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Rapamycin-Sensitive Late-LTP Is Enhanced in the Hippocampus of IL-6 Transgenic Mice

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Abstract

The neuroimmune factor IL-6 has been shown to regulate hippocampal long-term potentiation (LTP), an activity dependent enhancement of synaptic transmission that plays a central role in memory and learning. This IL-6 action was demonstrated with relatively short IL-6 exposure, and may reflect physiological actions of IL-6. IL-6 is also expressed chronically at elevated levels in the CNS under pathological conditions such as neurological disorders. Little is known about the effects IL-6 on LTP under such conditions, an issue that we are addressing by electrophysiological recordings from CA1 pyramidal neurons of hippocampal slices from transgenic mice that persistently express elevated levels of IL-6 in the CNS (IL-6 tg). The current studies examined the long-lasting phase of LTP (late LTP; L-LTP) and the potential involvement mTOR, a known regulator of L-LTP and a downstream partner of IL-6 signal transduction pathways. Results show that basal synaptic transmission and L-LTP were increased in hippocampal slices from IL-6 tg mice compared to slices from non-transgenic (non-tg) control mice. An inhibitor of mTOR, rapamycin, reduced L-LTP in slices from both genotypes, and eliminated the difference in magnitude of L-LTP between IL-6 and non-tg hippocampus. There were no genotypic effect of rapamycin on basal synaptic transmission, but synaptic responses during the LTP induction protocol were reduced in IL-6 tg slices, an effect that could contribute to the reduction of L-LTP in the IL-6 tg slices. These results indicate that persistently increased levels of IL-6 can lead to alterations in mTOR regulation of L-LTP, possibly affecting learning and memory.

Keywords

mTOR; synaptic plasticity; cytokine; neuroimmune; astrocyte; synaptic function

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Author Contributions

DG, AOE and PdG conceived of and designed the study. AOE and RH carried out the experiments. AOE and DG analyzed the data. AOE, DG and PdG wrote the manuscript.

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Introduction

Cells of the central nervous system (CNS), and particularly astrocytes and microglia, produce many of the same factors as immune cells, including the cytokine interleukin-6 (IL-6). IL-6 produced within the CNS is involved in both physiological and pathological processes, exerting advantageous as well as disadvantageous effects (Gadient and Otten, 1997; Gruol and Nelson, 1997; Spooren et al., 2011; Rothaug et al., 2016). Studies at the mRNA level have shown that IL-6 and IL-6 receptor (IL-6R) mRNA are expressed in many regions of the CNS, with high levels in the hippocampus, cerebellum, and neocortex (Schobitz et al., 1992; Gadient and Otten, 1994). In the hippocampus, localized increases in IL-6 mRNA were observed after intense synaptic activity produced by high frequency stimulation (HFS) used to induce long-term potentiation (LTP), a long-term increase in synaptic strength that plays an important role in learning and memory (Balschun et al., 2004; Peng et al., 2011; del Rey et al., 2013). Jankowsky *et al* (Jankowsky et al., 2000) reported that LTP induction by HFS at the Schaffer collateral to CA1 pyramidal neuron synapse of the rat hippocampus *in vivo* resulted in a 20-fold increase in IL-6 gene expression four hours after HFS. The increased IL-6 gene expression was localized to glial cells and some vascular cells, but not to neurons (Jankowsky et al., 2000). Similarly, Balschun *et al.* (Balschun et al., 2004) and del Rey et al. (del Rey et al., 2013) showed that expression of the IL-6 gene is markedly enhanced during LTP in studies at the perforant path to dentate gyrus granule neurons of rat hippocampus *in vivo* and at the Schaffer collateral to CA1 pyramidal neuron synapse in rat hippocampal slices *in vitro*.

LTP is characterized by an early phase (E-LTP; evident 60 min after the induction protocol), which does not require new protein synthesis for expression, and a late, long lasting maintenance phase (L-LTP; occurs 2–3 hours after tetanus), which requires new protein synthesis (Lynch, 2004). Studies using a variety of approaches indicate that IL-6 can serve as a negative regulator of both E-LTP and L-LTP in the hippocampus (Bellinger et al., 1995; Li et al., 1997; Tancredi et al., 2000; Balschun et al., 2004). For example, in studies by Balschun et al (2004) in the dentate gyrus, the endogenous production of IL-6 elicited by high frequency stimulation of the perforant pathway predominately affected the maintenance phase of LTP (i.e., L-LTP) in the granule neurons. When a neutralizing anti-IL-6 antibody was applied 90 minutes after the induction of LTP, L-LTP was prolonged, consistent with blockade of a negative L-LTP regulator (Balschun et al., 2004). In contrast, application of a neutralizing anti-IL-6 antibody 30 minutes before, or 5 minutes after induction of LTP had no effect on the L-LTP in the granule neurons (Balschun et al., 2004).

In hippocampal slices from IL-6 tg mice that chronically express elevated levels of IL-6 in the CNS, E-LTP at the perforant path to dentate gyrus granule neuron synapse was significantly reduced compared to hippocampal slices from wild-type control mice (Bellinger et al., 1995). This result indicates that IL-6 also acts as a negative regulator of E-LTP at this synapse under conditions of chronic IL-6 exposure. In contrast, in studies at the Schaffer collateral to CA1 pyramidal neuron synapse in hippocampal slices from IL-6 tg and non-tg mice, there was no genotypic difference in E-LTP, indicating that chronically express elevated levels of IL-6 does not appear to regulate E-LTP at this synapse (Nelson et al., 2012).

In vitro studies employing exogenous application of IL-6 support a role for IL-6 as a negative regulator of E-LTP under conditions of acute exposure. A marked decrease in E-LTP was observed at the Schaffer collateral to CA1 pyramidal neuron synapse in rat hippocampal slices exposed to exogenously applied IL-6 (Li et al., 1997; Tancredi et al., 2000). Post-tetanic potentiation (PTP), a shorter form of synaptic plasticity that is elicited by the LTP induction protocol (i.e., high frequency stimulation) and also involves an enhancement of the synaptic response, was also reduced, whereas basic synaptic transmission was not altered. The effect of IL-6 on PTP and E-LTP was blocked by pre-incubation of the slices with antibodies against the IL-6 receptor (IL-6R), consistent with the involvement of IL-6R in the effects of IL-6 on PTP and LTP (Li et al., 1997). The enhancement of the synaptic responses during PTP is thought to primarily involve presynaptic mechanisms, whereas the enhancement of the synaptic response during E-LTP and L-LTP is thought to primarily involve postsynaptic mechanisms. The inhibition of PTP and LTP by exogenous application of IL-6 was associated with changes in the two of IL-6 signal transduction partners, signal transducers activators of transcription 3 (STAT3) and p42/44 mitogen-activated protein kinase (MAPK; also known as extracellularly regulated kinase, ERK); STAT3 activation (i.e., phosphorylation) was increased and MAPK/ERK activation was reduced. Thus, IL-6 appears to negatively regulate different forms of synaptic plasticity by both presynaptic and postsynaptic mechanisms and multiple signaling pathways.

Several studies have reported that L-LTP in the hippocampus is regulated by mammalian target of rapamycin (mTOR) (Tang et al., 2002; Cammalleri et al., 2003; Tsokas et al., 2007; Stoica et al., 2011). mTOR is a conserved Ser/Thr kinase that is a regulator of mRNA translation and protein synthesis involved in a variety of cell functions (Bockaert and Marin, 2015). The role of mTOR in L-LTP appears to involve upregulation of translational signaling involved in local dendritic protein synthesis required for establishment of L-LTP (Cracco et al., 2005; Tsokas et al., 2005; Vickers et al., 2005). Consistent with such a role, mTOR signaling pathway components are present in hippocampal neurons at synaptic sites and are activated by synaptic activity (Tang et al., 2002; Cammalleri et al., 2003).

Rapamycin, the specific mTOR inhibitor, reduces the strength of L-LTP, whereas E-LTP, which does not require new protein synthesis for expression, is relatively unaffected (Tang et al., 2002; Cammalleri et al., 2003; Tsokas et al., 2007; Stoica et al., 2011). Rapamycin is only effective in reducing L-LTP if it is applied immediately prior to the LTP induction protocol, indicating that the induction phase is the rapamycin-sensitive phase of LTP (Cammalleri et al., 2003). Either enhancing or reducing activity of mTOR signaling causes memory deficits (Costa-Mattioli et al., 2009), whereas knockouts for several upstream and downstream effectors of mTOR results in impaired synaptic plasticity and memory consolidation (for review (Costa-Mattioli et al., 2009)).

mTOR is a downstream partner of IL-6 signal transduction. IL-6R is linked to activation of several signal transduction enzymes including Janus kinase (JAK), STAT3, phosphatidylinositol 3-kinase (PI3K), and MAPK/ERK (Eulendorf et al., 2012; Schaper and Rose-John, 2015). Both the PI3K and MAPK/ERK pathways can activate mTOR (Shi et al., 2002; Leibinger et al., 2016; Pinno et al., 2016). The potential for IL-6 to induce mTOR

activation raises the possibility that mTOR could be involved in actions of IL-6 under conditions of persistently elevated levels in the CNS. The common involvement of IL-6 and mTOR in certain CNS conditions supports this idea. For example, dysregulation of mTOR signaling can have serious outcomes including epilepsy and CNS hyperexcitability (Shu et al., 2010; Atzori et al., 2012), conditions that are also associated with elevated levels of IL-6 in the CNS (Vezzani et al., 2008; Han et al., 2016). The mTOR pathway is strongly activated after kainate-induced status epilepticus (Zeng et al., 2009). Chronic expression of elevated levels of IL-6 in the CNS in IL-6 tg mice results in synaptic hyperexcitability and enhanced sensitivity to kainate induce seizure activity (Samland et al., 2003; Nelson et al., 2012).

In the current study, we examined the effects of chronically expressed IL-6 on L-LTP in the hippocampus of IL-6 tg mice and the potential involvement of mTOR. The involvement of mTOR was identified by sensitivity of L-LTP to the mTOR inhibitor rapamycin. Results show that L-LTP is enhanced in hippocampal slices from IL-6 tg mice compared with slices from non-tg mice. Rapamycin reduced the level of L-LTP in hippocampal slices from both IL-6 tg and non-tg mice and eliminated the difference in magnitude of L-LTP between IL-6 and non-tg hippocampus. Rapamycin also produced a significant reduction in the magnitude of synaptic responses during the LTP induction protocol in slices from the IL-6 tg mice but not in slices from the non-tg mice. Rapamycin did not alter basal synaptic transmission or short-term synaptic plasticity in hippocampal slices from either genotype. These results are consistent with interactions between chronically expressed IL-6 and mTOR that enhance L-LTP in the IL-6 tg hippocampus, and raise the possibility that such interactions could play a role in altered learning and memory that occurs in neurological and neuropsychiatric disorders associated with elevated levels of IL-6 in the CNS.

Experimental Procedures

Animals

Heterozygote male and female IL-6-tg mice (n=16) from the 167 line (Campbell et al., 1993) and age-matched male and female non-tg littermate control mice (n=16) were used for all studies. Mice were 1.5–2.0 months of age (mean age $\pm$ SEM in months: IL-6 tg, 1.78 $\pm$ 0.05; non-tg, 1.78 $\pm$ 0.34). Methods for production of the IL-6 tg mice have been described previously (Campbell et al., 1993). Increased IL-6 expression was driven by a vector derived from the murine glial fibrillary acidic protein (GFAP) gene, which is primarily localized to astrocytes of the CNS. Full-length murine IL-6 cDNA was modified and inserted into the GFAP gene. The genes were then microinjected into fertilized eggs of F1 generation hybrid mice. The IL-6 tg line has been maintained for many years on a C57BL/6J background and is congenic. The IL-6 tg mice express moderately elevated levels of IL-6 in the CNS.

Standard methods for genotyping by PCR analysis of tail DNA were used to identify IL-6 tg and non-tg littermates. Animal procedures were performed as recommended in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal facilities and experimental protocols were as specified by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Slice preparation and electrophysiological recordings

Protocols for electrophysiological recordings of synaptic transmission and synaptic plasticity at the Schaffer collateral to CA1 pyramidal neuron synapse in hippocampal slices from IL-6 tg and non-tg mice were carried out as described previously (Nelson et al., 2012). Briefly, brains were rapidly removed from mice and stored in ice-cold artificial cerebrospinal fluid (ACSF) for ~ 1 min. ACSF was composed of (in mM): 130.0 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24.0 NaHCO₃, 2.0 CaCl₂, 1.3 MgSO₄, and 10.0 glucose (all chemicals from Sigma, St Louis, MO, USA). The hippocampus was isolated and hippocampal slices (400 µm) were cut using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK). Slices were allowed to recover (60 min) in a gas-fluid holding chamber maintained at approximately 33°C with constant perfusion of ACSF (perfusion rate of 0.55 – 1.0 ml/min). All solutions were gassed continuously with 95% O₂/5% CO₂ (pH 7.2–7.4).

The slices were transferred to a gas-fluid recording chamber (33°C) and incubated 10–20 min with constant ACSF superfusion at a rate of 2 ml/min to allow the slices to stabilize. Synaptic responses were elicited by electrical stimulation (50 µs duration; S88 Square Pulse Stimulator and PSIU6 Stimulus Isolation Unit, Grass Technologies, West Warwick, RI, USA) using a concentric bipolar stimulating electrode (FHC Inc., Bowdoin, ME, USA) placed at the Schaffer collateral afferent pathway near the CA2/CA1 border. Schaffer collateral stimulation at a range of intensities elicited field excitatory postsynaptic potentials (fEPSPs) and population spikes (PS) recorded extracellularly using ACSF filled recording electrodes (2–5 MΩ) placed within area CA1 in the stratum radiatum (dendritic region of pyramidal neurons) and stratum pyramidale (somatic region of pyramidal neurons), respectively. The signals were amplified with an Axoclamp-2A amplifier and acquired using pClamp 8.2 software (both from Axon Instruments of Molecular Devices, Sunnyvale, CA, USA).

Input/output (I/O) relationships for the fEPSP and PS were determined using a stimulation protocol of 3 Hz at increasing stimulus intensities from 60 µA (threshold intensity) up to 300 µA in increments of 10–20 µA until the maximum population spike amplitude was reached. Standard paired-pulse stimulation protocols were used to elicit short-term synaptic plasticity of the fEPSP and PS. For the fEPSP, stimulations were applied to the Schaffer collaterals that elicited a fEPSP that was 50% of the maximum fEPSP at paired-pulse intervals of 40, 100, and 200 ms. For the PS, stimulations were applied to the Schaffer collaterals at an intensity that elicited a PS that was 50% the maximum PS at paired-pulse intervals of 10 and 20 ms. Three paired-pulse responses were averaged in each slice with 30 s between acquisitions for each interval. Paired-pulse ratios (PPRs) were calculated for the fEPSP and PS by dividing the magnitude of the response to the 2nd stimulation by the magnitude of the response to the 1st stimulation of the paired-pulses.

LTP of the fEPSP was induced by a high frequency stimulation (HFS) protocol consisting of three stimulus trains of 100 pulses applied at 100 Hz with a 20 sec interval between each stimulus train. Both the test stimulus and HFS were applied to the Schaffer collaterals at a stimulus intensity that produced a half-maximum fEPSP. A pre-HFS baseline at the same stimulus intensity was recorded (1 pulse per minute for 5 minutes) prior to HFS protocol. Following HFS, responses to the test stimulation (1 pulse per minute) were recorded for

another 120 min. In addition to LTP, HFS induced post-tetanic potentiation (PTP; lasts 2–3 min and occurs immediately after HFS).

Drug treatment

After the initial I/O and paired-pulse protocols were carried out, the slices were treated with either rapamycin (1 μ M in 0.002 % DMSO) (Cell Signaling Technology, Inc.) or vehicle (0.002% DMSO) (Sigma-Aldrich, St. Louis, MO). A stability test was performed for 20 minutes, followed by I/O, paired-pulse, and then LTP protocols.

Analysis of electrophysiological data

The data were analyzed off-line with AxoGraph 4.6 software program (Molecular Devices). For all protocols, measurements were made of the fEPSP slope and PS amplitude. The fEPSP slope was determined on the rising phase of the fEPSP over a range (typically 40–60%) that allowed reliable measurements without interference from the presynaptic fiber volley (PSV) or the reflection from the somatic population spike, which occurs on the falling phase of the fEPSP. The amplitude of the PSV and PS were measured as the peak amplitude of the negative deflection that reflects that response from a baseline value. Baseline value for the PS was estimated from a tangent line fitted across the two positive peaks of the somatic synaptic response.

Western blot analysis for mTOR and phosphorylated mTOR

Western blot analysis was carried out using previously published methods (Hernandez et al., 2016). Hippocampi were removed from the brains of IL-6 tg and non-tg mice and snap frozen for storage. Proteins were extracted from whole hippocampi by sonication in cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% NP-40, a Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Mannheim, Germany), and a cocktail of phosphatase inhibitors (Na^+ pyrophosphate, β -glycerophosphate, NaF, Na^+ orthovanadate; all from Sigma-Aldrich). After incubation on ice for 30 minutes, supernatants were collected after centrifugation at 13,000g for 30 minutes at 4°C, and protein concentration determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Protein aliquots were stored at –80°C.

IL-6 tg and non-tg hippocampal protein samples (25 μ g) were subjected to SDS-PAGE using 4–12% Novex NuPAGE Bis-Tris gels (Invitrogen Life Technologies, Grand Island, NY), transferred to Immobilon-P membranes (Millipore, Billerica, MA), and evaluated for uniform transfer by Ponceau S staining (Pierce, Rockford, IL). After blocking (0.1% casein solution) (Pierce) membranes were incubated in primary antibody overnight (4°C), washed and then incubated (room temperature) in secondary antibody coupled to horseradish peroxidase. The primary mTOR antibodies used were purified rabbit polyclonal antibodies raised against a synthetic peptide (AB2972, 1:1000; Cell Signaling Technologies, Danvers, MA) or a synthetic phosphopeptide (AB2971, 1:1000; Cell Signaling Technologies) corresponding to residues surrounding Ser2481 of human mTOR. After stripping, membranes were reprobed with a monoclonal antibody to glial fibrillary acidic protein (GFAP)(MAB360; 1:10,000; Millipore), restriped and then probed with an antibody to β -actin (clone AC-15, 1:2000; Sigma-Aldrich). Protein bands were visualized by

chemiluminescence. NIH Image software (<http://rsb.info.nih.gov/ni-image/>) was used to quantify bands by densitometry measurements. To control for possible loading errors, the density reading for each band was normalized to the density reading of the band for β -actin in the same lane. Mean normalized values for hippocampi from non-tg mice were calculated for each gel and data from non-tg and IL-6 tg hippocampi were normalized to the non-tg mean value for the same gel. Data were combined according to genotype and treatment and reported as mean $\pm$ SEM.

Statistics

Statistical analyses were conducted using ANOVA or the Mann-Whitney test (for non-parametric data). Data were assessed for normality using the Kolmogorov-Smirnov (*K-S*) test and for variance by the F-test. For data analyzed by ANOVA, compiled data were expressed as the mean $\pm$ SEM. For non-parametric data, median, maximum and minimum values are shown. Statistical significance was set at $p < 0.05$. n = number of animals used (one slice/animal for electrophysiological studies).

Results

L-LTP is enhanced in hippocampus of IL-6 tg mice

LTP of the fEPSP was induced in the hippocampal slices by HFS, and monitored for approximately 2 hours following HFS (Fig. 1). For quantification, LTP was divided into an early phase (E-LTP; data points 48–52 min post HFS) and late-phase (L-LTP; data points 105–120 min post HFS). E-LTP has previously been studied in IL-6 tg hippocampal slices, but L-LTP has not (Nelson et al., 2012). Consistent with our previous results, under control conditions there was no significant difference in E-LTP between slices from IL-6 tg and non-tg mice ($F(1,16) = 0.36$, $p = 0.56$). In contrast, L-LTP of the fEPSP slope was significantly larger in hippocampal slices from the IL-6 tg mice compared to hippocampal slices from the non-tg mice ($F(1,16) = 5.87$, $p = 0.03$).

Rapamycin reduces the late phase of long-term synaptic plasticity

Effects of rapamycin on LTP were accessed by comparing values for the slope of the fEPSP between control and rapamycin-treated slices for the early and late time periods studied. In hippocampal slices from IL-6 tg mice, both E-LTP and L-LTP of the fEPSP slope were significantly reduced by rapamycin (E-LTP: $F(1,12) = 7.42$, $p = 0.019$; L-LTP: $U = 1.0$, $n_1 = 6$, $n_2 = 8$, $p = 0.003$, Mann Whitney) (Fig. 1A). In slices from non-tg mice, L-LTP was significantly reduced by rapamycin but E-LTP was not altered (L-LTP: $F(1,15) = 5.38$, $p = 0.03$; E-LTP ($F(1,15) = 2.13$, $p = 0.16$) (Fig. 1B). In the presence of rapamycin there was no genotypic difference in the magnitude of L-LTP ($F(1,12) = 2.67$, $p = 0.13$).

Effects of rapamycin on L-LTP were also assessed by comparing normalized I/O curves for the fEPSP slope. Normalized values for I/O curves were calculated from the ratio of the fEPSP 120 minutes after LTP induction to the value before LTP induction at each stimulus intensity. From these curves, it was evident that the normalized fEPSP slope was largest for the IL-6 tg hippocampus (Fig. 1C1). For quantification, a mean normalized value for the fEPSP slope across stimulus intensities (range 90 to 160 μ A) was calculated for each slice.

A comparison of mean normalized values between IL-6 and non-tg slices under control conditions (no rapamycin) showed a significantly larger fEPSP slope in slices from IL-6 tg mice compared to slices from non-tg mice ($U = 3.0$, $p = 0.004$, $n_1=8$, $n_2=8$, Mann Whitney) (Fig. 1C2), consistent with results from measurement of fEPSP slope for early and late time points reported above. Rapamycin significantly reduced the normalized I/O curves for slices from both IL-6 tg and non-tg mice (IL-6: $U = 4.0$, $n_1=7$, $n_2=8$, $p = 0.006$, Mann Whitney; non-tg: $F(1,14) = 8.6$, $p = 0.01$), and eliminated the genotypic difference in the normalized I/O curves observed under control conditions ($F(1,14) = 2.25$, $p = 0.16$) (Fig. 1C). Interestingly, in the hippocampal slices from IL-6 tg mice the normalized I/O curves showed that the enhanced fEPSP slope during L-LTP was largest at the low stimulus intensities and that the effect of rapamycin was also largest at low stimulus intensities (Fig. 1C1). These results suggest that the enhanced LTP in hippocampus from the IL-6 tg mice is likely to involve more than just an increase in AMPA receptor number, which would be expected to produce a similar increase at all stimulus intensities.

A comparison of normalized I/O curves for the PS amplitude (ratio of PS amplitude 120 minutes after induction of LTP to PS amplitude before LTP induction) showed similar amplitudes for LTP of the PS in IL-6 tg and non-tg slices under all conditions (Fig. 1D1). For quantification, a mean normalized value was calculated across stimulus intensities (range 110–180 μ A) for each slice. Mean normalized values for LTP of the PS were not altered by rapamycin in either genotype (IL-6 tg: $F(1,16) = 0.58$, $p = 0.46$; non-tg: $F(1,17) = 1.26$, $p = 0.28$) (Fig. 1D2). These results suggest that rapamycin has a greater impact at dendritic sites than at somatic sites of the CA1 pyramidal neurons, and that mTOR plays a larger role in LTP in hippocampus of the IL-tg mice than in the hippocampus of non-tg mice.

Rapamycin alters the fEPSP during LTP induction in IL-6 tg hippocampal slices

Studies show that rapamycin reduces L-LTP only when present during the induction protocol, while application before or after the induction protocol is ineffective (Cammalleri et al., 2003). To determine if rapamycin affects the fEPSP during the induction protocol, we measured the magnitude of the fEPSP slope during HFS. During HFS the fEPSP slope showed an initial facilitation followed by depression to levels below pre-HFS values, which stabilized after about the 14th pulse under all conditions for all three trains of 100 stimuli (Fig. 2). Therefore, the analysis focused on responses to the first 14 pulses of the three trains. Data from the three trains were averaged according to pulse number for each slice and the mean values were compared statistically. Under control conditions (vehicle superfusion) there was no significant difference in the magnitude of the fEPSP between hippocampal slices from IL-6 tg and non-tg mice during the first 14 pulses of HFS ($F(1,29) = 0.216$, $p = 0.65$, repeated measures ANOVA). There was also no difference in the magnitude of the fEPSP between control and rapamycin conditions for hippocampal slices from non-tg mice during the first 14 pulses of the HFS ($F(1,14) = 0.854$, $p = 0.37$, repeated measures ANOVA) (Fig. 2B). In contrast, fEPSPs in IL-6 tg hippocampal slices exposed to rapamycin were significantly depressed during the first 14 pulses ($F(1,13) = 5.625$, $p = 0.033$, repeated measures ANOVA) compared to fEPSP in hippocampal slices from IL-6 tg mice under control conditions (vehicle superfusion) (Fig. 2B). Thus, HSF revealed or induced an interaction between the effects of chronic expression of elevated levels of IL-6 and mTOR

that affected the magnitude of the fEPSP slope during HSF. This HSF/IL-6/mTOR interaction was not evident in the non-tg hippocampus. The HSF/IL-6/mTOR interaction that affected the amplitude of the fEPSP during HFS or other HSF/IL-6/mTOR interactions could be instrumental in the increased levels of L-LTP in the IL-6 tg hippocampus.

Rapamycin did not alter short-term synaptic plasticity

Repetitive stimulation of the Schaffer collaterals at low frequency using a paired-pulse protocol results in an increase in synaptic strength at the Schaffer collaterals to CA1 pyramidal neuron synapse, referred to as paired-pulse facilitation (PPF). PPF is evidenced by an increase in the amplitude of the second fEPSP of the pair relative to the first and is quantified by the ratio (PPR) of the slope for the fEPSP elicited by the 2nd stimulus divided by slope of the fEPSP elicited by the 1st stimulus. PPF was determined under baseline vehicle control conditions and then after superfusion of the slices with rapamycin (1 μ M) or vehicle for 20 min. PPF was greater than 1 for hippocampal slices from both IL-6 tg and non-tg mice under all conditions, consistent with an increase in synaptic strength. The magnitude of PPF was similar under vehicle control conditions and in the presence of rapamycin for hippocampal slices from both IL-6 tg and non-tg mice indicating no genotypic difference (IL-6 tg: $F(1,16) = 0.50$, $p = 0.40$; non-tg: $F(1,16) = 0.14$, $p = 0.72$, repeated measures ANOVA) (Fig. 3A, B).

The PS elicited by the fEPSPs of the paired pulses can also show paired-pulse plasticity, which can be either a facilitation or depression depending on the relative level of inhibition in the somatic region. The inhibitory influences result from recurrent inhibitory synaptic activity and intrinsic excitability characteristics of the soma-dendritic region. Paired-pulse plasticity of the PS was determined by the PPR (PS elicited by the 2nd stimulus/PS elicited by the 1st stimulus). For all conditions, PPR of the PS was greater than 1, indicating facilitation, and similar in magnitude for hippocampal slices from the IL-6 tg and non-tg mice. Thus, inhibitory influences were not prominent in the IL-6 tg and non-tg slices. The magnitude of PPR for the PS was similar under control conditions and in the presence of rapamycin for hippocampal slices from both IL-6 tg and non-tg mice (IL-6 tg: $F(1,16) = 0.14$, $p = 0.72$; non-tg: $F(1,16) = 0.30$, $p = 0.59$, repeated measures ANOVA)(Fig. 3C, D). These results indicate that mTOR pathways sensitive to rapamycin are not likely to play a role in short-term synaptic plasticity of the fEPSP or PS at the Schaffer collateral to CA1 pyramidal neuron synapse in hippocampal slices from either genotype.

Rapamycin did not alter basal synaptic responses

Effects of rapamycin on basic properties of synaptic transmission at the Schaffer collateral to CA1 pyramidal neuron synapse were also assessed, to determine if mTOR contributed to the enhancement of synaptic responses (fEPSP slope and PS amplitude) observed in hippocampal slices from IL-6 tg mice (Nelson et al., 2012) (Fig. 4). Effects on synaptic responses were assessed from changes in normalized I/O relationships for the fEPSP slope or PS amplitude. In these studies, the I/O protocol was performed first under baseline vehicle control conditions and then after superfused of the slices for 20 minutes with ACSF containing rapamycin (1 μ M) or vehicle. The normalized (rapamycin/control at each stimulus intensity) fEPSP slope and the PS amplitude for IL-6 tg and non-tg slices were

compared. No genotypic difference was observed for the fEPSP slope or PS amplitude (fEPSP slope: $F(1,16) = 0.60$, $p = 0.45$; PS amplitude: $F(1,16) = 0.57$, $p = 0.46$, repeated measures ANOVA). In addition, the amplitude of the presynaptic volley (PSV) and basal synaptic strength (fEPSP/PSV) were similar for IL-6 tg and non-tg slices and were not altered by rapamycin. Mean PSV amplitudes averaged over stimulus intensities 90–220 μ A were 0.81 ± 0.08 (n=9) mV and 0.84 ± 0.08 (n=9) mV for non-tg slices under control and rapamycin conditions respectively, and 0.75 ± 0.1 (n=9) mV and 0.71 ± 0.09 (n=9) mV for IL-6 tg slices under control and rapamycin conditions, respectively. Basal synaptic strength values averaged over stimulus intensities 90–220 μ A were 4.0 ± 0.33 (n=9) mV and 4.4 ± 0.30 (n=9) mV for non-tg slices under control and rapamycin conditions respectively, and 4.22 ± 0.24 (n=9) mV and 4.35 ± 0.27 (n=9) mV for IL-6 tg slices under control and rapamycin conditions, respectively. These results suggest that mTOR pathways sensitive to rapamycin are not likely to play a role in defining the magnitude of the baseline fEPSP slope or PS amplitude in either the IL-6 tg or non-tg hippocampus.

IL-6 tg and non-tg hippocampi have similar levels of mTOR and phospho-mTOR

Because IL-6 signal transduction can lead to activation of mTOR through phosphorylation, adaptive changes in the IL-6 tg hippocampus due to the persistent expression of IL-6 could involve alterations in the levels of mTOR or phospho-mTOR. To examine this possibility, the levels of mTOR and phospho-mTOR were determined by Western blot in hippocampus obtained from IL-6 tg and non-tg mice. No significant difference was observed in the levels of either of these forms of mTOR between IL-6 tg and non-tg hippocampus (Fig. 5).

Discussion

The studies described here show that hippocampus from IL-6 tg mice that persistently express elevated levels of IL-6 in the CNS exhibit enhanced L-LTP compared to L-LTP in hippocampus from non-tg mice. The mTOR pathway was identified as one mechanism involved in the enhanced L-LTP, because the inhibitor of this pathway, rapamycin, eliminated the difference in L-LTP between hippocampal slices from IL-6 tg and non-tg mice. Rapamycin also produced a significant depression of the fEPSP during the LTP induction protocol in hippocampal slices from the IL-6 tg mice, but not in hippocampal slices from non-tg mice. No effect of rapamycin on basal synaptic transmission or short-term synaptic plasticity induced by low- (e.g., PPF) or high-frequency (i.e., PTP) stimulation was observed for either genotype. The role of mTOR in the IL-6 tg hippocampus presumably results from adaptive changes induced by the persistently expressed IL-6 in the CNS of the IL-6 tg mice. These adaptive changes did not appear to involve baseline levels of mTOR or phospho-mTOR, as Western blot analysis indicated similar levels in IL-6 tg and non-tg hippocampus. Thus, HFS was instrumental to actions of mTOR that affected synaptic responses during HFS and L-LTP in hippocampal slices from the IL-6 tg mice. IL-6 is known to be a negative regulator of L-LTP, whereas the HSF/IL-6/mTOR interactions observed in this study resulted in an enhancement of L-LTP. The mechanisms responsible for the enhancement of synaptic responses during HFS and L-LTP remain to be resolved, but several possible contributors are noted below.

IL-6, mTOR, and synaptic plasticity

A number of studies have shown that inhibition of mTOR with rapamycin reduces hippocampal L-LTP without effects on basic properties of synaptic transmission, as was observed in our studies (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005; Tsokas et al., 2005; Vickers et al., 2005; Stoica et al., 2011). Rapamycin also appeared to affect the later stage of E-LTP in hippocampal slices from the IL-6 tg mice. However, this effect could represent a faster timecourse for detectable L-LTP expression in the hippocampus of the IL-6 tg mice such that L-LTP overlapped with E-LTP.

Studies have shown that to effectively reduce L-LTP, rapamycin must be present during the induction phase of LTP (Cammalleri et al., 2003). In our studies, the fEPSP showed an enhancement followed by a depression during HFS in both the IL-6 tg and non-tg slices. Rapamycin reduced these changes in the magnitude of the fEPSP in the IL-6 tg slices but not in the non-tg slices. Thus, rapamycin similarly affected the fEPSP during HFS and L-LTP in hippocampal slices from IL-6 tg mice but not in slices from non-tg mice, where only L-LTP was affected. This result and the fact that rapamycin must be applied during the LTP induction period to block L-LTP (Cammalleri et al., 2003) raises the possibility that mechanisms mediating excitatory synaptic responses during HFS play a role in the increased level of L-LTP in the IL-6 tg hippocampus.

The depressive effect of rapamycin on the fEPSP during HFS in the IL-6 tg slices was immediate (within 3–4 min from the start of HFS), whereas effects of rapamycin on LTP of the fEPSP were expressed with a delay, starting about 40 min after HFS. The immediate depressive effects of rapamycin during HFS primarily affected fEPSPs elicited by the first 14 pulses of each stimulus train, where an initial facilitation followed by depression was observed. A similar response pattern, an initial facilitation followed by depression, was shown previously to occur at the Schaffer collateral to CA1 pyramidal neuron synapse during rapid stimulation, and thought to result from presynaptic mechanisms involving the ready releasable pool of synaptic vesicles (Dobrunz and Stevens, 1997). Interestingly, excitatory synaptic transmission in CNS neurons from mice genetically modified to express increased mTOR signaling was enhanced due to presynaptic mechanisms including an increased size of the ready releasable pool of synaptic vesicles (Weston et al., 2012). Moreover, rapamycin treatment of wildtype neurons reduced the size of the ready releasable pool of synaptic vesicles (Weston et al., 2012). Thus, the consequences of elevated expression of IL-6 in the IL-6 tg hippocampus may include increased involvement of mTOR at presynaptic sites that regulate transmitter release during HFS. Increased involvement of mTOR in post-synaptic mechanisms could also be involved. Studies have shown that mTOR enhances L-LTP through effects on postsynaptic protein synthesis and that this protein synthesis is required for expression of L-LTP (Tang et al., 2002; Cracco et al., 2005; Tsokas et al., 2005; Vickers et al., 2005; Stoica et al., 2011). Thus the HFS/IL-6/mTOR interactions during HFS are likely to be important contributors to the enhanced L-LTP observed in the IL-6 tg hippocampus.

PPF of the fEPSP and PTP, which are examined by single or paired test pulses, were unaffected by rapamycin, suggesting that either the presynaptic mechanisms directly mediating these forms of plasticity do not involve mTOR, or that an involvement of mTOR

is not detectable using a single or paired-pulse paradigm that was used in the current studies. A variety of presynaptic components and mechanisms regulate transmitter release in a stimulus-dependent manner (Zucker, 1996; Regehr, 2012).

The presynaptic and postsynaptic mechanisms underlying the enhanced L-LTP in the hippocampal slices from the IL-6 tg mice presumably result from adaptive changes that occurred over time due to the persistently expressed elevated levels of IL-6. In addition to neuronal presynaptic and postsynaptic mechanisms, these changes could involve astrocytes. Previous studies showed that the LTP induction protocol induces endogenous IL-6 production in the rat hippocampus and that this production occurs with a delay (~ 90 min) and reduces L-LTP (Balschun et al., 2004). The increased IL-6 production appears to originate in the hippocampal astrocytes (Jankowsky et al., 2000). Interestingly, mTOR has been reported to negatively regulate IL-6 production in astrocytes (Codeluppi et al., 2014). Thus, the greater involvement of mTOR in the IL-6 tg hippocampus compared to the non-tg hippocampus during LTP induction could also involve mTOR in astrocytes and result in a reduction in HFS-induced IL-6 production by astrocytes and, consequently, reduced IL-6 inhibition of L-LTP in the IL-6 tg slices. Further studies will be needed to address this and other possibilities.

IL-6 signaling and synaptic plasticity

There are several possible signaling pathways through which IL-6 and IL-6R could influence mTOR signaling in the IL-6 tg hippocampus and consequently L-LTP. IL-6 has been shown to activate both JAK/STAT and MAPK/ERK signaling pathways in hippocampal neurons (Schumann et al., 1999). mTOR is a downstream signaling partner of both JAK/STAT and MAPK/ERK. Molecular components of JAK/STAT and MAPK/ERK pathways are present at synaptic sites in the hippocampus (Murata et al., 2000; Mao and Wang, 2016). Several behavioral studies have shown an involvement of STAT3 in memory and learning (Sparkman et al., 2006; Chiba et al., 2009; Burton and Johnson, 2012; Hao et al., 2014), providing support for the idea that STAT3 can play a role in L-LTP. MAPK/ERK activation has been shown to be required for LTP-dependent transcriptional regulation (Davis et al., 2000). The increase in mTOR activity in CA1 pyramidal neuron dendrites induced by HFS was shown to be MAPK/ERK dependent (Tsokas et al., 2007). Inhibition of the MAPK/ERK cascade by antagonists markedly attenuated the expression of LTP in area CA1 when the antagonists were applied before the induction paradigm, but not when applied after the induction paradigm (English and Sweatt, 1996, 1997). Similarly, antagonists for mTOR must be applied during the LTP induction period to block HFS activation of mTOR (Cammalleri et al., 2003). Thus, one or both of these signal transduction pathways (JAK/STAT, MAPK/ERK) could be involved in the IL-6/mTOR interaction observed in the current studies. Interestingly, both phospho-STAT3 and phospho-MAPK/ERK were significantly increased in hippocampus from IL-6 tg mice compared to hippocampus from non-tg mice (Nelson et al., 2012), which could contribute to the increased role of mTOR in L-LTP in the IL-6 tg hippocampus. However, the level of mTOR activation (i.e., pmTOR) was not increased in the hippocampus of the IL-6 tg mice. This result may reflect a requirement for the LTP induction protocol (HFS) for initiation of mTOR activation and IL-6/mTOR interactions that underlie the enhancement of L-LTP in the IL-6 tg mice. Future studies

where the level of mTOR activation is measured in hippocampal slices subjected to HFS will help to resolve this issue.

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Abbreviations

ANOVA	analysis of variance
AU	arbitrary units
ACSF	artificial cerebral-spinal fluid
CNS	central nervous system
DNA	deoxyribonucleic acid
E-LTP	early long-term potentiation
ELISA	enzyme-linked immunosorbent assay
ERK	extracellularly regulated kinase
fEPSP	field excitatory postsynaptic potential
HFS	high frequency stimulation
I/O	input/output
IL-6	interleukin-6
IL-6R	IL-6 receptor
JAK	Janus kinase
L-LTP	late long-term potentiation
LTP	long-term potentiation
mTOR	mammalian target of rapamycin
MAPK	mitogen-activated protein kinase
non-tg	non-transgenic
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PI3K	phosphatidylinositol 3-kinase
PCR	Polymerase Chain Reaction

PS	population spike
STAT3	signal transducers activators of transcription 3
MAPK	mitogen-activated protein kinase
ERK	extracellularly regulated kinase

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Highlights

- Transgenic mice that express elevated levels of IL-6 in the CNS show an increase in the late form of hippocampal LTP.
- Rapamycin, an antagonist of mTOR, reduced the difference in late-LTP between the IL-6 transgenic and non-transgenic hippocampus.
- Synaptic responses during the LTP induction protocol were reduced by rapamycin in IL-6 transgenic hippocampus.

Figure 1.

L-LTP in hippocampus from IL-6 tg and non-tg mice. A1, B1. Representative traces showing fEPSPs elicited by Schaffer collateral stimulation (at the arrow) in control and rapamycin-treated hippocampal slices from IL-6 tg (A) and non-tg (B) mice 1–5 minutes before (pre) and 120 minutes after (post) HFS. A2, B2. Graphs showing the amplitude of the fEPSP slope as a percentage of baseline levels before and after HFS (at the arrow) in vehicle (cont) and rapamycin (rap) treated slices from IL-6 tg (A2) and non-tg (B2) mice. A3, B3 Graph showing combined (mean or median) values for E-LTP (data points 48–52 min post HFS) and L-LTP (data points 105–115 min post HFS) in IL-6-tg (A3) and non-tg (B3) hippocampal slices. C1. Comparison of normalized I/O curves for the fEPSP slope in control or rapamycin-treated hippocampal slices from IL-6 tg and non-tg mice. Results were normalized in order to make a within slice comparison. Data at each stimulus intensity are expressed as the ratio of the fEPSP slope after HSF with respect to the fEPSP slope before HSF. C1. Graph showing combined (mean or median) normalized values for fEPSP slope for stimulus intensities 90–160 μ A. D1. Comparison of the I/O curves for the PS amplitude before and 120 minutes after LTP induction in control and rapamycin treated hippocampal

slices from IL-6 tg and non-tg mice. Data are expressed at each stimulus intensity as the ratio of the PS amplitude after LTP induction with respect to the PS amplitude before LTP induction. D2. Graph of mean values for the PS at stimulus intensities 90–160 μ A. * = significant difference between control and rapamycin treated slices within the same genotype (ANOVA or Mann Whitney test). In this and other figures, numbers in bars are the number of animals studied (1 slice/animal).

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Figure 2.

fEPSPs elicited by HFS in hippocampal slices from IL-6 tg and non-tg mice. A.

Representative recordings of fEPSPs elicited by the first 14 stimulations of the first train of HFS in control slices (vehicle superfusion) and in rapamycin treated slices for hippocampus from non-tg and IL-6 tg mice. B. Graphs showing mean values for the fEPSP slope during HFS. Arrows indicate time of stimulation. There was no genotypic difference in fEPSP slope before HSF. * = significantly different from control values for the same genotype (repeated measured ANOVA, $p < 0.05$).

Figure 3.

Rapamycin did not alter paired-pulse ratios. A, C. Representative traces showing responses to paired-pulse stimulation for the fEPSP (A) and PS (C) in hippocampal slices from non-tg and IL-6 tg mice before and after application of rapamycin. Arrows indicate the time of stimulus application. Traces #1 and #2 are superimposed responses to paired stimuli separated by a 40 ms (A) or 20 ms (C) interpulse interval. B, D. Graphs showing mean values for PPF of the fEPSP (B) and PPR of the PS (D) under control conditions and in the presence of rapamycin.

Figure 4.

Synaptic I/O relationships were not altered by rapamycin. A, C. Representative field potential recordings of fEPSP (A) and PS (C) elicited by a range of stimulus intensities in hippocampal slices from non-tg and IL-6 tg mice. Recordings are shown before and after addition of rapamycin to the recording saline. Arrows indicate the time of stimulus application. B. Graphs showing normalized results for the fEPSP expressed as a ratio of the fEPSP slope after application of rapamycin with respect to the fEPSP slope under baseline control conditions, calculated at each stimulus intensity. There was no significant difference between control fEPSP slope and the fEPSP slope after application of rapamycin in either the IL-6 tg or non-tg slices. D. Normalized results for the PS expressed as a ratio of the PS amplitude after application of rapamycin with respect to the PS amplitude under baseline control conditions, calculated at each stimulus intensity. Calibration bars are 2 mV by 5 ms.

Figure 5.

mTOR and pmTOR levels in IL-6 tg and non-tg hippocampus. Graph showing mean values for levels of mTOR and pmTOR in IL-6 tg and non-tg hippocampus assayed by Western blot. Insets above graphs show representative bands from Western blots for the proteins assayed. Also shown with the mTOR and pmTOR bands are bands for glial acidic fibrillary protein (GFAP) and β -actin run sequentially on the same Western blot as mTOR or pmTOR. As reported previously, GFAP levels are increased in the hippocampus of the IL-6-tg mice, whereas β -actin levels are not (Gruol et al., 2014).