand the molecular signaling underlying the FTI-induced enhancement of LTP, we assayed for activation of Akt as an indirect measure of PI3-K activity five minutes after HFS. Our previous investigation into the role of PI3-K in SV-mediated LTP enhancement revealed that a PI3-K inhibitor, LY-294002, selectively reduced LTP in SV-treated slices (Mans et al., 2010). This led us to hypothesize that inhibition of FTase facilitates recruitment of PI3-K for LTP induction, but PI3-K is not recruited in the presence of DMSO or GGTI-2133. When whole homogenates from region CA1 were subjected to Western blot analysis, we found that pre-treatment with FTI for one hour pre-HFS promoted the phosphorylation of Akt after tetanus, but treatment with DMSO or GGTI did not. While this result is in agreement with our hypothesis, it should be interpreted with caution. The primary limitation is the use of whole homogenate, rather than synaptic fractions. This approach prevents the requirement that samples be pooled between several animals to allow sufficient volume of protein, but it also includes nuclear and cytosolic Akt that is likely excluded from early
LTP signaling. The post-HFS increase in Akt activation we observe may therefore be independent of LTP induction mechanisms. Still, it is also possible that SV and FTI-277 enhance LTP by recruiting PI3-K to the induction process. It is known that insulin modulates hippocampal plasticity in a PI3-K-dependent manner, and that PI3-K is not required for LTP (Mans et al., 2010, Opazo et al., 2003). PI3-K may, in fact, act as a modulatory enzyme to supplement other kinases during the induction process. Certainly, further experimentation will be required to fully answer this question as it relates to statins and other prenylation inhibitors. Also, the activity of PDK1, the intermediary between PI3-K and Akt, may have its own regulatory influence that is not explored here.

**Conclusion**

The present study demonstrates that the SV-induced enhancement of LTP we observe in hippocampal slices from adult C57BL/6 mice is due to reduced production of FPP, but not GGPP, in the mevalonate pathway. Additionally, inhibition of farnesylation, but not geranylgeranylation type I can enhance LTP, and this effect is associated with the recruitment of Akt during the induction phase. The fact that over three hundred proteins have been identified as prenylation targets complicates any prediction of how statins and other prenylation inhibitors affect intracellular signaling. Predictions are further confounded by an incomplete understanding of how the two classes of prenylation, farnesylation and geranylgeranylation, regulate the vast number of small GTPases in the context of multiple cellular compartments and interacting processes. Nevertheless, the present study provides further evidence that isoprenylation, and particularly farnesylation, plays an important role in regulating synaptic plasticity, presents a ground-
work for future explorations into how statins enhance cognition at the cellular level and motivates the development of novel FTI’s as a potential treatment for cognitive disorders.

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CONCLUSIONS

The identification of cholesterol as a major risk factor for AD initiated numerous studies evaluating the cognitive benefits of HMG-CoARs, which inhibit the biosynthetic pathway for cholesterol. Unfortunately, epidemiological studies and clinical trials have proven remarkably inconsistent regarding the cognitive effects of statins on several outcome measures. These include the prevalence of AD, the severity of AD pathology, the prevalence of dementia in non-AD individuals and the rate of cognitive decline in statin-prescribed populations. Experiments in animal models of AD as well as non-diseased animals have likewise generated contradictory results. While the root of the inconsistencies are not immediately apparent, it is clear that carefully-designed studies are needed to elucidate the direct statin-mediated effects on processes underlying cognitive function.

The present study was undertaken to address the question of how statins affect LTP, a synaptic mechanism with great potential as a cellular correlate of learning and memory. There is evidence in humans and rodents that statins improve cognitive function, including a study showing that chronically administered SV enhances spatial learning and memory in non-transgenic mice as well as in a transgenic mouse model of AD (Li et al., 2006). These findings corroborate evidence in another study in which rats fed a statin diet performed better than controls in learning and memory assays, in addition to human epidemiological studies (Jick et al., 2000, Wolozin et al., 2000, Wolozin et al., 2007), which argue for positive effects of statins on cognition. Numerous positive pleiotropic effects have been observed in laboratory animals, both in vitro and in vivo.
and several appear to be mediated by isoprenoid intermediates of the cholesterol biosynthetic pathway rather than by cholesterol. Also, enhanced activation of Akt is commonly observed after statin treatment, and some pleiotropic effects are PI3K-dependent. In light of these results, several hypotheses are tested: 1) SV treatment for several hours in vitro can enhance the magnitude of LTP at CA3-CA1 synapses in C57/BL6 mice; 2) the effects of SV on LTP are derived from reduced isoprenylation; 3) PI3-K activation mediates SV-induced changes in synaptic plasticity.

Our current understanding of NMDAR-dependent LTP divides the phenomenon into “early”, protein-synthesis independent, and “late”, protein-synthesis dependent, phases (Malenka and Bear, 2004). The present study focuses on early LTP. In Mans et al. (2010), we demonstrated that incubation in SV for several hours (2-4), can enhance LTP magnitude at CA3-CA1 synapses in the hippocampi of young adult mice. Also, the enhancement occurred independent of changes in basal synaptic transmission, as neither paired-pulse ratios nor input-output response curves were significantly altered by SV treatment. These results are supportive of the hypothesis that learning improvements in mice fed SV for several months may be attributed to improvements in synaptic plasticity. SV displays strong lipophilic character, and the concentration of SV in plasma of statin-fed rats peaks at approximately two hours (Vickers et al., 1989), a time course consistent with the incubation duration employed here.

These results are in agreement with the hypothesis tested, and parallel the behavioral data that SV enhances hippocampal-dependent learning and memory, but they are seemingly in contrast with reports showing that hippocampal LTP is decreased by an acute treatment with a different statin, compactin, in mice (Kotti et al., 2006, 2008).
However, these results are not necessarily in conflict. The differences in experimental conditions may explain the discrepancy. Firstly, although all statins block HMG-CoAR, they possess subtle differences in their chemistry that endow them with different properties (Jones, 2003). While SV was used in the current study, compactin was used in the study of Kotti et al. (2006). Compactin is the first statin isolated, but it is not approved for human use because of toxicity concerns (Endo, 2004). Therefore, it is possible that the impact of compactin on neuronal function could be different from that of SV. Secondly, there is a difference in LTP induction protocols. While a high-frequency stimulation protocol was used here, Kotti et al. (2006) utilized a theta-burst protocol. It is now appreciated that different stimulation protocols induce LTP with varying molecular mechanistic requirements (Lisman, 2003). Other studies have reported inconsistencies between HFS- vs. theta-burst-induced LTP within a single experimental system (Hoffman et al., 2002, Costa and Grybko, 2005). Thirdly, the age of the animals is different between the two studies. Mature (3-4 months old) mice are used here, while Kotti et al (2006) used young (4-6 wks old) mice. The molecular mechanisms underlying LTP induction evolve during development (Esteban et al., 2003, Yasuda et al., 2003). C57BL/6 mice younger or older than 2 months have been shown to require different molecular mechanisms for expression of early LTP (Lu et al., 2007b). Therefore, it is possible that modulation of LTP by statin exposure depends upon the developmental state of the animals.

In a previous study, it was shown that enhancement of learning and memory by chronic SV treatment is associated with an increased level of activated (phosphorylated) Akt (p-Akt) in the brains of wild type mice (Li et al., 2006). The underlying intracellular
signaling cascades driving early LTP have been extensively investigated, yet the role of the PI3K/Akt pathway in the regulation of LTP is not well understood. While one study using rat hippocampal slices showed that PI3K/Akt activation is required for the expression but not the induction of LTP at CA3-CA1 synapses (Sanna et al., 2002), another study using mouse hippocampal slices demonstrated that PI3K/Akt activation contributes to the induction of LTP rather than the expression or maintenance of LTP at CA3–CA1 synapses (Opazo et al., 2003). The reasons for the discrepancy were not clear but might be attributable to differences in the concentration of PI3K inhibitors and in the animal species (rat vs. mouse) in these studies.

In the present study, it is found that inhibition of Akt phosphorylation by LY294002 had no effect on the expression of hippocampal LTP in either SV or vehicle-treated slices, but it significantly suppressed the induction of LTP in SV-treated slices such that the LTP magnitude was similar to that from slices treated with vehicle. Interestingly, LY294002 did not affect the induction of LTP in vehicle-treated slices under the experimental conditions used here. It is plausible that, in vehicle-treated slices, the inhibition of basal p-Akt by LY294002 was not sufficient to affect the magnitude of LTP, as other LTP-inducing mechanisms exist. However, in SV-treated slices, the level of p-Akt is upregulated, causing a greater contribution of p-Akt to the induction process and enhancing LTP. Thus, inhibition of Akt phosphorylation by LY294002 would produce a significant suppression of LTP in SV-treated slices. Taken together, the data indicate that enhanced activity in the PI3K/Akt pathway is a molecular mechanism contributing to the SV-induced increase in the magnitude of early LTP.
To continue probing the mechanism by which SV enhances LTP, experiments were performed to determine how key metabolites of the cholesterol biosynthetic pathway influence SV-induced plasticity enhancements. The first hypothesis tested in this regard was that statin-mediated LTP improvements derive from inhibition of HMG-CoAR, rather than a mechanism independent of SV’s inhibitory function. To this end, mevalonate, the immediate downstream product of HMG-CoAR, was replenished during the last forty minutes of a SV incubation lasting at least 100 minutes. Previous work demonstrated that a forty-minute treatment with LY294002 during the tail-end of a two-hour SV treatment is sufficient to block SV’s LTP enhancing ability (Mans et al., 2010). It was hypothesized that mevalonate replenishment would abolish SV-induced LTP enhancement. Indeed, mevalonate treatment completely reversed the effects of SV, bringing LTP down to the level of vehicle-treated slices. This result strongly supports the notion that the reduction of metabolites downstream of HMG-CoAR initiates SV-induced LTP augmentation.

Isoprenylation regulates the activity of hundreds of proteins in mammals, including those with their own regulatory duties, such as the numerous small GTPases. Thus, the availability of key isoprenoids, such as GGPP and FPP, can potentially influence a staggering array of signaling cascades, not excluding those integral to LTP induction. It was therefore hypothesized that the reduction of one or both key isoprenoid moieties downstream of mevalonate triggers LTP enhancement. Indeed, the restoration of FPP production via an endogenous salvage mechanism selectively reduced LTP in SV-treated slices. Interestingly, a high dose of the FPP precursor, farnesol, reduced LTP in vehicle-treated slices. This observation raises the intriguing possibility that FPP bi-
directionally modulates LTP magnitude, in which high amounts inhibit LTP, but reduced production (as occurs with SV treatment), enhances LTP.

Some effects of isoprenoid reduction, such as upregulation of Ras and Ras-related proteins, have been shown to be independent of FTase activity. In the current study, however, inhibiting FTase for at least one hour using FTI-277 preceding HFS produced LTP-enhancement similar to that caused by SV incubation. That FTI mimicked SV strongly supports a mechanism in which FPP-depletion-induced inhibition of farnesylation leads to enhanced NMDAR-dependent LTP. In fact, there are several lines of evidence that Ras, a farnesylated GTPase, negatively regulates NMDAR transmission. Specifically, it has been shown that an H-Ras deficient mouse exhibits both, enhanced NMDAR conductance and greater LTP in CA1 compared to wild-type mice (Manabe et al. 2000). Also, there is evidence that directly inhibiting a Ras effector protein, RACK1, increases NMDAR currents in hippocampal neurons, suggesting that Ras inhibition can augment NMDAR-dependent plasticity. Interestingly, abnormally high levels of activated Ras were found in a genetically-engineered mouse model of mental retardation (neurofibromatosis type 1), and learning deficits in this model were ameliorated by treatment with statin. Furthermore, elevated levels of FPP and GGPP have been found in the brains of Alzheimer’s disease patients (Eckert et al. 2009).

However, these results are seemingly in direct contradiction to the findings that Ras-MEK and Ras-PI3K-Akt signaling cascades are essential for AMPAR trafficking during LTP (Man et al., 2003; Qin et al., 2009; Hu et al., 2008; Jin and Feig, 2010; Patterson et al., 2010), and the related observation that FTase inhibition, which alters the intracellular localization of Ras and its ability to interact with effector kinases, can reduce
the magnitude of LTP under some conditions (O’Kane et al., 2004). When considering the effects of FTase inhibition, it is important to acknowledge that blocking isoprenylation does not necessarily preclude GTPase activation nor does it block the PI3K/Akt pathway. If this were indeed the case, then the PI3-K inhibitor LY-294002 would not selectively reduce LTP in SV-treated slices, nor would a hallmark effect of SV treatment, augmented PI3-K-dependent Akt activation, occur.

More experiments are required to verify that Ras GTPases modulate NMDA receptor transmission under the experimental conditions used here. Also, separate contributions of all Ras isoforms (K-ras, N-ras and H-ras), whose activities are differentially regulated (Omerovic and Prior 2008), and the actions of less-well studied GTPases must be considered. Finally, farnesylated proteins outside of the small GTPase superfamily should not be excluded when evaluating results described here.

Geranylgeranylation exerts its own regulatory effects, including modulation of the Rho GTPases (RhoA, RhoB, Rac1), which participate in cytoskeletal dynamics during LTP. Also, HFS triggers activation of hippocampal RhoA, RhoB and Rac1 (O’Kane et al. 2004). Moreover, theta-burst-induced LTP in rat CA1 has been shown to be impaired if either, Rac1 or an effector kinase for RhoA, ROCK, is inhibited (Rex et al. 2009). Therefore, it is surprising that experimental manipulations of geranylgeranylation in the current study, either by GGPP enrichment or GGTase Type 1 inhibition, did not affect LTP magnitude. However, inconsistencies pervade the literature regarding how Rho GTPases and/or geranylgeranylation affect LTP. In direct conflict with the study by Rex and colleagues (2009), O’Kane et al. (2003) show that inhibiting ROCK or GGTase Type-1 prenylation actually augments HFS-induced LTP.
It is now well appreciated that experimental conditions, especially the degree and pattern of post-synaptic depolarization, can dramatically alter the recruitment of signaling molecules during the induction phase of LTP (Opazo et al., 2003; Jin and Feig, 2010). The use of HFS rather than theta-burst is an obvious deviation from the protocol in Rex et al. (2010). As for the study by O’Kane and colleagues, it is notable that their baseline stimulations and tetanus were strong enough to evoke population spikes, but the stimulations during baseline and HFS used here were of sub-threshold intensity. It also important to consider that the levels of GGPP measured in the brains of mice are nearly three-fold higher than FPP (Eckert et al., 2009; Tong et al., 2008). Due to the high volume of GGPP present, GGTase inhibition under the present conditions may not have induced a change in the percentage of geranylgeranylated proteins of sufficient magnitude to affect LTP. There may be a threshold of prenylation necessary for normal function that was not crossed here. It is also possible that other GTPases, which may be farnesylated or geranylgeranylated, such as RhoB, have compensated for the effects induced by GGTI-2133. A more detailed biochemical analysis of GTPase localization, activation state and coupling to effector proteins will be necessary to fully understand how the current stimulation protocol and pharmacological manipulations of geranylgeranylation affect Rho GTPase activity. Also, small GTPases are known to retain some functionality while unprenylated. How these unprenylated GTPases function in the context of LTP is mostly unexplored. Finally, other post-translational modifications, such as palmitoylation, incur changes in protein activity, and they may contribute to these observations.
During our initial investigations into the role of PI3-K in statin-mediated LTP enhancement, it was revealed that a PI3-K inhibitor, LY-294002, selectively reduced LTP in SV-treated slices (Mans et al., 2010). The absence of an effect in vehicle-treated slices presents an intriguing possibility. Namely, the induction protocol used here may have been insufficient to recruit PI3K under control conditions, but the downstream effects of HMG-CoAR inhibition somehow facilitated the activation of PI3K during HFS. If this were the case, one would predict an increase in PI3-K activity during induction only in FTI-treated slices, since GGTI did not enhance LTP above control (DMSO).

When p-Akt levels from whole homogenates from region CA1 were analyzed by Western blot as an indirect measure of PI3-K activity, it was found that treatment with FTI for one hour pre-HFS promoted the phosphorylation of Akt five minutes after tetanus, but treatment with DMSO or GGTI did not. While this result is in agreement with the hypothesis tested, it should be interpreted with caution. The primary limitation is the use of whole homogenate, rather than synaptic fractions. This approach prevents the requirement that samples be pooled between several animals to allow sufficient volume of protein, but it also includes nuclear and cytosolic Akt that is likely excluded from early LTP signaling. The post-HFS increase in Akt activation we observe may therefore be independent of LTP induction mechanisms.

Also, the results may be confounded by a slight, statistically non-significant decrease in p-Akt that was observed in non-tetanized, FTI-treated slices. Slices that only experienced baseline stimulation served as estimators of basal p-Akt within each treatment group, since it is impossible to know the pre-HFS activation level in slices homogenized after HFS. The statistical analysis conducted here for each drug treatment
entailed normalizing p-Akt levels in tetanized slices to levels in non-tetanized slices from the same animal. It was then tested if tetanus caused a deviation from 1. There is the remote possibility that the perceived increase in p-Akt tetanus may be a statistical artifact arising from the slight decrease in non-tetanized controls. Still, it is also possible that SV and FTI-277 indeed enhance LTP by recruiting PI3-K to the induction process. It is known that insulin modulates hippocampal plasticity in a PI3-K-dependent manner, and that PI3-K is not absolutely required for LTP (Mans et al., 2010, Opazo et al., 2003). PI3-K may, in fact, act as a modulatory enzyme to supplement other kinases during the induction process. Of course, further experimentation will be required to fully answer this question as it relates to statins and other prenylation inhibitors. Also, the activity of PDK1, the intermediary between PI3-K and Akt, may have its own regulatory influence that is not explored here.

The present study demonstrates several important findings pertaining to statin-induced changes in synaptic plasticity in the normal, fully-developed, non-diseased brain. First, it is reported that a 2-4 hour exposure to SV in vitro enhances of the magnitude of LTP at CA3-CA1 hippocampal synapses, a circuit known to be critical for normal learning and memory. Second, it is shown that FPP, an isoprenoid derivative of mevalonate in the biosynthetic pathway for cholesterol, is capable of suppressing SV-mediated LTP enhancement. In accord with this result, inhibition of farnesylation mimics the ability of SV to enhance LTP. These results support a role for farnesylated proteins, such as Ras-GTPases, in the modulation of synaptic plasticity in the adult hippocampus. Furthermore, the effect of isoprenylation appears to be selective for farnesylated proteins,
as GGPP failed to suppress SV-induced increases in LTP magnitude, and inhibiting GGTase Type-I produced no change in LTP.

Finally, it is demonstrated that SV enhances LTP in CA1 by a PI3-K dependent mechanism, possibly by enhancing NMDAR conductance and recruiting PI3-K to the induction process. Interestingly, an FTase inhibitor, which increases LTP under the current conditions, also appears to facilitate activation of PI3-K immediately after HFS, further supporting a PI3-K dependent mechanism underlying SV-induced augmentation of LTP. In addition to establishing a solid framework for fully understanding the cellular and molecular mechanisms by which statins improve learning and memory in statin-fed mice, these findings contribute more insights into how isoprenylation modulates hippocampal synaptic plasticity.

As an extension of this work, both the amplitude of NMDAR currents and the magnitude of hippocampal LTP should be tested in vivo in statin-fed mice to avoid the confounds of slice preparation and in vitro dosage. Additionally, an intriguing experiment would entail determining the threshold for LTP induction in statin-fed mice. If NMDAR transmission and/or PI3-K recruitment are indeed enhanced, one would predict the threshold for LTP induction would be lower in statin-treated animals. Furthermore, in vivo pharmacological intervention during behavioral testing would contribute greatly to elucidating the molecular mechanisms underlying statin-induced improvements in learning and memory. Finally, the effects of statin-derived metabolites should also be tested in the context of synaptic physiology. Inconsistencies between studies using structurally distinct statins could be attributed to variable release of lipophilic moieties into the bloodstream that might influence synaptic function.
The results from the present study and others indicating cognitively beneficial effects of statins raise the question of whether statins should be prescribed to ameliorate cognitive decline. While there is mounting evidence that statins can improve human and rodent cognition, the results are not entirely consistent. There are studies showing no improvement after statin treatment as well as case reports of patients developing amnesia during statin treatment (reviewed in Introduction). To cope with these inconsistencies, one might propose evaluating learning and memory in each individual after a brief trial period. Individuals suffering amnesia could be withdrawn, and those benefitting could continue treatment. However, this seemingly safe approach may prove costly due to the potentially harmful withdrawal symptoms observed in statin users. Statin withdrawal is often characterized by a rebound of inflammatory responses (Li et al., 2006) and suppression of endothelial nitric oxide production (Laufs et al., 2000), both of which worsen coronary health. These rebound effects have not been conclusively linked to increases in cardiac events for patients with stable cardiac conditions (Hu, 2006), but in patients withdrawn from statins following stroke or other acute coronary syndromes, the risk of death, size of infarct and number of cardiac events have been shown to increase (Chen et al., 2009; Blanco et al., 2007; Heeschen et al., 2002). Therefore, it is perhaps premature to promote statin administration as either a front-line defense against age-related cognitive decline or as a supplement to improve learning and memory in non-impaired individuals.
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APPENDIX A
IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)
NOTICE OF APPROVAL

DATE: January 19, 2011
TO: LORI L. MCMAHON WAKEFIELD, Ph.D.
MCLM-964 6005
FAX: (205) 976-4028

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Protective Mechanisms of Statins in Alzheimer's Disease
Sponsor: NIH
Animal Project Number: 110108347

As of January 19, 2011, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
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<th>Species</th>
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<th>Number in Category</th>
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<td>200</td>
</tr>
<tr>
<td>Mice</td>
<td>B</td>
<td>50</td>
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</table>

Animal use must be renewed by January 18, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110108347 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7602.