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Review

Simvastatin treatment reduces the cholesterol content of membrane/lipid rafts, implicating the *N*-methyl-D-aspartate receptor in anxiety: a literature review

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Membrane/lipid rafts (MLRs) are plasmalemmal microdomains that are essential for neuronal signaling and synaptic development/stabilization. Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (statins) can disable the *N*-methyl-D-aspartate (NMDA) receptor through disruption of MLRs and, in turn, decrease NMDA-mediated anxiety. This hypothesis will contribute to understanding the critical roles of simvastatin in treating anxiety via the NMDA receptor.

Uniterms: Anxiolytic effects. Membrane/lipid rafts. *N*-methyl-D-aspartate. Simvastatin.

INTRODUCTION

Clinically, simvastatin (SIM) has been widely used to reduce serum low-density lipoprotein (LDL) cholesterol levels by inhibiting the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase (Owens, Byrnes, Mackman, 2014). However, SIM also has pleiotropic effects, such as anti-inflammatory, anti-oxidative and immunomodulatory effects (Eger *et al.*, 2016a, 2016b; Mihos, Pineda, Santana, 2014; Ungureanu *et al.*, 2003). Evidence illustrates that SIM reduces the risk of ischemic heart disease and cerebrovascular stroke and that it has potential applications in multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Daneschvar, Aronson, Smetana, 2015; Friedman *et al.*, 2013; Kataoka *et al.*, 2015; Ní Chróinín *et al.*, 2013; Pihl-Jensen, Tsakiri, Frederiksen, 2015; Sett, Robinson, Mistri, 2011). Despite growing evidence for the role of SIM in treating central nervous system (CNS) diseases, there is relatively little knowledge of its direct psychoneurological effects on central receptors and its association with behavioral effects.

In the CNS, cellular cholesterol homeostasis is independent of plasma cholesterol levels because blood-to-brain cholesterol transport is virtually nonexistent through the blood-brain barrier (Chobanian, Hollander, 1962; Dietschy, Turley, 2004). Statins that cross the blood-brain barrier could disrupt normal cholesterol turnover. As such, SIM, as a lipophilic statin that, can cross the blood-brain barrier easily, was recommended as the best candidate capable disrupting CNS cholesterol homeostasis (Lutjohann *et al.*, 2004; Thelen *et al.*, 2006). Changes in brain cholesterol metabolism have been reported in experimental animals and humans. 24(*S*)-Hydroxycholesterol has been used in many studies as an indicator of brain cholesterol turnover, as it is the by-product of cholesterol metabolism through brain-selective cholesterol 24-hydroxylase (CYP46A1) and is capable of crossing the blood-brain barrier for detection in systemic circulation. Following chronic SIM administration, numerous studies identified reductions in the plasma and cerebrospinal fluid concentrations of 24(*S*)-hydroxycholesterol (Locatelli *et al.*, 2002; Serrano-Pozo *et al.*, 2010; Thelen *et al.*, 2006). This is in-line with reduced cholesterol elimination in the brain as a result of prolonged statin treatment and suggests that statins affect cholesterol homeostasis in the brain. However, although the effect of SIM on the peripheral pool of cholesterol is well-established, its effects on CNS cholesterol are less clear.

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There are literature data demonstrating that SIM treatment diminished cholesterol levels in the brain and protected neurons *in vitro* against cell death induced by excessive stimulation of NMDA receptors (Kirsch, Eckert, Mueller, 2003; Zacco *et al.*, 2003). NMDA receptors have been reported to be associated with cholesterol-rich membrane microdomains known as membrane/lipid rafts (MLRs) that bind specific proteins (Nothdurfter *et al.*, 2013). MLRs have been proposed to function as platforms that allow the local accumulation of rafts-associated proteins, promoting interactions among protein complexes and modulating neurotransmitter signaling (Allen, Halverson-Tamboli, Rasenick, 2007; Resh, 2004). Accumulating evidence indicates that the precise localization of neurotransmitter receptors, transporters, ion channels, and other synaptic proteins in MLRs can be regulated by cholesterol and this regulation is critical for synaptic plasticity (Allen, Halverson-Tamboli, Rasenick, 2007; Sebastião *et al.*, 2013). Due to its impact on cholesterol synthesis and protein modification, SIM perturbs the composition and properties of MLRs (van der Most *et al.*, 2009). Thus, treatment with SIM might affect the functionality of proteins associated with MLRs, such as NMDA receptors (Ponce *et al.*, 2008). In fact, alterations in MLRs composition and structure have been associated with different pathologies, and therefore, drug-induced regulation of MLRs composition and structure can modulate cell signaling, offering potentially effective treatments for a variety of conditions (Escribá *et al.*, 2015; Marin *et al.*, 2013; Michel, Bakovic, 2007).

Our hypothesis provides strong evidence that NMDA receptor modulation after SIM treatment could be associated with cholesterol-rich membrane microdomains. It is well documented that NMDA receptors in the brain have a close correlation with anxiety-like activity (Bergink, van Megen, Westenberg, 2004; Chojnacka-Wójcik, Kłodzinska, Pilc, 2001). Modulation of NMDA receptors activity after SIM treatment could explain the drug's anxiolytic-like effects (Camargo *et al.*, 2013; Carrocini *et al.*, 2012; Cruz *et al.*, 2011; Kilic *et al.*, 2012; Pauleti *et al.*, 2013; Santos *et al.*, 2012; Wang *et al.*, 2009; Yan *et al.*, 2011). This hypothesis could be supported by the fact that altered levels of NMDA receptors in the hippocampus and amygdala directly influence anxiety behaviors (Barkus *et al.*, 2010; Blundell, Adamec, 2007; Masneuf *et al.*, 2014).

CHOLESTEROL IN THE CNS

Balanced cholesterol homeostasis is an important aspect of nervous system function (Mathews *et al.*, 2014). The majority of cholesterol is present in myelin sheaths and neuronal membranes, in which this lipid fulfills structural and functional tasks. Given the crucial role of cholesterol in regulating different neuronal processes, eukaryotes have developed sophisticated homeostatic mechanisms to preserve cholesterol levels in an optimal range in each brain region (Saher, Stumpf, 2015; Segatto *et al.*, 2013). Largely, cholesterol in the adult brain is metabolically inert. The most significant period of high cholesterol synthesis in the CNS occurs during active myelination, which occurs in early neural development through the action of oligodendrocytes (Dietschy, Turley, 2004). The rate of cholesterol synthesis decreases significantly after myelination has been completed; however, its synthesis continues at a low basal level in the mature adult brain. This occurs primarily through de novo cholesterol synthesis by astrocytes, although neuronal de novo synthesis and reutilization of free cholesterol following neuronal death are also contributory (Nieweg, Schaller, Pfrieger, 2009). Therefore, the CNS does not rely largely on cholesterol from systemic circulation due to limited metabolic turnover during adulthood and the brain's inherent capacity to synthesize its own cholesterol (Dietschy, Turley, 2004). Consequently, reductions in plasma cholesterol concentrations following statin treatment are unlikely to acutely disrupt in CNS cholesterol homeostasis (Lutjohann *et al.*, 2004; Thelen *et al.*, 2006). Thus, chronic statin therapy may be required before significant effects on CNS cholesterol levels are observed, with reductions in CNS cholesterol possible either directly through direct HMG-CoA reductase inhibition or indirectly via a "sink effect" (Cibičková, 2011). In addition, cholesterol depletion is reported to lead to the inhibition of Ca^{2+} entry induced by NMDA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), or kainate, inferring that MLRs contribute to the regulation of ionotropic glutamate receptor function (Frank *et al.*, 2008).

THE CONCEPT OF MLRS/NMDA RECEPTORS

MLRs consist of assemblies of cholesterol, sphingolipids including sphingomyelin and gangliosides, and certain types of proteins (Fielding, Fielding, 2003). They range from a few nanometers to a few hundred nanometers in diameter and comprise approximately 50% of the cellular membrane (Helms, Zurzolo, 2004). The most important properties of MLRs are that they are small, dynamic, and heterogeneous, and they can include or exclude proteins to variable extents (Hanzal-Bayer, Hancock, 2007; Simons, Toomre, 2000). MLRs have been implicated in various physiological cellular processes, such as protein membrane trafficking and signal transduction,

and they have been demonstrated to play roles in synaptic plasticity and contribute to neuropathologies, such as Alzheimer's disease, Parkinson's disease, and Huntington disease (Hanzal-Bayer, Hancock, 2007; Karasinska, Hayden, 2011; Korade, Kenworthy, 2008; Sebastião *et al.*, 2013; Simons, Toomre, 2000). Cholesterol is a key component of MLRs, as its depletion disrupts MLRs and leads to synaptic dysfunction or loss of synapses (Fielding, Fielding, 2003; Korade, Kenworthy, 2008; Sebastião *et al.*, 2013). Cholesterol is responsible for MLRs maintenance in a liquid-ordered phase (Marwali *et al.*, 2003). It maintains the raft assembly together and serves as a molecular spacer between hydrocarbon chains in sphingolipids (Simons, Toomre, 2000). Additionally, the interaction of cholesterol with distinct classes of membrane proteins within MLRs affects the assembly, stability, and function of the proteins (Lee, 2004). The two main types of MLRs in mammalian cells are planar MLRs and caveolae. As their names suggest, planar MLRs are continuous with the membrane, whereas caveolae form invaginations. Apart from their shape, caveolae and planar rafts can be distinguished by their marker proteins, namely: caveolins and flotillin, respectively. Flotillin and caveolins recruit other proteins to the raft, but they are structurally distinct (Allen, Halverson-Tamboli, Rasenick, 2007). The relative proportions of these components vary greatly depending on the cell and membrane type (Epand, 2008; Gallala, Breiden, Sandhoff, 2011). MLRs are specialized membrane structures that form an organized portion of the membrane with concentrated signaling molecules and links to the cytoskeleton (Byrum, Rodgers, 2015). Synapses, including both pre-synaptic and post-synaptic sites, are highly enriched in MLRs. MLRs both contribute to neurotransmitter exocytosis in pre-synaptic terminals, and post-synaptically modulate neuronal signaling through clustering of neurotransmitter receptors, ion channels, and components of downstream effectors. Several studies illustrated that MLRs concentrate many of the regulators and ion channels involved in Ca^{2+} signaling, suggesting significant roles of MLRs in modulating Ca^{2+} signaling (Pani, Singh, 2009).

The NMDA receptor is an ionotropic glutamate receptor. Its channel commonly has a high relative permeability to Ca^{2+}, and it is blocked in a voltage-dependent manner by magnesium ions such that at resting potential the response is substantially inhibited. Cholesterol depletion is reported to lead to an inhibition of Ca^{2+}entry induced by NMDA, AMPA, or kainate, inferring that MLRs contribute to the regulation of ionotropic glutamate receptor function (Egawa *et al.*, 2016; Frank *et al.*, 2008). Numerous post-synaptic density proteins such as NMDA (NR1, NR2A and NR2B), AMPA (GluR1 and GluR2), and metabotropic glutamate receptors (mGluRs) are associated with synaptic MLRs, providing a trans-synaptic link between post-synaptic density proteins and pre-synaptic active zones (Egawa *et al.*, 2016; Kumari, Castillo, Francesconi, 2013). Accumulating evidence suggests that MLRs clustering is a novel mechanism mediating and amplifying trans-membrane signaling in response to various stimuli in a variety of cell types, including lymphocytes, endothelial cells, and neurons (Dupree, Pomicter, 2010; Grassme *et al.*, 2001; Zhang *et al.*, 2006). These membrane platforms can recruit or aggregate various signaling molecules such as small G proteins, tyrosine kinases, and phosphatases, resulting in the activation of different signaling pathways (Zhang *et al.*, 2009).

MODULATION OF THE ISOPRENOID/CHOLESTEROL BIOSYNTHETIC PATHWAY

Statins are the drugs of choice for lowering LDL cholesterol and triglyceride levels in patients with hypercholesterolemia (Owens, Byrnes, Mackman, 2014). The mechanism of action of these drugs is competitive and selective inhibition of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, because it is responsible for the conversion of HMG-CoA to mevalonate, which is a precursor of cholesterol. Although inhibition of HMG-CoA reductase by statins reduces cholesterol synthesis, a concomitant effect of this functional blockade is decreased availability of isoprenoids, the major donors of prenyl groups for protein prenylation. A branch point downstream of mevalonate synthesis draws substrates away from cholesterol synthesis and yields the production of non-sterol isoprenoids such as farnesyl pyrophosphate (FPP), which can be converted into squalene and cholesterol but is also used for the production of geranylgeranyl pyrophosphate (GGPP), an important component for the localization and function of signaling proteins such as Ras (McTaggart, 2006). Small G-proteins (the Ras superfamily of small GTPases) comprise a large group of molecular switch proteins involved in several signaling pathways, reorganization of the cytoskeleton, and intracellular membrane trafficking. Although FPP and GGPP appear to mediate some of the effects of statins, it is likely that the downstream small GTPase family of signaling molecules also plays an important role (Houten, Frenkel, Waterham, 2003).

The fact that more than 300 proteins have been identified as prenylation targets complicates any prediction of the mechanism by which statins and other prenylation inhibitors affect intracellular signaling. Inhibition of HMG-CoA reductase results in

decreased levels of FPP and GGPP, potentially leading to decreased farnesylation and geranylgeranylation of proteins. Predictions are further confounded by an incomplete understanding of the mechanism by which the farnesylation and geranylgeranylation regulate the vast number of small GTPases in the context of multiple cellular compartments and interacting processes. The small GTPases, which include the two major families Ras and Rho, are differentially regulated by farnesylation and geranylgeranylation, respectively, although some exceptions exist (McTaggart, 2006). By facilitating the coupling of extracellular signals to intracellular kinase activity, both families of GTPases regulate intracellular functions.

The inhibition of farnesylation by SIM has been associated with the enhancement of long-term potentiation between neurons in mice (Mans, McMahon, Li, 2012). This study also found that the protective effect of SIM treatment was abolished following replenishment of FPP but not GGPP. Paradoxically, it has been suggested in other studies that the constant production of GGPP, but not FPP or cholesterol, is required for neurite outgrowth and maintenance, long-term potentiation, and learning, possibly suggesting divergent neuroprotective effects associated with these two isoprenoid intermediates (Kotti *et al.*, 2006). Given the different roles of these compounds, known differences in FPP/GGPP ratios across various brain regions may subsequently result in different local SIM-induced effects within these regions. The mechanisms underlying the differential distribution of FPP and GGPP across the brain and the interplay with the effects of SIM are unknown. However, studies supporting the isoprenoid hypothesis were performed using extremely high statin-concentrations; thus, their clinical significance is debatable. The question whether inhibition of prenylation occurs in the brain therefore remains open. We believe that more detailed research into the pharmacology of statins, particularly the concentrations they reach in the CNS and the level at which they block the production of cholesterol and various isoprenoids in different cell types, may solve this question.

However, the impact of SIM treatment on NMDA receptor function/trafficking is most likely related to the effects of SIM on protein prenylation, in particular proteinfarnesylation. Indeed, activation of the small GTPase H-Ras, which depends on farnesylation for function, decreases the surface distribution of the NR2 subunit of the NMDA receptor (Suvarna *et al.*, 2005). Ruocco *et al.* (2007) demonstrated that inhibition of H-Ras farnesylation by farnesyltransferase inhibitor treatment inhibits NMDA-mediated excitotoxicity in the rat brain. By contrast, treatment of hippocampal slices for several hours with SIM increases the magnitude of NMDA receptor-dependent long-term potentiation, a mechanism thought to mediate memory at the cellular level, in the CA1 region in the brains of young adult C57BL/6 mice (Figure 1) (Mans, Mcmahon, Li, 2012). Additionally, chronic SIM treatment in rats stimulates the production of brain-derived neurotrophic factor in the hippocampus after traumatic brain injury by activating Akt-mediated signaling (Wu *et al.*, 2008), as well as increases NMDA receptor levels, promotes neurogenesis, and increases cerebral blood flow by up-regulating vascular growth factor, synaptophysin, Akt, and ERK (Chen *et al.*, 2003; Wang *et al.*, 2009). However, numerous pre-clinical studies led to the hypothesis that hypofunctional NMDA receptors may also play an important role in the mechanism of anxiolytic actions after SIM treatment (Camargo *et al.*, 2013; Carrocini *et al.*, 2012; Cruz *et al.*, 2011; Pauleti *et al.*, 2013; Santos *et al.*, 2012; Wang *et al.*, 2009; Yan *et al.*, 2011). Accordingly, the functional consequence of such regulation on NMDA receptor function varies on the basis of both the identity of the G protein coupled receptor, and the cell type in which relevant receptors are expressed. Certainly, further studies are needed to identify particular small GTPases and related signaling pathways that mediate the neuronal effects of SIM.

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INVOLVEMENT OF THE NMDA RECEPTOR COMPLEX IN ANXIOLYTIC-LIKE EFFECTS

The results of a number of studies performed in recent years indicate that glutamatergic neurotransmission

Simvastatin → Farnesyl Pyrophosphate ↓ → H-Ras ↓ → NMDA receptor ↑

FIGURE 1 - Effects of simvastatin (SIM) on the N-methyl-D-aspartate (NMDA) biosynthesis pathway. Inhibition of H-Ras by SIM suggests increased NMDA receptor levels.

via ionotropic receptors is involved in the pathophysiology of anxiety (Masneuf *et al.*, 2014). The ionotropic glutamate receptor family comprises ligand-gated channels divided into three groups named after their selective agonists, namely NMDA, AMPA, and kainate and their density is high in cortical and limbic regions implicated in the mediation of fear and anxiety (Bergink, van Megen, Westenberg, 2004; Réus *et al.*, 2015). Several binding sites for structurally different ligands have been recognized within the NMDA receptor, including a high affinity site for glutamate and NMDA, a glycine site physiologically activated by glycine or D-serine, and a polyamine site for putrescine, spermine and spermidine (Hirose *et al.*, 2015; Mothet, Le Bail, Billard, 2015; Sirrieh, Maclean, Jayaraman, 2015). To activate the NMDA receptor, both glutamate and glycine sites should be occupied simultaneously, and depolarization of the membrane by AMPA receptors at the same synapse is required (Dutta *et al.*, 2015). Under depolarizing conditions, the blockage of ion channels is relieved and the inflow of Na^+ and Ca^{2+} ions through the NMDA receptor pore is permitted (Seong, Behnia, Carter, 2014). Several studies revealed that MLRs concentrate many of the regulators and ion channels involved in Ca^{2+} signaling, suggesting significant roles of MLRs in modulating Ca^{2+} signaling (Marques-da-Silva, Gutierrez-Merino, 2012, 2014).

Pre-clinical data indicate that a number of different classes of NMDA receptor antagonists, acting at specific sites on the NMDA receptor complex, produced anxiolytic-like activity in tests of anxiety in rodents. It has been reported that a close correlation exists between the regulation of NMDA receptors in the brain and anxiety-like behavior (Bergink, van Megen, Westenberg, 2004). By applying the selective NMDA receptor antagonist (±)-2-amino-5-phosphonopentanoic acid into the hippocampus, a profound increase in activity was observed in the open arms of an elevated plus-maze, strongly suggesting that the up-regulation of NMDA receptors due to the blockage in the hippocampus mediates an anxiolytic-like effect (Nascimento Häckl, Carobrez, 2007). The elevated plus-maze is a widely used animal model of anxiety that is based on two conflicting tendencies, namely the rodent's drive to explore a novel environment and its aversion to open spaces. Thus, anxious animals will spend most of their time in the closed arms, whereas less anxious animals will explore open areas for longer period of time (Pellow *et al.*, 1985). A similar result was also found in Rainnie's study illustrating that long-lasting anxiety-like behavior in rats was eliminated by applying an NMDA receptor antagonist into the basolateral complex of the amygdala (Rainnie *et al.*, 2004). It appears that treatment with an NMDA receptor antagonist such as dizocilpine in rodents could reduce anxiety behavior (Ma, Leung, 2007). Moreover, the anxiolytic effect obtained after the microinjection of spermine into the periaqueductal gray matter was blocked by pretreatment with a polyamines antagonist, such as arcaine or ifenprodil, at the same site (Cruz, Carobrez, 2006). These results demonstrate that other modulatory sites on the NMDA receptor complex may be targeted to alter anxiety in addition to direct antagonism of the NMDA pore region or glutamate-binding site. Reduction of the availability of NMDA receptors to interact with NMDA or its physiological ligand glutamate is one mechanism that might mediate the anxiolytic activity of SIM. Our results also provide strong evidence that chronic high-dose SIM administration has NMDA antagonist-like effects, which would partially explain the anxiolytic effects of this drug (Camargo *et al.*, 2013; Carrocini *et al.*, 2012; Cruz *et al.*, 2011; Pauleti *et al.*, 2013; Santos *et al.*, 2012).

SIM TREATMENT EXERTS ANXIOLYTIC-LIKE EFFECTS

Our results suggest that sub-chronic treatment with SIM reduced anxiety levels in male Wistar rats when combined with environmental enrichment (Cruz *et al.*, 2011; Camargo *et al.*, 2013). Music exposure combined with SIM treatment increased the percentage of time spent and entries into the open arms of the elevated plus-maze (Table 1). These two studies reached the same conclusion that SIM combined with music has anxiolytic-like effects in the elevated plus-maze. A possible mechanism mediating these effects could involve NMDA receptor modulation. These results indicate that music can serve as an effective adjuvant in rats treated with SIM and that this species could potentially be used in other pre-clinical models utilizing musical interventions (Cruz *et al.*, 2015).

Prolonged administration of different classes of drugs may produce convergent effects on different neurotransmitter systems or signaling targets. Unfortunately, little information is available regarding neurotransmitter substrates that are critical for the behavioral effects of different types of drugs in animal models. In addition, data accumulated in the last decade indicate that NMDA receptors may be involved in the pathophysiology of depression and the mechanism of action of antidepressants (Szasz *et al.*, 2007). These data suggest that the inhibitory effect of fluoxetine is exerted directly on NMDA receptors, contributing to the therapeutic effects of this drug. Pre-clinical data demonstrated that blocking the NMDA receptor complex

TABLE I – Effect of simvastatin (SIM, 1 and 10 mg/kg oral) on anxiety-like behavior in rats under each sound condition (silence and music) in the elevated plus-maze – percent time spent in the open arm and percent frequency of open arm entries. Values are expressed as the mean ± standard error of the mean; n = 10, *p<0.05, **p<0.01, and ***p<0.001. Statistically significant compared to the vehicle-silence group (analysis of variance [ANOVA] followed by the Newman-Keuls test)

	Treatment	%Time spent in the open arm (ANOVA → F = 4.160; p<0.01)	% Frequency of open arm entries (ANOVA → F = 5.483; p<0.001)
Silence	Vehicle	11.5 ± 2.1	22.5 ± 6.2
	SIM 1 mg/kg	16.6 ± 1.7	28.4 ± 6.6
	SIM 10 mg/kg	33.1 ± 3.6	31.1 ± 6.4
Music	Vehicle	25.3 ± 2.9	28.6 ± 5.0
	SIM 1 mg/kg	40.0 ± 5.2*	43.7 ± 4.7***
	SIM 10 mg/kg	36.3 ± 3.0*	34.9 ± 4.9**

Source: (Camargo *et al.*, 2013).

produced anxiolytic and antidepressant activity in animal tests (Pittenger, Sanacora, Krystal, 2007; Riaza Bermudo-Soriano *et al.*, 2012). The results of our experiments suggest that sub-chronic treatment with SIM reduces anxiety levels in rats when administered with fluoxetine (Santos *et al.*, 2012). The experimental groups orally administered 10 mg/kg SIM combined with 10 mg/kg fluoxetine exhibited increased time spent in the open arms of the elevated plus-maze (Table II). These results strongly indicate that SIM treatment combined with fluoxetine improves the ability to cope with aversive situations, thus leading to a reduced anxiety level and playing an important role in the synergistic effect of combination therapy (Santos *et al.*, 2012; Gougol *et al.*, 2015). A possible mechanism mediating these effects may involve modulation of NMDA receptors by the cholesterol distribution within brain cell membranes (Kirsch, Eckert, Mueller, 2003; Ponce *et al.*, 2008). However, the possible involvement of MLRs in NMDA receptor function and its modulation by fluoxetine could not be confirmed. In addition, the impairment of MLRs integrity by cholesterol depletion did not affect the modulatory potency of desipramine at the NMDA receptor, suggesting that the membrane localization of this receptor and compound plays a minor role for its modulation by this antidepressant (Nothdurfter *et al.*, 2013).

Benzodiazepines are the most frequently used psychotropic agents and the mainstay of drug treatment for anxiety disorders. The elevated plus-maze test is a task that reliably detects the anxiolytic effect of clinically

TABLE II – Behavioral responses of rats in the elevated plus-maze following sub-chronic simvastatin (SIM) treatment combined with fluoxetine (Flu) - percent time spent in the open arm and percent frequency of open arm entries. Values are expressed as the mean ± standard error of the mean; n = 8, **p<0.01. Statistically significant compared to the vehicle group (analysis of variance [ANOVA] followed by the Newman-Keuls test)

Treatment	%Time spent in the open arm (ANOVA → F = 4.979; p<0.001)	% Frequency of open arm entries (ANOVA → F = 2.207; p>0.05)
Vehicle	10.5 ± 5.0	48.0 ± 4.0
SIM 1 mg/kg	18.0 ± 7.0	56.5 ± 3.5
SIM 10 mg/kg	28.5 ± 2.0	54.8 ± 6.0
Flu 2 mg/kg	17.7 ± 8.0	51.7 ± 9.0
SIM 1 mg/kg + Flu 2 mg/kg	11.8 ± 2.0	42.1 ± 3.0
SIM 10 mg/kg + Flu 2 mg/kg	27.4 ± 3.0	54.8 ± 2.5
Flu 10 mg/kg	27.3 ± 9.0	53.3 ± 8.0
SIM 1 mg/kg + Flu 10 mg/kg	13.9 ± 4.0	41.8 ± 4.0
SIM 10 mg/kg + Flu 10 mg/kg	41.2 ± 7.0**	48.0 ± 4,5

Source: (Santos *et al.*, 2012)

relevant benzodiazepines, such as diazepam that display anxiolytic-like behavioral profile including increases in the percentage of entries into and time spent in the open arms of the maze (Zhou *et al.*, 2015). Our results suggest that sub-chronic treatment with SIM reduces anxiety levels in mice when combined with diazepam (Pauleti *et al.*, 2013). Diazepam combined with SIM increases the percentage of time spent and entries into the open arms of the elevated plus-maze (Table III). Additionally, SIM could potentiate the anxiolytic-like effects of diazepam. In mammals, the involvement of excitatory glutamate and inhibitory gamma (γ)-aminobutyric acid (GABA) in the amygdala is particularly important for controlling levels of fear and anxiety (Bian, 2013). The NMDA receptor has also been directly implicated in the emergence of gamma rhythms, as NMDA receptor antagonists can disrupt or potentiate gamma rhythms *in vivo* (Pinault, 2008). Pre-synaptically, NMDA receptors may participate in neurotransmitter release, a process dependent on Ca^{2+} ions, whereas post-synaptically, their location on GABAergic neurons may contribute to the regulation of inhibitory tone and normal oscillatory activity in the brain (Deutsch *et al.*, 2010). Therefore, NMDA receptors contribute to the regulation of central inhibitory tone by influencing the firing of GABA-inhibitory neurons. In addition to NMDA receptors, other receptors on the surface of GABA-inhibitory neurons may represent promising pharmacotherapeutic targets. Consistent with this hypothesis, blockade of the NMDA receptor complex and/or activation of benzodiazepine receptors mediated anxiolytic activity in elevated plus-maze tests induced, by the co-administration of SIM and diazepam. Additionally, the localization of NMDA receptors within the MLRs appears to depend on the respective $GABA_A$ receptor subunit composition (Nothdurfter *et al.*, 2013).

Several studies identified ethanol as a potent and selective inhibitor of NMDA receptors, and prolonged ethanol exposition leads to a compensatory up-regulation of these receptors, resulting in enhanced NMDA receptor-mediated functions after ethanol withdrawal. These alterations are believed to contribute to the development of ethanol tolerance and, dependence as well as the acute and delayed signs of ethanol withdrawal (Nagy, 2004). In particular, the up-regulation of different subunits of the NMDA receptor may cause hyperexcitability of the CNS during withdrawal or negative emotions such as dysphoria, irritability, anxiety, and depression (Blanco-Gandía *et al.*, 2015). In accordance with numerous data in the literature, ethanol withdrawal induces an anxiogenic effect in the elevated plus-maze (Kumar *et al.*, 2013; Van Skike, Diaz-Granados, Matthews, 2015). However, these findings contrast our previous data indicating that lower doses of ethanol produce anxiolytic-like effects (Cruz *et al.*, 2012a). Therefore, negative modulators of NMDA receptors may be useful for the pharmacotherapy of alcoholism, attenuating both the physical symptoms and

TABLE III - Behavioral responses of mice in the elevated plus-maze following sub-chronic simvastatin (SIM) treatment combined with diazepam (DZP) - percent time spent in the open arms and percent frequency of arms entries. Values are expressed as the mean ± standard error of the mean; n = 8, *p<0.05, **p<0.01, and ***p<0.001. Statistically significant compared to the vehicle group (analysis of variance [ANOVA] followed by the Newman-Keuls test)

Treatment	%Time spent in the open arm (ANOVA → F = 6.858; p<0.001)	% Frequency of open arm entries (ANOVA → F = 9.614; p<0.001)
Vehicle	1.5 ± 0.5	3.2 ± 1.0
SIM 1 mg/kg	12.1 ± 1.0**	31.8 ± 4.5*
SIM 10 mg/kg	10.0 ± 0.8**	14.0 ± 5.0
DZP 0.5 mg/kg	28.9 ± 2.0**	52.2 ± 8.0**
SIM 1 mg/kg + DZP 0.5 mg/kg	27.8 ± 1.5**	48.1 ± 6.0**
SIM 10 mg/kg + DZP 0.5 mg/kg	41.6 ± 1.6**	52.4 ± 4.0**
DZP 1 mg/kg	50.3 ± 2.0***	74.8 ± 11.0***
SIM 1 mg/kg + DZP 1 mg/kg	57.0 ± 2.5***	45.4 ± 13.0**
SIM 10 mg/kg + DZP 1 mg/kg	88.6 ± 2.7***	92.5 ± 7.0***
DZP 2 mg/kg	60.2 ± 3.0***	69.7 ± 6.8***
SIM 1 mg/kg + DZP 2 mg/kg	57.0 ± 5.0***	60.2 ± 7.3***
SIM 10 mg/kg + DZP 2 mg/kg	73.4 ± 3.5***	75.6 ± 5.0***

Source: (Pauleti *et al.*, 2013)

some affective and motivational components of alcohol withdrawal. The results of our experiments suggest that sub-chronic treatment with SIM reduces anxiety-like behavior in ethanol-withdrawn rats (Carrocini *et al.*, 2012). These symptoms were observed as increases in the percentage of time spent in and entries into the open arms of the elevated plus-maze (Table IV). The evidence supporting the effects of SIM treatment on NMDA receptor binding density in the brain reveals a possible NMDA antagonist-like effect, which provides an exciting and potential paradigm for decreasing anxiety (Yan *et al.*, 2011; Wang *et al.*, 2009). Cholesterol depletion is reported to lead to an inhibition of Ca^{2+} entry induced by NMDA, AMPA, or kainate, inferring that MLRs contribute to the regulation of ionotropic glutamate receptor function (Frank *et al.*, 2008). Therefore, treatment with SIM might affect the functionality of proteins associated with MLRs, such as NMDA receptors.

These findings will contribute to a better understanding of the critical roles of SIM in treating anxiety, demonstrating that reducing cholesterol levels protects against NMDA-induced anxiety, probably by suppressing the association of NMDA receptors with MLRs. Changes in brain cholesterol metabolism have been reported in experimental animals and humans, sometimes following short-term statin treatment. It is possible that SIM affects neuronal cholesterol levels soon after the beginning of treatment (Locatelli *et al.*, 2002; Thelen *et al.*, 2006).

ORCHESTRATION OF NMDA RECEPTOR SIGNALING BY SIM

Statins have been demonstrated to act through cholesterol-dependent and independent mechanisms, and they can affect several tissue functions and modulate specific signal transduction pathways that could explain their pleiotropic effects. Cholesterol appears to be essential for the stability and functionality of MLRs. The inhibition of HMG-CoA reductase by SIM disrupts the distribution of signaling molecules within rafts, resulting in a reduced association of an NMDA receptor subunit with MLRs, although no change was found in the total level of this NMDA receptor subunit (Kirsch, Eckert, Mueller, 2003; Ponce *et al.*, 2008; Wang *et al.*, 2009). As a possible mechanism of the anxiolytic-like effect of SIM based on our data, we propose that the reduction of the level of membrane cholesterol decreases transporter-mediated glutamate release from nerve terminals (Camargo *et al.*, 2013; Carrocini *et al.*, 2012; Cruz *et al.*, 2011; Pauleti *et al.*, 2013; Santos *et al.*, 2012). This does not contradict the NMDA-dependent and non-sterol mechanisms of action of SIM (there may be additive or synergetic effect). It is well documented that NMDA receptors in the brain have a close correlation with anxiety-like activity (Bergink, van Megen, Westenberg, 2004; Réus *et al.*, 2015). It is highly probable that inhibition of NMDA receptor down-regulation in the hippocampus and basolateral complex of the amygdala mediate the anxiolytic effects of SIM (Yan *et al.*, 2011, Wang *et al.*, 2009). Accordingly, NMDA, AMPA, and metabotropic glutamate receptors are regulated by MLRs-related pathways (Francesconi, Kumari, Zukin, 2009; Hou *et al.*, 2008; Swanwick *et al.*, 2009).

However, the cholesterol synthesis pathway also has several by-products, including non-sterol isoprenoids that are important in cellular functioning (McTaggart, 2006). The impact of SIM treatment on NMDA receptor function/trafficking is most likely related to its effects on protein prenylation, in particular protein farnesylation (Mans, Mcmahon, Li, 2012). It is worth noting that SIM treatment can enhance NMDA receptor activity by

TABLE IV - Behavioral responses in the elevated plus-maze following sub-chronic simvastatin (SIM) treatment in rats with ethanol (EtOH) withdrawal syndrome - percentage of time spent in the open arm and percentage of frequency of open arm entries. Values are expressed as the mean ± standard error of the mean; n = 10, *p<0.05. Statistically significant compared to the vehicle group (analysis of variance [ANOVA] followed by the Newman-Keuls test)

Treatment	%Time spent in the open arm (ANOVA → F = 3.183; p<0.05)	% Frequency of open arm entries (ANOVA → F = 1.469; p>0.05)
Vehicle	6.9 ± 2.0	19.6 ± 3.8
SIM 1 mg/kg	18.6 ± 4.6*	25.9 ± 4.3
SIM 10 mg/kg	12.1 ± 2.8	32.7 ± 4.3
EtOH	4.0 ± 1.6	28.1 ± 4.0
SIM 1 mg/kg + EtOH	23.4 ± 4.6*	30.9 ± 5.2
SIM 10 mg/kg + EtOH	17.3 ± 4.9	22.1 ± 4.8

Source: (Carrocini *et al.* (2012)

reducing FPP levels (Chen *et al.*, 2016; Mans, Mcmahon, Li, 2012; Parent *et al.*, 2014). These data strongly implicate SIM-induced changes in neuronal function via isoprenoid-mediated modulation of small GTPases. Indeed, activation of the small GTPase H-Ras, which depends on farnesylation for function, decreases the surface distribution of the NR2 subunit of the NMDA receptor (Suvarna *et al.*, 2005). Accordingly, deletion of H-Ras increases NMDA receptor-dependent synaptic conductance (Manabe *et al.*, 2000).

Studies supporting the isoprenoid hypothesis were performed using extremely high statin concentrations; thus, their clinical significance is debatable. The question whether inhibition of prenylation occurs in the brain therefore remains open. On the contrary, although the effect of statins on the peripheral pool of cholesterol is well-established, their effects on CNS cholesterol are less clear. The CNS does not rely largely on cholesterol from systemic circulation due to limited metabolic turnover during adulthood and the brain's inherent capacity to synthesize its own cholesterol (Dietschy, Turley, 2004). As such, reductions in plasma cholesterol concentrations following statin treatment are unlikely to acutely disrupt in CNS cholesterol homeostasis (Lutjohann *et al.*, 2004; Thelen *et al.*, 2006). Unlike cholesterol in plasma, which has a half-life of only a few days, brain cholesterol has a half-life of 6 months to 5 years (Dietschy, Turley, 2004). Thus, chronic statin therapy may be required before significant effects on CNS cholesterol are observed, with reductions in CNS cholesterol content possibly occurring either directly through direct HMG-CoA reductase inhibition or indirectly via a "sink effect" (Cibičková, 2011). There exists much controversy regarding the effects of statins on neuronal function (Cruz *et al.*, 2012b). We believe that more detailed research into the pharmacology of statins, particularly the concentrations they reach in the CNS and the level at which they block the production of cholesterol and various isoprenoids in different cell types, may solve this question.

In summary, if our speculation is correct, this finding presents a novel point that sub-chronic treatment with SIM results in an anxiolytic effect, probably by reducing the association of NMDA receptors with MLRs. These novel results could have significant implications for our understanding of the influence of cholesterol-lowering agents such as SIM on the organization and function of the NMDA receptor, an important neurotransmitter receptor. In this study, we reviewed the mechanism by which membrane lipids, which play roles in the membrane's function as a barrier and signaling medium, contribute to anxiety. Understanding the molecular mechanisms by which lipids change and affect this disorder will be of great benefit to biology and pharmacology in cases in which lipids and lipid-affecting drugs might be used as clinically. However, more evidence is required before any SIM therapy can be recommended clinically in the treatment or prevention of anxiety.

CONFLICT OF INTEREST

No conflict of interest has been declared.

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