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An Analog of Thyrotropin-Releasing Hormone (TRH) is Neuroprotective Against Glutamate-Induced Toxicity In Fetal Rat Hippocampal Neurons In Vitro

Michael C. Veronesi⁴, Michael Yard^{1,4}, James Jackson², Debomoy K. Lahiri^{1,4}, and Michael J. Kubek^{1,3,4}

¹ Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN 46202

² Department of Pharmacology & Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202

³ Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202

⁴ Program in Medical Neurobiology, Indiana University School of Medicine, Indianapolis, IN 46202

Abstract

TRH has been found to be efficacious in treating certain neurodegenerative disorders such as epilepsy, Alzheimer's disease, neurotrauma and depression, however, its mechanism of action is poorly understood. Since Glutamate (Glu) toxicity has been implicated in these disorders, we utilized primary enriched cultures of rat fetal (E 17) hippocampal neurons to test the hypothesis that an analog of TRH, 3-Methyl-Histidine TRH (3Me-H TRH), given concurrently with Glu would protect such neurons against cell damage and cell death. Cell viability was assessed via Trypan Blue exclusion cell counts and neuronal damage was determined by assaying lactic acid dehydrogenase (LDH) released in the conditioned media. Fetal hippocampal neurons were cultured in neurobasal media for 7 days. On day 7, neurons (10⁶/well) were treated with: control media, 10 μ M 3Me-H TRH, 500 μ M Glu, or 500 μ M Glu with either 10, 1, 0.1, 0.01 or 0.001 μ M 3Me-H TRH. Both media and neurons were harvested 16 hr after treatment. Prolonged exposure to 10 μ M 3Me-H TRH was not toxic to the cells, whereas, neurons exposed to 500 μ M Glu resulted in maximal cell death. Notably, 10, 1 and 0.1 μ M 3Me-H TRH, when co-treated with 500 μ M Glu protected fetal neurons against cell death in a concentration-dependent manner. These results provide support for an important neuroprotective effect of TRH/analogues against glutamate toxicity in primary hippocampal neuronal culture, and implicate a potentially beneficial role of TRH/analogues in neurodegenerative diseases.

Keywords

neuroprotection; Thyrotropin-Releasing Hormone; TRH analog; Glutamate; hippocampus

Corresponding author: Michael J. Kubek, Address: Indiana University School of Medicine, 635 Barnhill Drive, Room MS5035, Department of Anatomy and Cell biology, Indianapolis, IN 46202, Email Address: mj kubek@iupui.edu, Telephone #: 317-274-8204, Facsimile #: 317-278-2040, URL: www.iupui.edu/~kubeklab.

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1. Introduction

Evidence for a neuromodulatory role of Thyrotropin-releasing Hormone (TRH) within the central nervous system has increased considerably over the past twenty-five years. TRH is associated with regulation of the hypothalamic-pituitary-thyroid axis but also functions as a neuropeptide in certain key areas of the brain and other neural tissues [16,50]. TRH is synthesized in neurons, packaged into and stored in vesicles along with classical neurotransmitters. It is released at synaptic terminals and binds, with high affinity, to specific TRH receptors on neurons [50]. TRH and TRH receptors are found in abundance in certain extrahypothalamic brain loci, particularly in the hippocampus, and suggested to have a potential role in neuromodulation [16,37,50]. An increasing body of evidence implicates TRH as an anticonvulsant in regulation of seizure susceptibility [26,27,32,33,36,38,64], and as a neuroprotective agent against Alzheimer's disease [39,40,55], neurotrauma [8,12,13,52] and brain ischemia [63]. These neuropathologies have been closely linked with abnormally high levels of Glutamate (Glu) release, the principle excitatory neurotransmitter throughout the CNS [55].

The TRH analog, 3Me-H TRH (pGlu-3-His-Pro-NH₂), is ten times more biologically active than TRH due to its greater binding affinity for the TRH receptor [57,59]. Additionally, 3Me-H TRH is more metabolically stable than TRH as the addition of the 3Me substitution renders the tri-peptide more resistant to enzymatic degradation [20].

Neurophysiologically, Glu acts by binding to and activating a number of Glu receptors at the synaptic level and controls either fast acting ionotropic channels or longer acting metabotropic second messenger mediated events [6,51,58]. Group I metabotropic receptors have a diverse means of propagating Glu excitotoxicity, which extends over a far greater time period and causes more severe detrimental effects than any of the ionotropic Glu receptors [51]. Thus, the aim of the present study was to investigate the effect of an analog of TRH (3Me-H TRH) on Glu-induced toxicity utilizing a primary fetal (E17) hippocampal cell culture system. We report here that 3Me-H TRH has concentration-dependent neuroprotective effects against Glu toxicity in cultured fetal neurons.

2. Results

2.1 Glutamate toxicity

As shown in Figure 1, all concentrations of Glu exhibited significant reduction in cell viability and a corresponding increase in cell damage relative to untreated controls ($p < 0.05$). Since there was no significant difference between 500 μ M and higher Glu concentrations detected in the cell counts, we utilized 500 μ M in our 3Me-H TRH protection studies. The linear regression lines for comparisons between cell counts vs. Glu treatment (Fig. 1a) in the cell viability assay and LDH release vs. Glu treatment (Fig. 1b) in the LDH assay were 0.8605 and 0.9638 respectively. These results indicate a strong relationship between cell death/damage with administration of increasing Glu concentrations.

2.2 3Me-H TRH Effects on Glutamate-Induced Toxicity

Exposure of primary fetal neurons to 10 μ M 3Me-H TRH for 16 hrs did not cause toxicity when compared to the negative controls ($p < 0.05$) (Fig. 2). In fact, treatment with 10 μ M 3Me-H TRH caused significantly less damage (LDH release) than untreated controls ($p < 0.05$) (Fig. 2b) and a trend toward sustained neuronal viability, although not significant, is also apparent (cell counts) (Fig. 2a). Neurons treated with 10 μ M 3Me-H TRH + Glu resulted in a significant inhibition of cell death (Fig. 2a) and damage (Fig. 2b) when compared to Glu treatment alone. A concentration response in protection was also achieved, according to the cell counts, down

to the 0.1 μM 3Me-H TRH range (Fig. 2a). Linear regression analysis revealed a strong relationship between increasing 3Me-H TRH and maintenance of cell viability ($y = -3.857x + 53.494$, $R^2 = 0.9143$). There was no difference in cell counts between 0.01, 0.001 μM 3Me-H TRH co-treated with 500 μM Glu in comparison with the 500 μM Glu treatment alone (Fig 2a). The LDH assay demonstrated significant 3Me-H TRH protection of Glu damaged neurons at the 10 μM and 1 μM concentrations, while regression analysis revealed a robust relationship between increasing 3Me-H TRH and reduction of cell damage ($y = 0.0123x + 0.0709$, $R^2 = 0.7877$) (Fig 2b).

3. Discussion

The main finding of this study was that an analog of TRH inhibited the effects of Glu-induced toxicity in cultured primary hippocampal neurons in a concentration-dependent manner when assessed by quantitative cell viability and cell damage criteria. Expression of Glu receptors takes approximately a week of development in culture in E17 embryonic hippocampal neurons [3] and, once the Glu receptors are expressed, the cells can become susceptible to excitotoxic shock. This is shown in Figure 1a, wherein a negative, linear relationship exists between viable neurons and increasing Glu concentrations, and in Figure 1b, which shows that loss of neuronal membrane integrity (LDH release) is linearly related to increasing Glu in the cultures. LDH is a ubiquitous cytoplasmic oxidoreductase that oxidizes lactate to pyruvate in the presence of reduced nicotinamide adenine dinucleotide (NADH) [35]. LDH release is associated with plasma membrane damage and is highly correlated with neuronal cell death as assessed by Trypan Blue staining *in vitro* [5,54]. Glutamate-induced neurodegeneration involves a dynamic process associated with elements of both necrosis and apoptosis [2]. The period of peak TRH receptor expression in the rat central nervous system occurs shortly after the first week of birth [1], and TRH receptor mRNA has been localized primarily to ventral hippocampal granule cells, as well as pyramidal and subicular neurons [22]. Since the ontogeny of the TRH receptor parallels the development of Glu susceptibility and the media is known to select for neurons, this culture procedure should facilitate studies aimed at more clearly defining the neuroprotective mechanism of TRH/analog.

Administration of 10 μM of 3Me-H TRH, over 16 hrs, has no apparent adverse effects on fetal hippocampal cells. It is possible that 3Me-H TRH may act trophically to sustain more durable and healthy neurons [25]. Importantly, we observed considerable neuronal death after application of 500 μM of Glu for 16 hrs. This effect was significantly diminished with co-administration of 3Me-H TRH in the 0.1 (20 %) to 10 (39 %) μM range. At concentrations lower than 0.1 μM 3Me-H TRH, protection became less discernable, especially with respect to LDH release, although a trend towards protection is still evident. It is possible that a more complex ligand-receptor interaction occurs with different 3Me-H TRH levels causing altered outcomes upon receptor binding. A supporting example is the finding that NPY reverses its inhibition of Glu release at higher concentrations [19]. Therefore, a more in-depth understanding of the pharmacokinetics of TRH neuronal receptors would be warranted in future studies. It is also possible that the LDH assay is not sensitive enough to detect small differences in levels of LDH. Nevertheless, 0.1 μM approaches the K_d of the TRH receptor, [29] and 10 μM is known to be saturating [23,59]. Therefore, these concentrations provide potential for treatment against pathologies that cause an excess of Glu release.

Taken together, our findings demonstrate that TRH/analog facilitates neuronal viability and protects against neuronal overexcitability. This supports and extends the earlier studies of Jaworska-Feil and coworkers who reported TRH and RGH 2202, a TRH analog, significantly reduced the effects of Kainate-induced excitotoxicity and neuronal loss in the CA₁ and CA₃ regions of the mouse hippocampus [26]. Others have demonstrated concentration-dependant protection by TRH against Glu-induced excitotoxicity in hippocampal slices using a

morphological approach [53]. Furthermore, TRH has been shown to significantly attenuate the kainate-induced LDH release in primary, cortical, cell cultures from rat embryos [26]. Similarly, preliminary studies in our lab revealed that co-application of 3Me-H TRH with Glu protected cultured pituitary adenoma cells (GH3) and pheochromocytoma cells (PC-12) from Glu-induced toxicity [66]. Moreover, TRH administration after eight hours of toxic Glu exposure was found to rescue both GH₃ and PC-12 cells from apoptotic death [65].

Overall, these findings suggest that TRH/analogues protect hippocampal neurons against activation of multiple Glu receptors, since Glu activates ionotropic as well as metabotropic receptors [51]. The mechanism of TRH's neuroprotective role in the central nervous system is not well understood. However, our present findings indicate that it involves modulation of excess Glu effects. TRH/analogues most likely does not act directly at Glu receptors to exert its neuroprotective response but may act through its own selective G-protein coupled receptor (GPCR) and/or via an unknown receptor-independent mechanism [9,10,14]. TRH has been shown to inhibit Glu-stimulated increases in intracellular Calcium $[Ca^{2+}]_i$ in cultured rat cortical cells [34] and selectively depresses Glu-stimulated neuronal activity [56]. Also, TRH produced a decrease in $[Ca^{2+}]_i$ by initiating a 5–20-fold Ca^{2+} efflux in GH3 cultures and HEK 293 cells transfected with TRH receptor cDNA [47].

Sustained exposure of TRH to its GPCR receptors activates phospholipase C (PLC) through $G_{\alpha_{q/11}}$ signaling causing an increase in $[Ca^{2+}]_i$, activation of Protein Kinase C (PKC), and homologous receptor desensitization/downregulation [18,28]. The mGluR 1,5 glutamate receptors use the same signaling cascade [4,46,48,51]. Paradoxically, as shown herein and elsewhere TRH appears not to be toxic [49,53] while mGluR1 mediated PKC activation, along with increases in $[Ca^{2+}]_i$, is known to potentiate the NMDA receptor [60], which has been implicated in Glu excitotoxicity [42,43,51]. Interestingly, it has been shown that when two GPCR's share the same signaling G-protein, downregulation of the homologous receptor can result in desensitization/ downregulation of a heterologous receptor that shares the same signaling G-protein (see [44] for review). For example, heterologous activation of PKC and CaMKII after stimulation of G_{α_q} - coupled M₁ muscarinic receptors can induce rapid desensitization/ downregulation of the mGluR1a receptor in a manner that protects cells against both acute and chronic Glu overstimulation [15,17,21,45]. Moreover, desensitization of mGluR1 receptors has been shown to protect neurons against cell death [7]. Since prolonged exposure to TRH results in desensitization/ downregulation of $G_{\alpha_{q/11}}$ in vitro [61,65], it is possible that TRH/analogues could be neuroprotective, in part, through heterologous downregulation of Group I metabotropic Glu receptors [30].

Finally, the finding that TRH neurons in vivo co-express the mRNA coding for VGLUT2 [24] (a vesicular transporter specific to Glut neurons) combined with recent results from our laboratory that TRH inhibits potassium-stimulated Glu and Aspartate release from hippocampal slices in vitro, raises the possibility that TRH/analogues may be involved in the modulation of vesicular release of Glu [49].

On the other hand, TRH analogues, 3-ARA-57 2-ARA-35b, and 606, that are structurally related to an active TRH diketopiperazine metabolite, protected primary neuronal-glial co-cultures from Glu toxicity by preventing necrosis and apoptosis [13,31]. Thus, TRH/analogues may be acting through anti-apoptotic mechanisms to reverse Glu-induced apoptosis that does not depend solely on a receptor-mediated cascade [12,13,41].

In conclusion, we have demonstrated that TRH/analogues protect cultured, primary, fetal hippocampal cells from Glu-induced toxicity in a concentration-dependent manner *in vitro*. This paradigm should permit in-depth investigations into the downstream molecular mechanism(s) involved in this TRH effect. Moreover, these results further provide impetus for

investigating the potential for TRH-like compounds in treatment of diseases that involve imbalances in Glu homeostasis, including epilepsy, neurotrauma, stroke, ischemia and Alzheimer's dementia [11,16,36,50,62,63].

4. Materials and Methods

4.1 Hippocampal Dissection, Processing and Plating

Timed-pregnant Sprague Dawley rats (17 day) were rapidly asphyxiated via CO₂. Fetuses were removed under sterile conditions using scissors by cutting along the length of the uterus to release the inner sacs from the outer protective layer. Severing of umbilical cord allowed placement of fetus into a dish containing Hank's Buffered Salt Solution (HBSS, Invitrogen, CA). Fetal heads were removed by sharp dissection and placed in a dish containing HBSS. The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Heads were anchored to the bottom of a wax coated dish filled with HBSS for dissection under low-power magnification. A single cut was made using fine-tipped scissors from posterior to anterior near the nose through the skull and meninges on the dorsal aspect of the head, exposing the brain. The brain was removed, fully intact, and the cortical hemispheres were separated along the midline with the forceps. Gentle removal of the overlying cortex revealed the C-shaped hippocampus in each cortical hemisphere. Forceps were used to remove the hippocampus. 10 to 14 brains were dissected per experiment yielding a total of 20–28 hippocampi. All animal procedures were approved by the IACUC and are in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). The hippocampal tissue was centrifuged at 800–900 rpm to isolate the neuronal tissue. HBSS was slowly aspirated from the hippocampal tissue pellet. Two milliliters of Media 1 (see 2.2) were then added to the hippocampal tissue and the tissue was triturated using a fire polished glass pipette. Cells were then counted on a hemacytometer (Hausser Scientific, Horsham PA) under a microscope (20x) and diluted to a density of approximately 10⁶ viable cells per 2 ml Media 1. Cells were plated onto 0.2mg/ml poly-D-Lysine (Sigma, St Louis, MO) coated six-well plates (10⁶ cells/well) and placed into an incubator (37°C, 95% O₂, 5 % CO₂).

4.2 Culture Media

Neurons were grown in serum free medium containing neurobasal supplemented with 2% B-27 (Gibco, Grand Island, NY). This combination has been shown to reduce glia to less than 0.5% [3]. Media 1 consisted of neurobasal medium, B-27 supplement and 200mM L-Glutamine (Sigma) plus basic fibroblast growth factor (BFGF) (50 µM/12 ml) and Normocin (2 µg/ml), an antibiotic, as additional components, which further optimize neuronal growth and select against microbial contamination. [3].

4.3 Cell Harvest

Neurons were cultured for 7 days followed by a 16 hr treatment period. After treatment, 1 ml aliquots of conditioned media were pipetted from each well for Lactate Dehydrogenase (LDH) analysis. Phosphate Buffered Saline (1 ml, pH 7.4) was added to each well and a latex policeman was used to detach the treated cells from the culture plate. The cell suspension was then pipetted into Eppendorf tubes for viability determination by Trypan Blue dye.

4.4 Cell Viability Assay

Immediately following the harvest, 20µl Trypan Blue solution (Sigma-Aldrich, St. Louis MO) was added to 20µl of cell suspension. The cells were counted in a hemacytometer under a 20X magnified light microscope. Cells that were stained blue were considered dead cells and were

not included in the cell count, while cells containing a translucent clear appearance with a spherical morphology were considered live cells and, therefore, were included in the cell count. The depth of the chamber in the hemacytometer was 0.1 mm and the area counted was 1 mm². Therefore, the number of cells in suspension were derived from the number of cells counted in 10⁻⁴ ml (0.1 mm x 1.0 mm² = 0.1 mm³ = 10⁻⁴ ml). Cell death was calculated by comparing the percent survival of untreated, control neurons to the percent survival of drug treated neurons. The resulting equation was: #cells counted / 1x10⁻⁴ ml x 1ml suspension = N cells.

4.5 LDH Assay

Lactate Dehydrogenase (LDH) was measured by an assay kit (Sigma, St Louis) per the manufacturer instructions. LDH was determined colorimetrically by the amount of NADH produced at specific 490nm absorbance using a spectrophotometer (Beckman/Coulter). Standard curves were constructed using LDH enzyme standard (Roche, Indianapolis, IN) (0, 2, 4, 6, 8, 10 ng) in triplicate. Unknowns were diluted such that density readings were on the linear part of the standard curve and corrected for dilution.

4.6 Glutamate Toxicity Determination

After 7 days in culture, the differentiated neurons were removed from the incubator and the media was carefully aspirated from the culture dishes to remove cellular debris. A stock solution of Glu (Sigma, St. Louis) was diluted in Media 1 to yield Glu concentrations of 125, 250, 500, 750 or 1000 µM for treatment. Following 16 hr treatments, cell counts and LDH release were measured to determine the concentration that achieved maximal neuronal death.

4.7 Effect of 3Me-H TRH on Glutamate-Induced Toxicity

3Me-H TRH (Peninsula Laboratories, San Carlos, Ca) dilutions were prepared from a stock solution of 0.1M 3Me-H TRH in distilled water. The stock 3Me-H TRH was diluted with Media 1 to provide a concentration range from 0.001 to 10 µM. To determine if 3Me-H TRH was toxic to the neurons, we treated the cultures with the highest concentration of 3Me-H TRH in the study (10 µM) and compared the results of cell counts and LDH assay to an unconditioned Media 1 treated control group. For 3Me-H TRH protection studies, different concentrations of 3Me-H TRH were co-administered with 500 µM Glu for 16 hrs. LDH and neuron viability results were compared to a negative (untreated) control (Media 1 alone) and positive control (500 µM Glu alone) cultures.

4.8 Data Analysis

Results are expressed as mean $\pm$ SEM and represent between 8 and 50 replications for each data point. Means were analyzed by Student's two tailed t-tests with p<0.05 being considered significant. A linear regression line (R²), using the least-squares method, and its resulting slope (y=mx+b), was calculated for each comparison. A regression value close to 1 indicates a strong relationship between x and y.

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Figure 1. Effect of glutamate on cell viability (a) and damage (b) in cultured fetal (E17) hippocampal neuronal cultures.

Cultured fetal (E17) hippocampal neurons were either untreated (C) or exposed to varying concentrations of Glu for 16 hrs. Subsequently, cell viability was assessed using the Trypan Blue exclusion method (a) and cell damage was determined by measuring LDH release (b). Linear regression analysis revealed a strong negative relationship ($y = -3.993x + 46.52$, $R^2 = .8605$) between cell viability and increasing concentrations of Glu (a). Additionally, linear regression analysis revealed a strong positive relationship ($y = .0278x + .0669$, $R^2 = .9638$) between neuronal damage and increasing concentrations of Glu (b). 500 μ M Glu achieved maximal killing at 42% relative to control (semi-transparent box) and was, therefore, utilized

in the 3MeH-TRH protection studies. Data are expressed as mean $\pm$ SEM and represent between 8 and 50 replications for each treatment.

Figure 2. Effect of 3Me-H TRH on glutamate toxicity as measured by cell counts (a) and LDH release (b) in cultured fetal hippocampal neurons.

Cultured fetal (E17) hippocampal neurons were either untreated or exposed to varying concentrations of 3Me-H TRH and/or 500 μM Glu for 16 hrs. After 16 hrs, cell viability was assessed using the Trypan Blue exclusion method (a) and cell damage was determined by measuring LDH release from cells using the LDH Assay (b). 10 μM 3Me-H TRH alone did not exert a toxic effect on cell viability (a) and significantly ($p < 0.05$) inhibited cell damage (b) relative to untreated control (**). 10, 1 and 0.1 μM 3Me-H TRH, co-treated with 500 μM Glu, significantly ($p < 0.05$) protected neurons from cell death relative to the negative control (a) as indicated by an asterisk (*). 10 and 1 μM 3Me-H TRH significantly ($p < 0.05$) protected neurons

from cell damage relative to the negative control (b) as indicated by an asterisk (*). Linear regression analysis revealed a strong relationship between presence of 3MeH TRH and maintenance of cell viability ($y = -3.857x + 53.494$, $R^2 = .9143$) as shown in (a) and also revealed a strong relationship between presence of 3Me-H TRH and inhibition of cell damage ($y = 0.0123x + 0.0709$, $R^2 = .7877$) as shown (b). Data are expressed as mean $\pm$ SEM and represent between 8 and 50 replications for each treatment