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Effects of Rapamycin on Gene expression, Morphology, and Electrophysiological Properties of Rat Hippocampal Neurons

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Summary

Purpose—We assayed the effects of rapamycin, an immunomodulatory agent known to inhibit the activity of the mammalian target of rapamycin (mTOR) cascade, on candidate gene expression and single unit firing properties in cultured rat hippocampal neurons as a strategy to define the effects of rapamycin on neuronal gene transcription and excitability.

Methods—Rapamycin was added (100 nM) to cultured hippocampal neurons on days 3 and 14. Neuronal somatic size and dendritic length were assayed by immunohistochemistry and digital imaging. Radiolabeled mRNA was amplified from single hippocampal pyramidal neurons and used to probe cDNA arrays containing over 100 distinct candidate genes including cytoskeletal element, growth factor, transcription factor, neurotransmitter, and ion channel genes. In addition, the effects of rapamycin (200 nM) on spontaneous neuronal activity and voltage-dependent currents was assessed.

Results—There were no effects of rapamycin on cell size or dendrite length. Rapamycin altered expression of distinct mRNAs in each gene family on days 3 and 14 in culture. Single unit recordings from neurons exposed to rapamycin exhibited no change from baseline. When spontaneous activity was increased by blocking GABA-mediated inhibition with bicuculline, a fraction of the neurons exhibited a decreased duration of spontaneous bursts and a decrease in synaptic inputs. Rapamycin did not appear to alter voltage dependent Na⁺ or K⁺ currents underlying action potentials.

Conclusions—These data demonstrate that rapamycin does not produce neurotoxicity nor alter dendritic growth and complexity *in vitro* and does not significantly alter voltage gated sodium and potassium currents. Rapamycin does affect neuronal gene transcription *in vitro*. Use of rapamycin in clinical trials for patients with tuberous sclerosis complex should involve vigilance for possible effects on seizure frequency and neurocognitive function.

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1. Introduction

Rapamycin is an antibiotic immunosuppressant compound known to directly antagonize the mammalian target of rapamycin (mTOR) cascade which lies downstream of the insulin like growth factor cascade and leads to activation of several downstream kinase proteins including p70S6 kinase that phosphorylate proteins known to regulate cell growth (Schmelzle and Hall, 2000; Potter et al., 2001; Hay and Sonenberg, 2004; Soliman, 2005; Martin and Hall, 2005). Recent evidence suggests that loss of function mutations in the tuberous sclerosis complex genes (*TSC1* and *TSC2*) leads to constitutive activation of the mTOR cascade (Arrazola et al., 2002; Inoki et al., 2002; Kenerson et al., 2002; Onda et al., 2002; Tee et al., 2002; El-Hashemite et al., 2003; Zhang et al., 2003) and disturbs the regulation of neuronal morphology and function mediated by TSC1 and TSC2 (Tavazoie et al., 2005) resulting in abnormal neuronal organization and seizures (Uhlmann et al., 2002). Rapamycin has been proposed as a treatment modality for tuberous sclerosis complex (TSC), a multisystem, autosomal dominant disorder characterized by abnormal brain development, epilepsy, and potentially life threatening disorders such as pulmonary lymphangioleiomyomatosis (Johnson and Tattersfield, 2002; Crino et al., 2006).

Rapamycin is a potent translational modifier in neurons although its effects on gene transcription and neuronal development are poorly understood. In fact, no study has documented effects of rapamycin on neuronal gene transcription. Rapamycin mediates protein synthesis stimulated by brain derived neurotrophic factor (Takei et al., 2001; Schratt et al., 2004). Rapamycin has also been shown to have direct neurophysiological effects, although some of the data have been controversial. The single channel activity of the Ca²⁺-dependent K⁺ channel is modulated by rapamycin through its direct association with the FK506 binding protein-12 (FKBP12) (Terashima et al., 1998). Rapamycin might also influence mTOR mediated propagation of inhibitory transmission via the glycine- and GABA-receptor clustering protein gephyrin (Sabatini et al., 1999; Sassoè-Pogetto et al., 2000; Kneussel, 2002). In one model, rapamycin did not enhance the firing of spontaneous action potentials (Victor et al., 1995; Beaumont et al., 2002; Norris et al., 2002), perhaps because unlike other calcineurin inhibitors, cyclosporine A and tacrolimus, rapamycin does not inhibit the Ca²⁺-calmodulin-dependent phosphatase calcineurin.

Perhaps most controversial is rapamycin's effect on long term potentiation (LTP). One study demonstrated that low dose rapamycin leads to NMDA-dependent induction of long-term potentiation (LTP) in CA1 neurons of rat hippocampal slices when paired with weak presynaptic stimulation (Terashima et al., 2000). In contrast, others have shown that rapamycin impairs LTP by inhibiting mTOR through reduced protein synthesis (Casadio et al., 1999; Tang et al., 2002). Finally, a third study demonstrated that rapamycin did not affect LTP at baseline, and did not prevent the inhibition of LTP by H₂O₂ (20 µM) (Kamsler and Segal, 2003).

While clinical experience in organ transplant patients suggests that neurological sequelae of rapamycin therapy are rare, there is little clinical or pre-clinical data on the effects of rapamycin in patients with epilepsy, autism, or cognitive disability, or on the developing nervous system *in vivo*. The latter is particularly important, as rapamycin is being proposed for use in children with TSC. One group recently reported rapamycin-induced regression of astrocytomas in five patients with TSC. Four of these patients remained seizure-free during the treatment period; no information is given on seizure frequency of the fifth patient or on other neurological side effects (Franz et al., 2006). In the light of planned clinical trials for rapamycin in young patients, we investigated the effect of rapamycin on cell morphology, gene expression, and seizure-like firing patterns in cultured rat hippocampal neurons at post-natal time periods using morphometry, single cell mRNA amplification, and single unit

recoding. These experiments provide a strategy to define potential effects of rapamycin on neuronal function that might complicate treatment of TSC patients in future trials.

2. Materials and Methods

2.1 Dissociated Hippocampal Cell Cultures and Treatment

Standard culture techniques were as previously described (Wilcox et al., 1994; Cummings et al., 1996). Briefly, hippocampi from E19 rat embryos were dissected from anesthetized pregnant Sprague-Dawley rats and trypsinized in Dulbecco's Minimum Essential Medium (DMEM; Whittaker Bioproducts) containing 0.027% trypsin at 4°C for 20 minutes. They were triturated in a media consisting of DMEM (Whittaker Bioproducts) supplemented with 10% bovine calf serum (Hyclone Lab), 10% Ham's F12 with glutamine (Whittaker Bioproducts), and 50 U/mL penicillin-streptomycin (Sigma). Dissociated cells were plated on poly-L-lysine coated glass coverslips in 35 mm petri dishes and cultured at 37°C in a humidified 5% CO₂ incubator. Dissociated cells were plated at a density of 100,000 cells/mL in Neurobasal medium (GIBCO) supplemented by B27 (GIBCO). Because glia overgrowth is rare in these cultures, no mitotic inhibitors were used to inhibit glial growth. There were also no antibiotics used. The cultures were fed by replacing one third of the media weekly.

2.2 Rapamycin Treatment

Hippocampal cells were treated with rapamycin (dissolved in methanol) for 24 hours, at 3 and 14 days in culture. The final concentrations assayed were 100nM based on previous studies in hippocampal cultures (Tang et al., 2002; Sabatini et al., 1999). Cells were then washed with serum free media and rapidly fixed in ice cold 4% paraformaldehyde prior to gene expression analysis. In control experiments, either methanol alone or the same volume of media was added to the cultures.

2.3 Immunohistochemistry

Hippocampal neurons were immunolabeled with NeuN (mouse monoclonal, 1:1000 dilution) for cell counting experiments or MAP2 (mouse monoclonal, 1:10, Sigma) to measure dendrite length. Primary antibody labeling was performed for 2 hours at 4°C. Immunolabeling was visualized using the avidin-biotin conjugation method (Vectastain ABC Kit; Vector Labs; Berlingame, CA) and 3,3'-diaminobenzidine or with immunofluorescence with a TRITC-conjugated secondary antibody.

2.4 Quantitative Cell Counting, Cell Size, and Dendrite Length Analysis

NeuN labeled pyramidal cells were identified using morphometric parameters (cell diameter and large apical dendritic segment) for quantitative cell counting analysis following treatment with rapamycin or buffered saline solution at 3 and 14 days in culture. Five representative contiguous digital photos were obtained (20X magnification) from each coverslip using image acquisition and analysis software (Spot RT CCD camera, Diagnostic Instruments, Inc. and Phase 3 Imaging System integrated with Image Pro Plus; Media Cybernetics, Silver Spring MD). The three images spanned a 2mm² region of interest (ROI). NeuN labeled pyramidal cell were counted in each digital photo in each ROI. For each cell counted on the coverslips, the cell area was simultaneously determined. For the dendritic length assay, approximately 100 MAP2 labeled pyramidal cells were photographed in the treated and untreated coverslips and the length of each apical segment was determined from the proximal somatodendritic junction to the distal tip by digital imaging.

2.5 Aspiration of Single Cells and mRNA Amplification

Single NeuN labeled neurons exhibiting a pyramidal morphology with a single large apical dendrite cells were aspirated (n=30 each cell type) under light microscopy using plastic micropipette and a joystick micromanipulator (Eppendorf). Aspirated single cells were transferred to a microfuge tube containing reagents necessary for cDNA synthesis (including an oligo-dT(24) primer coupled to a T7 RNA polymerase promoter) and incubated at 40°C for 90 minutes. cDNA synthesis was performed in reaction buffer (10 mM HEPES buffer pH 7.4, 120 mM KCl, 1 mM MgCl₂, 250 μM dATP, dCTP, dGTP, TTP) with avian myeloblastosis reverse transcriptase (AMVRT. 0.5U/ • 1, **Seikagaku America**).

Double stranded template cDNA was generated with T4 DNA polymerase I (Boehringer-Mannheim) and mRNA was amplified from the double stranded cDNA template with T7 RNA polymerase (Epicentre Technologies)(Kacharina et al., 1999). Amplified mRNA served as a template for a second round of cDNA synthesis with AMVRT, dNTPs, and N(6) random hexamers (Boehringer-Mannheim). cDNA generated from amplified mRNA was made double stranded and served as a template for a second mRNA amplification incorporating ³²PCTP.

2.6 Candidate Gene Expression Analysis and cDNA Arrays

Gene expression analysis was performed using lab generated arrays containing linearized, full-length plasmid cDNAs encoding genes of interest including cytoskeletal element, growth factor, transcription factor, neurotransmitter, and ion channel genes. Lab generated arrays were screened in duplicate with amplified, radiolabeled mRNA from single microdissected cells (n=120 arrays per timepoint). β-actin and GAPDH were included as “housekeeping genes” to serve as positive hybridization controls. pBlueScript (PBS) and pUC18 plasmid cDNAs were used to define background levels of hybridization on each array platform.

Lab generated arrays were hybridized (24 hours) in 6X SSPE buffer, 5X Denhardt's solution, 50% formamide, 0.1% SDS, and salmon sperm DNA 200 μg/ml at 42°C, washed in 2X SSC, and apposed to phosphorimaging screen cassette for 24–48 hours to generate an autoradiograph.

2.7 Data and Statistical Analysis

The hybridization intensity of each mRNA-cDNA hybrid was determined by densitometry of the slot blot phosphorimage generated from single cell mRNA probes (ImageQuant5.0 software). Non-specific (background) hybridization to pBluescript (PBS) plasmid cDNA or pUC18 was subtracted from that of each mRNA-cDNA hybrid. The relative abundance of each mRNA was determined by averaging the hybridization intensity of all the mRNA-cDNA hybrids on the array and then expressing each mRNA-cDNA hybrid as a percentage of this average. Differences in relative abundance were determined using a one-way ANOVA. To control the experiment-wise error rate for the multiple univariate ANOVAs to be performed, a Bonferroni correction was applied to each univariate ANOVA and if a significant difference was found, individual post-hoc comparisons will be made using the Fischer's test (p<0.05 was considered significant).

2.8 Electrophysiology

Using the whole-cell patch clamp technique, spontaneous ionic currents were recorded from neurons in 2 week old cultures that had been grown in serum-free NB/B27 media. All recordings were performed in the voltage clamp mode at room temperature with 4–6 MΩ electrodes made from borosilicate glass capillaries (Kimax). The external bath solution

contained a HEPES-buffered saline (HBS) solution containing 145 mM NaCl, 3 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 8 mM glucose, and 1 mM MgCl₂. The recording solution within the electrode contained 127.5 mM K-gluconate, 12.5 mM KCl, 10 mM HEPES, 10 mM EGTA, 2.5 mM Mg-ATP, 1 mM CaCl₂, 1 mM EGTA and 10 mM D-glucose (osm = 290 mOsm). In the standard experiment, the cells were recorded for 10 min at baseline, then for the same time in the external bath solution containing 10 μ M bicuculline. (Bicuculline is a GABA_A receptor antagonist that is a potent convulsant in vivo and in hippocampal slices and produces an increase in neuronal firing associated with enhanced burst firing in hippocampal neurons in dissociated cell culture.) Rapamycin (dissolved in methanol 100%, from Cell Signaling, Beverly, MA) was then added to bicuc-bath in a final concentration of 200 nM. After a 10 minute incubation in rapamycin, the neurons were recorded up to 150 minutes. Control experiments were performed by adding only the same amount of solvent (i.e., methanol).

All chemicals were from Sigma, St.Louis MO, if not otherwise stated. Data acquisition and subsequent data analysis were performed with pCLAMP software (Axon Instruments) and stored on VHS video tape.

3. Results

3.1 Cell Size and Dendritic Length

Quantitative cell counts did not reveal significant differences in the numbers or morphology of neurons in the rapamycin treated or control cultures (24 hour incubations at 37°C; 48,365 $\pm$ 188 versus 49,112 $\pm$ 203 neurons per coverslip (Fig. 1). Similarly, there was no difference in mean cell soma area in neurons treated with rapamycin compared with control cells. Finally, MAP2 labeling demonstrated intact dendritic processes in neurons exposed to rapamycin at each dose (Fig. 1) and the mean length or branch number of dendritic processes was not statistically significant in cells treated with rapamycin (Fig. 2). Thus, in these cultures of developing neurons, 24 hour exposure to rapamycin did not appear to affect neuronal survival or gross dendritic morphology.

3.2 Rapamycin Effects on Gene Expression

At 3 and 14 days in culture, 24 hour exposure to rapamycin resulted in altered expression of a number of distinct mRNAs in each gene family (Fig. 3). At 3 days in culture, rapamycin treatment caused a change in expression of 13 mRNAs within cytoskeletal element, growth factor, transcription factor, neurotransmitter, and ion channel gene families ($p < 0.05$). Reduced expression of nestin, aFGF, BMP6, CREB, c-jun, OTX1, EAAC1, Kir 4.1 and Kir5.1 and increased expression of erbB4, netrin1, and mGluR4 were observed. Interestingly, rapamycin treatment induced a decrease in tuberlin mRNA expression.

At 14 days in culture, 24 hour exposure to rapamycin led to altered expression of 18 mRNAs within cytoskeletal element, growth factor, transcription factor, neurotransmitter, and ion channel gene families, some of which were different than those altered at 3 days in culture. Reduced expression of c-ret, PDGFR β , TGF β 2, TGF β 1, and EAAC1 and increased expression of LIF, IL-6, LIFR, IFG-1, GLT-1, HES, CREB, c-jun, mGluR4, mGluR5, KCNQ2, and Kir1.1 were observed following rapamycin treatment. Tuberlin mRNA levels were unchanged in contrast to the effects on the younger neurons.

3.3 Effects of Rapamycin on Neuronal Network Activity and Firing Properties in Vitro

In order to assess the affects of rapamycin on spontaneous neuronal activity, whole cell patch clamp recordings (n=41) were made from dissociated rat hippocampal neurons in culture. These neurons receive both excitatory and inhibitory synaptic inputs from multiple

neighboring cells and fire spontaneously with both single action potentials and short bursts of action potentials. Addition of rapamycin (200 nM) to the bath appeared to either produce no change in firing frequency or a slight decrease in the frequency of the spontaneous bursts (Fig 4A). When the GABA_A receptor antagonist, bicuculline (10 μ M), was added to the control bath, action potential frequency was markedly increased and burst firing was increased in many, but not all cultures. The addition of rapamycin (200 nM) in some cells decreased the burst firing and appeared to reduce overall network excitability (Fig. 4B), although in other cultures, rapamycin produced no significant change in the bicuculline-induced bursting pattern, even after an hour of exposure (Fig 4C). Thus, rapamycin did not increase network excitability, but in some cases reduced network excitability. Control experiments with the addition of the solvent of rapamycin only (methanol) did not show any alteration of the spiking behavior of the cells.

The lack of effect of rapamycin on action potential characteristics noted seen in the experiments with spontaneous firing was more directly examined by recording voltage dependent Na and K currents in the cells when synaptic activity was inhibited by a combination of agents that blocked GABA_A, AMPA, and NMDA receptors. Figure 5A illustrates that the I/V plots for voltage dependent Na and K currents (recorded simultaneously in 5 neurons) were unchanged after the addition of rapamycin. Because of the almost identical IV curves in control and rapamycin media, we did not further investigate the detailed properties of these currents.

4. Discussion

Rapamycin is a potential new therapeutic agent for the treatment of TSC. Although previous widespread clinical use as an immunomodulatory agent in post-transplant patients has not documented significant effects on the central nervous system (Kuypers, 2005), the molecular and pharmacological effects of rapamycin on neuronal function may have implications for TSC patients with neurological disease such as epilepsy, autism, and cognitive disabilities (Bolton et al., 2002). In addition, the use of rapamycin in children could have effects on the developing nervous system not seen in adults. In our experiments, rapamycin did not appear toxic to neuronal cells in culture and did not alter the growth of cells *in vitro*. However, we do demonstrate that administration of rapamycin to cultured hippocampal neurons *in vitro* can lead to alterations in expression of numerous genes in several gene families and that these changes in gene expression can be age-dependent. We also show that rapamycin does not lead to significantly altered neuronal voltage-dependent Na or K currents or spontaneous firing properties. On the other hand, the enhanced neural network activity seen when GABA-mediated inhibition was diminished was reduced in some cells by rapamycin. These effects suggest that rapamycin may have effects on neuronal function but that enhanced neuronal excitability is not a consequence of rapamycin treatment. The results of a recent pilot study on five patients with TSC treated with rapamycin which demonstrated a regression of astrocytomas supported this assumption (Franz et al, 2006). In view of planned larger human trials evaluating rapamycin to slow or prevent formation of tubers in TSC patients, it was important to confirm this “no effect” hypothesis. Of course, if rapamycin does exhibit an anti-seizure effect *in vivo*, this would be an extra, unexpected benefit of its use in TSC.

Since rapamycin mainly acts through its influence on protein synthesis which peaks only after hours or a few days, an immediate effect might seem very unlikely. Indeed, no morphological effects were observed following a 24 hour exposure to rapamycin although previous studies clearly demonstrate alterations in cell morphology following more prolonged rapamycin treatment (Tavazoie et al., 2005).

Rapamycin had differential effects on gene transcription at 3 and 14 days in culture suggesting that cellular maturation may contribute to the effects of rapamycin on mRNA expression. For example, the expression profile of several genes including CREB and c-jun, was altered at day 3 compared with day 14 in culture. For select genes, e.g., EAAC1 and mGluR4, the expression profiles were similar at both time points. Of particular interest, the expression of several genes including EAAC1, (the neuronal glutamate transporter), nestin, erbB4, and c-jun were altered opposite to what has been reported in single cells microdissected from tubers, consistent with an effect of rapamycin on the mTOR pathway. For example, EAAC1 mRNA is upregulated in cortical tubers (White et al., 2001) but is downregulated by rapamycin. Conversely, erbB4 and c-jun mRNA expression is diminished in cortical tubers (Baybis et al., 2004) but expression is enhanced by rapamycin. These alterations may have effects on neural function in TSC and could possibly lead to improvement in neurological function in TSC. Future studies to define how rapamycin can affect gene transcription in fully mature neurons are warranted.

Taken together, our data indicate that rapamycin had relatively little acute influence on the electrophysiological properties of dissociated rat hippocampal neurons (during the first hour of exposure). However, the effects of more prolonged treatment remain to be determined. Nevertheless, the clinical experiences with rapamycin in transplantation medicine and a small series of patients with TSC did not reveal a significant frequency of neurological adverse events, especially seizures or cognitive (memory) impairment.

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Figure 1. Rapamycin exposure for 24 hours at day 3 or 14 in culture does not affect cell morphology (MAP2 immunolabeling, 40× magnification). Note similar morphological appearance and dendritic arborizations in control (a, 3 days and c, 14 days) versus rapamycin treated neurons (b, 3 days and d, at 14 days in culture).

Figure 2.

Morphometric analysis of maximal cell soma diameter, average dendritic length, and dendritic branching at 3 (blue) and 14 (red) days in culture. Top, modified Scholl analysis of dendritic branching (MAP2 immunolabeling) at 3 days in culture; y-axis depicts mean number of dendritic branch intersections. Bottom left, circles depict representative measured cells. Lines depict computer graphic drawing of MAP2 labeled dendrite selected for measurement. Bottom middle, subtle reduction in cell size in rapamycin treated cells at 3 and 14 days did not achieve statistical significance; y-axis, mean soma diameter microns². Bottom right, there was no difference in mean dendrite length between control and rapamycin treated cells; y-axis, microns.

Figure 3.

Graphic depiction of alterations in gene expression following rapamycin treatment at 3 (blue) and 14 (red) days in culture. Bars (and lines above) represent mean $\pm$ standard error of gene expression. Numbers on Y-axis depict fold change in gene expression.

Figure 4.

Recordings of spontaneous cellular activity from hippocampal neurons in culture. Whole cell patch clamp recordings in voltage clamp mode from 3 week old neurons grown in serum-free cell culture.

A (Left) Spontaneous activity in control solution and **(Right)** after addition of rapamycin (200 nM). Note the cell had bursts of synaptic currents and more isolated synaptic currents. The insert below the traces illustrates one burst of inward synaptic currents expanded to a faster time scale. Under the conditions being used for recording, both excitatory and

inhibitory synaptic currents would be inward at the holding potential employed in these experiments.

B (Left) Spontaneous activity after addition of bicuculline (10 μ M) to the external bath. Note the marked increased frequency of synaptic currents. **(Right)** Addition of rapamycin (200 nM) produces a marked decrease in bursting and spontaneous synaptic currents. .

C (Left) Spontaneous activity from another cell after addition of bicuculline (10 μ M). This neuron showed less frequent bursts of synaptic activity with much less activity between bursts. **(Right)** Addition of rapamycin (200 nM) did not appear to alter the network activity.

I-V curves for peak inward Na and outward K currents

Figure 5.

I-V plots of Na⁺ and K⁺ currents for five hippocampal neurons. Voltage steps of 20 mV from a holding potential of -80 mV to +60 mV. Experiments were performed in the presence of receptor antagonists for GABA-A (bicuculline (10 μ M)); AMPA (2-amino-5-phosphonopentanoic acid (APV) 50 μ M); NMDA (1,2,3,4-tetrahydro-6-nitro-2,3-di-oxo-benzo[f]quinoxaline-7-sulfonamide (NBQX) 10 μ M). Each point represents mean and SD for 5 neurons. Note lack of affect of rapamycin on the IV plots for either current.