NEUROPROTECTIVE EFFECT OF THYROTROPIN-RELEASING HORMONE (TRH) AGAINST GLUTAMATE TOXICITY *IN VITRO*

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I. INTRODUCTION

Over-activation of both fast, ionotropic and slower metabotropic glutamate receptors during acute central nervous system (CNS) insults has been implicated in the neuropathophysiology of epilepsy, Alzheimer's disease, and CNS trauma (Croucher and Bradford, 1989); (Lee et al., 2000); (Choi et al., 1987a). Glutamate-mediated neurotransmission and excitotoxicity have thus been the defining examples of the duality of the signaling mechanisms which serve the nervous system in health, but which often become the very mediators of neuronal cell degeneration in disease settings (Lee et al., 1999).

Thyrotropin-releasing hormone (TRH) is a well-described neuroendocrine hormone in control of thyroid hormone function, although studies have shown that it may also serve as an important modulator of CNS function. TRH is known to be part of the regulation of the hypothalamic-pituitary-thyroid axis, and also is involved as a neuropeptide in the CNS and other neural tissues (Gary et al., 2003); (Nillni and Sevarino, 1999).

Clinically, TRH has shown therapeutic potential against many neurodegenerative disorders (Kubek and Garg, 2002) suggesting a close association of TRH with excitatory activity. TRH has shown efficacy in affective and cognitive disorders, alcohol withdrawal, and in selected neurodegenerative disorders such as intractable epilepsies, progressive cerebellar ataxia, and acute neurotrauma (Faden et al., 1982b); (Gary et al., 2003); (Kubek and Garg, 2002); (Nie et al., 2005); (Nillni and Sevarino, 1999). However, the mechanism of the TRH neuroprotective effect has not been completely elucidated.

One mechanism of protection against excitatory amino acid (EAA)/glutamate-induced neurotoxicity could include slowing the progression of processes involving the glutamatergic second messenger cascade, which may in fact stop the progression of cell injury and death *in vivo* that occur as a result of this cellular signaling (Choi and Mallet, 1999); (Gary et al., 2003). In addition to the ionotropic glutamate receptors, both glutamate and TRH use metabotropic G-protein coupled receptors to initiate their second messenger signal transduction cascade. Moreover, group I metabotropic glutamate receptors (mGluRs), which induce toxicity, and TRH share identical signal transduction cascades, as described in the following sections.

Receptor downregulation of shared cellular G-protein subunits has been shown as a possible mechanism for heterologous receptor desensitization (Drmota et al., 1998); (Milligan and Green, 1997). This concept will be explored in subsequent sections.

Summaries of the role of glutamate as a neurotransmitter and its association with neurological disorders are presented first, followed by a description of the hippocampus, focusing on TRH and glutamate receptor distribution. TRH biosynthesis, inactivation, and receptor distribution are briefly described, followed by a summary of the role of TRH in neuroprotection. Lastly, since the focus for this work involves the modulation of glutamate excitotoxicity by TRH, our current understanding of heterologous receptor desensitization is presented.

A. Glutamate as a Neurotransmitter

Watkins notes in his 2006 review that in the late 1950s and early 1960s, the first evidence that the amino acid glutamate may serve as a neurotransmitter in the central

nervous system (CNS) came when it and other acidic amino acids were found to serve an excitatory function in many types of central nervous system neurons. An early indicator of the role of glutamate in electrophysiological processes was the observation in the 1950s that glutamate injection into the brain or carotid arteries produced convulsions. It was thus speculated that glutamate was a transmitter in the mammalian CNS (Watkins and Jane, 2006). These findings spurred a massive research effort that eventually established glutamate as the primary excitatory neurotransmitter in the vertebrate CNS.

Glutamatergic synaptic transmission was shown to involve the opening of ligand-gated cation channels via ionotropic glutamate receptors, (iGluRs) that were identified as NMDA, AMPA, and kainate receptor subtypes. Glutamate, it is now known, plays a major role both as a fast excitatory transmitter, and it has been demonstrated that most central neuronal circuits involve glutamatergic neurotransmission at some level (Watkins, 2000); (Conn, 2003).

Until the mid-1980s, the actions of glutamate in the mammalian brain were thought to be mediated exclusively by activation of the glutamate-gated cation channels, which mediate the vast majority of fast excitatory synaptic transmissions in the mammalian brain. Fast synaptic transmission through networks of neurons can be modulated by activation of receptors coupled to second messenger systems through GTP-binding proteins. In a network of neurons connected by glutamatergic synapses, for example, it was generally held that glutamate would elicit fast synaptic responses by activating members of the ionotropic glutamate receptor family. Neuromodulators from extrinsic afferents (e.g., acetylcholine, serotonin, and norepinephrine) could then

modulate transmission through the network of glutamatergic neurons by activating GTP-binding protein-linked receptors and second messenger systems (Watkins and Jane, 2006).

Activation of G-protein coupled receptors (GPCRs) through extrinsic neuromodulatory pathways can elicit a wide variety of effects in glutamatergic circuits, including increases or decreases in neurotransmitter release, changes in neuronal excitability, and changes in rate and patterns of action potential firing, among other changes. These modulatory effects can dramatically increase or decrease net transmission of information through a neuronal circuit (Conn, 2003).

Substances are defined as neurotransmitters if they are substances released at a synapse by one neuron and affect another cell (effector) in a specific functional manner, and therefore they must have a receptor. Glutamate has been shown to depolarize CNS neurons, and systemic administration of glutamate to animals of various species causes acute degeneration of neurons in the retina or several areas of the brain that lack blood-brain barriers (Olney et al., 1971); (Olney and Sharpe, 1969).

Of the amino acids in the adult CNS, L-glutamate and L-aspartate are the most likely candidates for neurotransmitter action at excitatory amino acid receptors, and are used by some of the most widely distributed neuronal types. Both are present in high concentrations in the CNS, and are released in a Ca^{2+}-dependent manner upon electrical stimulation *in vitro*. Both have powerful excitatory effects on neurons when iontophoresed *in vivo*, and high-affinity uptake systems are located on nerve terminals of many neuronal pathways (Watkins, 2000); (Watkins and Jane, 2006).

Additionally, both glutamate and aspartate are acidic amino acid neurotransmitters, which re-enter the cell by a transporter driven by the high extracellular concentrations of sodium (Na^+) and the high intracellular concentrations of potassium (K^+). Sodium enters the cell along with the amino acids, and potassium leaves the cell via a classic antiport mechanism. Thus, glutamate/aspartate entry is indirectly powered by the ATP-driven Na^+-K^+-ase (sodium pump), which creates the high ion concentration gradients. Selective binding sites can be demonstrated by both autoradiographic and pharmacological techniques *in vitro*. Notably, glutamate is the only L-amino acid that has been shown to activate mGluRs (Frauli et al., 2006).

The unequivocal identification of glutamate and aspartate as neurotransmitters was hampered by their involvement in many other functions. For example, glutamate is incorporated into proteins; is involved in fatty acid synthesis; contributes to the regulation of ammonia levels and the control of osmotic and anion balance; and serves as a precursor for GABA and various Krebs cycle intermediates. Not surprisingly, then, glutamate is the most abundant amino acid in the CNS, with a concentration almost six times that of the principal inhibitory neurotransmitter, GABA. The transmitter pool of glutamate may constitute approximately thirty percent of the total glutamate concentration in the CNS (Watkins, 2000); (Watkins and Jane, 2006).

Glutamate and aspartate are potent neuroexcitants, and excess excitatory amino acid activity is suspected in the pathophysiology of many CNS disorders, including the neurodegenerative disorders noted previously (Choi et al., 1987); (Olney et al., 1986a). Historically, the evidence that glutamate and aspartate function as neurotransmitters has

come from the observation that at low concentrations both amino acids function to excite most cells in the CNS. Under physiological conditions the major role of synaptically released glutamate is to interact with receptors on adjacent cells. Receptor activation opens post-synaptic cation channels, which depolarizes the post-synaptic membrane and increases the likelihood that a post-synaptic cell will fire an action potential (Schoepp et al., 1990b).

Glutamate and aspartate physiological studies have revealed that their interactions with receptors are complex, and several subtypes of glutamate receptors are known to exist throughout the CNS. In functional terms, ionotropic glutamate receptors are classified as AMPA- or NMDA-preferring. Both classes are present on most neurons of the CNS, with a few notable exceptions. There is evidence to suggest that AMPA and NMDA receptors are clustered together within the same postsynaptic densities on many cell types. This close apposition results in the simultaneous activation of these receptors. The integration of current flow through the two-receptor systems endows the cell with a powerful means of regulating neuronal excitation (Olney 2000); (Watkins, 2006).

1. Biosynthesis of Glutamate

Glutamate is synthesized and stored within the synaptic endings of the nerve terminal. Glutamate and aspartate are nonessential amino acids that do not cross the blood-brain barrier; therefore, they are not supplied to the brain by the circulation. Instead, they are synthesized from glucose and a variety of other precursors. Synthetic and metabolic enzymes for glutamate and aspartate have been localized to the two main compartments of the brain, neurons and glial cells. Both the synthesis and metabolism of

glutamate and aspartate are more dependent on the interaction between the nerve terminals and the glial cells than are either the synthesis or metabolism of other groups of neurotransmitters (Watkins and Jane, 2006); (Dingledine, 1983).

Glutamic acid is in a metabolic pool with α-ketoglutaric acid and glutamine. Glutamate is released from nerve terminals and, to a large extent, is taken up into glial cells where it is converted to glutamine. Glutamine is believed to be cycled back to the nerve terminal, where it participates in the replenishment of the transmitter pool of glutamate and GABA. Synthesis of transmitter pools of glutamate is likely to involve two major synthetic pathways. In one pathway, the conversion of glutamine to glutamate involves the enzymatic action of glutaminase within the mitochondrial compartment (Watkins and Jane, 2006).

The second pathway involves glutamate formation by a process of transamination, as follows. Newly-synthesized glutamate is packaged and stored in high concentration within synaptic vesicles. After release of glutamate from the nerve terminal into the synaptic cleft, it is taken up into glial cells and converted into glutamine, which itself is then cycled back to the nerve terminal, where it participates in the replenishment of transmitter stores of glutamate (Dingledine, 1983).

Thus, glucose and glutamine are the precursors of glutamate, which is an essential component of cellular metabolism. Ingested proteins are broken down by digestion into amino acids, which are then degraded via transamination, in which the amino group of an amino acid is transferred to an α-ketoacid, typically catalyzed by a transaminase. Notably, α-ketoglutarate, an α-ketoacid, is an intermediate in the citric acid cycle

(Krebs's Cycle), which forms glutamate as the product of transamination (Watkins and Jane, 2006).

Glutamate also plays an important role in the body's disposal of excess or waste nitrogen. Glutamate undergoes deamination, an oxidative reaction catalyzed by glutamate dehydrogenase, resulting in α-ketoglutarate and ammonia, which is then excreted predominantly as urea (Watkins, 2000); (Dingledine, 1983).

Another area of current research involves vesicular glutamate transporters (VGLUTs). Danik and colleagues recently reported their work with vesicular glutamate transporters, describing the VGLUT1 and VGLUT2 distribution both anatomically and temporally during brain development. They found, using RT-PCR, that VGLUT1 and VGLUT2 mRNAs were co-expressed in most of the sampled neurons harvested from rat hippocampus, cortex, and cerebellum at postnatal day P14, but not at P60. Similarly, changes in the concentrations of both VGLUT1 and VGLUT2 mRNA were found to occur in these and other brain areas between P14 and P60, via semi-quantitative RT-PCR as well as via quantitative ribonuclease protection assays (Danik et al., 2005).

Danik found that VGLUT1 and VGLUT2 co-expression in the hippocampal formation is supported further by *in situ* hybridization data, which indicated that almost 100% of the sampled cells from the hippocampal CA1 – CA3 pyramidal and granule cell layers were highly positive for both transcripts until P14 (Danik et al., 2005).

2. Inactivation of Glutamate

Glutamate stimulation is terminated by a chloride-independent membrane transport, which reabsorbs glutamate and aspartate across the pre-synaptic membrane.

Glutamate released into synapses is either reabsorbed directly into neurons by the ion-exchange transport system, or is removed by astrocytes (glial cells), which convert the glutamate into glutamine (a molecule which cannot cause excitotoxicity). Both the pre- and post-synaptic neurons at glutamic acid synapses have glutamic acid-reuptake systems, which quickly lower glutamic acid concentration (Watkins and Jane, 2006).

The acceptance of L-glutamate as a neurotransmitter required that a means to terminate the excitatory signal be identified. Early studies detailing the presence of uptake systems capable of transporting glutamate and aspartate into neurons and glia represented a key step in the overall task of demonstrating that L-glutamate was an endogenous excitatory neurotransmitter. Rapid transport out of the synaptic cleft provided a plausible mechanism, particularly in the absence of a classic degradative enzyme (Bridges and Esslinger, 2005). This transport activity also served as one of the first biochemical markers of excitatory synapses (Cotman et al., 1981). Two of the properties that emerged from these studies that clearly distinguished the uptake of L-glutamate into CNS preparations were a high-affinity for substrate as well as a requirement for sodium (Logan and Snyder, 1972); (Bridges and Esslinger, 2005).

Early indications that glutamate uptake in the CNS was not mediated by a single homogenous system were primarily identified via comparative pharmacological studies, where the potency of inhibitors were observed to vary among CNS preparations (Roberts and Watkins, 1975); (Balcar et al., 1978); (Schousboe and Waagepetersen, 2006); (Takamoto et al., 2002); (Robinson et al., 1993). This issue was resolved when three different high-affinity, sodium-dependent glutamate transporters were cloned and expressed: GLAST (Storck et al., 1992) and GLT-1 (Pines et al., 1992) from rat brain and

EAAC1 (Kanai and Hediger, 1992) from rabbit intestine. Isolation of the homologous transporters from human brain occurred two years later. These were referred to as excitatory amino acid transporters (EAAT) 1, 2, and 3, respectively (Arriza et al., 1994). Subsequently, screens of cDNA libraries from human cerebellum and retina have resulted in the isolation of EAAT4 (Fairman et al., 1995) and EAAT5 (Arriza et al., 1997), respectively. Sequence comparisons among the 5 EAATs indicated that about 50–60% homology exists among the subtypes (Seal and Amara, 1999); (Bridges and Esslinger, 2005).

3. Receptor Distribution in the Hippocampus

The glutamate receptor family is comprised of two major subfamilies, and within these subfamilies are additional categories and subcategories. Glutamate binds to two types of receptors in the synaptic cleft: metabotropic and ionotropic. Notably, metabotropic receptors (mGluRs) do not form an ion channel pore; rather, they are indirectly linked on the plasma membrane with ion-channels through second messenger systems, as described below (Pin and Duvoisin, 1995); (Conn and Pin, 1997); (Schoepp and Conn, 1993).

Direct evidence for the existence of glutamate receptors directly coupled to second messenger systems via G-proteins began to appear in the mid-1980s, when Sladezeck and colleagues were the first to report that glutamate analogues could activate receptors coupled to activation of phosphoinositide hydrolysis in striatal neurons (Sladeczek et al., 1985). Nicoletti and coworkers then demonstrated a similar response in hippocampal slices (Nicoletti et al., 1986b) and cerebellar granule cells, (Nicoletti et al.,

1986a). Sugiyama and colleagues reported in 1987 that expression of rat brain mRNA in *Xenopus* frog oocytes led to the expression of a novel glutamate receptor that is coupled to activation of phosphoinositide hydrolysis (Sugiyama et al., 1987); (Conn, 2003).

At least eight receptor subtypes have now been identified within the metabotropic family, some of which achieve signal transduction through G-protein coupling to a phosphoinositide second messenger system, as described below, and others to an adenylate cyclase system (Schoepp et al., 1994). Metabotropic glutamate receptors (mGluRs) are seven transmembrane G-protein coupled receptors which are found in pre- and postsynaptic neurons in synapses of the hippocampus, cerebellum, and the cerebral cortex, as well as other parts of the brain and peripheral tissues, and are implicated in the regulation of many physiological and pathological processes in the CNS, including synaptic plasticity, learning and memory, motor coordination, pain transmission, and neurodegeneration.

These mGluR subtypes are classified into three major groups on the basis of sequence homologies, coupling to second messenger systems, and selectivities for various agonists (Conn and Pin, 1997). Group I mGluRs, which include mGluR1 and mGluR5, couple primarily to G_{α} and increases in phosphoinositide hydrolysis. Group II mGluRs (mGlu2 and mGlu3), and group III mGluRs (mGlu4, 6, 7, and 8) couple to $G_{i/o}$ and associated effector pathways such as inhibition of adenylyl cyclase (Conn, 2003). This second messenger cascade of the mGluRs is important when one considers the potential of downregulating the heterotrimeric G-proteins through the use of substances,

such as TRH, which use the same second messenger cascade, namely G_{α}, as will be described.

The ionotropic receptors, originally identified by Watkins (Watkins et al., 1990), are N-Methyl-D-Aspartate (NMDA) and non-NMDA (AMPA and kainate, KA) receptors. NMDA receptors are most densely concentrated in the cerebral cortex (hippocampus, especially the CA1 region), amygdala, and basal ganglia. They are particularly vulnerable to glutamate excitotoxicity; i.e., damaging effects due to excessive excitatory neurotransmitter release. The mRNAs encoding NMDA receptor subunits are differentially distributed (Kew and Kemp, 2005); (Watkins and Jane, 2006).

The distribution of gluatamate receptors within the hippocampus specifically is described in a subsequent section.

4. Receptor Physiology

Neurophysiologically, glutamate acts by binding to and activating a number of Glu receptors at the synaptic level, and controls either the fast-acting ionotropic channels or the longer-acting metabotropic second messenger-mediated events (Conn and Pin, 1997); (Pin and Duvoisin, 1995); (Schoepp et al., 1999).

Regarding the fast-acting ionotropic glutamate receptors, it should be noted that NMDA is not naturally found in biological systems, but it binds specifically to the ionotropic NMDA glutamate receptor with higher specificity than glutamate. The NMDA receptor is actually the only known receptor that is regulated both by a ligand (glutamate) and by voltage. Five binding sites have been discovered, which regulate NMDA receptor

activity, including sites for (1) glutamate (2) glycine (3) magnesium (4) zinc and (5) a site that binds the hallucinogenic substance phencyclidine (PCP).

Conversely, the delayed process has been studied primarily in cerebrocortical neuronal cultures, and has been monitored primarily by biochemical measurement of lactic dehydrogenase (LDH) release as an indicator of cellular membrane damage. This has been described as a calcium-dependent and NMDA receptor-mediated process, which leads to cell death (confirmed via LDH release) in the 16-24 hr time interval. Romano adapted the chick embryonic retina model to allow incubation for up to 24 hrs and employed both LDH analysis and histological analysis to monitor cell death (Romano et al., 1995a); (Romano et al., 1995b).

The results of the study by Romano did not support the concept of a delayed cell death process that is separate and distinct from excitotoxic cell death. Rather, by morphological analysis, the cells show signs of acute but quite advanced degeneration within two hours after exposure to an excitotoxic agonist, but release of LDH is a slowly evolving phenomenon that occurs on a delayed basis primarily in the 16-24 hr interval after the degenerating neurons have shown morphological signs of advanced deterioration for many hours (Chen et al., 1999); (Olney et al., 2002).

The potential interaction between group I mGluRs and NMDA receptors in the mediation of post-traumatic neuronal death was studied using an *in vitro* trauma model. Treatment with group I mGluR antagonists provided significant neuroprotection either in the presence or absence of an NMDA receptor antagonist. Conversely, treatment with only a group I mGluR agonist significantly increased the amount of damage; and this

damage was significantly, but incompletely, reduced in the presence of an NMDA receptor antagonist. It was thus shown that the effects of group I mGluR activation on post-traumatic cell death are mediated only in part through NMDA receptor modulation, and suggested at the time that group I mGluR antagonists may have therapeutic potential (Mukhin et al., 1997).

Glutamate is involved in many important neurological functions, but this discussion will concentrate on its role in excitotoxicty, and further develop that role through the experiments, which were conducted in cell, slice, and neuronal systems. The major inhibitory and excitatory neurotransmitters in the mammalian CNS are GABA and glutamate, respectively. Schoepp states that the balance of excitation/inhibition within neuronal circuits is highly dependent on postsynaptic activation of ionotropic receptors for these ligands ($GABA_A$ or AMPA/kainate/NMDA receptors).

The related proteins, $GABA_B$ and mGluRs, function in nervous tissue to recognize these ligands, but they serve a more modulatory role in the control of excitation/inhibition (Schoepp, 2001). Glutamate is the transmitter of the vast majority of the fast excitatory synapses in the mammalian CNS and plays an important role in a wide variety of CNS functions (Hollmann and Heinemann, 1994).

The search for mGluR-related cDNA resulted in the isolation of seven other genes and several splice variants that encode mGluRs (Tanabe et al., 1992); (Pin et al., 1992); (Nakajima et al., 1993); (Okamoto et al., 1994); (Saugstad et al., 1997); (Joly et al., 1995); (Minakami et al., 1994); (Duvoisin et al., 1995); (Iversen et al., 1994); (Laurie et al., 1996). These receptors are named mGluR1 through mGluR8 (Conn and Pin, 1997).

These eight different types of mGluRs are divided into groups I, II, and III. Metabotropic glutamate receptors ($mGluR_{1-8}$) function to modulate brain excitability via pre-synaptic, post-synaptic, and glial mechanisms, and each consists of G-protein coupled receptors (GPCRs). There is reportedly an approximately 70% sequence homology within each mGluR receptor group; whereas, between the subgroups there is lesser (40%) homology (Schoepp, 2001).

In general, it is believed that mGlu receptors have evolved as modulatory mechanisms to control CNS excitability. Group I mGlu receptors include mGluR1 and mGluR5, which when expressed are coupled via G_q to phospholipase-C. Group II (mGluR2 and mGluR3) and Group III (mGluR4, 6, 7, and 8) receptors are coupled to G_i and inhibit stimulated cAMP formation when expressed in cell lines (Schoepp, 2001).

The mGluRs in group I, including mGluR1 and mGluR5, are stimulated most strongly by the excitatory amino acid analog L-quisqualate. They are present presynaptically, postsynaptically, and also on glia. Stimulating these receptors causes an associated phospholipase-C (PLC) molecule to hydrolyze phosphoinositide phospholipids (IP3).

Group I metabotropic receptors have a variety of means whereby they propogate glutamate excitotoxicty, extending over more time than ionotropic events, thus resulting in more serious damage than that caused by the activation of the ionotropic receptors (Pin and Duvoisin, 1995). Additionally, there is biochemical and electrophysiological evidence that groups I, II, and III play a role in the modulation of glutamate release (Anwyl, 1999); (Cartmell and Schoepp, 2000).

The group I mGlu receptors are primarily postsynaptic, functioning to enhance cellular excitability via interactions with other postsynaptic processes, such as ionotropic receptors and ion channels (Bordi and Ugolini, 1999); (Schoepp, 2001). Some biochemical evidence exists for a presynaptic role for the mGlu receptors, but group II and group III mGlu receptor subtypes predominate on presynaptic sites, where they regulate glutamate release (Schoepp, 2001).

According to Conn and Pin, the discovery of mGluRs dramatically altered the traditional view of glutamatergic neurotransmission, since activation of mGluRs can modulate activity in glutamatergic circuits in a manner previously associated only with neuromodulators from nonglutamatergic afferents. Unlike receptors for monoamines and other neuromodulators, however, the mGluRs are believed to provide a mechanism by which glutamate modulates synaptic activity at the same site at which it elicits fast synaptic responses (Conn and Pin, 1997).

Additionally, mGluRs have the potential to participate in a wide variety of functions of the CNS, due to the widespread distribution of glutamatergic synapses. Finally, the wide diversity and heterogeneous distribution of mGluR subtypes provide an opportunity for developing pharmacologic agents that selectively interact with mGluRs involved in only one or a limited number of CNS functions. Thus, it is widely believed that gaining a detailed understanding of the specific roles of mGluRs will probably have a major impact on development of novel treatment strategies for a variety of neurologic disorders (Conn and Pin, 1997).

The first mGluR cDNA was cloned independently by two groups that used the same functional expression assay and is now generally named mGluR1a (Masu et al., 1991); (Houamed et al., 1991). Its deduced amino acid sequence revealed that this receptor shares no sequence homology with any other G-protein coupled receptor (GPCR), which suggests that it could be a member of a new receptor gene family. Pharmacologic studies suggested that several G-protein coupled glutamate receptors existed (Schoepp et al., 1990a).

B. Neurological Disorders Associated with Glutamate Toxicity

Glutamate and its structurally related ligands, in addition to their powerful excitatory effects at glutamate receptors, are potent neurotoxins. Glutamate and other amino acids were first recognized as neurotoxic agents in the 1970s, when they were given orally to immature animals. Acute neurodegeneration was observed in those areas not well protected by the blood-brain barrier, notably the arcuate nucleus of the hypothalamus.

Glutamate and specific excitatory amino acid (EAA) analogs cause acute neuronal degeneration by excessive stimulation of postsynaptic EAA receptors through which glutamate functions physiologically as a transmitter (Olney, 1989); (Olney, 2003). It is often maintained that calcium influx or intracellular calcium mobilization may be the single most important factor responsible for triggering the cascade of pathophysiological events that lead to excitotoxic cell death (Olney et al., 1999); (Kew and Kemp, 2005).

Based on early *in vitro* studies, two separate forms of excitotoxic cell death have been identified, an acute form and a delayed form. Acute excitotoxic cell death has been described as occurring within minutes or a few hours, and is associated with uptake of sodium, chloride, and water, resulting in rapid swelling of the neuron with cell death occurring by lysis, studied primarily by morphological methods in isolated chick embryo retinas (Olney, 1982).

Choi and colleagues in the late 1980s demonstrated the ionic dependence of glutamate toxicity, demonstrating that morphological changes occur in neuronal culture after exposure to toxic glutamate, separated into two components. The first component is marked by neuronal swelling, occurs early, and is dependent on extracellular chloride and sodium ions. The second component, which occurs later, is distinguished by neuronal disintegration, and is dependent on extracellular calcium.

Choi stated that, while either component could produce neuronal injury, the calcium-dependent mechanism predominated at exposure to lower gluatamate levels (Choi, 1987). Choi demonstrated a dose-dependent effect of glutamate in cortical cell cultures, using micromolar concentrations, which were less than 1% of the millimolar concentrations described more than fifty years ago as present normally in whole cortex (Waelsch, 1951); (Choi et al., 1987).

The mechanisms of neurodegeneration are divergent and activation of most classes of glutamate receptor has been implicated. Neurodegeneration following an ischemic insult may involve mechanisms resembling a pathological exaggeration of LTP-type phenomena; i.e., the neuronal insult results partially from AMPA receptor activation

with simultaneous NMDA receptor activation and Ca^{2+} influx. A close correlation exists between neurotoxic potency and affinity of glutamate receptors for a range of agonists. Thus, the more able a compound is to depolarize neurons, the greater the likelihood of that agent causing neuronal toxicity (Allen et al., 1999); (Simon et al., 1984).

Many acute CNS injuries, by definition, involve an excitotoxic mechanism, since excitotoxicity is of primary significance in triggering the cell death process. It is the classical type of excitotoxic mechanism that is believed to be involved in acute CNS injury syndromes, and excitotoxins of both exogenous and endogenous origin have been implicated (Olney, 2002). Excitotoxicity is a phenomenon typically encountered in neurons following a stimulation that exceeds the physiologic range with respect to duration or intensity. Typical excitotoxic mediators are acetylcholine or, most importantly in the CNS, glutamate.

Many chronic neurodegenerative diseases also have been found to have an excitotoxic component (Leist et al., 1999); (Choi, 1992); (Meldrum and Garthwaite, 1990). Additionally, a causal contribution of excitotoxicity to neuronal damage has been established in stroke or head trauma (Lipton and Nicotera, 1998); (Tamargo et al., 1993), as well as in acute poisoning phenomena, such as exposure to carbon monoxide (Ishimaru et al., 1999); (Olney et al., 2000); (Koliatsos et al., 2004).

Generally, excitotoxicity is induced by conditions favoring glutamate accumulation in the extracellular space, and it is enhanced by conditions (such as energy depletion) that hinder cellular protective mechanisms (Novelli and Tasker, 2000). Typical conditions leading to increased extracellular glutamate concentrations (Bullock et al.,

1995); (Rothman, 1900); (Drejer et al., 1985) are depolarization of neurons, energy depletion due to hypoglycemia or hypoxia (O'Dowd et al., 2000); (Wieloch et al., 1984), or defects in the glutamate reuptake systems (Volterra, 1994).

A number of major acute CNS injury syndromes, including those associated with stroke, hypoglycemia, and status epilepticus, are believed to be triggered by excessive activation of glutamate receptors by endogenous excitotoxins, primarily glutamate, but probably also aspartate, and possibly sulfur-containing amino acids, thus called *excitatory amino acid (EAA) toxicity*. Excessive release and/or impaired uptake of endogenous excitotoxins occur in each of these conditions that lead to an extracellular accumulation of these toxins, and to acute degeneration of neurons (Olney, 1969); (Olney et al., 1971); (Lucas et al., 1985).

Thus, excess glutamate triggers excitotoxicity, causing neuronal damage and eventual cell death, particularly when NMDA receptors are activated. This may be due to high intracellular Ca^{2+} exceeding storage capacity and damaging mitochondria, leading to apoptosis, or to Glu/Ca^{2+}-mediated promotion of transcription factors for pro-apoptotic genes, or to downregulation of transcription factors for anti-apoptotic genes. This contribution of synaptic activity to neurotoxicity, the release of glutamate and receptor stimulation on membranes post-synaptically, was noted by direct observations, which showed that glutamate is neurotoxic *in vivo* when injected directly (Olney, 1969); (Olney et al., 1971); (Lucas et al., 1985).

Inhibition of the neurotransmission by Mg^{2+} or glutamate antagonists was found to protect neurons from hypoxic damage (Rothman and Olney, 1995), and electrically

stimulating neurons in the hippocampus stimulated glutamate-induced increases of calcium concentrations within the same cell (Tong et al., 1989). Thus, there appears to be a very close reciprocal relationship between increased extracellular glutamate concentrations and increased calcium concentration, which may result in neuronal death under excitotoxic conditions (Koliatsos et al., 2006).

The initial thought that calcium may be excitotoxic dates back to Fleckenstein's suggestion in 1968 that excessive entry of calcium into cardiomyocytes may be the underlying mechanism of cardiac pathology following ischemia (Fleckenstein-Grun et al., 1992). Since the 1980s, the relationship between calcium and cell death has been examined intensively, especially in hepatocytes, the kidney, and the brain (Nicotera et al., 1986); (Nicotera et al., 2000); (Nicotera and Leist, 1997); (Trump et al., 1997); (Trump and Berezesky, 1996); (Siesjo, 1989); (Siesjo et al., 1989). The key role of calcium in excitotoxicity is suggested by three different lines of evidence (Siesjo, 1989); (Dubinsky and Oxford, 1985); (Choi, 1994).

First, there is an obvious increase in calcium concentration both in *in vivo* and *in vitro* models of excitotoxic cell death, observed in ischemic brain (Simon et al., 1984b); (Simon et al., 1984a) or in brain slices exposed to NMDA receptor agonists or anoxia (Meldrum and Garthwaite, 1990b); (Kass and Lipton, 1986). Also, glutamate-stimulated calcium influx has been shown directly in cultured neurons (Bruno et al., 1998), and increased calcium has been detected repeatedly after NMDA receptor stimulation, using fluorescent probes. *In vivo* microelectrode measurements have demonstrated an 80-90%

decline of extracellular calcium during ischemia, with a corresponding increase of intracellular calcium (Kristian et al., 1993); (Kristian and Siesjo, 1998).

Secondly, decreasing calcium entry into the neuron by removal of extracellular calcium (Choi and Hartley, 1993); (Hartley and Choi, 1989); (Gabellini et al., 1994); (Meldrum and Garthwaite, 1990); (Rothman and Olney, 1995), depletion of NMDA receptors, pharmacological inhibition of glutamate receptor subtypes or of VDCCs (Sucher et al., 1991b); (Sucher et al., 1991a) prevented death in many attempts at excitotoxicity (Koliatsos et al., 1993).

Third, prevention of neurotoxicity by the inhibition of calcium-dependent downstream effects most strongly supports a causal role for calcium in neurotoxicity. Intracellular calcium chelators have been found to protect against both ischemic damage *in vivo* and excitotoxic neuronal damage *in vitro* (Tymianski et al., 1993); (Tymianski et al., 1994).

Furthermore, cultured neurons are protected against excitatory amino acid toxicity by inhibiting effectors of calcium toxicity such as calmodulin inhibition (Marcaida et al., 1997); (Marcaida et al., 1996).

1. Dementias

Glutamate overstimulation occurs as part of the ischemic cascade, and has been associated with dementias, including Alzheimer's disease.

Alzheimer's disease is a neurodegenerative disease with poorly defined pathogenic mechanisms. The actions of amyloid-beta (Aß) peptides derived from

amyloid precursor proteins (APP) (Mattson, 2006) have been implicated as a cause of neuropathological changes. The neurodegeneration observed at autopsy shows apoptotic features. Apoptosis is also evoked *in vitro* by exposure of neuronal cultures to Aß (Forloni et al., 1989). The mechanism of Aß toxicity *in vitro* may involve excessive calcium entry into cells. Antigenic changes of cytoskeletal elements typical of Alzheimer's Disease are mimicked by excessive calcium influx into cultured hippocampal neurons (Mattson, 2006) and blocking receptor-mediated calcium entry prevents Aß toxicity.

Additionally, Aß-induced disturbances of calcium homeostasis in hippocampal neurons is corrected by pretreatment of those cultures with various peptide mediators such as fibroblast growth factor (Mattson, 2006) or tumor necrosis factor (Leist et al., 1997); (Leist and Nicotera, 1998b). An hypothesis by Olney and colleagues explains neurodegeneration in Alzheimer's disease. This hypothesis posits an interaction between genetic factors (unfavorable ApoE genotype and Aß-linked genetic mutations) and a form of excitotoxicity that occurs spontaneously in the brain under conditions in which ionotropic NMDA glutamate receptors are blocked or impaired (Olney et al., 1997).

2. Affective Disorders and Schizophrenia

Many recent studies have indicated that modulating the glutamatergic system may be an efficient method of achieving an antidepressant effect. Palucha and colleagues presented data suggesting that mGluRs, since they are involved with long lasting modulatory effects on glutamatergic neurotransmission, are a potential target for the development of drugs which may be useful in the treatment of several CNS disorders,

including depression and anxiety. Palucha and Pilc note that the mGluR5 antagonists and group II mGluR antagonists seem to be the most promising compounds, with potential antidepressant-like activity in rodents. They do note that the efficacy of mGluR ligands in the clinical setting, however, is still an unanswered question (Palucha, 2006); (Palucha and Pilc, 2002); (Palucha and Pilc, 2005).

Wieronska and colleagues have shown that the level of mGluRs in rat brains after chronic mild stress was increased in the CA1 and decreased in the CA3 region of the hippocampus, via Western blotting assays for the level of the mGluR5 protein. Their results indicated that mGluR5 may be involved with the mechanism of depression (Wieronska et al., 2001).

Recent research suggests a combined glutamate/dopamine hypothesis to explain the pathophysiology of schizophrenia. This hypothesis, like the hypothesis pertaining to Alzheimer's disease, describes NMDA glutamate receptor hypofunction as a key mechanism.

An integrative model that has also been addressed is the glutamate receptor dysfunction model of schizophrenia (Olney et al., 1999). It should be noted that a psychosis similar to schizophrenia is induced by phencyclidine (PCP), a non-competitive NMDA receptor blocker (Arnold et al., 1973) which causes hallucinations and delusions, as well as an associated apathetic state and a formal thought disorder, which are both distinctive schizophrenic features. Interestingly, ketamine, an anesthetic agent that binds to the PCP site of the NMDA receptor, rarely induces psychosis in children, while psychosis occurs upon emergence from anesthesia in many young and middle-aged adults. This age dependency of the response to glutamate antagonists is also seen in rats,

which become susceptible to the neurotoxic effects of NMDA antagonists between puberty and adulthood (Arnold, 1999).

The precise nature of glutamatergic dysfunction in schizophrenia is yet to be determined, however, since postmortem findings are difficult to interpret and are extremely diverse. Decreased glutamatergic synaptic activity has been suggested by reports of increased glutamate uptake and kainate receptor binding in the prefrontal cortex of patients with schizophrenia (Deakin et al., 1989); whereas, kainate receptor binding has been reported to be decreased in the putamen, hippocampus, and parahippocampal gyrus (Kerwin et al., 1988).

In an animal model that explores glutamate excitotoxicity in relation to other proposed neuropathological features of schizophrenia, Bardgett and colleagues studied the effects of ventricular kainate injection in rats (Csernansky et al., 1996); (Bardgett et al., 1995). They observed a dose-dependent loss of neurons that was regionally specific for the ventral and dorsal hippocampus, thalamus, and piriform cortex. An increase in dopamine receptor binding was also noted in the nucleus accumbens, which probably represented a neurochemical response to denervation of limbic control afferents after their destruction; and behavioral hyperactivity. Thus, by creating a diffusely excitotoxic state, the investigators were able to induce specific limbic system neuropathology and neurochemical alterations similar to those seen in human postmortem studies.

3. Epilepsy

Involvement of excitatory amino acids in epilepsy is well documented. A large number of animal models of epilepsy have indicated a causal role for the glutamate

receptor family. An increased sensitivity to the action of glutamate at NMDA receptors is seen in hippocampal slices from kindled rats and in cortical slices from, or adjacent to, cortical foci in human epilepsy (Hwa and Avoli, 1991). This leads to an enhanced entry of Ca^{2+} into neurons during synaptic activity that can be detected with ion-specific microelectrodes (Pumain et al., 1987). Excessive stimulation of glutamatergic pathways or pharmacological manipulation resulting in glutamate receptor activation can precipitate seizures. Evidence from human tissue supports the role of amino acids in epilepsy.

Epilepsy is one of the most common neurological disorders affecting mankind. It is a syndrome of unpredictable spontaneous recurrence of seizures, first described in Hippocrates's "On the Sacred Disease" over 2500 years ago. Its prevalence ranges from 1.5 to 19.5 per 1000, depending on the geographical area and the ethnic group (Hauser et al., 1998). Compared with marked improvements in diagnostic and treatment modalities, however, the understanding of the basic pathophysiological mechanisms of epilepsy remains rather elusive.

The term "epileptic brain damage" has traditionally been used to refer to the selective pattern of damage found in patients with chronic epilepsy. More recently, it is also used to refer to the similar pattern of damage described in animals or humans after prolonged seizures. Since Bouchet and Cazauvieilh first noted that one or both Ammon's horns in the brains of some epileptic patients were small and "hard," the association of epilepsy with selective neuronal death and structural changes in the hippocampus has been recognized by many neuropathologists.

Glutamate has absolutely been implicated in epileptic seizures. Microinjection of glutamate into neurons produces spontaneous depolarizations around one second apart, and this firing pattern is similar to what is known as paroxysmal depolarizing shift in epileptic attacks. It has been suggested that a drop in resting membrane potential at seizure foci could cause spontaneous opening of voltage-operated calcium channels (VOCCs), leading to glutamate release and further depolarization. It is likely that both apoptosis and necrosis may occur in the same neuronal population in association with the epileptic damage. The mode of neuronal death may be determined by the severity of the insult, different environmental factors (growth factors, synaptic connections) and the susceptibility of each neuron, involving glutamate receptor subtypes/subunits, threshold of activation, and calcium-buffering capacity (Bonfoco et al., 1995).

Excessive electrical discharges induce massive release of excitatory neurotransmitters that activate receptors, open ionic channels, and presumably induce an excessive increase in intracellular calcium. Excitotoxicity appears to mediate neuronal death induced by epileptic seizures for three reasons.

First, the pattern of selective damage has a close relationship with the network across which seizure activity is propagated; for example, limbic seizures produce damage in a wide variety of nuclei involved in limbic seizure activity (Collins and Olney, 1982). Second, the cytopathological changes occurring in vulnerable hippocampal or cortical neurons are quite similar to the changes induced by glutamate, kainate, or other excitotoxic agents. Third, NMDA antagonists can protect against the acute neuronal

damage induced by epileptic seizures in various animal models (Fariello and Garant, 1992).

Thus, it appears that selective neuronal death likely plays an important role in this process in humans. Further insight into the mechanisms involved may provide a window of opportunity for the prevention of epilepsy.

4. Trauma

A number of major acute CNS injury syndromes, as indicated, including those associated with stroke (Rothman and Olney, 1986); (Faden et al., 1982a); (Ikonomidou et al., 1996), trauma (McDonald et al., 2000), hypoglycemia, and status epilepticus are believed to be triggered by excess activation of glutamate receptors by endogenous excitotoxins; primarily glutamate, but probably also aspartate, and possibly certain sulfur-containing amino acids. Excessive release and/or impaired uptake of endogenous excitotoxins occur in each of these conditions, leading to extracellular accumulation of excitotoxins at EAA receptors, and subsequent acute degeneration of neurons by the described excitotoxic mechanism (Rothman and Olney, 1986); (Faden et al., 1982a).

In head trauma, increased intracranial pressure is a major source of morbidity and mortality. An excitotoxic mechanism may play a major role in raising the intracranial pressure. An excessive stimulation of EAA receptors causes the hyperstimulated neurons and the surrounding astroglia to accumulate intracellular fluid, transferring a large volume of fluid from the vascular to the intracellular compartment. Depending on the severity of the trauma, hundreds of thousands or millions of CNS cells will participate in this pathological process (Olney et al., 1986a); (Faden et al., 2005); (Faden, 1993).

5. Stroke and Other Disorders

Excitotoxicity caused by glutamate toxicity is a major destructive process seen in strokes and other forms of brain ischemia, although the relationship is complex. Prolonged periods of anoxic insult to neuronal tissue, such as during cardiac arrest or thrombotic stroke, often result in ischemic cell damage and neurotoxicity. Oxygen depletion precipitates a depletion of energy stores within neuronal and glial cell compartments, with a concomitant acidosis and release of free radicals. The depletion of energy stores affects cellular metabolism, energy-dependent ion pumps, and the ability of cells to maintain resting membrane potential.

Consequently, depolarization of cells results in action potentials, and the subsequent release of glutamate from pre-synaptic terminals. The released glutamate then activates postsynaptic AMPA and NMDA receptors. The entry of Ca^{2+} through the NMDA receptor complex and voltage-sensitive Ca^{2+} channels increases the intracellular concentration of Ca^{2+}. This elevation of intracellular Ca^{2+} will trigger a cascade of second-messenger systems, many of which remain activated long after the initial stimulus is removed (Obrenovitch, 1999); (Obrenovitch et al., 2000); (Obrenovitch and Richards, 1995); (Obrenovitch and Urenjak, 1998).

This inability of the neurons to maintain a resting potential precipitates a positive feedback loop that leads to neuronal cell injury or death. The two regions most heavily damaged by interruption in blood supply are the hippocampus and striatum.

The demonstration that neuronal injury could be reduced by antagonists of excitatory neurotransmission during recovery from global ischemia has been a major factor driving neuroprotection research. These studies provided convincing evidence that

ischemic neuronal injury was reversible well after reperfusion, allowing for a "therapeutic window." Global ischemia represents only one type of clinical ischemia, affecting a region of the brain, which, while critical, represents a small portion of the human brain. Early studies concluded that ischemic injury occurs when energy failure results in a massive release of glutamate into the extracellular space.

Several subsequent studies have shown that the protective effects of glutamate receptor antagonists can be observed long after the acute ischemic glutamate release has ended, and the classic signs of glutamate toxicity are not observed early in ischemia (Obrenovitch et al., 2000); (Obrenovitch, 1999); (Obrenovitch and Richards, 1995); (Obrenovitch and Urenjak, 1998). Some researchers suggest that the protective effects of glutamate antagonists may be due to hypothermia rather than receptor blockade under certain conditions (Vornov et al., 1998); (Vornov and Coyle, 1991).

As an aside, neurolathyrism is a crippling motor neuron disorder caused by chronic ingestion of a legume which contains beta-N-oxalylamino-L-alanine (BOAA or ODAP), an acidic amino acid with powerful excitotoxic properties mediated through non-NMDA glutamate receptors (Olney et al., 1986b). This spastic disorder occurs in East Africa and India. The action of BOAA, identified as the toxin in this chickpea, acts at AMPA receptors in the spinal cord, resulting in observed degeneration of lower and upper motor neurons.

If chronic exposure to BOAA, an exogenous excitotoxin, acts selectively at non-NMDA glutamate receptors and can cause motor neurons to degenerate slowly over months or years, it may be possible for *endogenous* glutamate acting at the same

receptors to cause chronic degeneration of motor neurons in idiopathic motor neuron disorders such as amyotrophic lateral sclerosis (Olney, 1990a); (Olney, 1990b).

Interestingly, one alternative hypothesis for the pathogenesis of Parkinson's Disease, in which nigro-striatal dopaminergic neurons are lost, is that excitotoxic mechanisms, possibly acting in concert with oxidative stress (Coyle et al., 1981) or impaired energy metabolism (Albin and Greenamyre, 1992) may play a role. These factors have been postulated to underlie a number of chronic, neuron phenotype-specific neurodegenerative disorders.

The evidence, however, is minimal, although the study by Turski is often quoted, whereby NMDA antagonists protected the substantia nigra from direct injection of DA-specific neuronal toxins (Turski et al., 1987). The finding could not be replicated, however, and the number of NMDA receptor binding sites in the substantia nigra is low (Sonsalla et al., 1998). Other work is continuing in this field, involving spontaneous degeneration of the substantia nigra neurons in a mutant Weaver mouse, which may involve excitotoxic mechanisms (Slesinger et al., 1996).

Both exogenous and endogenous excitotoxins are implicated in acute CNS injury syndromes. Domoate encephalopathy is an excellent example of an acute CNS injury syndrome caused by human exposure to an exogenous excitotoxin. In 1987, an outbreak of food poisoning in Canada affected 145 people, some of whom did not survive (Olney et al., 1990). Some survivors sustained permanent brain damage and continued to show signs of cognitive deficits, especially impairment of memory, many months after the initial incident. All of the afflicted individuals had eaten mussels which had high concentrations of domoate, an excitotoxic analog of glutamate similar to kainate, which

produces persistent seizures (status epilepticus) in adult rats (Stewart et al., 1990). Status epilepticus was a prominent feature in the symptomatology displayed by the poisoned individuals who ate the mussels. Primarily aged adults displayed the seizure activity after the domoate poisoning, leading to the conclusion that the aged brain may actually display a kainate receptor, which is a potentially sensitive mediator of excitotoxicity.

Notably, the brain damage induced by kainate in rats can be prevented by pretreatment with an NMDA antagonist (Clifford et al., 1990). Thus, it has been postulated that although the persistent seizure activity induced by either domoate or kainate is triggered by activation of kainate receptors, it is the excessive seizure-mediated release of an excitotoxic transmitter, presumably glutamate, at NMDA receptors that is apparently responsible for most of the brain damage that ensues (Olney, 2002); (Olney and Farber, 1995).

Additionally, disorders of excitatory amino acid transmission have been implicated in amyotrophic lateral sclerosis (ALS) and in the chronic neurodegenerative diseases olivopontocerebellar atrophy and Huntington's chorea.

C. Thyrotropin-Releasing Hormone (TRH) Protirelin

1. Biosynthesis of TRH

Protirelin (TRH) (**Figure 1**) is a 3-amino-acid neuropeptide with the highest density of its receptors (TRH R-1 and TRH R-2) throughout the CNS, highly expressed in pituitary, hippocampal dentate gyrus, and amygdalae (Heuer et al., 2000); (Kubek et al., 1977); (Nillni and Sevarino, 1999); (Sharif, 1989). TRH and its analogues have been

shown to act as modulators in many classical neurotransmitter systems, including norepinephrine (Keller et al., 1974); (Rastogi et al., 1981), acetylcholine (Narumi et al., 1983); (Toide et al., 1993), GABA (Mouginot et al., 1991), serotonin (Rastogi et al., 1981); (Sattin et al., 1992), dopamine (Bennett et al., 1989); (Marsden et al., 1989), and glutamate (Renaud and Martin, 1975).

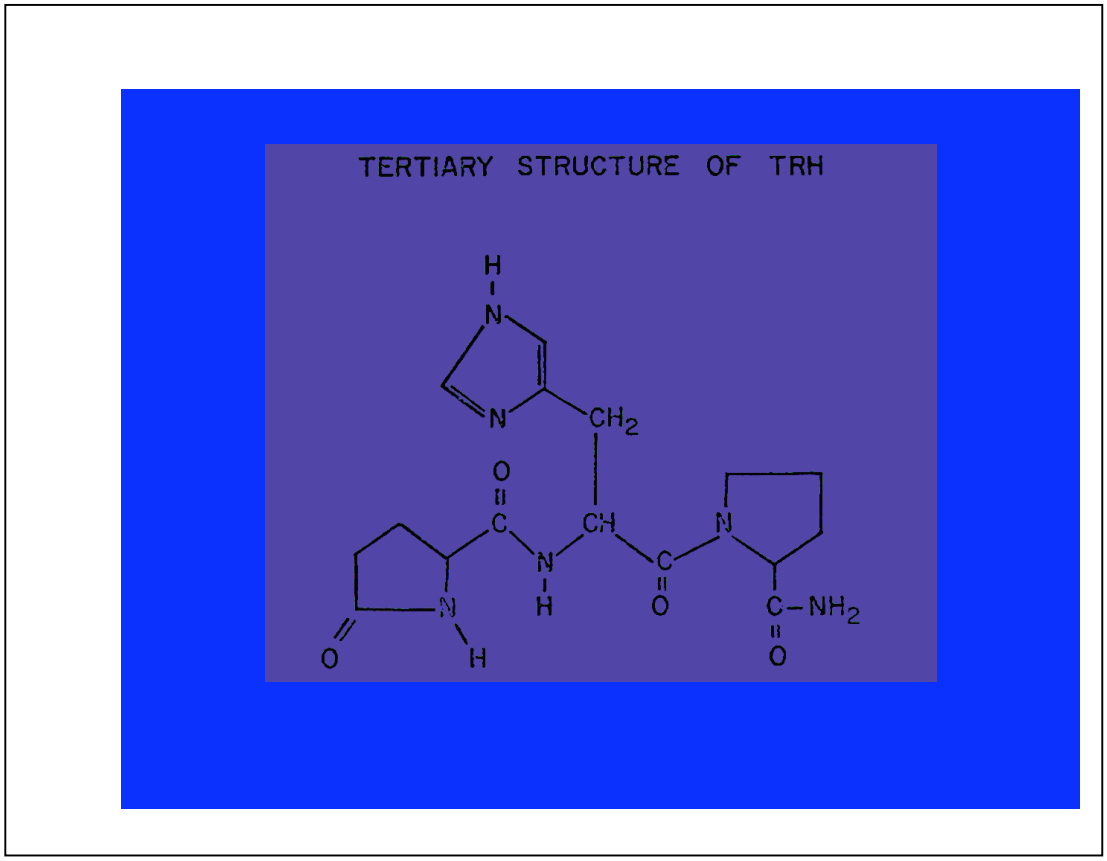


Figure 1. (Courtesy of Prof. M. Kubek)

It was discovered in the 1970s that TRH is produced in the paraventricular nucleus of the hypothalamus (PVN), and stimulates the biosynthesis and secretion of

thyroid-stimulating hormone (TSH) from the anterior pituitary (Harris et al., 1978); (Hall et al., 1970). TSH then stimulates thyroid hormone biosynthesis and release (Morley, 1981). TRH is central in regulating the hypothalamic-pituitary-thyroid (HPT) axis. It influences the release of other hormones, including PRL, GH, vasopressin, and insulin (Bowers and Schally, 1970); (Bowers et al., 1971); (Wilber and Utiger, 1968); (Takahara et al., 1974), and the classic neurotransmitters noradrenaline and adrenaline (Griffiths, 1985).

Notably, TRH is present in many brain loci outside of the hypothalamus, supporting a potential role as a neuromodulator or neurotransmitter outside of traditional HPT axis function (Metcalf, 1974); (Hedner et al., 1981). For example, TRH is implicated as a modulator of seizure activity (Kubek et al., 1989) as well as gastrointestinal function (Tache et al., 1989).

TRH has been found outside the central nervous system (CNS) in the gastrointestinal tract, pancreas, reproductive tissues (placenta, ovary, testis, seminal vesicles, and prostate) as well as in retina, and blood elements (Lechan, 1987). Nillni states in his 1999 review that the widespread distribution of TRH within and outside the CNS supports a diverse range of roles for this molecule, roles likely to involve many functions outside of the traditional HPT axis (Nillni and Sevarino, 1999).

TRH is thus not only associated with regulation of the HPT (hypothalamic-pituitary-thyroid) axis, but has also been found to function as a neuropeptide in specific areas of the brain and other tissues (Gary et al., 2003); (Nillni and Sevarino, 1999). TRH is synthesized in neurons, and then packaged into and stored in vesicles, along with the catecholamine and other classical neurotransmitters. TRH is then released at synaptic

terminals and binds with high affinity to specific neuronal receptors (Nillni and Sevarino, 1999).

In summary, the fact that TRH and its receptors have been found in certain regions of the CNS, including a high density of both in the hippocampus, and have been hypothesized to function in neuromodulation (Gary et al, 2003); (Kubek, et al., 1977); (Nillni and Sevarino, 1999) was critical to the design of this study.

Nillni discusses the biosynthesis of TRH in his 1999 review. He begins with the processing of pro-TRH. The discovery of the rat prepro-TRH sequence in 1986 was critical for understanding the processing of pro-TRH to TRH and non-TRH peptides. Rat prepro-TRH is a 29-kDa polypeptide composed of 255 amino acids. The rat precursor contains an N-terminal 25-amino acid leader sequence, 5 copies of the TRH progenitor sequence Gln-His-Pro-Gly flanked by paired basic amino acids (Lys-Arg or Arg-Arg), 4 non-TRH peptides lying between the TRH progenitors, an N-terminal flanking peptide, and a C-terminal flanking peptide (Lechan et al., 1986b); (Lechan et al., 1986a).

The N-terminal flanking peptide (prepro-$TRH_{25–50}$-R-R-prepro-$TRH_{53–74}$) is further cleaved at the C-terminal side of the arginine pair site to render prepro-$TRH_{25–50}$ and prepro-$TRH_{53–74}$, thus yielding a total of 7 pro-TRH-derived peptides (Nillni and Sevarino, 1999).

The biosynthesis of TRH and other pro-TRH-derived peptides follows the same prohormone-processing mechanisms, starting with mRNA-directed ribosomal translation, followed by posttranslational-limited proteolysis of the larger precursor, proTRH. This process occurs while pro-TRH is transported from the trans-Golgi network (TGN) to newly-formed immature secretory granules (ISGs) (Cruz and Nillni, 1996). These

granules then mature and are targeted to sites of secretion at the plasma membrane of the cell. Rat, mouse, and human pro-TRH, similar to other peptide hormone precursors such as pro-enkephalin, contain multiple copies of one of its peptide products, in this case, the progenitor for TRH, Gln-His-Pro-Gly (Nillni et al., 1993).

Most of the products derived from pro-TRH are targeted into the regulatory secretory pathway (RSP). Cleavage of the precursor to generate biologically active TRH occurs at paired basic residues by the action of two serine proteases, which are members of the family of prohormone convertases, PC1 and PC2 (Nillni et al., 1995); (Friedman et al., 1995); (Schaner et al., 1997), followed by the action of carboxypeptidase-e (CPE) to remove the basic residue(s). Gln-His-Pro-Gly is then amidated by the action of glycine α-amidating monooxygenase (PAM), which uses the C-terminal Gly as the amide donor, and the Gln residue undergoes cyclization to a pGlu residue to yield TRH (Nillni and Sevarino, 1999).

2. Inactivation of TRH

TRH is degraded by separate enzymes, which are cytosolic and membrane bound, resulting in several physiologically active metabolites (O'Cuinn et al., 1990). In the cytosol, TRH degradation occurs via cleavage by pyroglutamate aminopeptidase, generating pyroglutamic acid and His-ProNH_2 (Browne and O'Cuinn, 1983); (Bauer and Kleinkauf, 1980). Occasionally, this His-ProNH_2 metabolizes/cyclizes to cyclo(his-pro), which does not degrade any further (Peterkofsky et al., 1982); (Prasad et al., 1982).

Additionally, deamidation may be catalyzed by another enzyme, a post-proline-cleaving endopeptidase. In this instance, cleavage forms free-acid TRH, which was used in this study since it is an inactive metabolite of TRH (Emerson et al., 1980).

Nillini stated in his 1999 review that the rapid degradation of TRH after release from cells represents a significant drawback in its potential use as a therapeutic agent. To overcome these difficulties, various analogs have been synthesized and evaluated by many groups of investigators, including O'Leary and colleagues in 1995. Specific enzymes that act on TRH are found in many tissues including the brain, spinal cord, pituitary, liver, kidney, pancreas, adrenal glands, and blood. These enzymes not only inactivate TRH, but act in concert with biosynthetic processes to determine the steady state levels of TRH, and its metabolites.

The four key enzymes mentioned above which breakdown TRH: PAP I, PAP II, and thyroliberinase, give rise to the stable cyclized metabolite CHP (also known as histidyl-proline-diketopiperazine or His-Pro-DKP), and prolyl endopeptidase gives rise to the deamidated free acid, TRH-OH (Yanagisawa et al., 1980). In the CNS, the soluble PAP I and prolyl endopeptidase, and the membrane-bound PAP II, are the principal enzymes acting to metabolize TRH (Kelly, 1995).

TRH degradation in serum and many peripheral tissues is through the serum enzyme thyroliberinase (Nillni and Sevarino, 1999). As will be discussed, we used the stable analogue of TRH, 3Me-H-TRH (pGlu-3-His-Pro-NH2), since it is much more biologically active than TRH due to its binding affinity to the TRH receptor (Rivier et al., 1972); (Sharif et al., 1990). The 3Me addition also makes the analog more resistant to enzymatic degradation (Griffiths et al., 1989).

Nillni states that the physiological significance of the soluble enzymes PAP I and prolyl endopeptidase within the brain and spinal cord are unclear, since in the case of neurotransmitter inactivation, TRH is probably degraded outside the neuron by ectoenzymes located on the cell surface, or within lysosomes after endocytosis. Membrane-bound ectoenzymes that are specific for TRH, such as PAP II, are better located for hydrolysis of synaptically released peptides. Soluble enzymes are better situated to control degradation of TRH during its transport in the hypophyseal portal vessels and systemic TRH. The exact mechanisms that control the amount of TRH that ultimately reaches the pituitary remain to be elucidated (Nillni and Sevarino, 1999).

3. Receptor Distribution in the Hippocampus

TRH receptor type 1 (TRH-R1) was cloned originally from mouse pituitary cells (Straub et al., 1990) and then from rat (Zhao et al., 1993); (de la Pena et al., 1992); (Sellar et al., 1993), human (Matre et al., 1993); (Yamada et al., 1993); (Duthie et al., 1993), chicken (Sun et al., 1998), and bovine (Takata et al., 1998) tissues. TRH receptor type 2 (TRH-R2) was cloned from rat central nervous system (Itadani et al., 1998); (Cao et al., 1998); (O'Dowd et al., 2000).

Both receptor subtypes exhibit similar binding affinities for many TRH analogs, as well as similar activation of phosphoinositide-calcium signal transduction pathway by TRH (Wang and Gershengorn, 1999). Homogenate analysis and autoradiographical techniques have been used to measure the distribution of TRH receptors in the brain (Pilotte et al., 1984); (Sharif and Burt, 1985); (Manaker et al., 1985); (Mantyh and Hunt, 1985); (Pazos et al., 1985); (Burt and Snyder, 1975); (Manaker et al., 1986).

Furthermore, TRH receptor density has been studied in anterior pituitary cells and in transfected oocytes from frogs, in which case the downregulation involved changes in degradation or synthesis of the receptors (Hinkle and Tashjian, Jr., 1975); (Halpern and Hinkle, 1981); (Oron et al., 1987). Heuer and colleagues showed that TRH receptor mRNA is localized mainly in the granule cells of the ventral hippocampus, as well as in pyramidal and subicular neurons (Heuer et al., 2000).

Anatomically, a high density of TRH receptors is distributed in the anterior pituitary, spinal cord and limbic system, especially the hippocampal dentate gyrus. Thus, the simplest explanation for TRH inhibition is through stimulation of inhibitory cells such as GABA neurons in the dentate gyrus (Manaker et al., 1985); (Sharif, 1989); (Sharif et al., 1990). TRH-containing neurons have been located in the granule cell layer of the hippocampal dentate gyrus, pyramidal cells of the cornu ammonis (CA), the amygdala, and the piriform and entorhinal cortices (Hokfelt et al., 1975); (Merchenthaler et al., 1989); (Kubek et al., 1993); (Low et al., 1989a).

It has been shown that TRH mRNA and TRH receptor mRNA exist in granule cells of the dentate gyrus, which are excitatory and contain both ionotropic and mGluR subclasses (Heuer et al., 1998); (Blumcke et al., 1996); (Calza et al., 1992).

Regional differences for TRH receptors have been observed. Specifically, TRH-R2 was highly abundant in the precommisural hippocampus, but no specific TRH-R2 signal was detected in the granular or pyramidal cell layers. Heuer reported that a strong TRH-R1 expression was noted in the ventral dentate gyrus, and a subpopulation of neurons containing TRH-R1 was found scattered within the granular layer of the dentate gyrus and the stratum radiatum (Heuer et al., 2000).

Heuer also noted that Ammon's horn was devoid of TRH mRNA signals, but both receptor isoforms were found in the subiculum. Additionally, he found a robust and specific labeling for TRH mRNA in a distinct subpopulation of dentate gyrus granular cells, which had not been previously reported (Segerson et al., 1987); (Heuer et al., 2000).

Thus, TRH-R1 and TRH-R2 receptors and group I metabotropic glutamate receptors $mGluR_{1,5}$ are co-localized in the granule cells of the hippocampal dentate gyrus, and are the only receptor subclass to share the same signaling cascade ($G_{\alpha q/11}$) in these neurons.

4. Receptor Physiology

TRH functions through a seven transmembrane (metabotropic) G-protein coupled receptor as its second messenger system (Yu and Hinkle, 1998). Notably, both TRH and metabotropic group I ($mGluR_{1,5}$) receptors are expressed in the dentate granule cells of the hippocampal dentate gyrus. The TRH receptor is similar to the G-protein coupled family of receptors, in that there is are seven transmembrane-spanning domains and two glycosylation sites in the N-terminus. The third intracellular loop is an amphipathic helix, which binds a G-protein. Cysteine residues, which may form a disulfide bond, are present in extracellular loops 1 and 2; and 15 of the last 52 amino acids are serine or threonine residues. The most likely candidates for protein kinase C phosphorylation are 3 residues in loops 3 and 4 residues in the C-terminus. The mouse and rat pituitary receptors have been cloned and sequenced (Straub et al., 1990); (Zhao et al., 1993).

The $G_{\alpha q/11}$ subunit, which is shared by both TRH and group I mGluRs, is critical when one considers the proposed mechanism of heterologous receptor downregulation as one mechanism whereby TRH exerts its neuroprotective effect on cells, slices, and neuronal cultures.

The binding of TRH to its receptor activates phospholipase C by coupling through a GTP-dependent protein, as shown by pituitary cell studies (Martin et al., 1986). Mechanistically, phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) to the second messengers myoinositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Gershengorn, 1989). IP_3 mobilizes intracellular calcium; whereas, DAG activates protein kinase C (PKC), which is a phosphorylating enzyme (Berridge and Irvine, 1984). Additionally, TRH may also stimulate the production of cyclic AMP, which may be a consequence of calcium/calmodulin or PKC-mediated adenylate cyclase regulation (Martin, 1989); (Mori et al., 1991); (Iriuchijima and Mori, 1989).

D. The Hippocampus

1. Anatomic Description

The hippocampal formation is identified on the medial surface of the temporal lobes and extends along the floor of the temporal horn of the lateral ventricle. Three major subdivisions are described as the dentate gyrus, the hippocampus proper, and the subicular complex, which is part of the parahippocampal gyrus that is contiguous with the hippocampal fields. Ammon's horn, or cornu ammonis (thus CA), is a classic anatomic

term describing the hippocampus proper. CA is the term most widely used to describe the subfields of the hippocampus: CA1, CA2, CA3, and CA4 (dentate hilus) (**Figure 2.**)

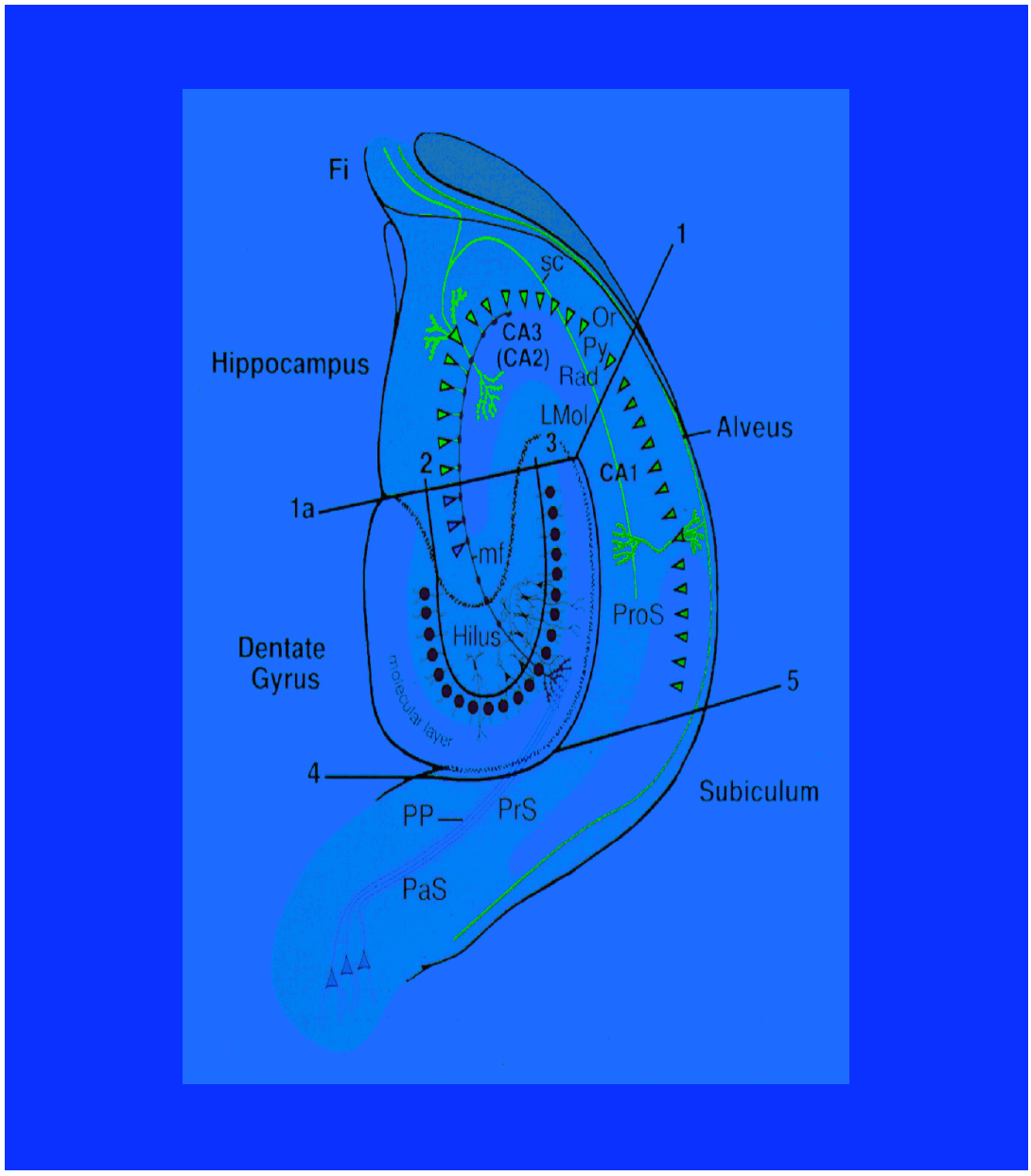


Figure 2. (Courtesy of Prof. M. Kubek)

The trisynaptic pathway is an internal circuit mechanism unique to the hippocampus. Viewed in coronal section, the granule cell layer of the dentate gyrus is considered to be the first stage of the trisynaptic pathway, and is the target for most of the entorhinal afferents. These afferents reach the granule cells via the perforant pathway, whose axons perforate the hippocampal fissure.

The second stage of the trisynaptic pathway is the CA3 subfield. Pyramidal cells of this region are the principal targets of granule cell axons. CA3 pyramidal cell axons give rise to extensive axonal projections, which extend to the CA1 subfield, as originally described by Schaffer in the nineteenth century. The CA1 subfield represents the third and last stage of the intrahippocampal trisynaptic loop, and serves as the target for CA3 pyramidal cell axons (Schaffer collaterals); (Benes and Berretta, 2000).

The intrahippocampal pathways and neuroanatomy of the hippocampus are thus well described, and contain almost all of the major peptide and neurotransmitter systems. The hippocampus is used as a model system for mechanisms of synaptic excitation and inhibition since it has three major excitatory pathways running from the subiculum to the CA1 region. The perforant pathway runs from the subiculum to the granule cells in the dentate gyrus hilus. The granule cell axons form the mossy fiber pathway that runs to the pyramidal cells the CA3. Finally, the pyramidal cells in the CA3 region send excitatory collaterals, Schaffer collaterals, to the pyramidal cells in CA1 (Storm-Mathisen, 1977).

High affinity uptake of glutamate and aspartate is localized in the terminals of the perforant pathway, mossy fibers and pyramidal cell axons. Hippocampal efferents also take up aspartate and glutamate. Peak concentrations of gamma-aminobutyrate (GABA) occur near the pyramidal and granular cell bodies, corresponding to the site of the

inhibitory basket cell terminals, and in the outer parts of the molecular layers. A further description of the distribution of glutamate receptors in the hippocampus is in a subsequent section (Storm-Mathisen, 1977).

Cholinergic septo-hippocampal afferents are also present. Serotonin, noradrenaline, dopamine and histamine are located/synthesized in afferent fiber systems. The hippocampal formation also contains nerve elements reacting with antibodies against neuroactive peptides, such as enkephalin, substance P, somatostatin and gastrin/cholecystokinin (Storm-Mathisen, 1977).

On transverse section, the hippocampus is divided into four subfields: CA1, CA2, CA3, and dentate hilus. CA2 is between CA1 and CA3 but is not well demarcated. The areas of CA3 adjacent to the CA2 region are termed CA3a. The CA3b region is approximately the middle third and CA3c is the terminal portion between the superior and inferior blades of the dentate gyrus.

The main cells of the hippocampus are the pyramidal cells, with both their basal and apical dendrites. The axons of the pyramidal cells branch into collaterals that project within and outside the hippocampus. The collateral axons of the CA3 pyramidal cells that synapse on the apical dendrites of the CA1 pyramidal cells are called Schaffer collaterals. The second major cell type are basket cells which contain GAD (glutamic acid decarboxylase), the gamma-aminobutyric acid (GABA) synthesizing enzyme, and exert an inhibitory action on the pyramidal cells.

The dentate gyrus is a three-layered structure consisting of the molecular layer (stratum moleculare), granular layer (stratum granulosum), and the polymorphic layer, from superficial to deep. The dentate granule cells are the main cell type of the dentate

gyrus. Mossy cells and other interneurons are found in the polymorphic layer in the dentate hilus (CA4).

The axons of granule cells, known as mossy fibers, contain zinc, and make excitatory synaptic contacts with the proximal portions of the apical dendrites of the CA3 pyramidal cells in the stratum lucidum. Mossy fibers also innervate the soma and dendrites of polymorphic interneurons (basket cells and mossy cells) in the dentate hilus (Teyler and DiScenna, 1984).

The dendrites of granule cells branch into the molecular layer where the perforant pathway (the main hippocampal afferent system originating from the entorhinal cortex) makes synaptic contacts. The granule cells then project via mossy fibers to the CA3 pyramidal cells, which then send Schaffer collaterals to CA1 pyramidal cells. These CA1 pyramidal cells then project to the subicular complex, which will then project to the entorhinal cortex (Teyler and DiScenna, 1984).

The dentate granule cells are glutamatergic excitatory neurons, which contain dynorphin and cholecystokinin (CCK) in the terminal boutons of their axons (mossy fibers). Inhibitory interneurons containing GAD exist throughout the hippocampus. Basket cells are GABAergic inhibitory interneurons, which are also immunoreactive for CCK. Somatostatin (SS) and neuropeptide-Y (NPY) immunoreactive interneurons are found in the hilar region and the stratum oriens of the CA1 subfields. The mossy cells are found in the dentate hilar region, and have excitatory synapses onto the GABA interneurons (basket cells). This provides tonic baseline inhibition and mediates phasic feedback inhibition to the granule cells (Teyler and DiScenna, 1984).

2. Receptor Distribution

Although glutamate receptors are ubiquitously found in the cortex, there are some notable differences in the distribution of receptor subtypes (Choi, 2003); (Cotman and Monaghan, 1988). In the neocortex, NMDA receptors are particularly dense within the outer two layers; whereas, high-affinity kainate receptors predominate in layers 5 and 6.

In the hippocampus, which was the focus of these experiments, the trisynaptic loop contains glutamate and aspartate excitatory amino acid neurotransmitters; although different EAA receptors are found along the loop (Storm-Matheson, 1981); (White et al., 1977). High densities of NMDA and kainate (KA) receptors are found in the dentate gyral molecular layer. In contrast, NMDA receptors are absent in the CA3 stratum lucidum, which is the mossy fiber termination zone. The stratum oriens and radiatum of subfield CA1, which receive the Schaffer collaterals, contain high densities of both AMPA and NMDA receptors (Monaghan et al., 1983); (Monaghan and Cotman, 1985); (Unnerstall and Wamsley, 1983).

Regarding group II mGluR distribution in the hippocampus, it has been shown in general that $mGluR_2$ receptors are localized on preterminal axons of glutamate neurons, and serve as pre-synaptic modulatory receptors in the hippocampus, where they function via a negative feedback mechanism to suppress further release of glutamate. Conversely, $mGluR_3$ receptors are found most often post-synaptically on neurons and expressed in glia, where their functional role is less clear (Shigemoto et al., 1997).

Notably, the pre-synaptic localization of the $mGluR_2$ to glutamate preterminal axons has been most clearly defined within the hippocampal formation, where $mGluR_2$

immunoreactivity illustrates a primary localization to subfields corresponding to the medial perforant and mossy fiber pathways (Shigemoto et al., 1997). Unilateral lesions of the entorhinal cortex, which contains cell bodies of the perforant path neurons, lead to ipsilateral loss of $mGluR_{2/3}$ immunoreactivity (Shigemoto et al., 1997).

Unlike wild-type animals, knockout mice deficient in $mGluR_2$ receptors showed no immunostaining of $mGluR_2$ within the stratum lucidum of CA3 (mossy fiber terminal field) or the stratum lacunosum moleculare of CA1 (medial perforant path terminal field) of hippocampus (Yokoi et al., 1996). In addition, in $mGluR_2$-deficient mice, long-term depression induced by low-frequency stimulation of the mossy fiber CA3 synapses was abolished, indicating that pre-synaptic $mGluR_2$ receptors are essential for inducing long-term depression at this synapse, illustrating an important role in the modulation of neuronal excitability. However, Schoepp states that $mGluR_2$ knockout animals appear to be functionally normal, with no alteration in basal synaptic transmission. Thus, $mGluR_2$ receptors are not believed to play a prominent role in the acute regulation of excitatory synaptic transmission (Schoepp, 2001).

Regarding the distribution of group III mGluR subtypes ($mGluR_{1,5}$) within the hippocampus, it was reported in 1981 by Koerner and colleagues that the L-2-amino-4-phosphonobutyric acid (L-AP4), an acidic amino acid analog, selectively suppressed glutamate excitations pre-synaptically in the hippocampal lateral perforant pathway. This inhibitory activity of L-AP4 on glutamate excitations was also observed in other preparations, including the mossy fiber synapse, lateral olfactory tract, and spinal cord (Koerner and Cotman, 1981); (Schoepp, 2001); (Thomsen et al., 1992); (Thomsen, 1997).

Until the 1990s, pre-synaptic inhibition induced by L-AP4 was ascribed to what was labeled the *L-AP4 receptor*. After the cloning of the group III mGluRs, which Nakanishi defined by their sensitivity to L-AP4, it was recognized that certain group III mGluR subtypes might be responsible for L-AP4-induced suppression of glutamate release (Nakanishi, 1992). The $mGluR_7$, $mGluR_8$, and possibly $mGluR_4$ receptors have been suggested as candidates for the pre-synaptic effects of L-AP4 in the brain (Thomsen, 1997); (Schoepp, 2001).

In certain subfields of the hippocampus, both $mGluR_4$ and $mGluR_8$ receptors have been shown to be expressed to a certain extent, and their possible role in synaptic transmission at hippocampal pathways has been reported (Bradley et al., 1998); (Bradley et al., 1996); (Shigemoto et al., 1997); (Schoepp, 2001). Studies have shown selective labeling of $mGluR_8$ receptors to the terminal fields of the lateral perforant pathway (CA3 stratum lacunosum moleculare), and loss of this labeling following perforant path lesions (Shigemoto et al., 1997). While $mGluR_8$ receptors are relatively restricted to the terminal subfields of the dentate gyrus, the initial work of Bradley and colleagues demonstrated that $mGluR_{4a}$ staining was in cell bodies and dendrites of pyramidal neurons, granule cells, and scattered interneurons throughout the hippocampus (Bradley et al., 1996).

Three years later, however, this same group, using a more specific antibody, suggested a more limited hippocampal distribution of $mGluR_{4a}$. High expression was noted in the molecular layer of the dentate gyrus, as well as in the stratum moleculare of CA1 and the stratum oriens of the CA3 area, which was not present in the $mGluR_{4a}$ knockout mouse (Bradley et al., 1998); (Bradley et al., 1999). Via electron microscopy,

$mGluR_4$ receptors are found post-synaptically at asymmetrical (presumably glutamatergic) synapses, and pre-synaptically at both asymmetrical and symmetrical (presumably GABAergic) synapses (Bradley et al., 1999); (Schoepp, 2001).

Thus, $mGluR_4$ receptors may have pre- and postsynaptic functions, and they may be involved in both homo- and heterosynaptic modulation in these brain regions. Gerlai and colleagues have reported a possible role for $mGluR_4$ receptors in hippocampal processing of spatial information (Gerlai et al., 1998); (Schoepp, 2001).

Bradley reported that $mGluR_7$ receptors are highly expressed throughout the forebrain, brainstem, and spinal cord regions of the CNS (Bradley et al., 1998). In particular, $mGluR_7$ receptors may represent an autoreceptor in certain synapses that provide negative feedback to limit further release of glutamate under normal physiological conditions of excitatory synaptic transmission (Schoepp, 2001). Shigemoto and colleagues showed that $mGluR_{7a}$ receptor immunoreactivity in the rat hippocampus was restricted to the pre-synaptic grid, or the site of vesicle fusion. The lower affinity of glutamate for $mGluR_7$ receptors was consistent with its localization in the synaptic cleft and function as an autoreceptor (Shigemoto et al., 1997); (Schoepp, 2001).

In general, $mGluR_7$ receptor protein and mRNA are relatively more widespread in distribution throughout the neuro-axis when compared with other pre-synaptic mGluRs (e.g., $mGluR_2$, $mGluR_4$, and $mGluR_8$), possibly indicating a more prominent role in normal regulation of synaptic glutamate release. Nevertheless, like other mGluRs, $mGluR_7$ expression is more concentrated in certain areas and appears to be specifically targeted to certain synapses. Thus, not all glutamatergic neurons appear to express (or

need) an $mGluR_7$ autoreceptor regulatory mechanism to maintain normal excitatory functions (Schoepp, 2001).

The expression of $mGluR_7$ receptors in glutamatergic nerve terminals of the perforant path is supported by loss of $mGluR_{7a}$ immunoreactivity following lesions of entorhinal cortex (Shigemoto et al., 1997). Additionally, colchicine lesions of the dentate granule cells also produced loss of $mGluR_7$ receptor immunoreactivity in the CA3 of the hippocampus, indicating a pre-synaptic role of $mGluR_7$ in the mossy fiber pathway. Terminals of pyramidal neurons, which were pre-synaptic to the population of interneurons expressing postsynaptic $mGluR_1$ receptors, expressed ~10-fold higher levels of $mGluR_7$ receptors when compared with terminals making synaptic contacts with other pyramidal neurons or interneurons, suggesting that $mGluR_7$ receptors may regulate release of glutamate at certain synapses based on what other receptors are expressed post-synaptically (Schoepp, 2001).

Boudin and colleagues suggested that the targeting of the $mGluR_{7a}$ receptor to the pre-synaptic membrane is dependent upon binding to PICK1, a PDZ domain-binding protein. The PDZ domain-binding site for the $mGluR_{7a}$ receptor is within the extreme carboxyl terminus of the receptor, and this sequence appears to confer PICK1 binding and receptor targeting, as $mGluR_2$, another pre-synaptic mGlu receptor, did not bind to PICK1 and an $mGluR_{7a}$ receptor mutant lacking critical amino acids led to lack of pre-synaptic receptor clustering in hippocampal neurons (Boudin et al., 2000); (Schoepp, 2001).

As stated previously, glutamate acts through both fast ionotropic (AMPA, NMDA, kainate) and slower G-protein coupled metabotropic receptors (mGluRs). The

metabotropic receptors ($mGluR_{1\text{-}8}$) generate changes that are slower as well as longer lasting in neuronal heterotrimeric G-protein signaling cascades. The different metabotropic glutamate receptors are the foremost receptors, which have been shown to mediate extrasynaptic glutamatergic signaling, and are distributed throughout the hippocampal subfields (Blumcke et al., 1996); (Vignes et al., 1998); (van Hooft et al., 2000); (Conn and Niswender, 2006).

The first physiological evidence for the remote effects of synaptically released glutamate in the hippocampus, however, came from examining NMDA receptors, which have an affinity for glutamate of the same order of magnitude as the metabotropic ones. The NMDA receptors are activated at low micromolar (μM) concentrations, with a $K_D \approx$ 100-fold lower than AMPA receptors (Patneau and Mayer, 1990); (Kullmann et al., 1999a).

Glutamate can act at remote receptors in the hippocampus, and thus does not act as a point-to-point messenger, exclusively. Kullmann and colleagues noted that it is difficult to determine whether the modulatory effects of glutamate are actually confined to a small spatial and temporal domain around the synaptic release event, or are instead spread diffusely to nearby receptors, or even to neighboring synapses. Glutamate may thus function as a diffuse neuromodulator, in addition to its conventional role in fast excitatory transmission. Given that glutamate is also an excitotoxin, Kullmann and others speculated that the feedback regulation via metabotropic receptors may thus act to protect neurons from excessive excitation (Kullmann et al., 1999a); (Kullmann, 1999b).

Blumcke and colleagues showed that immunoreactivity for $mGluR_{2/3}$, members of class II mGluRs, was present in all principle neurons in the hippocampal dentate gyrus, as well as in the CA4, CA3 and CA2 regions. Pyramidal cells of the CAI region exhibited only weak labeling for $mGluR_{2/3}$ in the study; additionally, glial cells were also $mGluR_{2/3}$-immunoreactive. The reaction obtained with an antiserum directed against $mGluR_{4a}$, a member of class III mGluRs, was confined to the mossy fiber projection field in CA3 stratum lucidum (Blumcke et al., 1996).

The experiments detailed in this thesis investigated the phenomenon of heterologous receptor downregulation, involving the second messenger cascade shared by both the metabotropic glutamate receptors (specifically $mGluR_{1,5}$) as well as the TRH receptors localized in the hippocampus.

E. Heterologous Receptor Desensitization

1. Background

Synaptic receptors have two major functions: recognition of specific transmitters and activation of effectors. The receptor first recognizes and binds a transmitter in the external environment of the cell; then, as a consequence of binding, the receptor alters the biochemical state of the cell.

Milligan described the phenomenon by which cells and tissues develop reduced sensitivity over time to the continued presence of a stimulus at constant intensity as "desensitization." In homologous desensitization, treatment with an agonist results in

decreased sensitivity to the agonist, but not to other signals that work through distinct receptors. Milligan and colleagues proposed receptor-mediated downregulation of the shared cellular G-protein subunits as a mechanism for the development of sustained *heterologous receptor desensitization* (Milligan and Green, 1997). This is described as a more general type of desensitization, such that reduced sensitivity to *several different agonists* develops.

TRH administration has been shown to induce the sub-cellular redistribution and down-regulation of its intracellular signaling protein, $G_{\alpha q/11}$. Svoboda and colleagues showed that prolonged exposure to TRH resulted in selective and robust redistribution and down-regulation of $G_{\alpha q/11}$ when both the TRH receptor, TRH-R1, and $G_{\alpha q/11}$ are overexpressed in human embryonic kidney 293 (HEK293) cells (Svoboda et al., 1996).

Furthermore, Kim and colleagues showed that sustained exposure to TRH downregulates G_q proteins in HEK293 and GH-3 cells, again, the same heterotrimeric G-protein used by glutamate in its metabotropic second messenger cascade (Kim et al., 1994). In these experiments, it should be noted that the TRH receptor R1 as well as $G_{\alpha q/11}$ were overexpressed in the HEK 293 cells.

This desensitization of G-protein coupled receptors is considered to be a mechanism of adaptation to the continuous or repeated presence of stimuli, through mechanisms which are complex, and may involve receptor phosphorylation, G-protein uncoupling, internalization, and ultimately intracellular downregulation (Ferguson, 2001); (Claing et al., 2002); (Mundell et al., 2004). The metabotropic glutamate receptor family provides a mechanism by which activation by glutamate can regulate a number of

important neuronal and glial functions that are not typically modulated by ligand-gated ion channels (Conn, 2003).

At least two families contributed to the desensitization of the G-protein coupled receptors (GPCRs), second messenger-dependent protein kinases and GPCR-specific G-protein-coupled receptor kinases (GRKs). It has been shown that within seconds of receptor stimulation these kinases phosphorylated serine and threonine residues within the GPCR intracellular domain, uncoupling receptors from the heterotrimeric G-protein (Ferguson, 2001); (Ferguson et al., 1996); (Premont et al., 1995); (Krupnick and Benovic, 1998).

We attempted to prove that TRH could modulate levels of $G_{\alpha q/11}$ in a complex neuronal milieu, *in vitro*, as will be described.

2. Group I ($mGluR_{1,5}$) Receptors

As described previously, the mGluRs have been grouped on the basis of their sequence homology, transduction mechanisms, and agonist selectivity. Regarding distribution of mGluRs in the rat hippocampus, principal cells of all hippocampal subfields were found to be immunoreactive for $mGluR_5$ by Lujan and colleagues, which was in agreement with the expression of mRNA previously described by Abe, et al. (Lujan et al., 1996); (Abe et al., 1992). Additionally, Lujan found that immunolabeling for $mGluR_1$ of granule cells in the dentate gyrus and pyramidal cells of the CA3 but not the CA1 area was in agreement with the *in situ* hybridization results of Shigemoto earlier (Lujan et al., 1996); (Shigemoto et al., 1993). These results led Lujan to conclude that

most CA3 pyramidal and granule cells express both receptors coupled to PLC; whereas, CA1 pyramidal cells only express $mGluR_5$ (Lujan et al., 1996).

Frauli and colleagues have reported that, of the twenty known classical L-amino acids, only glutamate itself directly activates metabotropic glutamate receptors. They showed that the effects of other amino acids, namely cysteine, aspartate, and asparagines, are due to an indirect action involving glutamate transporters/exchangers, rather than direct activation of the receptors (Frauli et al., 2006).

Frauli demonstrated that glutamate transporter systems allow mGluRs to "sense" large increases in the extracellular concentration of certain amino acids (Frauli et al., 2006). Additionally, Mundell and colleagues have demonstrated the heterologous desensitization of metabotropic glutamate receptor splice variants $mGluR_{1a}$ and $mGluR_{1b}$ after activation of endogenous $G_{q/11}$-coupled receptors in human embryonic kidney (HEK293) cells (Mundell et al., 2004); (Mundell and Benovic, 2000).

Mailleux and colleagues reported the cellular distribution of G proteins G_{α} and $G_{11\alpha}$ by immunocytochemistry in 1992 by using an antipeptide antiserum directed against the C-terminal decapeptide conserved between the two G-proteins, and showed by Western blot that the brain neuronal localization of G_{α} and $G_{11\alpha}$ match that of phospholipase-C (PLC), as well as 1,4,5-triphosphate receptor and 1,4,5-triphosphate-3-kinase (Mailleux et al., 1993); (Mailleux et al., 1992).

Upon guanine nucleotide-binding regulatory protein-mediated activation of PLC, phosphatidyl inositol 4,5-bisphosphate (PIP_2) is converted into two second-messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The immunoreactivity was found in neurons where the G-proteins were enriched, at the periphery of the perikarya

and in the dendrites. In the hippocampus, after selective lesioning of the pyramidal cells with quinolinic acid, a disappearance of staining in the CA1 layer was noted (Mailleux et al., 1992).

Blaabjerg and colleagues actually showed that treatment with the DHPG *protects* neurons from NMDA-induced excitotoxicity in a concentration-dependent manner (Blaabjerg et al., 2003), but the neuroprotection occurred after the initial prolonged exposure to the agonist, and was associated with inhibition of the NMDA receptor-mediated inward current. In addition to changes in Ca^{2+} homeostasis, production of second-messengers IP3 and DAG and activation of protein kinase C (PKC) are associated with mGluR activation (Meldrum, 2000). Additionally, it has been shown that group I mGluRs can have effects at the gene level (Mao and Wang, 2003).

Blaabjerg used the cDNA microarray technique to determine whether changes in gene expression may be involved in regulation of the group I mGluR-induced neuroprotection, and compared gene expression patterns in DHPG-treated organotypic hippocampal slice cultures with untreated control cultures. Blaabjerg states that it is possible that DHPG-induced increase in certain proteins may lead to internalization of NMDA and AMPA receptors, and that internalization has been reported to occur in primary hippocampal cultures treated with DHPG (Snyder et al., 2001) and might explain the neuroprotection and reduction of NMDA-mediated currents after DHPG treatment.

Blaabjerg concluded that neuroprotection by group I mGluR activation may not be restricted to a single pathway, but may involve multiple mechanisms acting together (Blaabjerg et al., 2003). In contrast to the technique Blaabjerg used, exposing

hippocampal slice cultures to DHPG for 2 hrs prior to exposure to NMDA, we exposed hippocampal slices simultaneously to DHPG + NMDA, which was found to be toxic in the slice system, as will be described in sections that follow.

F. Molecular Markers

1. Caspase-3

Caspase-3 activation is involved in cell death in hippocampal mixed neuronal cultures treated with glutamate. Caspase-3 (also referred to as Cpp32, Yama, and apopain) is one of the critical enzymes of apoptosis, and is thus the most studied of mammalian caspases. Caspase-3 is a member of the CED-3 subfamily, and can activate pro-caspases 2, 6, 7, and 9, and specifically cleave most caspase substrates, which have been described.

In addition, caspase-3 plays a central role in mediating chromatin condensation and DNA fragmentation, as well as cell blebbing (Nicholson and Thornberry, 1997); (Kamada et al., 2005); (Cohen, 1997); (Porter et al., 1997); (Porter and Janicke, 1999).

Brecht and colleagues demonstrated activation of caspase-3 and DNA fragmentation after glutamate toxicity in cultured primary hippocampal neurons, in a dose-dependent and time-dependent manner. In the same cultures, caspase-3 was absent in astrocytes. Additionally, hippocampal neurons displayed an individual vulnerability to glutamate, in that CA1 neurons were more sensitive than CA2/4 or dentate gyrus neurons. Neurons in close proximity to astrocytes tolerated glutamate better than neurons

without nearby astrocytes (Brecht et al., 2001). We used caspase-3 as a cellular marker for apoptotic death in hippocampal neuronal cultures.

2. Secreted Amyloid Precursor Protein (sAPP) Alpha

The toxicity of Aß is strongly enhanced by conditions found during ischemic brain injury, such as raised concentrations of EAA and hypoglycemia. It may be speculated that the pathology of Alzheimer's disease is related to a prolonged, chronic form of excitotoxicity following the conversion of APP to Aß. Accordingly, the unprocessed APP protein seems to have a dampening effect on intracellular calcium concentration, by opening neuronal potassium channels, which results in hyperpolarization. In agreement with these findings, soluble APP protects neurons from Aß toxicity. The 22C11 antibody is used to detect sAPP as described in the sections that follow (Leist and Nicotera, 1998a).

A currently favored hypothesis is that the amyloid beta peptide (Aß), synthesized as the larger beta-amyloid precursor protein (APP), is the trigger that induces a cascade of events leading to the loss of cholinergic neurons and the progressive dementia that defines Alzheimer's disease (AD). One of therapeutic aims in AD is to reduce levels of toxic Aß. Cholinergic agonists lower Aß levels and increase the levels of APP processed by alternate alpha-secretase pathways (sAPPα). Since cholinesterase inhibitors (ChE-Is) are the only approved FDA therapeutics for AD, the effects of ChE-Is on levels of secreted APP and Aß were examined during the preparation of this thesis.

Experiments on primary rat fetal cortical neuronal cells indicated that ChE-Is might affect APP processing in multiple ways, as follows. First, drugs like phenserine

lowered sAPP and Aß levels in the neuronal cells. Second, agents like galantamine showed a trend towards an increase in sAPPα without changing Aß levels. Third, drugs like donepezil insignificantly affected sAPPα but reduced Aß levels.

Characterization of these drugs' actions on APP synthesis and Aß biogenesis demonstrates the amyloid-modifying properties of specific anticholinesterases. Drug doses tested to achieve APP inhibition were higher than cholinesterase-inhibition but still non-toxic. It is possible that treatment with drugs such as those listed may not only treat the symptoms of AD, but may perhaps slow disease progression by reducing Aß levels. We found that the administration of TRH to cells and slices treated with various cholinesterase inhibitors did not significantly affect the levels of sAPP. These experiments are further described in the sections that follow.

II. HYPOTHESES

The hypotheses to be tested are: 1) TRH protects against glutamate-induced toxicity in vitro; and 2) TRH signaling is selectively downregulated as part of this neuroprotective mechanism.

III. EXPERIMENTAL DESIGN

A. Rationale for using GH-3 Cells in Neuroprotection Studies

We used pituitary adenoma (GH-3) cells as the first system in which the hypothesized neuroprotective effect of TRH was investigated, due to their reported TRH receptor profiles (de la Pena et al., 1992). The vast majority of receptors on GH-3 cells are, in fact, well characterized, although the presence of mGluRs is controversial. Administration of glutamate has been shown to stimulate pituitary hormone secretion. This effect was thought to depend on action at a central site in the brain but, alternatively, it could also result from a direct action on the pituitary.

It has been reported that addition of glutamate to rat pituitary cells in primary culture is able to induce secretion of prolactin. Recent studies have shown that glutamate stimulates calcium entry through L-type calcium channels, and that this effect is secondary to membrane depolarization induced by electrogenic uptake through a NAP-dependent *high-affinity glutamate transporter* present in GH3 cells (Villalobos et al., 1996).

The goal of this portion of the study was to determine if TRH could affect cell survival when added to cultured GH-3 cells either prior to, during, or after glutamate-induced toxicity. GH-3 cells were examined due to their known concentration of TRH receptors and less-characterized glutamate receptor population. TRH has been shown to activate calcium efflux in GH-3 cells (Nelson and Hinkle, 1994); (Drummond et al., 1989); (Gershengorn et al., 1983).

Renaud and colleagues showed an inhibition by TRH of glutamate-stimulated activity in a neuronal cell system (Renaud et al., 1979), and Koenig and colleagues showed that TRH inhibited glutamate-stimulated calcium uptake in cortical cells (Koenig et al., 1996). Thus, these experiments were designed to determine if TRH added to GH-3 cell culture systems would exhibit a protective role against glutamate-induced excitotoxicity. If it did so, it may involve modulation of glutamate-mediated ionic and/or secondary events. The neuroprotective effect of TRH in neuronal systems, using cultured fetal neurons (E17) will also be addressed.

It has been shown that glutamate is a potent neurotoxin in cell culture (Choi et al., 1987b), and that TRH stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in GH-3 cell membranes. Notably, the stimulation of the phosphoinositide-phospholipase C (PI-PLC) activity can be blocked by incubation of GH-3 membranes with polyclonal antibodies directed against a peptide derived from the C-terminal region of $G_{\alpha q}$ and G_{α}. This suggested that the TRH-dependent stimulation of PI-PLC in GH-3 cells is mediated through the G-protein alpha subunits, $G_{\alpha q}$ and/or $G_{\alpha 11}$ (Aragay et al., 1992).

In GH-3 rat pituitary cells, it has been shown that exposure of GH-3 cells to TRH resulted in a marked down-regulation of $G_q\alpha/G_{11\alpha}$. In GH3 cells, both $G_q\alpha$ and $G_{11\alpha}$, which are expressed at similar levels, were observed to be down-regulated equivalently by treatment with TRH (Kim et al., 1994). In other experiments, as described, prolonged exposure to TRH resulted in selective and substantial redistribution and down-regulation of $G_{\alpha q/11}$ when the TRH receptor (TRH-R1) and $G_{\alpha q/11}$ are over-expressed in HEK 293 cells (Svoboda and Milligan, 1994).

It has been noted that glutamate increases Ca^{2+} via the action of glutamate transporters in GH-3 cells (Villalobos et al., 2002); (Villalobos and Garcia-Sancho, 1995). Additionally, Kim and colleagues showed that stimulation of human embryonic kidney cells, which expressed the TRH receptor after transfection with TRH-R cDNA (HEK-293), and stimulation of GH-3 cells by TRH caused downregulation of $G_{\alpha q}$ and $G_{\alpha 11}$ proteins. This was extremely important, as we addressed the possible mechanism of TRH action in GH-3 cells (Kim et al., 1994).

In this study, it was to be determined whether TRH can affect cell survival when added in cultured cells either prior to, during, or after 8 hrs of 100 nM glutamate-induced toxicity. The cells used were pituitary adenoma (GH-3) cells (3×10^6/plate) in six separate experiments. Cell viability and integrity were assessed by trypan blue exclusion assay and LDH release.

1. Effect of TRH against Glutamate-Induced Toxicity in GH-3 Cells

a. Effect of TRH on GH-3 Cell Viability

Initially, it needed to be shown that TRH was non-toxic against GH-3 cells, for the dose utilized, as well as for the time required for the experiment.

b. Dose Effect of Glutamate on GH-3 Cell Viability

A toxic dose of glutamate needed to be utilized in the GH-3 cell experiments, and a dose response curve needed to be generated, in order to determine the $LD_{50,}$ at which dose and time 50% of the GH-3 cells were dead and/or damaged, as assayed by LDH analysis and trypan blue exclusion assay.

c. Effect of TRH *Before* Glutamate Toxicity in GH-3 Cells

After the LD_{50} of glutamate in GH-3 cells was determined, we treated the GH-3 cells with TRH *before* the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could exert a prophylactic neuroprotective effect against excitatory amino acid-induced toxicity.

d. Effect of TRH *During* Glutamate Toxicity in GH-3 Cell

Using the LD_{50} of glutamate in GH-3 cells, we treated the GH-3 cells with TRH *during* the period when the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could exert a neuroprotective effect against excitatory amino acid-induced toxicity simultaneously with the toxic insult.

e. Effect of TRH *After* Glutamate Toxicity in GH-3 Cells

Again using the LD_{50} of glutamate in GH-3 cells, we treated the GH-3 cells with TRH *after* the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could rescue the cells from excitatory amino acid-induced toxicity.

f. Effect of Sustained Release TRH Formulation on Glutamate Toxicity in GH-3 Cells

We used TRH nanoparticles in GH-3 cells to analyze another method of delivery of TRH within the brain in order to determine whether TRH could protect against glutamate-induced toxicity.

B. Rationale for using PC12 Cells in Neuroprotection Studies

We used neuron-like pheochromacytoma (PC12) cells as a negative control in the experimental design due to their known glutamate receptor profile and their uncharacterized TRH receptor population. Glutamate treatment of PC12 cells has been shown to result in the accumulation of inositol phosphates, suggesting that group I metabotropic glutamate receptors ($mGluR_1$ and $mGluR_5$) are present, positively coupled to phospholipase C (Kane et al., 1998). Additionally, PC12 survival through a possible mechanism of glutamate receptor suppression has been noted (Uemura et al., 2003).

In this study, it was to be determined whether TRH can affect cell survival when added in cultured cells either prior to, during, or after 8hrs of 100 nM glutamate-induced toxicity. The cells used were neuron-like pheochromocytoma (PC12) cells (3×10^6/plate) in six separate experiments. Cell viability and integrity were assessed by trypan blue exclusion assay and LDH release.

1. Effect of TRH on Glutamate-Induced Toxicity in PC12 Cells

a. Dose Effect of TRH on PC12 Cell Viability

Initially, it needed to be shown that TRH was non-toxic against PC12 cells, for the dose utilized, as well as for the time required for the experiment.

b. Dose Effect of Glutamate on PC12 Cell Viability

A toxic dose of glutamate needed to be utilized in the PC12 cell experiments, and a dose response curve was generated, in order to determine the $LD_{50,}$ at which dose and

time 50% of the PC12 cells were dead and/or damaged, as assayed by LDH analysis and trypan blue exclusion assay.

c. Effect of TRH *Before* Glutamate Toxicity in PC12 Cells

After the LD_{50} of glutamate in PC12 cells was determined, we treated the cells with TRH *before* the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could exert a prophylactic neuroprotective effect against excitatory amino acid-induced toxicity in PC12 cells, despite the fact that PC12 cells are not known to have TRH receptors.

d. Effect of TRH *During* Glutamate Toxicity in PC12 Cells

Using the LD_{50} of glutamate in PC12 cells, we treated the cells with TRH *during* the period when the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could exert a neuroprotective effect against excitatory amino acid-induced toxicity simultaneously with the toxic insult, again despite the fact that PC12 cells are not known to have TRH receptors.

e. Effect of TRH *After* Glutamate Toxicity in PC12 Cells

Again using the LD_{50} of glutamate in PC12 cells, we treated the cells with TRH *after* the cells were exposed to 30 minutes of toxic glutamate, in order to determine if TRH could rescue the cells from excitatory amino acid-induced toxicity, despite the fact that PC12 cells are not known to have TRH receptors.

C. Rationale for using Hippocampal Slices and Anterior Pituitaries in Neuroprotection Studies

We used hippocampal slices in order to increase the complexity of the experimental design, and to get closer to the cellular environment, which would be encountered *in vivo*. CNS slice preparations have become very useful in studying numerous scientific questions.

In particular, hippocampal slice studies have been used to define the role of excitatory amino acids and their derivatives in the induction of, or the protection against, neuronal damage (Schurr et al., 1984); (Schurr et al., 1995), although we are among the first to maintain hippocampal slices for an extended period of time (15 hrs). Schurr and colleagues demonstrated that rat hippocampal slices, which are sensitive and inexpensive, are very well suited as an *in vitro* system for use in the screening of both agonists and antagonists of excitatory amino acid receptors (Schurr et al., 1995).

The hippocampal slices were used due both to the presence of neurons with the required receptor profiles, as well as the presence of glia, thus increasing the complexity of the experimental design, and therefore reflecting the complexity of the neuronal environment, which exists *in vivo*.

Pitfalls of the slice superfusion have been described as well (Reid et al., 1988), including possible problems due to the thickness of the slices in the experimental design, and viability of the slices over the course of the experiment, as well as the fact that the superfusion medium must be tightly controlled by the experimental design. In this

system, the goal was to determine if TRH can protect against glutamate excitotoxicity in superfused hippocampal slice or anterior pituitary preparations.

Rat dentate gyrus slices were prepared according to previously described methods (Low et al., 1989a); (Knoblach et al., 1989), and were used to determine if prolonged exposure to TRH or its analogues resulted in specific downregulation of $G_{\alpha q/11}$. If so, this would demonstrate that TRH can modulate levels of $G_{\alpha q/11}$ in a complex neuronal milieu *in vitro* (Reid et al., 1988); (Schoepp and Desai, 1995); (Schurr et al., 1984); (Schurr et al., 1985); (Schurr et al., 1987).

We used anterior pituitaries in the superfusion studies as a positive control in the slice superfusion studies, due to the presence of a high density of known TRH receptors (Schally and Arimura, 1977); (Schally and Bowers, 1 A.D.); (Nillni and Sevarino, 1999). The granule cells of the hippocampal dentate gyrus (DG) were examined, since they possess both metabotropic glutamate receptors (mGluRs) and TRH receptors, all of which utilize $G_{\alpha q/11}$ in their second messenger signal cascade post-synaptically (Heuer et al., 2000); (Gershengorn and Osman, 1996); (Calza et al., 1992); (Cao et al., 1998); (Itadani et al., 1998); (Sun et al., 2003).

Anterior pituitaries were also used as a positive control due to the fact that the downregulation of $G_{\alpha q/11}$ in the pituitary after TRH administration has been demonstrated (Hinkle, 1989). Glutamate has been shown to induce pituitary hormones *in vivo* (Terry et al., 1985); (Mason et al., 1988).

Another key reason that we used slices and pituitaries was to be able to examine the tissue using morphological analysis, comparing damaged tissue with undamaged

tissue, and then verifying those results with Western blots, which would be used to further elucidate the hypothesized mechanism of neuroprotection in the experimental design.

TRH administration has been shown to induce the sub-cellular redistribution and down-regulation of its intracellular signaling protein, $G_{\alpha q/11}$. TRH added to anterior pituitary cells that were co-transfected with G-protein and the TRH receptor resulted in substantial redistribution and reduction in the total cellular levels of this G-protein in response to activation of phospholipase-C (PLC)-coupled receptor (Milligan et al., 1993); (Svoboda et al., 1996). Furthermore, TRH levels are markedly increased and TRH receptors significantly downregulated after kindled seizures (Kubek et al., 1993).

1. Effect of Superfusion Time on Slice Viability

a. Assessment by LDH Assay

It was essential to determine that slices and whole pituitaries would be viable for the entire period of the superfusion experiments, in order to be able to accurately assess the neuroprotective effect of TRH and its analogues against excitatory amino acid-induced toxicity. The analysis of LDH release into the superfusate, collected at specific time points, allowed us to determine if and when cells were experiencing membrane damage.

b. Assessment by Morphology

It was also essential to determine visually that slices and whole pituitaries would be viable for the entire period of the superfusion experiments, in order to be able to

accurately assess the neuroprotective effect of TRH and its analogues against excitatory amino acid-induced toxicity. The morphological analysis of the slices, collected at specific time points, and examined microscopically, allowed us to determine visually if the tissue was experiencing swelling and/or shrinkage over time and/or with various drugs.

c. Assessment by Caspase-3 Assay

Caspase-3 assays were conducted on the slices and anterior pituitaries as another way to determine that slices and whole pituitaries were (or were not) viable for the entire period of the superfusion experiments, in order to be able to accurately assess the neuroprotective effect of TRH and its analogues against excitatory amino acid-induced toxicity. The enzymatic analysis of the slices, collected at specific time points, and compared to negative and positive superfusion slice controls, allowed us to determine if the tissue was experiencing apoptotic damage over time and/or with various drugs.

2. Effect of TRH on Glutamate Toxicity in the Anterior Pituitary and in Hippocampal Slices

a. Dose Effect of N-Methyl-D-Aspartic Acid (NMDA) on Superfused Hippocampal Slice Viability

We over-stimulated hippocampal tissue slices with N-methyl-D-aspartate (NMDA), a selective ionotropic glutamate receptor agonist, in order to cause damage to the hippocampal slices.

b. Dose Effect of NMDA + (S)-3,5-Dihydroxyphenylglycine (S-DHPG) on Superfused Hippocampal Slice Viability

We also over-stimulated tissue slices with a combination of N-methyl-D-aspartate (NMDA), a selective ionotropic glutamate receptor agonist, and with dihydroxyphenylglycine (DHPG), a selective group I metabotropic glutamate receptor agonist, and with a combination of both, in order to cause damage to the hippocampal slices.

c. Effect of TRH on Superfused Hippocampal Slice Viability after Exposure to Toxic Dose of NMDA + DHPG

TRH and its analogues were added during the exposure of superfused hippocampal slices to toxic NMDA and DHPG doses to determine its neuroprotective effects in the slice system.

d. Effect of TRH on Levels of Secreted Amyloid Precursor Protein (sAPP) in Superfused Hippocampal Slices

Alzheimer's disease (AD) is characterized by the significant loss of cholinergic markers and depositions of the amyloid-beta peptide (Aß), which is derived from the Aß precursor protein (APP). Currently, acetylcholinesterase inhibitors and memantine are the FDA-approved drugs but they are used only for symptomatic relief.

The aim of this investigation was to study the effect of a highly selective acetylcholinesterase inhibitor drug, phenserine, on the level of the beta-amyloid precursor protein (APP) in whole hippocampal slices following superfusion. Utilizing whole hippocampi serially sectioned from Sprague-Dawley rats, the effects of varying doses of phenserine (1, 3 and 10 μm) on secreted APP (sAPP) were investigated

e. Effect of TRH on Superfused Anterior Pituitary Viability

Initially, it needed to be shown that TRH was non-toxic against anterior pituitaries, for the dose utilized, as well as for the time required for the experiment.

f. Effect of TRH on DHPG/NMDA-Treated Superfused Hippocampal Slices

Using toxic dose of DHPG + NMDA in hippocampal slices, we treated the slices with TRH *during* the period when the cells were exposed to a toxic dose of DHPG + NMDA, in order to determine if TRH could exert a neuroprotective effect against excitatory amino acid-induced toxicity simultaneously with the toxic insult.

g. Effect of TRH on $G_{\alpha q/11}$ Levels in Hippocampal Dentate Gyrus Slices

We focused on heterologous receptor down regulation as a mechanism, which may be responsible for the neuroprotective effect of TRH noted in our slice studies.

IV. MATERIALS and METHODS

A. Hippocampal Subregional Dissection

The procedure previously described by Low, and used in the Kubek lab was utilized for the hippocampal slice superfusion experiments (Low et al., 1989b). Rats were decapitated, and hippocampi were removed from the brain, keeping the dorsal (septal), ventral (temporal) orientation intact, and placed on an ice-cold aluminum plate. The dissection of the hippocampi started with a slice transversely through the center, separating them into two halves. Transverse sections of 500 μm were obtained, using microforceps and a scalpel with a stainless steel #5100 eye blade (George Tiemann, Philadelphia, PA).

Hippocampal structures were identified using a low-power dissecting microscope, with the dentate gyrus as the key feature. The first cut was made at 90 degrees to the ends of the dentate gyrus, and the next cut (a continuation of cut 1) was made parallel to, and immediately outside this region. This isolated the CA3 subregion of Ammon's Horn, as described in the Kubek lab previously.

Next, cuts 2 and 3 were made inside the cell bodies of the granule layer, isolating CA4 of Ammon's Horn, as well as the hilar region of the dentate gyrus. Cut 4 was then made along the fissure between the dentate gyrus and Ammon's Horn, in order to isolate the dentate gyrus for superfusion.

The final cut was then made through the arc of Ammon's Horn, at the transition point between CA1 and the subiculum. Microforceps were used to place the dentate gyrus subregions on tared tin foil squares measuring 2 inches x 2 inches, weights were

obtained, and the subregions were then subjected to superfusion. It should be noted that tissue was manipulated as minimally as possible prior to superfusion, and that the dissection did not take longer than 30 min to complete for 2 animals, since TRH has been shown to be stable for at least 2 hours (Kubek et al., 1977).

Furthermore, the tissue obtained from the superfusion wells after the superfusion procedure was complete was separated into sections to be (1) frozen at -70°C for future Western blotting; (2) frozen at -20°C for LDH assay; or (3) fixed in 10% paraformaldehdye for morphological analysis via cresyl violet staining, as described.

B. Superfusion of Hippocampal Slices

The ability to maintain viable brain slices over an extended period of time is essential for several molecular and morphological analyses, which require tissue to be viable for hours or even days. We began with the hypothesis that rat hippocampal slices could be maintained for more than the 12-16 hours previously described.

We measured the viability of superfused and non-superfused hippocampal slices for a period of 60 hours via (1) lactic dehydrogenase (LDH) release assays, as a measure of cell damage; (2) caspase-3 assays, as a measure of apoptosis; and (3) Western blots, as a measure of protein expression.

In these hippocampal time course experiments, young adult WISTAR rats (4-5 weeks old) were sacrificed via live decapitation, and the hippocampi (60-80 mg) were removed and dissected on ice into 500μm thin sections, while bathed with cold 0.9% normal saline. These thin hippocampal slices were randomized into 4 groups and allowed to equilibrate for 2 hours in oxygenated low calcium (1.0 mM) Kreb's Ringer

Bicarbonate buffer, at a pH of 7.4, warmed to 37°C, superfused through the tissue at a rate of 0.5 ml/min via a peristaltic pump.

Next, slices and 2 ml of effluent were collected after 0, 12, 24, 36, 48, and 60 hours of superfusion at 0.5 ml/min with a continuously oxygenated normal calcium (2.5 mM) Kreb's Ringer Bicarbonate buffer, at a physiological pH of 7.4 and a temperature of 37°C.

The tissue was immediately frozen in two vials at -70°C, and effluent was placed at 4°C, until the assays were performed. Simultaneously, random tissue samples were removed after the 2 hours of initial oxygenation in low calcium (Ca^{++}) KRB and placed immediately in a non-superfusion environment, at 37°C in normal calcium oxygenated buffer. These non-superfused slices and 2 ml of KRB were also collected after 0, 12, 24, 36, 48, and 60 hours of incubation. Slices were immediately frozen at -70°C, and media was placed at 4°C until the assays were performed.

Rat dentate gyrus slices (500 μm) or anterior pituitaries were equilibrated for 2 hours in low Ca^{++} (1.0 mM) KRB, then superfused for 12 hours in either normal Ca^{++} (2.5 mM) with 10 μM TRH, 3-Me-His-TRH, or Free Acid-TRH-containing KRB.

The DG slices were equilibrated in low Ca^{++}-containing phosphate buffer (KRB), in order to determine baseline LDH release and morphology, and were then superfused for various times in either normal Ca^{++}-containing KRB with TRH, or with glutamate analogues.

Tissue slices were over-stimulated with N-methyl-D-aspartate (NMDA), a selective ionotropic glutamate receptor agonist, and with dihydroxyphenylglycine (DHPG), a selective group I metabotropic glutamate receptor agonist, and with a

combination of both, in order to cause damage to the hippocampal slices. TRH and its analogues were added during the NMDA and DHPG exposure to determine its neuroprotective effects in the slice system.

LDH in the superfusate were compared to controls over time and treatment. Cresyl violet histological stains were conducted to determine qualitatively whether any difference exists morphologically between slices superfused over time or with drugs.

C. Cell Culture Technique for GH-3 Cells

Rat pituitary adenoma (GH-3) cells from ATCC were cultured in RPMI 1640 (Fisher) with 0.5% FBS (Fisher), 15% HS (Fisher) without antibiotic/antimycotic, and maintained in a 5% CO_2 incubator at 37°C until equal confluence was attained. Culture flasks coated with 0.2 mg/ml poly-D-lysine for the initial 7-10 day growth period were used.

Subsequently, cells were harvested using pancreatin digestion, and equal cell aliquots were plated at a density of 3×10^6 cells per 60mm Petri dish. A 16-hour cycle of treatment with glutamate (glu), TRH, or a combination of glu and TRH was utilized: 8 hours of treatment in low serum media (LSM), followed by wash with low serum media alone, and an additional 8 hours of treatment.

The specific protocol for the 16 hours was as follows:

FIRST EIGHT HOURS

PLATE 1: low serum media (LSM) + 100 nM glu (G)

PLATE 2: LSM + 100 nM glu (G)

PLATE 3: LSM + 100 nM glu + 10 μM TRH (GT)

PLATE 4: LSM + 100 nM glu (G)

PLATE 5: LSM + 10 μM TRH (T)

PLATE 6: LSM + 10 μM TRH (T)

PLATE 7: LSM + 10 μM TRH (T)

PLATE 8: LSM ONLY (M)

SECOND EIGHT HOURS

PLATE 1: LSM + 100 nM glu (G/G)

PLATE 2: LSM + 10 μM TRH (G/T)

PLATE 3: LSM + 100 nM glu + 10 μM TRH (GT/GT)

PLATE 4: LSM ONLY (G/M)

PLATE 5: LSM + 100 nM glu (T/G)

PLATE 6: LSM + 10 μM TRH (T/T)

PLATE 7: LSM ONLY (T/M)

PLATE 8: LSM + 100 nM glu (M/G)

At 0, 4, 8, 12, and 16 hours, 50 μl aliquots of conditioned media were collected for lactic dehydrogenase (LDH) analysis by a calorimetric tetrazolium dye method

(Sigma) to assess cell membrane viability. Cells were harvested on ice after 16 hours, and cell counts were performed via Trypan Blue exclusion analysis to determine cell viability.

D. Cell Culture Technique for PC12 Cells

Rat neuron-like pheochromacytoma (PC12) cells from ATCC were cultured in RPMI 1640 (Fisher) with 0.5% FBS (Fisher), 15% HS (Fisher) without antibiotic/antimycotic, and maintained in a 5% CO_2 incubator at 37°C until equal confluence was attained. Culture flasks coated with 0.2 mg/ml poly-D-lysine for the initial 7-10 day growth period were used.

Subsequently, cells were harvested without digestion, using sterile spatulas, and equal cell aliquots were plated at a density of 3 X 10^6 cells per 60mm Petri dish. A 16-hour cycle of treatment was utilized: 8 hours of treatment in low serum media, followed by wash with low serum media alone, and an additional 8 hours of treatment.

The specific protocol for the PC12 treatments was as follows:

FIRST EIGHT HOURS

PLATE 1: low serum media (LSM) + 100 nM glu (G)

PLATE 2: LSM + 100 nM glu (G)

PLATE 3: LSM + 100 nM glu + 10 μM TRH (GT)

PLATE 4: LSM + 100 nM glu (G)

PLATE 5: LSM + 10 μM TRH (T)

PLATE 6: LSM + 10 μM TRH (T)

PLATE 7: LSM + 10 μM TRH (T)

PLATE 8: LSM ONLY (M)

SECOND EIGHT HOURS

PLATE 1: LSM + 100 nM glu (G/G)

PLATE 2: LSM + 10 μM TRH (G/T)

PLATE 3: LSM + 100 nM glu + 10 μM TRH (GT/GT)

PLATE 4: LSM ONLY (G/M)

PLATE 5: LSM + 100 nM glu (T/G)

PLATE 6: LSM + 10 μM TRH (T/T)

PLATE 7: LSM ONLY (T/M)

PLATE 8: LSM + 100 nM glu (M/G)

At 0, 4, 8, 12, and 16 hours, 50 μl aliquots of conditioned media were collected for lactic dehydrogenase (LDH) analysis by a calorimetric tetrazolium dye method (Sigma) to assess cell membrane viability. Cells were harvested on ice after 16 hrs, and cell counts were performed via Trypan Blue exclusion analysis to determine cell viability.

E. Western Blot Analysis

Western blots were performed on the tissue extract as follows: 100 μg of protein was aliquoted from each sample extract, and speed vacuumed to dryness. 75 μl of water

and 25 µl of 4X Sample Buffer were added to obtain 1.0 µg protein/1.0 µl for each sample. Boil for 5 minutes. PAGE in 12% agarose gel was performed using 30 µg of protein per well. The gel was run at 200V for 48 minutes. Western blot transfer to nitrocellulose membrane was performed via running in transfer buffer at 110V for 60 minutes.

Ponceau stain was used to visualize with acetic acid to verify that serum protein transfer occurred in equal amounts per well. Blot photographs were made using the GDS system. Ponceau was washed with TBST buffer for 10 minutes x 3, and nonspecific binding was blocked with 3% gelatin incubation for 1-2 hours.

Blots were washed with TBST for 10 minutes x 3, and probed O/N with primary antibody of choice. The blots were rinsed with TBST for 10 minutes x 3, and were hybridized with secondary antibody for 1-2 hours. Blot was rinsed with TBST buffer for 10 minutes x 2 and with TBS buffer for 10 minutes x 1.

Next, biotinylated avidin (VECTASTAIN kit) was added for 1 hour. Blot was rinsed with TBS buffer for 10 minutes x 3, and developed with horseradish peroxidase. Finally, densitometric analyses of Western Blots with Scion Image program were performed.

We used the Western blot technique described by Mallieux and colleagues (Mailleux et al., 1992) to determine the effect of TRH administration on the levels of G-proteins $G_{q\alpha}$ /$G_{11\alpha}$, using hippocampal slice homogenates and antiserum against the G-proteins. A linear range of G-protein immunoreactivity was used as a standard against which the actual amount of G-protein present in the hippocampal homogenate could be determined.

F. LDH Assay

The toxic effects of unknown compounds have been measured *in vitro* by counting viable cells after staining with a vital dye. Other methods that have been used include the measurement of DNA synthesis by radioisotope incorporation, cell counting by automated counters, and other methods relying on various dyes and cellular activity. The lactate dehydrogenase (LDH) assay (Sigma) is a means of measuring either the number of cells via total cytoplasmic LDH, or of membrane integrity as a function of the amount of cytoplasmic LDH released into the medium (Sigma).

In this study, the latter measurement is that of membrane integrity as a function of the amount of cytoplasmic LDH released into the medium, whether it is the effluent in a slice superfusion study or the conditioned low serum medium (LSM) used in cell experiments, was utilized (Koh and Choi, 1987).

The LDH assay is reported to be simple and accurate, yielding reproducible results. The LDH assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is then utilized in the stoichiometric conversion of tetrazolium dye. The resulting colored compound is then measured via spectrophotometric analysis at an absorption wavelength of 490 nanometers, using a spectrophotometer (Beckman/Coulter).

If the cells are lysed prior to assaying the medium, an increase or decrease in cell numbers results in a concomitant change in the amount of substrate converted (Sigma). This indicates the degree of inhibition of cell growth (cytotoxicity) caused by the test material. If cell-free aliquots of medium from cultures, which have been exposed to different treatments, are assayed as in this study, then the amount of LDH activity can be

used as an indicator of relative cell viability as well as a function of membrane integrity (Decker and Lohmann-Matthes, 1988); (Legrand et al., 1992); (Sigma Product Info, TOX-7).

LDH assays were performed on both superfused and non-superfused samples, as follows. For the effluent/KRB samples, triple 45 µl aliquots of effluent were incubated for 30 minutes with 5 µl of water and 25 µl of a Master Mix (SIGMA TOX-7 LDH ASSAY) containing LDH dye, LDH substrate, and LDH enzyme in darkness, using a 96-well multiplate. Absorbance of each sample was read using a microplate reader at 490 nm. For the tissue samples, tissue extract was obtained via placing the superfused tissue in 200 µl of ice cold IP Buffer (protease inhibitor), and homogenizing with a Polytron homogenizer at 50% speed for 10 seconds. The homogenate was cold centrifuged for 15 minutes at 10,000 rpm, and protein estimation was performed on the homogenate using the Bradford method.

For the extract samples, triple 5 µl aliquots of extract were incubated for 30 minutes with 5 µl of water and 25 µl of a Master Mix containing LDH dye, LDH substrate, and LDH enzyme in darkness, using a 96-well multiplate. Absorbance of each sample was read using a microplate reader at 490 nm. LDH values were recorded for extract analysis as LDH/µg total protein.

Several possible sources of error exist. The reconstituted enzyme preparation is stable when stored frozen. Storage at 4°C may result in loss of activity and yield erroneous results. Additionally, uneven separation of culture fluid in wells of multiwell plates may cause erroneous results. Finally, NADH is subject to photodegradation.

Excessive exposure of the assay reaction to light can reduce both sensitivity and accuracy.

Standard curves were constructed using LDH enzyme standard (Roche), using 0, 2, 4, 6, 8, 10, 12, 14, 16, 22, and 24 ng in triplicate. Unknowns were diluted so that colorimetric density readings, at specific 490 nm absorbance, were on the linear part of the standard curve, and corrected for dilution.

G. Caspase-3 Assay

Caspase-3 assays were performed as follows. The morning of the assay, slices were weighed and homogenized in 200 µl of Promega cell lysis buffer (#G726A, composition protected via proprietary rights) using a Polytron tissue homogenizer set at 50% speed for 10 seconds. The resultant suspension was centrifuged at 11,000 rpm for 20 minutes, and a protein assay was performed. (Approximately 25-100 µg of total protein is used for the assay).

The assay was then performed in a total volume of 100 µl in 96-well plates. Replicate wells were prepared containing blank (no tissue extract, but all other reactants added). Replicate wells of samples were prepared by adding 32 µl reaction buffer (312.5 mM HEPES, 31.25% w/v sucrose, 0.3125% w/v CHAPS), 2 µl of DMSO, 10 µl of 100mM DTT, and 27 µl of each tissue extract, as well as 27 µl of H_2O. Finally, 2 µl of the DEVD-pNA Substrate (10 mM stock) was added to all wells (Brecht et al., 2001).

The plates were incubated for 4 hours at 37°C. (Note that overnight incubation at 22-25°C is also possible, since sample absorbance should not change, although background absorbance may increase). Absorbance was read using a microplate reader at

405nm. Note that (1) our absorbance range (corrected for protein) was from 0.087-0.193 for Time 0 thru 48 hours of superfusion (T-0 thru T-48), and a value of 0.633 at 60 hours; and (2) the Promega catalog number used for the caspase assay is #G7220.

H. Trypan Blue Analysis

Immediately following the harvest of cells (GH-3, PC12, or fetal neurons), 20 µl of Trypan Blue solution (Sigma-Aldrich) was added to 20 µl of cellular suspension. Cell counting was conducted in a hemacytometer at 20X magnification, using a light microscope. Dead cells stained blue, while viable cells maintained a translucent, clear appearance, as well as a spherical morphology. Only the viable cells were included in the cell count.

The hemacytometer chamber had a depth of 0.1 mm, and the counting area was 1 mm^2. Thus, the number of cells in suspension was calculated from the number of cells counted in 10^{-4} ml (0.1 mm x 1.0 mm^2 = 0.1 mm^3 = 10^{-4} ml). Cell death was calculated using the survival percentage of untreated control cells and comparing that figure to the survival percentage of treated cells. The resulting equation was: # cells counted/1 x 10^{-4} ml x 1 ml suspension = N cells).

I. Morphology

Morphological analysis of superfused hippocampal slices was performed using cresyl violet histological staining. Cresyl violet stain was prepared by mixing 0.5 g cresyl violet in 100 ml double distilled water (DDW), and allowing it to "ripen" for 48 hrs prior to filtering. A 50/50 mixture of Canada Balsam/xylene was used for the analysis, as well,

in a solution consisting of 50 ml Canada Balsam plus 50 ml xylene. The hippocampal slices were fixed in 10% paraformaldehyde and then cut via serial frozen sections on a microtome to a thickness of 10 μm. The resultant slices were placed on a microscope slide and allowed to dry overnight. The slices were then subjected to the following staining protocol, prior to being photographed under a microscope at various magnifications.

3 min DDW rinse

45 sec cresyl violet stain

1 min DDW rinse

2 min DDW rinse (fresh)

1 min 95% ethanol

1 min 100% ethanol

1 min xylene

2 min balsam/xylene

4 min 100% ethanol (fresh)

4 min 100% ethanol (fresh)

5 min xylene (fresh)

5 min xylene (fresh)

Permount coverslip added

Allow to dry horizontally

Examine microscopically

V. RESULTS

A. Effect of TRH on Glutamate-Induced Toxicity in GH-3 Cells

1. Toxic Dose of Glutamate in GH-3 Cells via LDH and Trypan Blue Analyses

The LD_{50} for glutamate in GH-3 cell cultures was found to be 100 nM. These results were confirmed via both trypan blue exclusion analysis as well as by LDH assay, as a measure of cell viability and cell damage, respectively.

2. Non-Toxicity of TRH in GH-3 Cells via LDH and Trypan Blue Analyses

TRH (10μM/8 hrs) treatment was non-toxic by both trypan blue exclusion analysis and LDH assay. (**Figure 3** shows LDH Assay) This was essential in order to show that the cell damage was due to glutamate, and that the resultant neuroprotection was due to TRH, although the mechanism for neuroprotective effect was not known until further experimentation occurred, as will be addressed in subsequent sections.

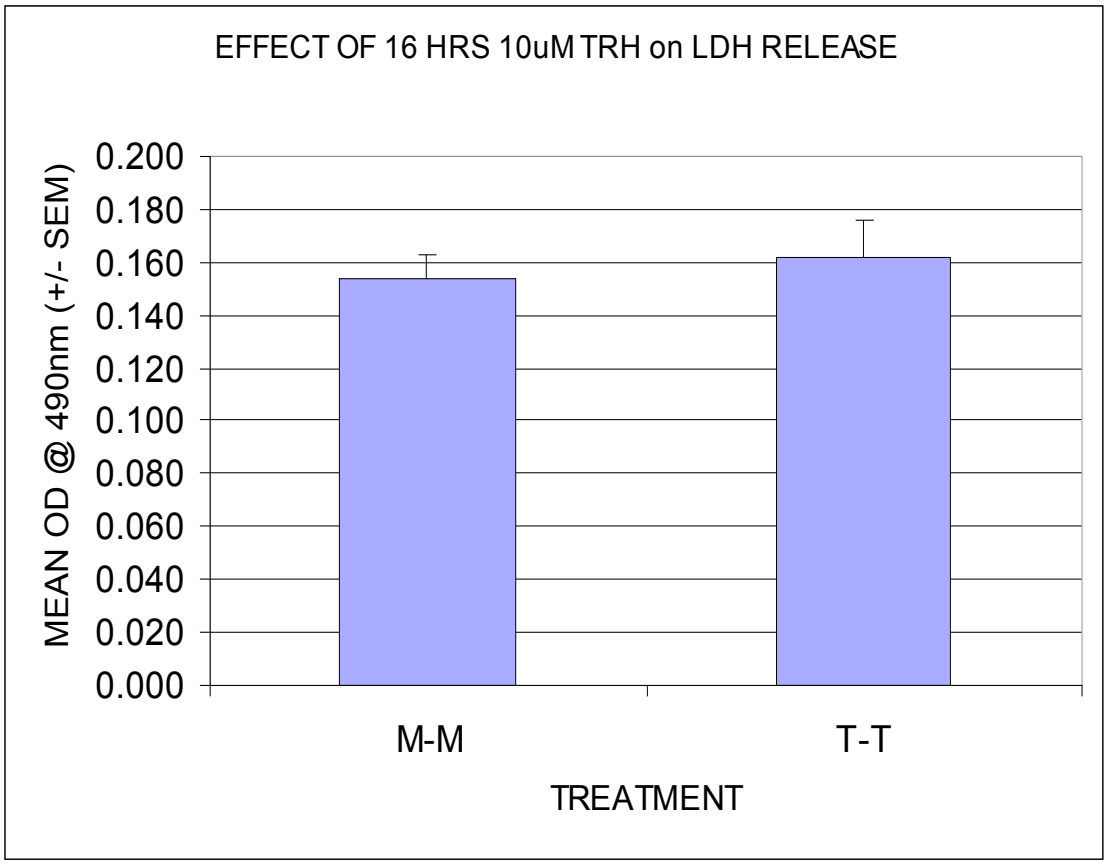

EFFECT OF 16 HRS 10uM TRH on LDH RELEASE
MEAN OD @ 490nm (+/- SEM)
0.200
0.180
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
M-M
T-T
TREATMENT

Figure 3.

3. Effect of TRH *Before* Glutamate Toxicity in GH-3 Cells

TRH *pretreatment* resulted in a significant reduction (compared to control) in GH-3 cells, in cell death (16%, $P<0.05$, $P<0.05$) and damage (GH-3: 68%, $P<0.05$). (**Figures 4 and 5**)

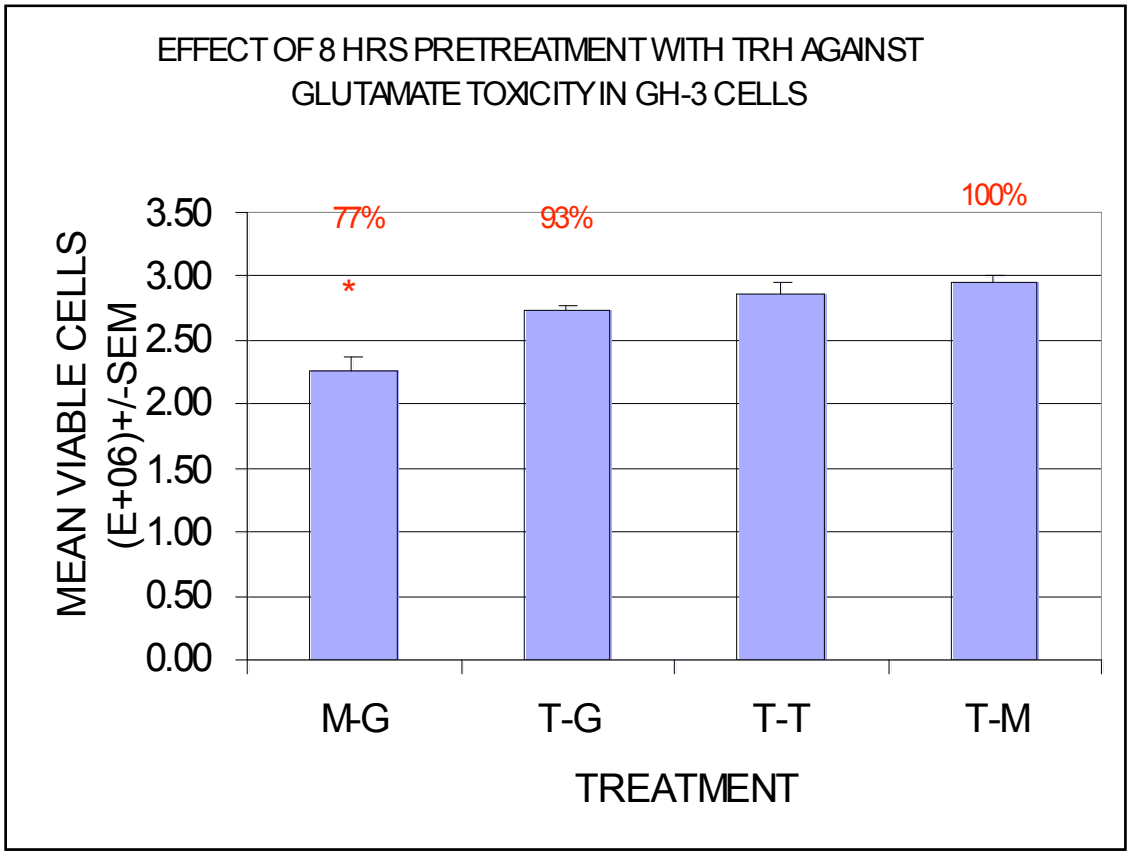


Figure 4.

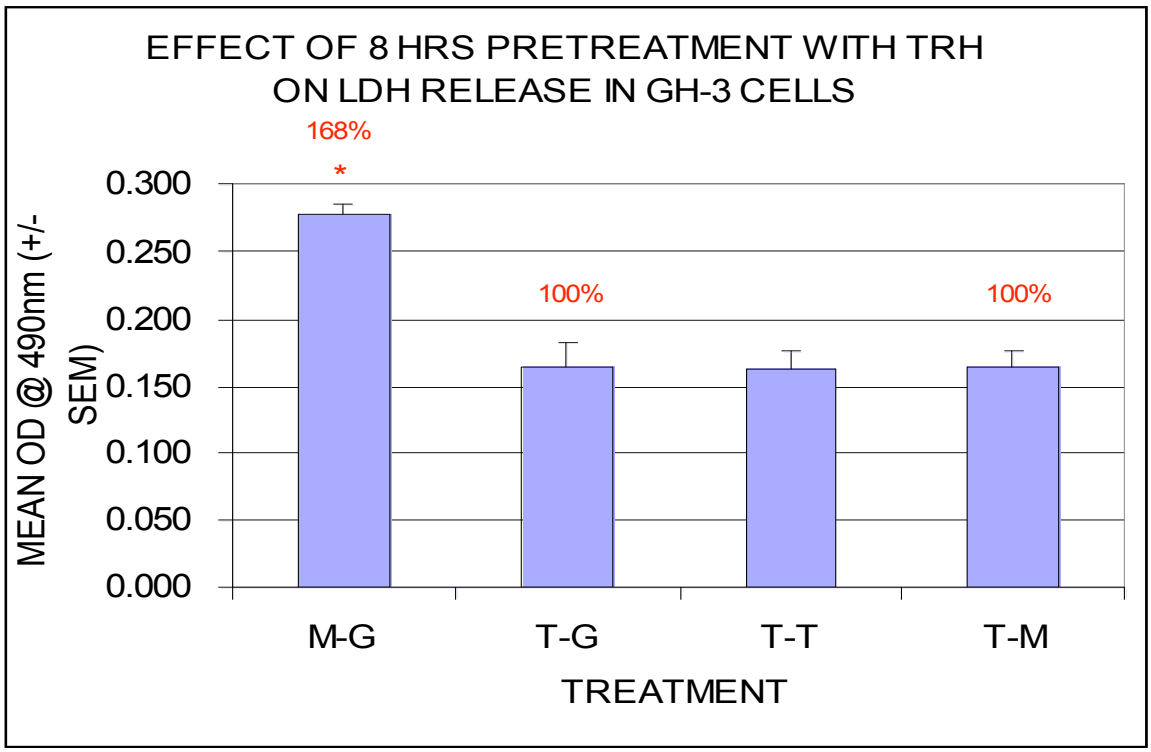

EFFECT OF 8 HRS PRETREATMENT WITH TRH
ON LDH RELEASE IN GH-3 CELLS
168%
*
100%
100%
0.300
0.250
0.200
0.150
0.100
0.050
0.000
MEAN OD @ 490nm (+/- SEM)
M-G
T-G
T-T
T-M
TREATMENT

Figure 5.

4. Effect of TRH *During* Glutamate Toxicity in GH-3 Cells

TRH treatment *during* glutamate (glut) toxicity resulted in a reduction in cell death (GH-3: 33%, $p<0.05$, $P<0.05$) and damage (GH-3: 102%, $P<0.05$).

(**Figures 6 and 7**)

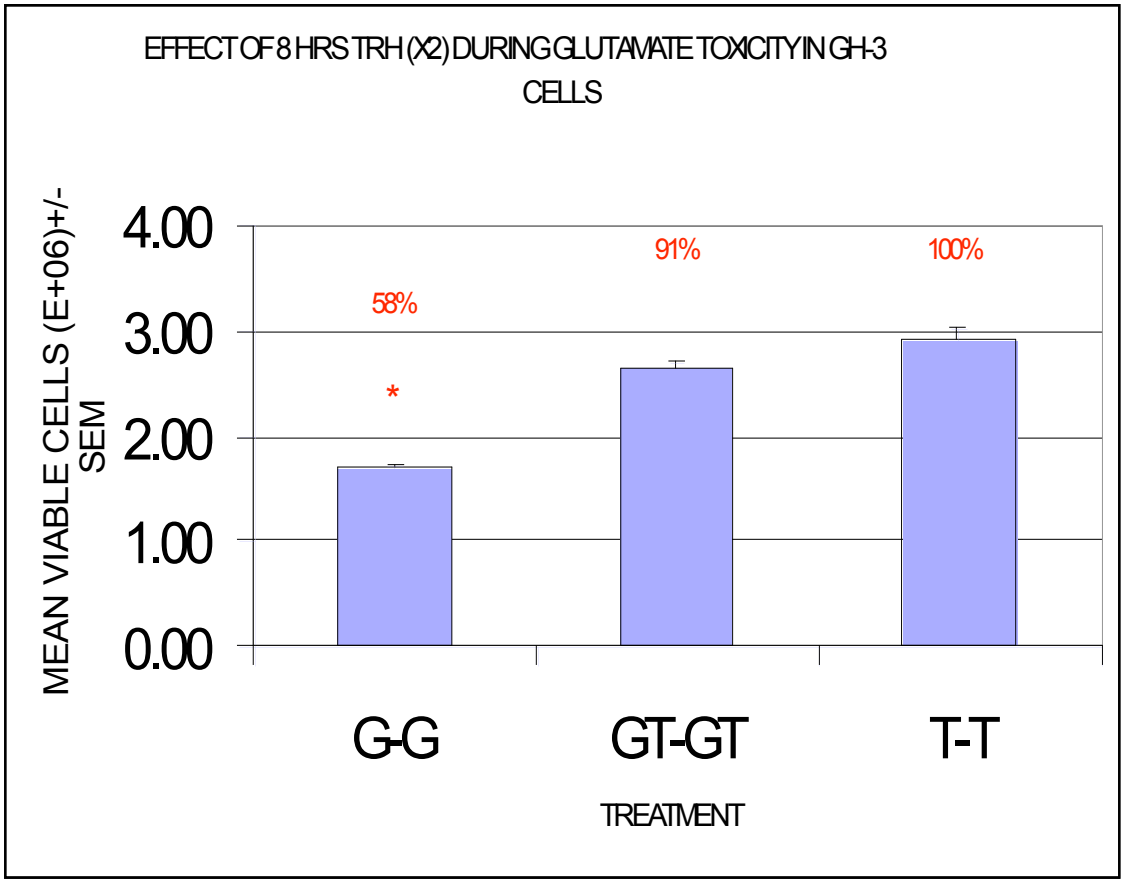


Figure 6.

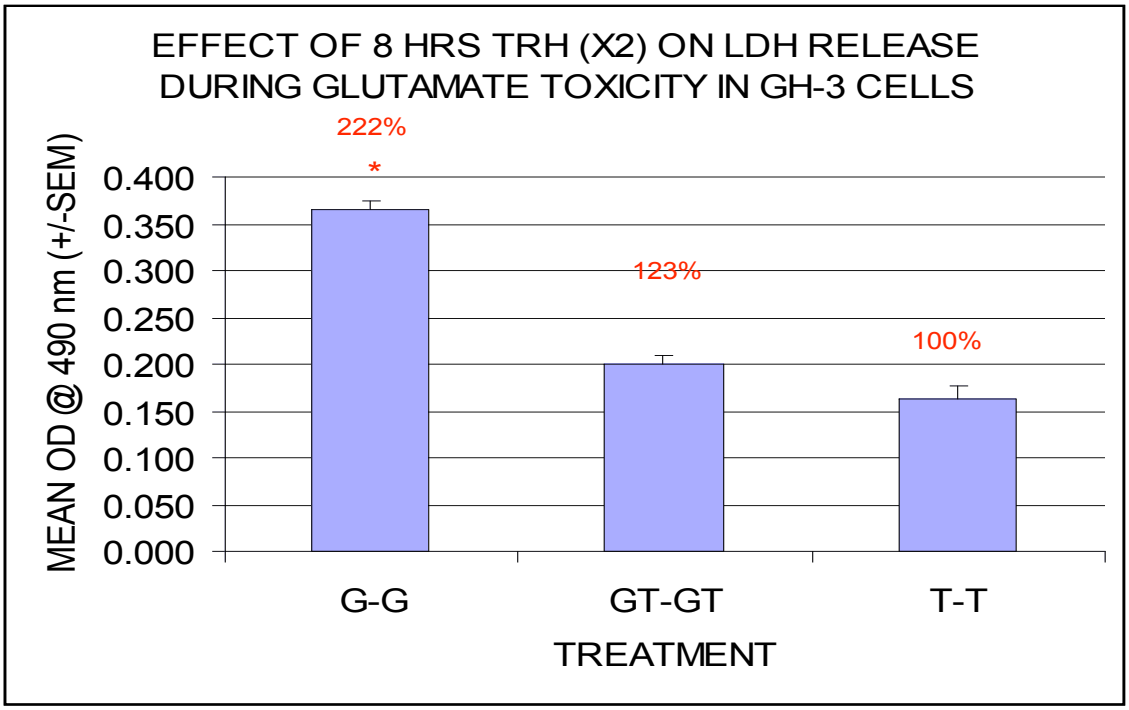

EFFECT OF 8 HRS TRH (X2) ON LDH RELEASE
DURING GLUTAMATE TOXICITY IN GH-3 CELLS
222%
*
123%
100%
MEAN OD @ 490 nm (+/-SEM)
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
G-G
GT-GT
T-T
TREATMENT

Figure 7.

5. Effect of TRH *After* Glutamate Toxicity in GH-3 Cells

TRH given 8hrs *after* glut insult resulted in cellular rescue in GH-3 (GH-3: 9%, P<0.05) and also in damage reduction (GH-3: 44%, P<0.05). (**Figures 8 and 9**)

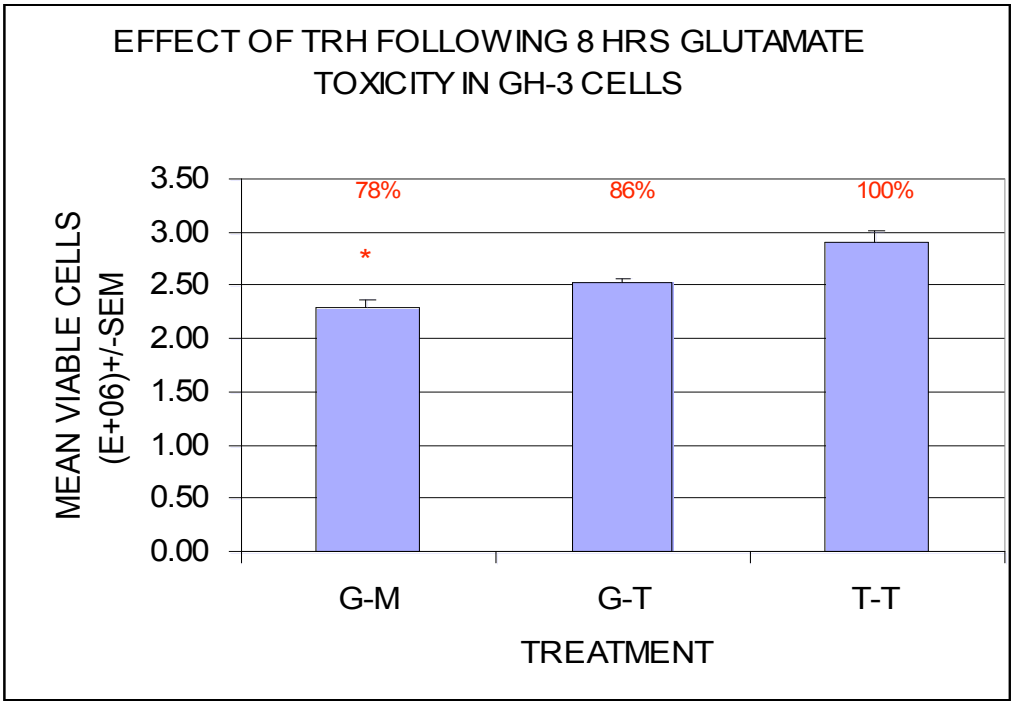


Figure 8.

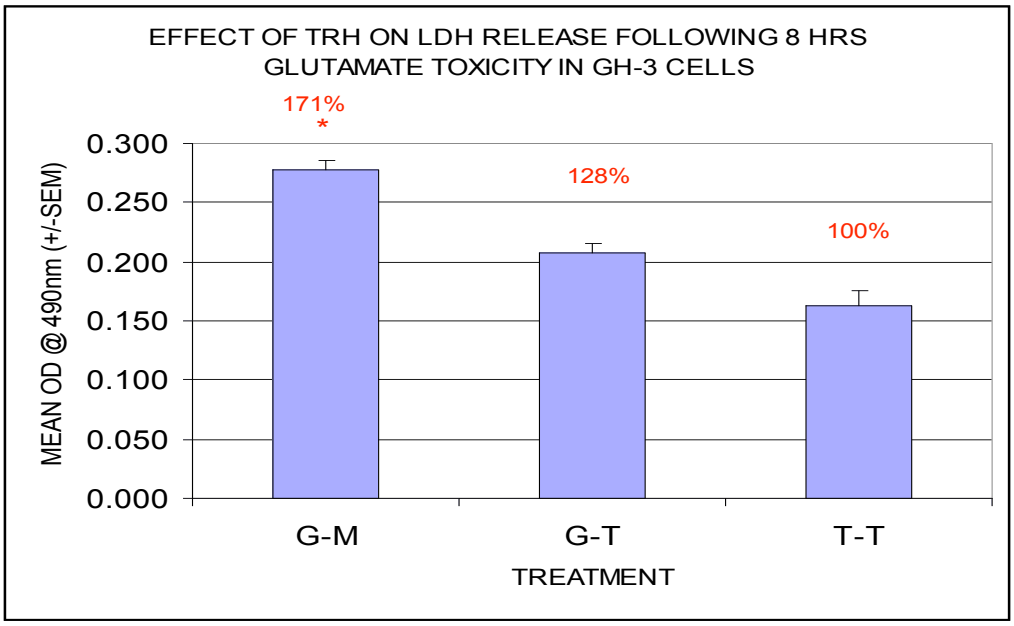


Figure 9.

B. Effect of TRH on Glutamate-Induced Toxicity in PC12 Cells

1. Toxic Dose of Glutamate in PC12 Cells via LDH and Trypan Blue Analyses

The LD_{50} for glutamate in PC12 cell cultures was found to be 100 nM. These results were confirmed via both trypan blue exclusion analysis as well as by LDH assay, as a measure of cell viability and cell damage, respectively.

2. Non-Toxicity of TRH in PC12 Cells via LDH and Trypan Blue Analyses

TRH (10µM/8 hrs) treatment was non-toxic by both trypan blue exclusion analysis and LDH assay. (**Figure 10**)

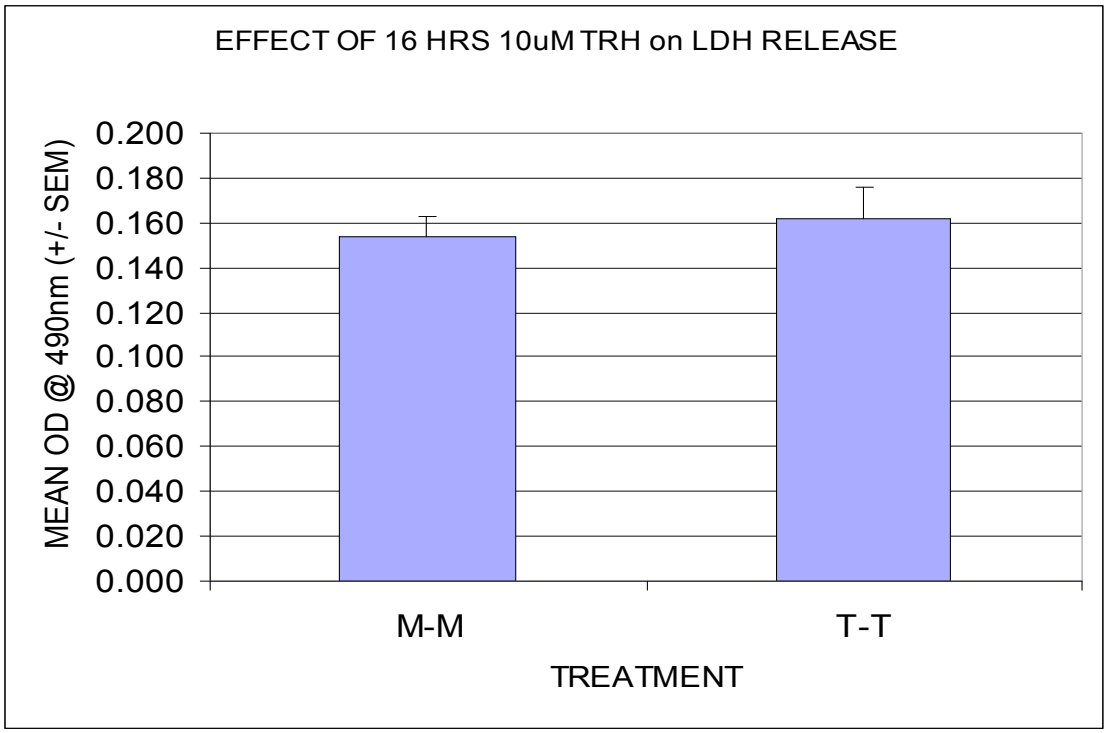


Figure 10.

3. Effect of TRH *Before* Glutamate Toxicity in PC12 Cells

TRH *pretreatment* resulted in a significant reduction (compared to control) in PC12 cells, in cell death (PC12: 21%, $P<0.05$) and damage (PC12: 11%, $p<0.05$). (**Figures 11 and 12**)

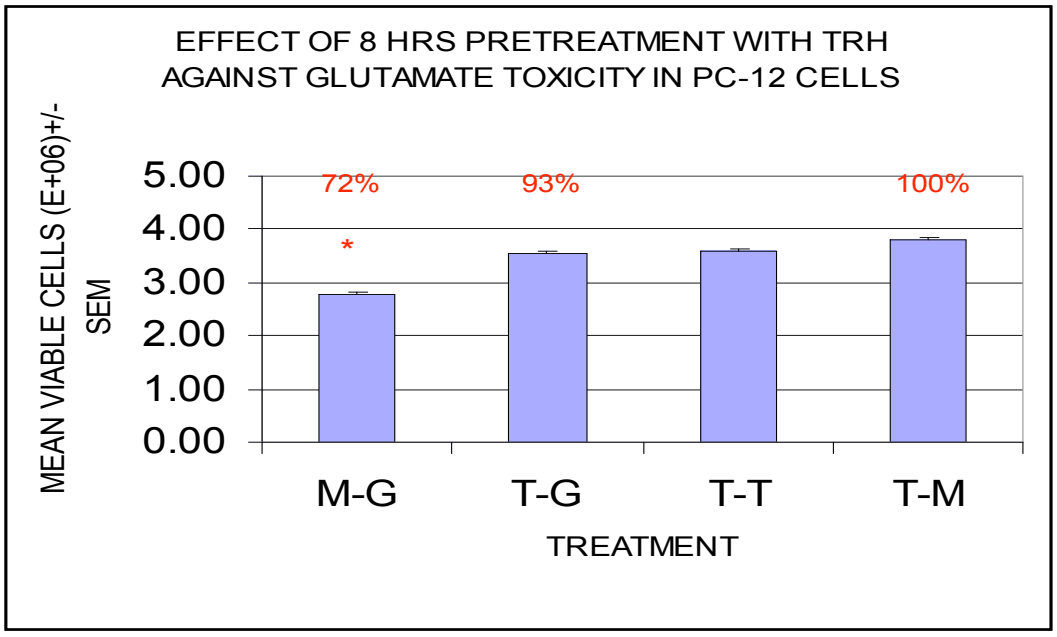


Figure 11.

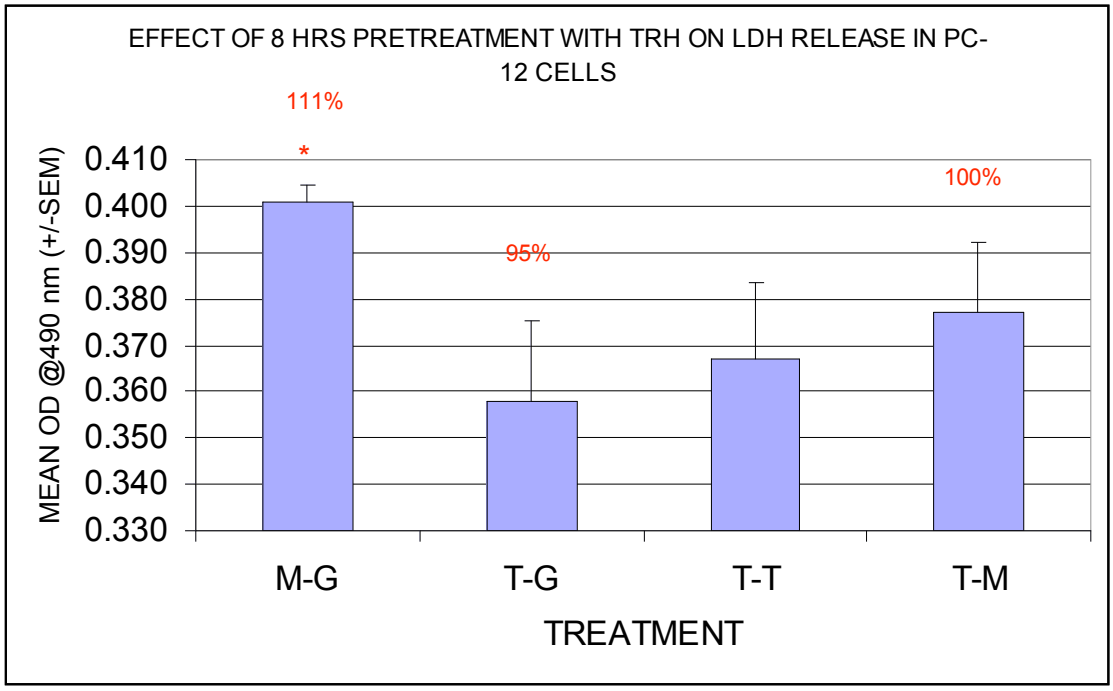

EFFECT OF 8 HRS PRETREATMENT WITH TRH ON LDH RELEASE IN PC-12 CELLS
111%
*
95%
100%
MEAN OD @490 nm (+/-SEM)
0.410
0.400
0.390
0.380
0.370
0.360
0.350
0.340
0.330
M-G
T-G
T-T
T-M
TREATMENT

Figure 12.

4. Effect of TRH *During* Glutamate Toxicity in PC12 Cells

TRH treatment *during* glut toxicity resulted in a reduction in cell death (PC12: 27%, P<0.05) and damage (PC-12: 10%, p<0.05). (**Figures 13 and 14**)

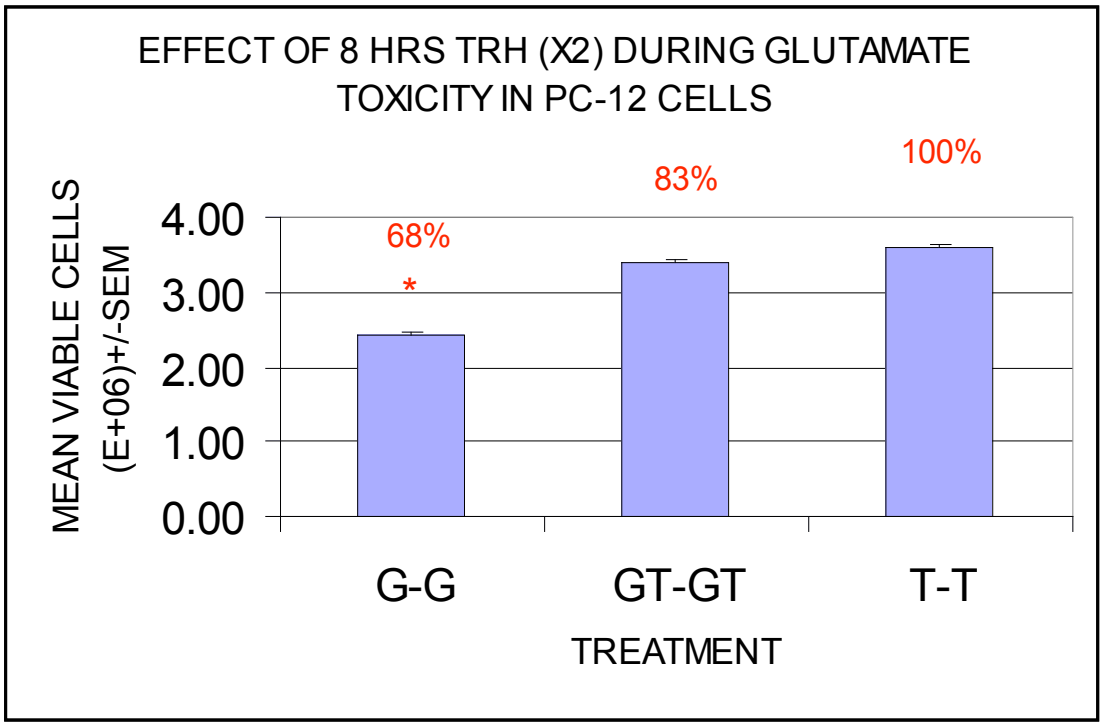


Figure 13.

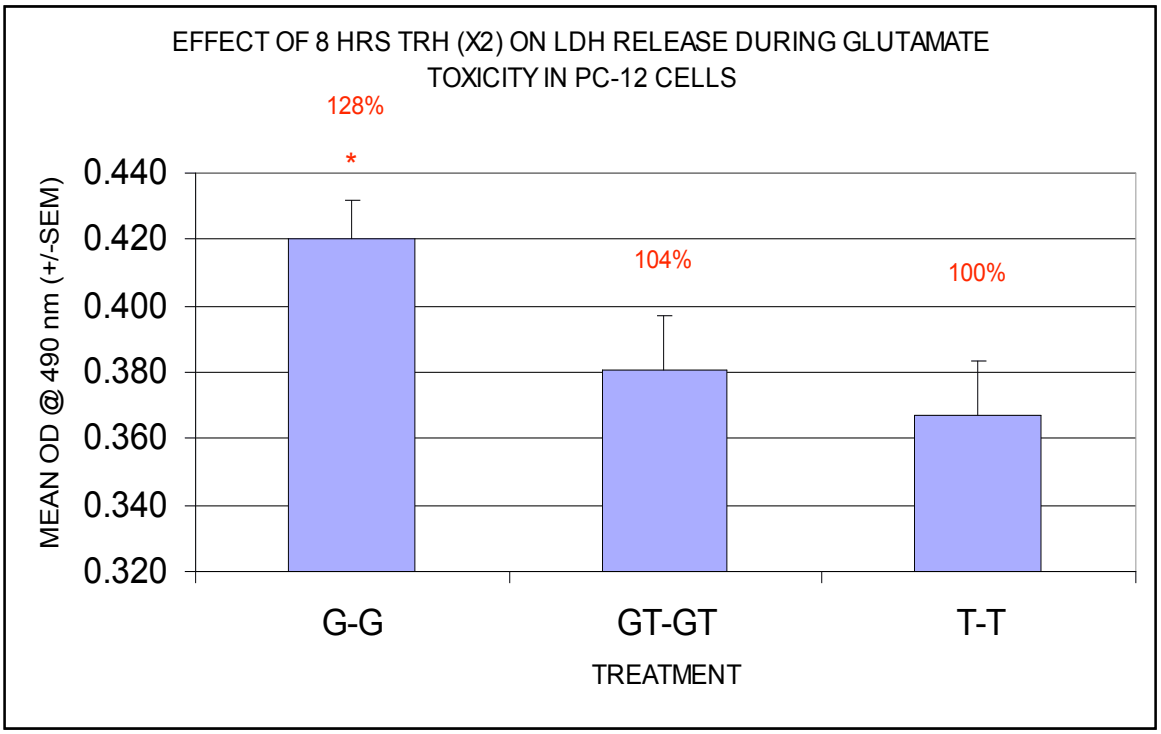

EFFECT OF 8 HRS TRH (X2) ON LDH RELEASE DURING GLUTAMATE TOXICITY IN PC-12 CELLS
128%
*
104%
100%
MEAN OD @ 490 nm (+/-SEM)
0.440
0.420
0.400
0.380
0.360
0.340
0.320
G-G
GT-GT
T-T
TREATMENT

Figure 14.

5. Effect of TRH *After* Glutamate Toxicity in PC12 Cells

TRH given 8 hours *after* glutamate insult resulted in cellular rescue in PC12 cells, (PC12: 15%, P<0.05) but *not in damage reduction*. (**Figures 15 and 16**)

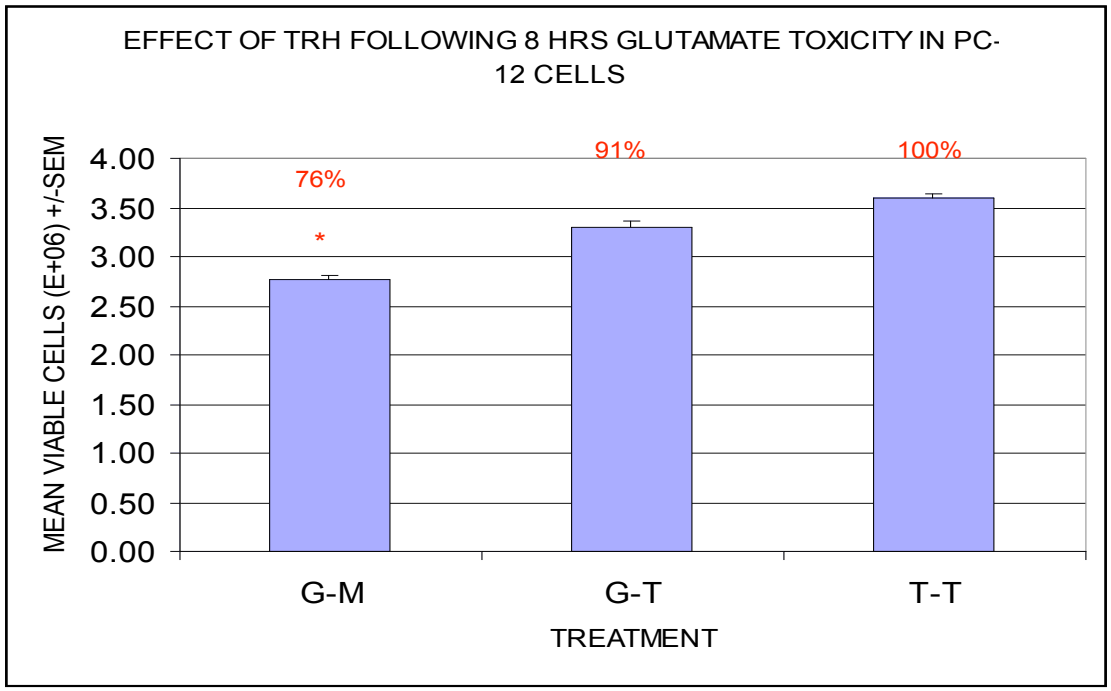


Figure 15.

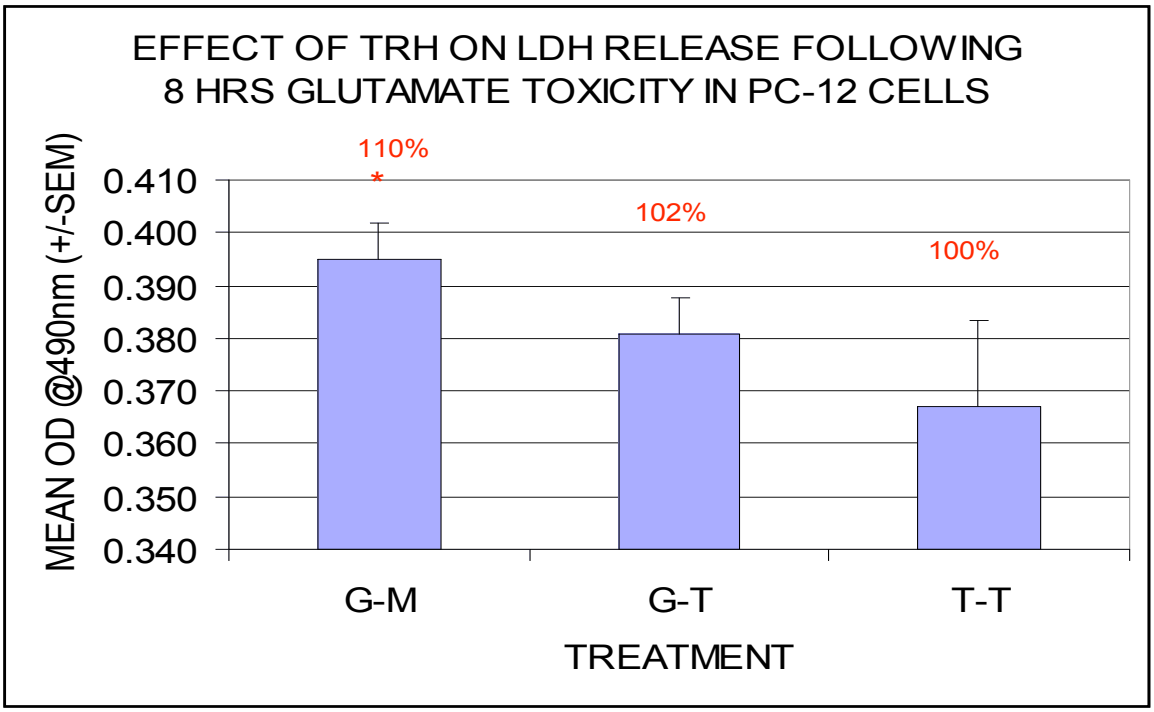

EFFECT OF TRH ON LDH RELEASE FOLLOWING
8 HRS GLUTAMATE TOXICITY IN PC-12 CELLS
MEAN OD @490nm (+/-SEM)
0.410
0.400
0.390
0.380
0.370
0.360
0.350
0.340
110%
*
102%
100%
G-M
G-T
T-T
TREATMENT

Figure 16.

C. Assessment of Superfusion Time on Hippocampal Slice Viability

1. LDH Assay

Hippocampal slices were superfused with oxygenated normal (2.0 mM) calcium-containing KRB for 48 hours. Relative LDH release in 50 µl superfusate expressed as OD @ 490 nm after 0, 4, 6, 8, 12, 15, 36, and 48 hours. *P<0.05 T-0 versus T-36, T-48 from three combined experiments. No significant difference was noted in superfusate at earlier time points.

There was no detectable increase in LDH release from T-0 to T-15 hrs in superfused hippocampal slices; however, there was a significant elevation in LDH release from superfused hippocampal slices at T-36 and T-48 hrs. (**Figure 17**)

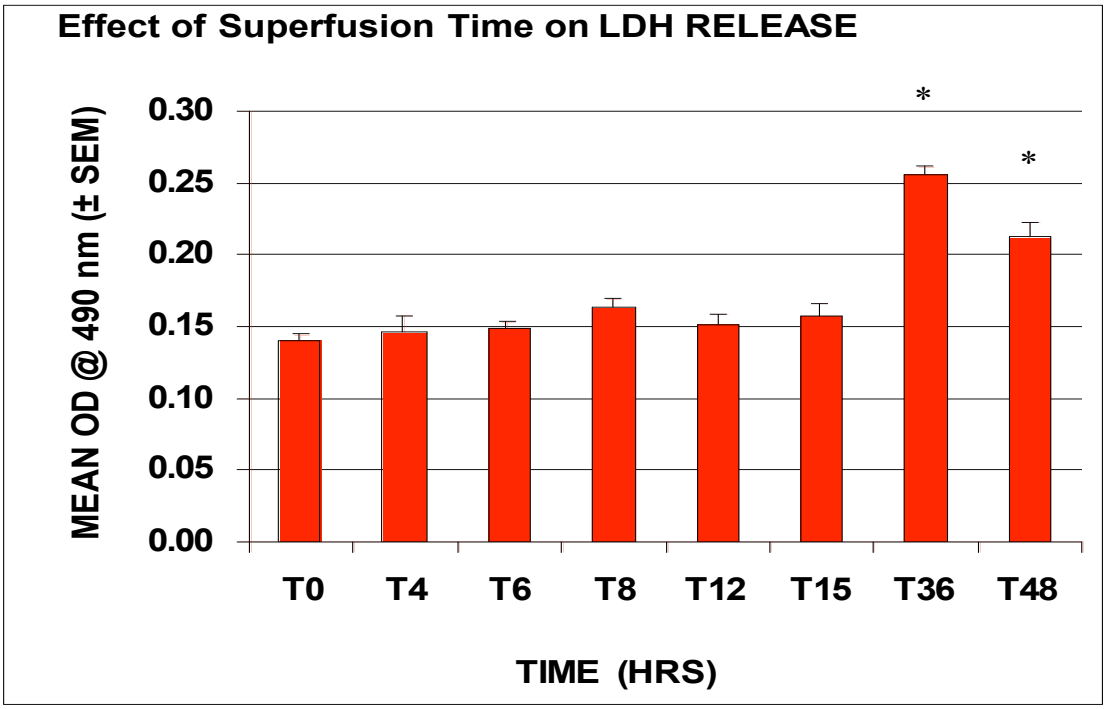


Figure 17.

2. Effect of TRH and its Analogs on LDH Release in Superfused Dentate Gyrus

DG slices were superfused with KRB (control) or KRB containing 10 μM TRH (T10), 10 μM 3-Me-HIS-TRH (M10), or 10 μM Free-Acid-TRH (FA10) for 12 hours. C = controls, T10 = TRH 10 μM, M10 = 3-Me-HIS-TRH 10 μM, FA10 = Free-Acid-TRH 10 μM. As a positive LDH control, slices were exposed to 30 μM NMDA for 12 hours, or subjected to freeze-thawing prior to superfusion. Relative LDH release in 50 μl superfusate expressed as OD @ 490 nm after 0, 4, 6, 8, 12, and 15 hours. *$P<0.05$ C versus F/T, NMDA from three combined experiments. No significant difference was noted in slices treated with T10, M10, or FA10. (**Figure 18**)

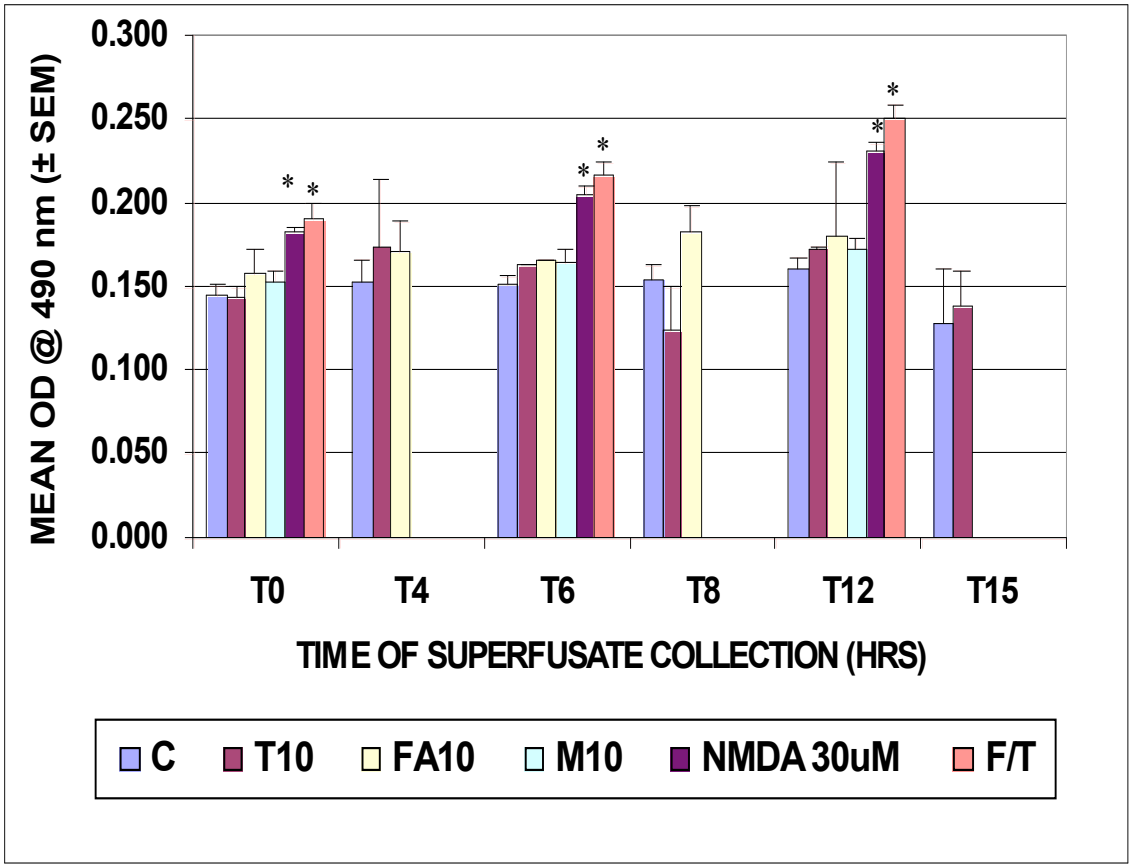

Effect of TRH or its Analogs on LDH Release in Superfused Dentate Gyri
MEAN OD @ 490 nm (± SEM)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
T0
T4
T6
T8
T12
T15
TIME OF SUPERFUSATE COLLECTION (HRS)
C
T10
FA10
M10
NMDA 30uM
F/T

Figure 18.

3. Caspase-3 Assay

Hippocampal slices were superfused with oxygenated normal KRB for 60 hours. A small increase of caspase activity occurred over time with a substantial increase after 48 hours of superfusion. Relative caspase-3 activity/µg protein of tissue lysate expressed as OD @ 405 nm after 0, 12, 24, 36, 48, and 60 hours. *P<0.05 T-0 versus T-48 & 60 (N = 4). (**Figure 19**)

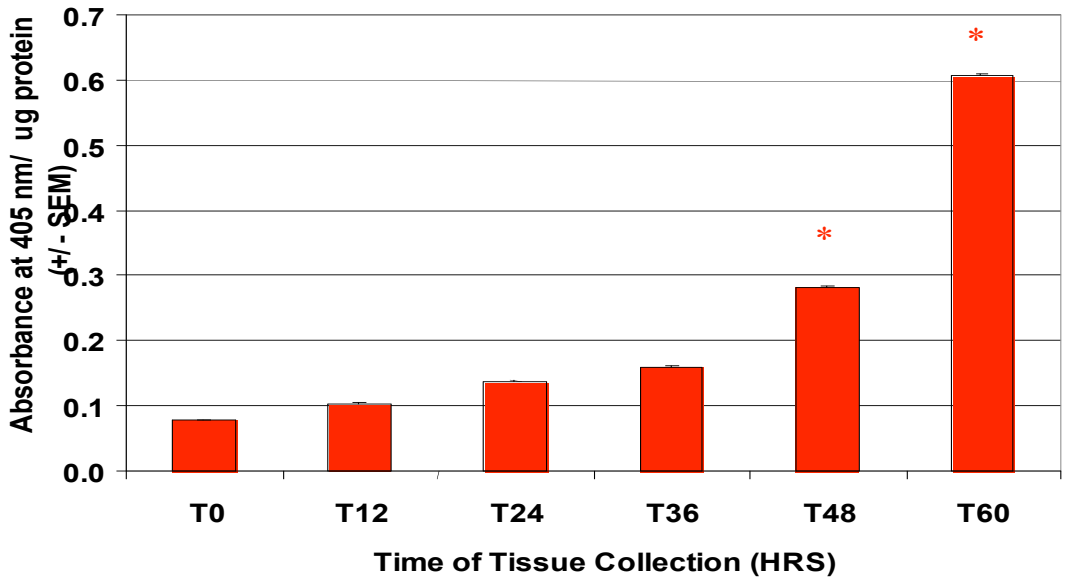


Figure 19.

D. Effect of TRH on Glutamate-Induced Toxicity in the Anterior Pituitary and Hippocampal Slices

1. Effect of NMDA in Superfused Hippocampal Slices

Our preliminary results show that a 30-minute exposure of superfused hippocampal slices or anterior pituitaries to 30 μM NMDA, followed by 12 hours of superfusion, caused significant damage demonstrated both morphologically and by LDH release assays, versus untreated controls.

2. Effect of NMDA + DHPG in Superfused Hippocampal Slices

Our preliminary results show that a 30-minute exposure to a combination of 10 μM DHPG / 30 μM NMDA, 100 μM DHPG / 30 μM NMDA, and to either 10 μM DHPG or 30 μM NMDA alone, caused significant damage, demonstrated by morphological analyses and LDH release assays, after 12 hours of superfusion.

Interestingly, 30-minute exposure to a combination of 5 μM DHPG / 30 μM NMDA, followed by 12 hours of superfusion resulted in significantly less damage than that seen in the slices exposed to toxic doses of DHPG + NMDA. Future studies will examine the neuroprotective effect of TRH against hippocampal slices or anterior pituitaries damaged by toxic doses of DHPG + NMDA.

3. Effect of TRH on Levels of sAPP in Superfused Hippocampal Slices

The hippocampal sections were bathed in a superfusion buffer of oxygenated low calcium KRB for 2 hours to allow LDH to stabilize, and then were superfused in an oxygenated normal calcium buffer with varying concentrations of phenserine for 8 and 15 hours.

Effluent samples were collected at times 0, 4, and 8 hours for the 8-hour experiment, and at times 0, 8, and 15 hours for the 15-hour experiment, to verify that cell death was not occurring. The slices were collected at the endpoint, homogenized in 300 µl IP buffer and 40 µg of each sample was loaded onto a 12% PAGE for Western blotting analysis with 22C11. (**Figure 20**)

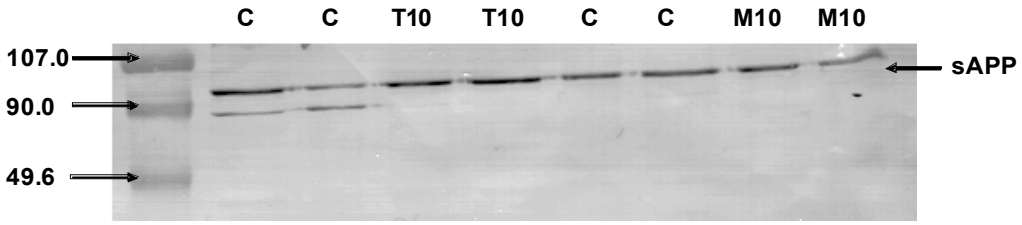


Figure 20.

4. Effect of TRH on Superfused Hippocampal Slices

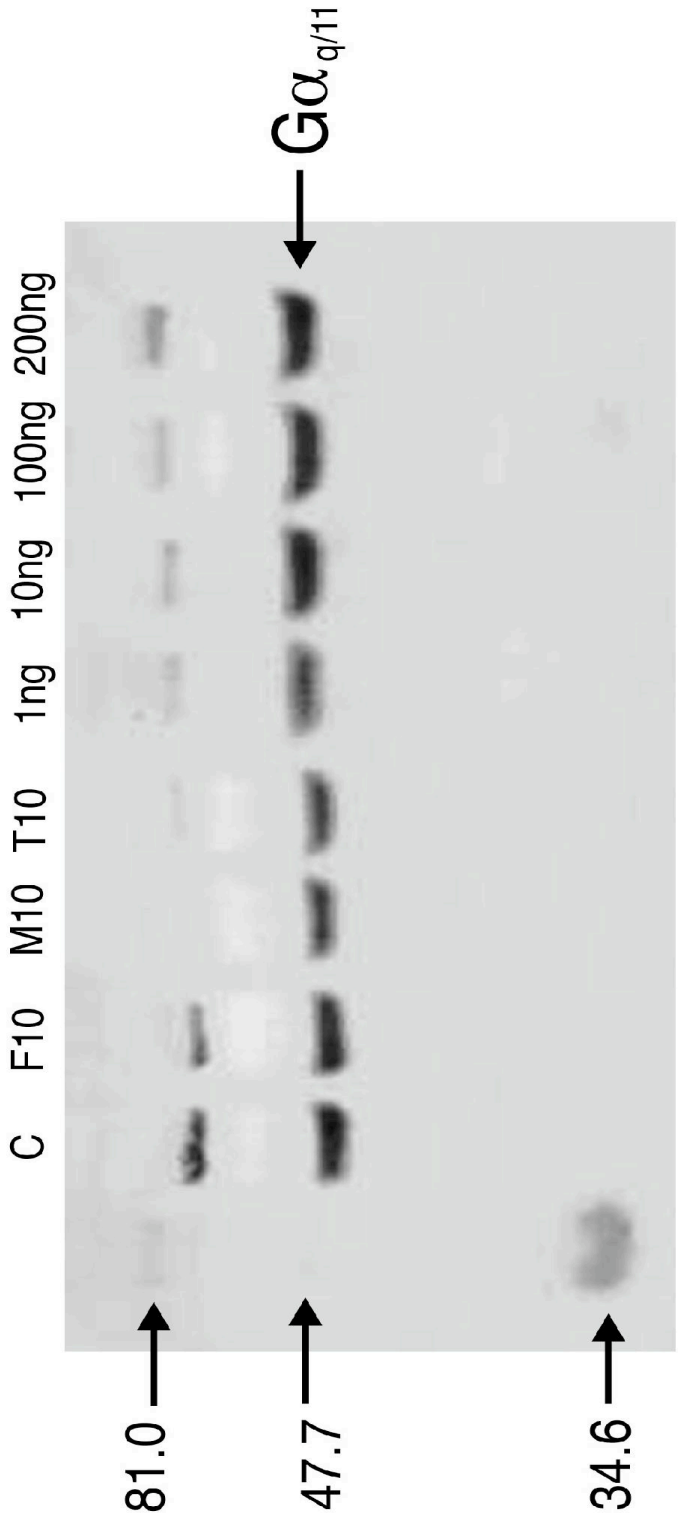
Rat dentate gyrus slices (500 μm) were equilibrated for 2 hours in low Ca^{++} (1.0 mM) KRB, then superfused for 12 hours in either normal Ca^{++} (2.5 mM) with 10 μM TRH, 3-Me-His-TRH, or Free-Acid-TRH-containing KRB. LDH in the superfusate was not significantly different from controls over time or treatment. Morphology via cresyl violet staining revealed no apparent difference between slices superfused over time or with drugs.

5. Effect of TRH on Superfused Anterior Pituitaries

Rat anterior pituitaries were equilibrated for 2 hours in low Ca^{++} (1.0 mM) KRB, then superfused for 12 hours in either normal Ca^{++} (2.5 mM) with 10 μM TRH, 3-Me-His-TRH, or Free-Acid-TRH-containing KRB. LDH in the superfusate was not significantly different from controls over time or treatment. Morphology via cresyl violet staining revealed no apparent difference between slices superfused over time or with drugs.

a. **Figures 21 and 22** demonstrate that prolonged superfusion (12 hrs) of dentate gyrus slices with 10 μM TRH or 10 μM 3-Me-His-TRH (3-Me-HTRH) significantly reduced $G_{\alpha q/11}$ levels by 79% and 93%, respectively.

Figure 21.



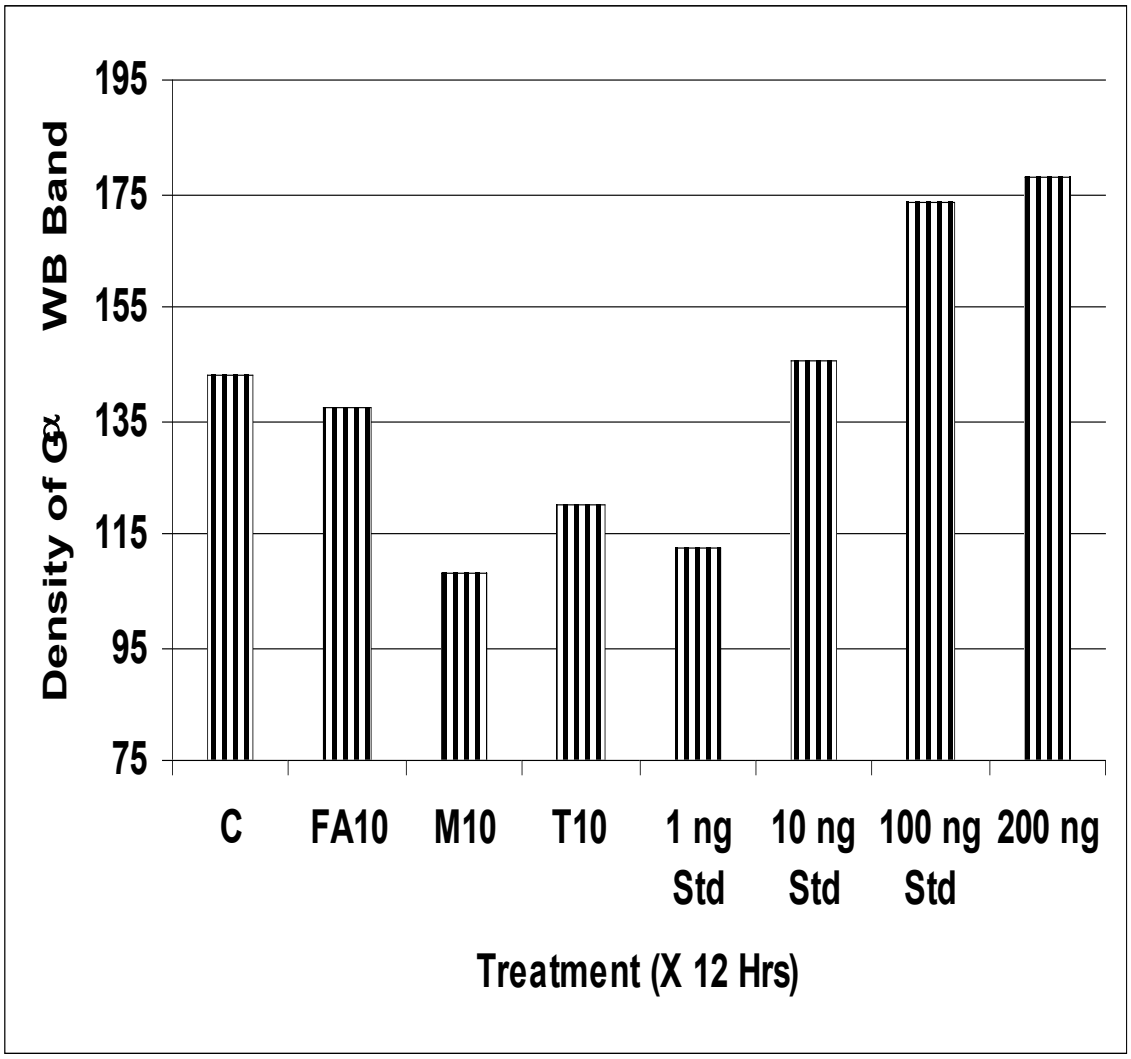

195
175
155
135
115
95
75
Density of Gα WB Band
C
FA10
M10
T10
1 ng Std
10 ng Std
100 ng Std
200 ng
Treatment (X 12 Hrs)

Figure 22.

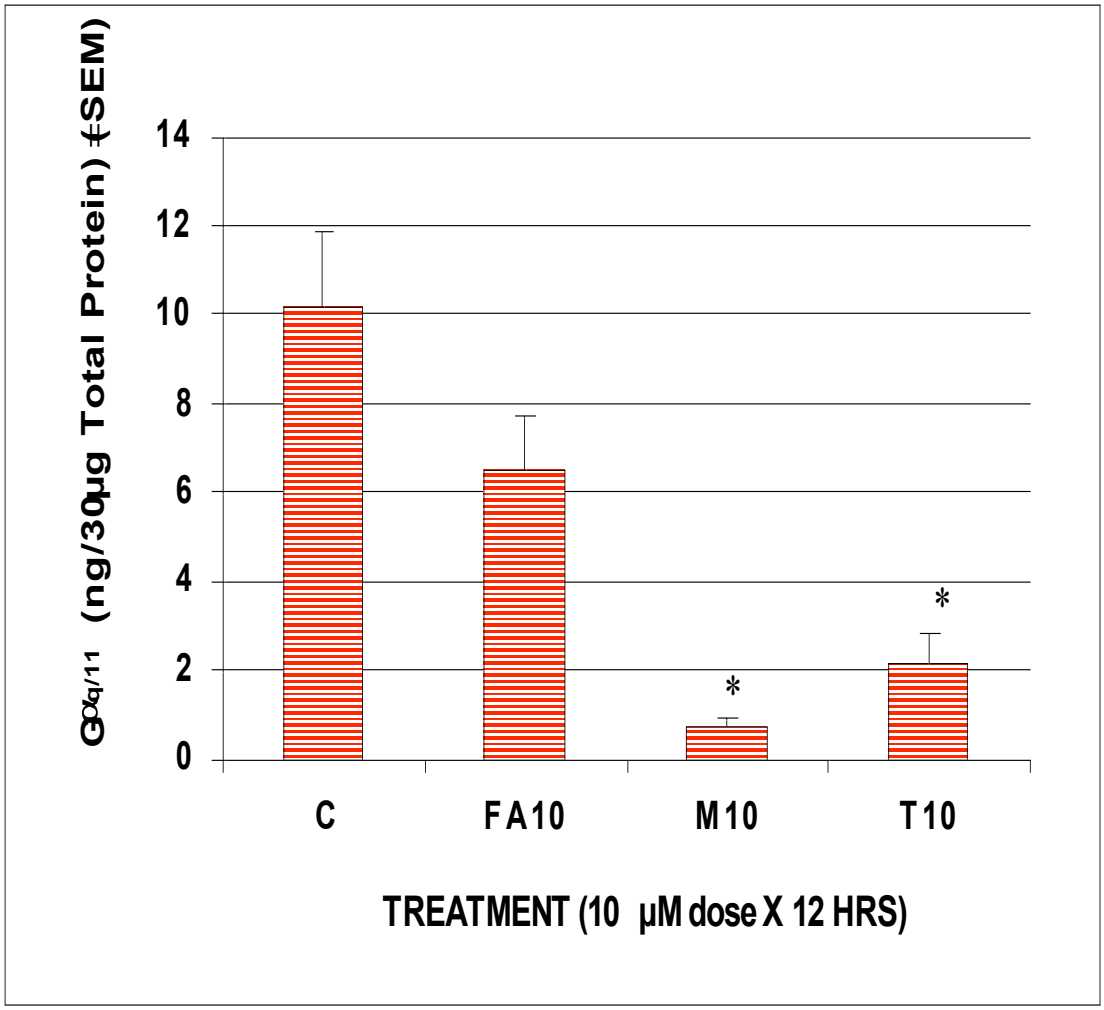

Effect of TRH or its Analogs on [Gα q/11] in Dentate Gyri Extracts
Gαq/11 (ng/30µg Total Protein) ±SEM)
14
12
10
8
6
4
2
0
*
*
C
FA10
M10
T10
TREATMENT (10 µM dose X 12 HRS)

Figure 23.

b. **Figures 24 and 25** show that prolonged superfusion (12 hours) of anterior pituitaries with 10 μM 3-Me-HTRH reduced $G_{\alpha q/11}$ density by 10%.

Figure 24.

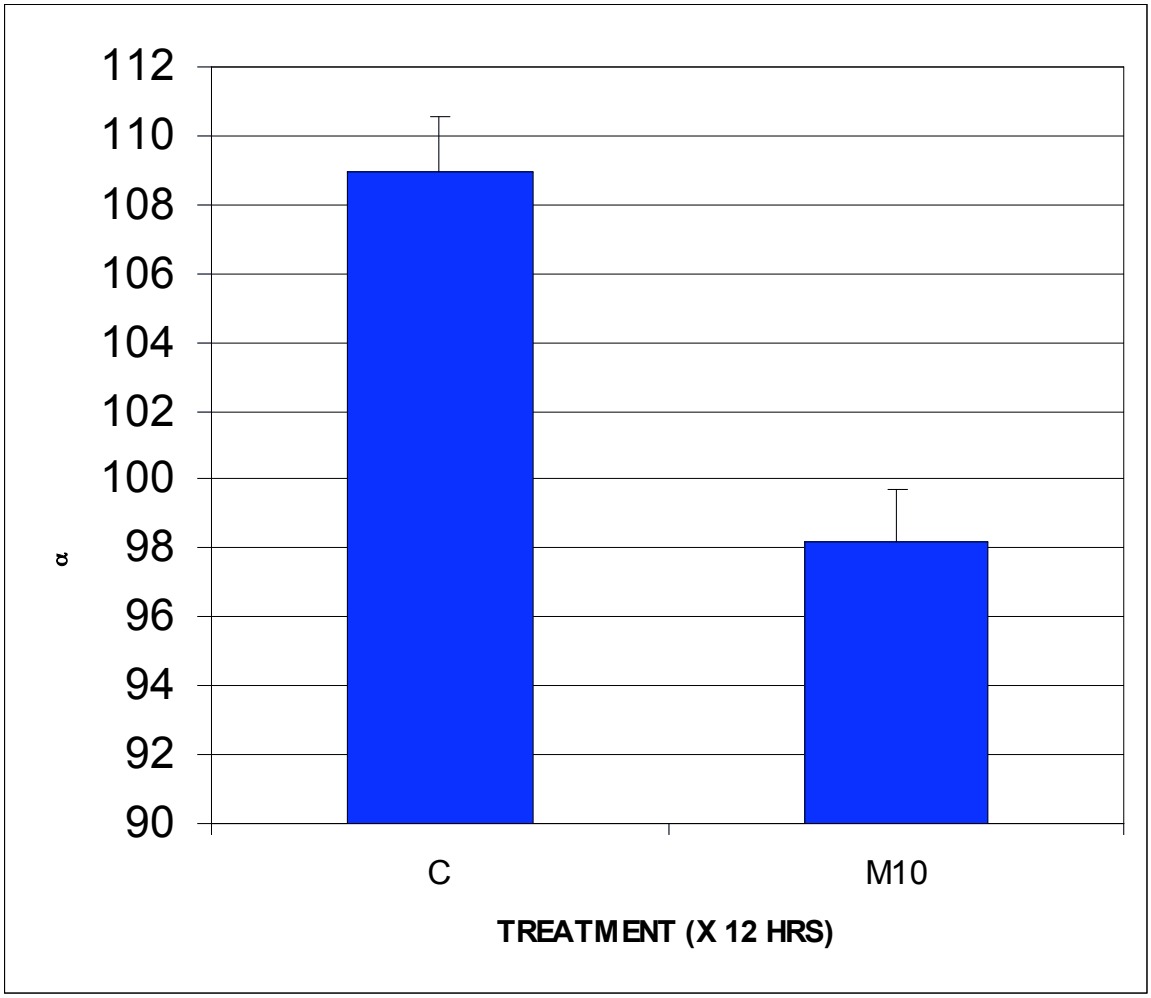


Figure 25.

c. Hippocampal dentate gyrus slices superfused for 12 hours with 10 μM Free-Acid-TRH showed **no significant change in $G_{\alpha q/11}$ levels**.

6. Effect of TRH on NMDA/DHPG-Damaged Superfused Hippocampal Slices

Data in **Figures 26, 27, 28 and 29** show that superfused slices, which were treated with 3-Me-HTRH for 15 hours after 30 minutes exposure to toxic NMDA and DHPG, showed significantly less death and damage than slices not treated with 3-Me-HTRH.

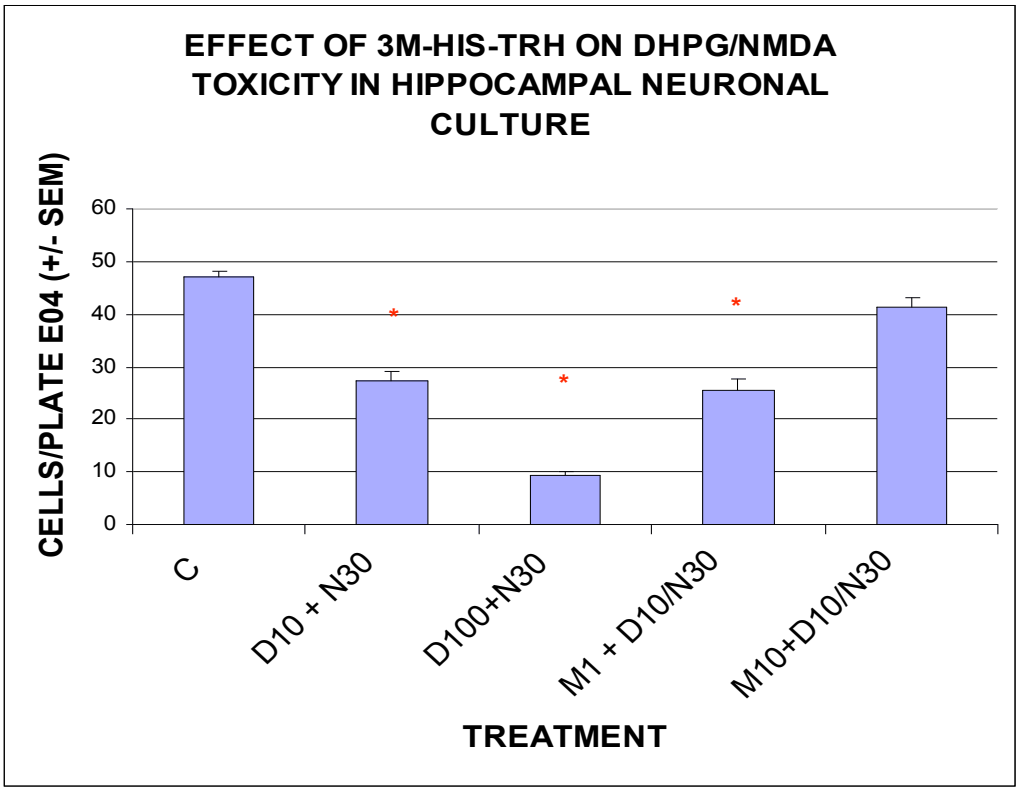


Figure 26.

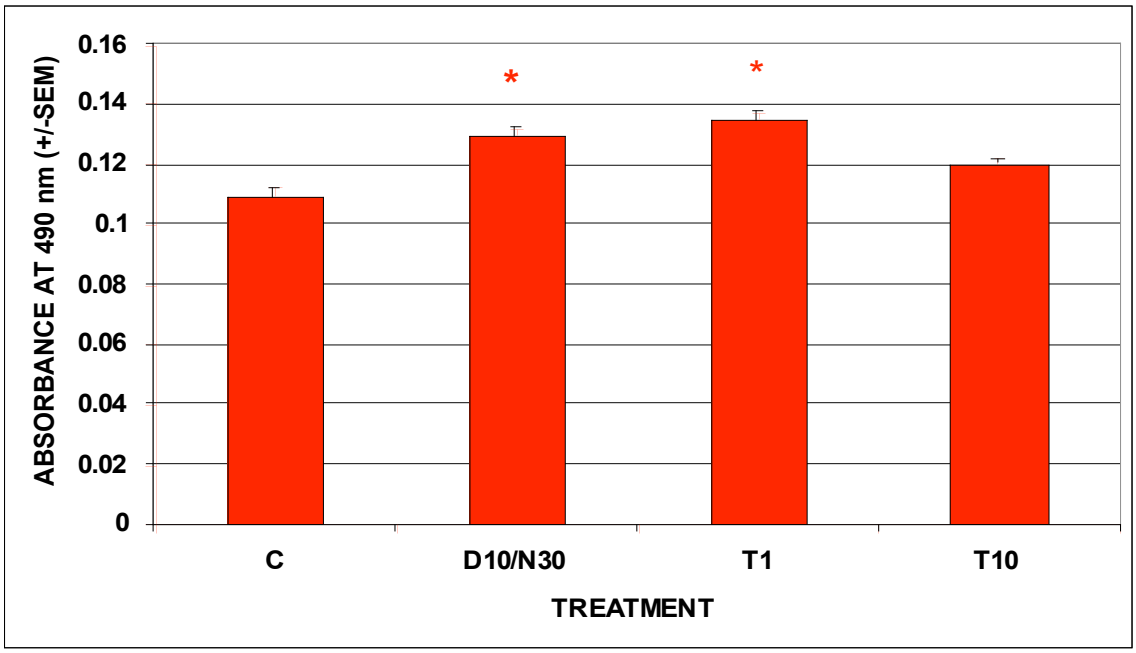


Figure 27.

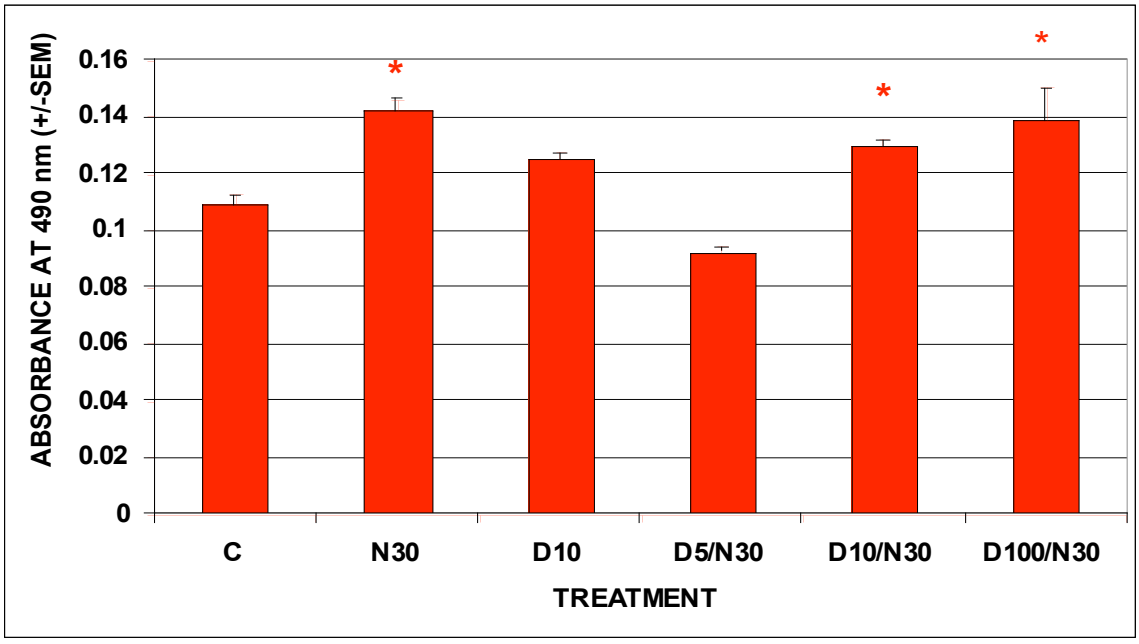


Figure 28.

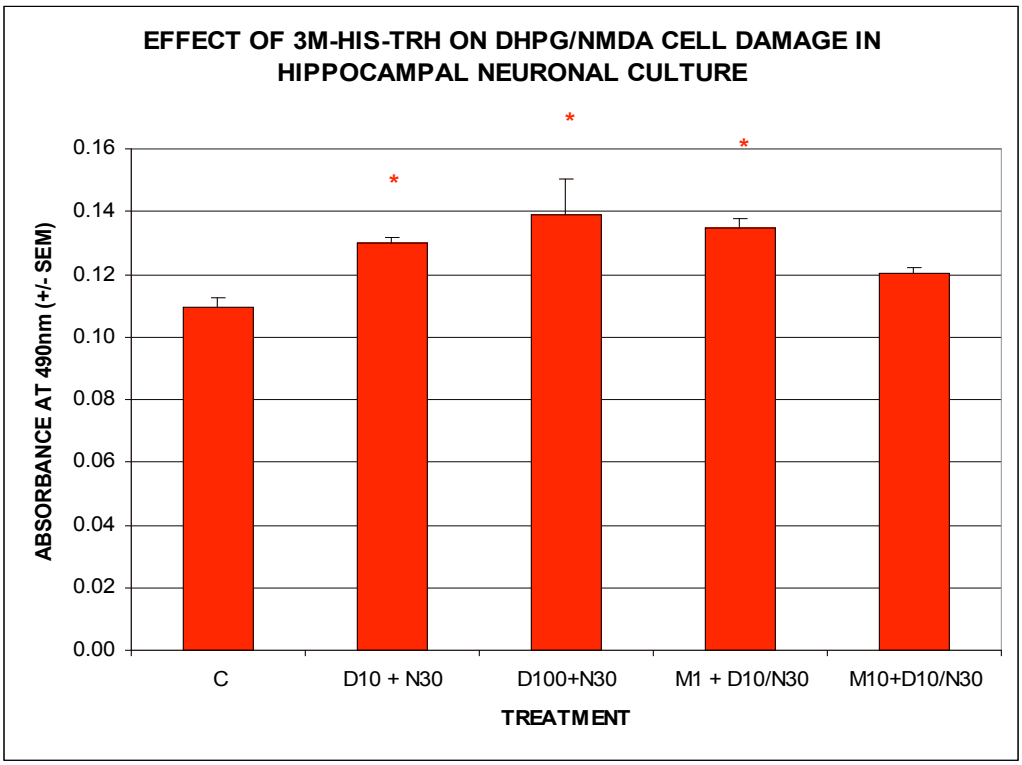


Figure 29.

7. Effect of TRH on $G_{\alpha q/11}$ Levels in Superfused Hippocampal Slices and the Anterior Pituitary

After superfusion, slices were homogenized and subjected to Western blotting with G_α standards. Blots were scanned and densities determined semi-quantitatively via image analysis. Slices superfused with 3-Me-HTRH or TRH showed a significant reduction in G_α compared to controls. Superfusion with Free-Acid-TRH showed no change. Similar results were seen with anterior pituitaries.

8. Effect of NMDA + DHPG on Cell Viability in Fetal Hippocampal Neurons

To further investigate the effect of TRH on excitoxicity in the hippocampus, fetal hippocampal neurons were harvested (Appendix). Trypan blue exclusion analyses were performed on fetal hippocampal neurons that had been exposed to a combination of DHPG (5 µM, D5; 10 µM, D10; or 100 µM, D100) + NMDA (30 µM, N30) or N30 and D10 for 30 minutes, followed by 18 hours of culture in neurobasal medium. Exposure to a combination of D10/N30, D100/N30, and to N30 and D10 alone resulted in significantly less cell viability (58%, 20%, 65%, 92% versus untreated control $P<0.05$). Exposure to D5/N30 was not significant. C= untreated control. (**Figure 30**)

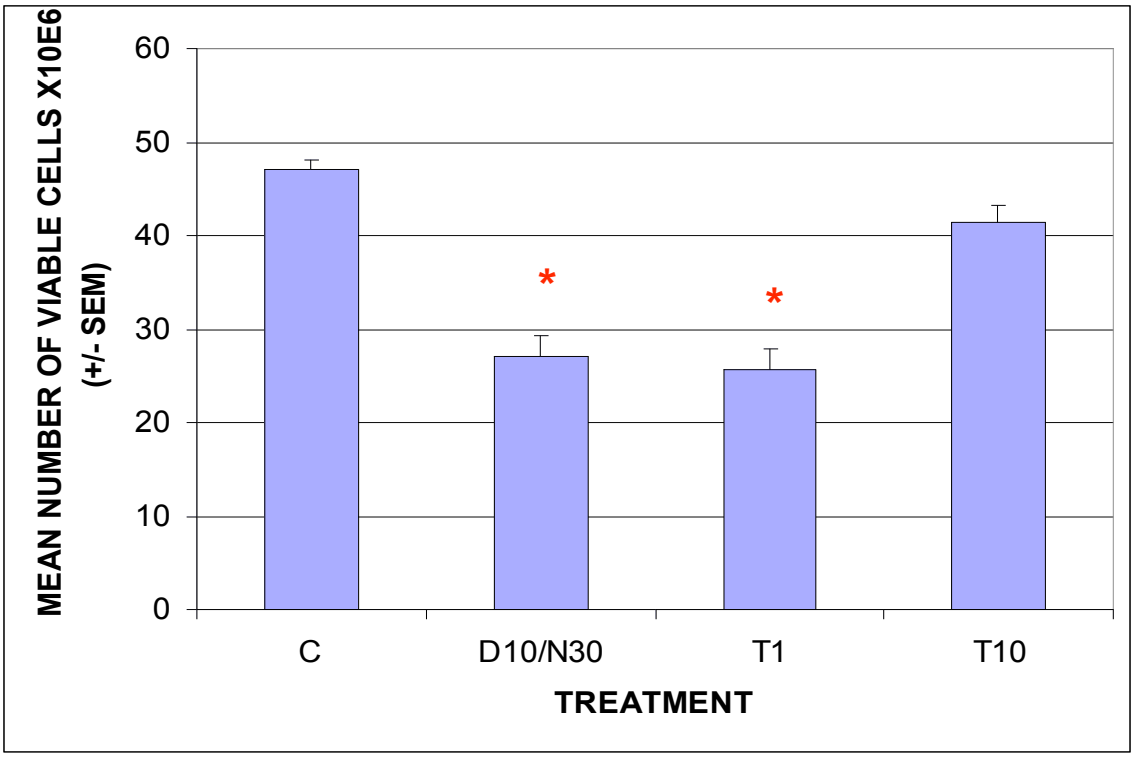


Figure 30.

9. Effect of NMDA + DHPG on LDH Release from Fetal Hippocampal Neurons

LDH release analyses were performed on media from fetal hippocampal neurons that had been exposed to a combination of DHPG (5 μM, D5; 10 μM, D10; or 100 μM, D100) + NMDA (30 μM, N30) or N30 and D10 for 30 min, followed by 18 hours of culture in neurobasal medium. Exposure to a combination of D10/N30, D100/N30, and to N30 and D10 alone resulted in significantly increased cell damage (119%, 128%, 130%, 115% versus untreated control $P<0.05$). Exposure to D5/N30 significantly reduced cell damage ($P<0.05$). C= untreated control. (**Figure 31**)

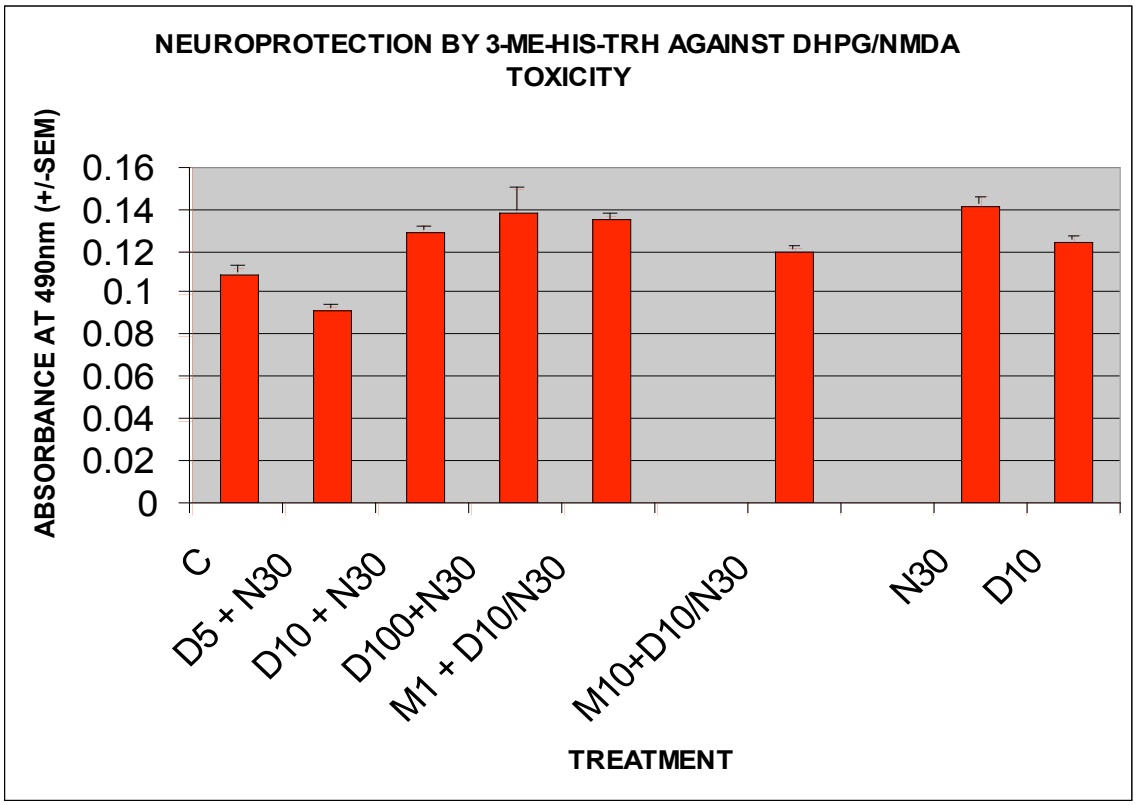


Figure 31.

10. Effect of TRH on Cell Viability in Fetal Hippocampal Neurons.

Trypan blue exclusion analyses were performed on fetal hippocampal neurons that had been exposed to a combination of DHPG (10 μM, D10) + NMDA (30 μM, N30); D10 + N30 + 3-Me-HTRH (1 μM or 10 μM) for 30 minutes followed by 18 hours of culture with 3-Me-HTRH (1 μM, T1 or 10 μM, T10). Concurrent and continued treatment with TRH (10 μM but not 1 μM) resulted in significantly less neuronal death (12%, 54% versus control $P<0.05$), respectively. C = untreated control; D10/N30 = treated control. (**Figure 32**)

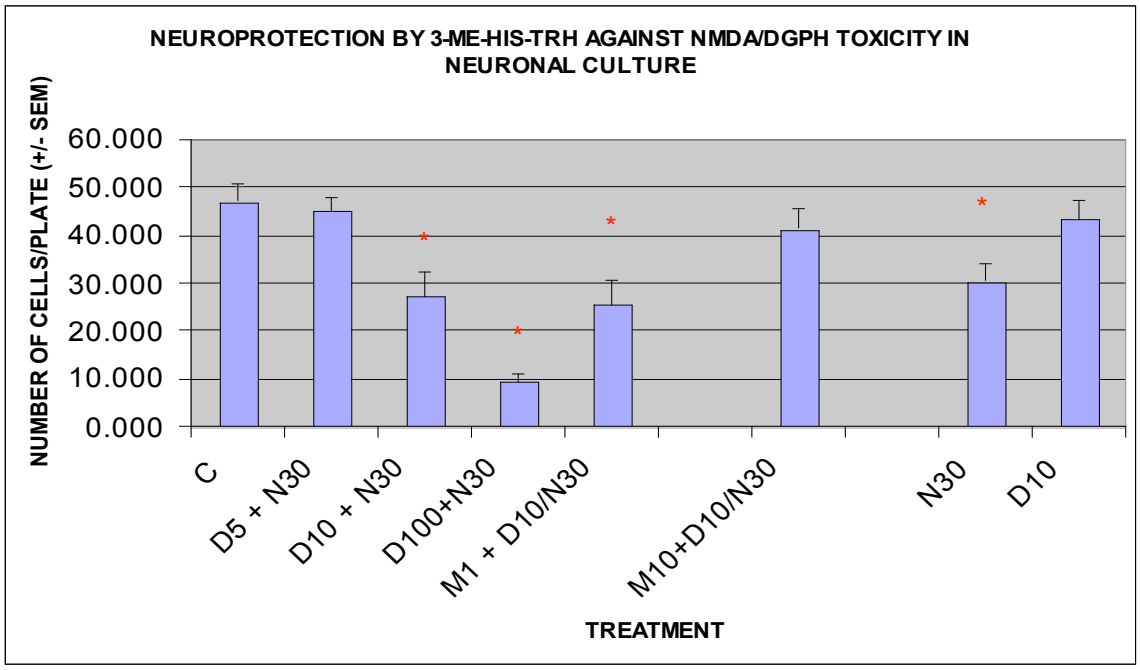


Figure 32.

11. Effect of TRH on LDH Release from Fetal Hippocampal Neurons

LDH release assays were performed on media from fetal hippocampal on fetal hippocampal neurons that had been exposed to a combination of DHPG (10 μM, D10) + NMDA (30 μM, N30); D10 + N30 + 3-Me-HTRH (1 μM or 10 μM) for 30 minutes followed by 18 hours of culture with 3-Me-HTRH (1 μM, T1; or 10 μM, T10). Concurrent and continued treatment with TRH (10 μM but not 1 μM) resulted in significantly less cell damage (110%, 123% versus control $P<0.05$), respectively. C = untreated control; D10/N30 = treated control. (**Figure 33**)

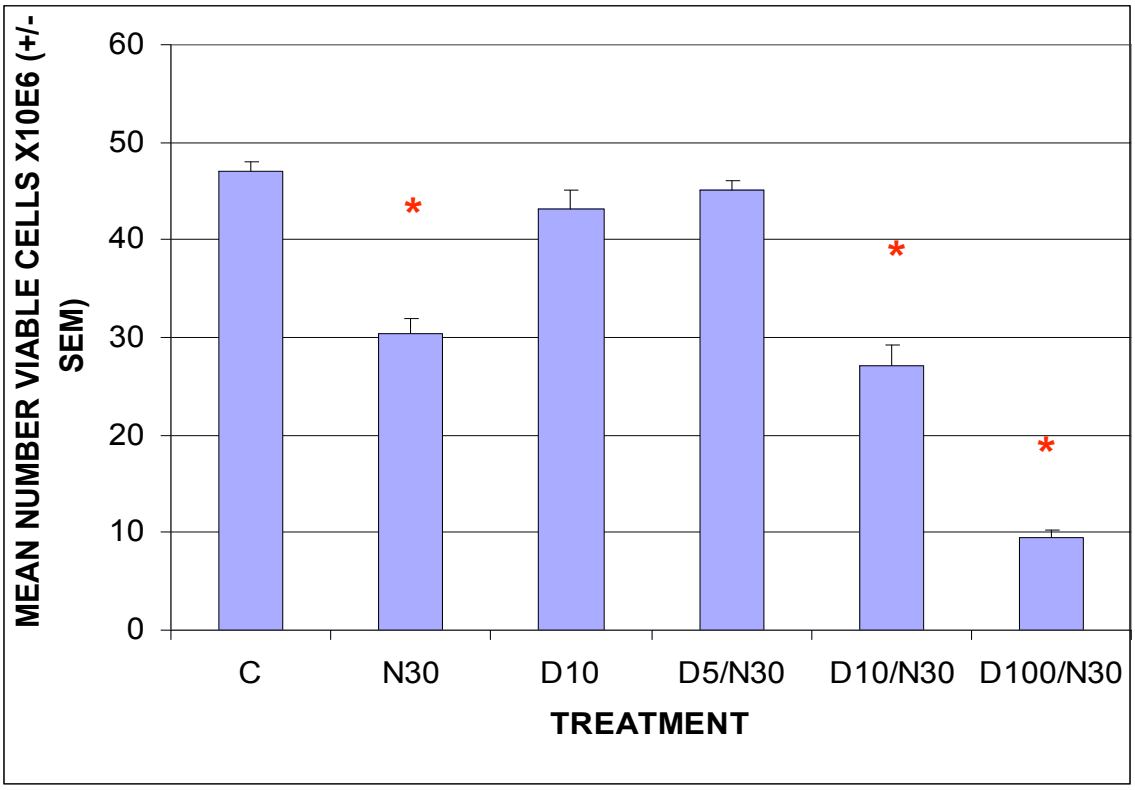


Figure 33.

VI. DISCUSSION

Regarding the cell studies, *pre-treatment* of GH-3 and PC12 cells with 10 μM TRH 8 hours prior to 8 hours of 100 nM glutamate exposure significantly reduced cell death and cell damage as assessed by viable cell counts and LDH assays, respectively.

Furthermore, *concurrent treatment* of GH-3 and PC12 cells with 10 μM TRH at the same time as toxic glutamate (100 nM) exposure (16 hours) significantly reduced cell death and cell damage as assessed by viable cell counts and LDH assay, respectively.

Finally, in the cell study, it was shown that *delayed treatment* of GH-3 and PC12 cells with 10 μM TRH 8 hours after 8 hours toxic exposure to glutamate (100 nM) significantly reduced cell death and cell damage as assessed by viable cell counts and LDH assay, respectively.

Regarding the slice superfusion studies, the results show that prolonged exposure to TRH results in downregulation of G_{α} in the hippocampus. It is important to note that superfused hippocampal slices remain viable for up to 15 hours, as assessed by LDH release and caspase activity, as well as morphological analysis, and neither TRH nor selected analogues are toxic to hippocampal slices when superfused for 15 hours when assessed by LDH release.

Levels of $G_{\alpha q/11}$ were significantly and substantially lower in slices treated with either TRH or its high affinity analogue, 3-Me-HTRH. These data thus suggest that the neuroprotective role of TRH in part may involve heterologous receptor downregulation of metabotropic glutamate receptors.

Regarding the neuron studies, *concurrent and continued treatment* of fetal hippocampal neurons with 10 μM 3Me-HTRH (a stable analogue of TRH) for 18 hours

can protect against toxic exposure to a combination of ionotropic + metabotropic glutamate agonists (10 μM DHPG + 30 μM NMDA) *in vitro*.

The preliminary superfusion results with AChE-I indicate that phenserine superfusion reduces APP level in the hippocampal slice model, suggesting that the drug may inhibit the amyloidogenic pathway. Notably, levels of sAPP were unchanged in slices treated with TRH or its analogues, suggesting no effect of TRH on secretase activity.

A. TRH and Glutamate Toxicity

TRH modulates glutamate action by previously unknown mechanisms (Renaud and Martin, 1975); (Renaud, 1978); (Renaud et al., 1979). In GH-3 and PC-12 cells, the protective role of TRH may involve modulation of glutamate-mediated ionic and/or secondary events, even though these cells most likely express different TRH/glutamate receptor and glutamate transporter profiles.

One mechanism of TRH protective action in hippocampal tissue may involve heterologous downregulation of the $G_{\alpha q/11}$ subunit of the heterotrimeric G-protein as a modulator of glutamate overexcitability.

TRH receptors (TRH-R1, R2) and group I glutamate receptors ($mGluR_{1,5}$) are co-localized on granule cells of the dentate gyrus, and are the only receptor subclass to share this same signaling cascade ($G_{\alpha q/11}$).

Since the $G_{\alpha q/11}$ component of the heterotrimeric G-protein is shared by both TRH and group I metabotropic glutamate receptors, receptor-mediated downregulation of

shared cellular G-protein subunits has been proposed as a mechanism for the development of sustained *heterologous* receptor desensitization (Milligan and Green, 1997).

B. Heterologous Receptor Downregulation as a Possible Mechanism of TRH Neuroprotection

The protective action of TRH may involve heterologous downregulation of the $G_{\alpha q/11}$ subunit of the heterotrimeric G-protein as a modulator of glutamate overexcitability. TRH has been shown to activate calcium efflux in GH-3 cells (Nelson and Hinkle, 1994); (Drummond et al., 1989).

The role of group I metabotropic glutamate receptors in synaptic overexcitability may be described as follows. Pre-synaptic glutamate binds to group I receptors, resulting in inhibition of group II and group III receptors, which will further result in increased calcium influx and excess pre-synaptic glutamate release. Post-synaptically, this excess glutamate blocks group II and group III modulation of receptor-mediated calcium influx and potassium efflux, resulting in overexcitability.

A proposed mechanism of TRH modulation of glutamate overexcitability is that the TRH receptor complex internalizes upon binding to TRH, thus resulting in a rapid removal of G_{α} from the plasma membrane. This is an initial heterologous downregulation of the α subunit of the heterotrimeric G-protein of group I glutamate receptors pre- and post-synaptically. This group I receptor downregulation (uncoupling) results in disinhibition (upregulation) of group II and group III glutamate receptors that block

calcium influx and function to *modulate* excess pre-synaptic glutamate release and post-synaptic glutamate overexcitability.

C. Potential Methods for Delivery of TRH to the Hippocampus

One additional study performed during the preparation of this thesis involved the hypothesis that sustained-release TRH is protective against glutamate toxicity in GH-3 cells. Poly(lactic) acid (PLA) TRH polyanhydride nanoparticles (TRH-NPs) were used that allow sustained release of known concentrations of TRH. PLA TRH-NP (2% TRH) were not toxic to the cultured GH-3 cells after 24 hours exposure, followed by 8 hrs maintenance in low serum media (LSM) prior to harvesting, as indicated by trypan blue exclusion (cell viability) and LDH analysis (cell damage). No difference in either cell viability or cell damage was detected in cells treated with blank-NPs or TRH-NPs, versus controls in which the GH-3 cells were maintained in LSM alone for 32 hours.

Additionally, treating the GH-3 cells with TRH-NPs either for 24 hours *before* 8 hours toxic glutamate exposure or for 24 hours *after* 8 hours glutamate exposure demonstrated (1) protection against cell damage, as indicated by LDH analysis; and (2) increased cell viability, as indicated by trypan blue exclusion, versus GH-3 cells exposed to glutamate alone. The protective effect of TRH-NPs against glutamate toxicity was more apparent when the cells were treated with the TRH-NPs *before* exposure to glutamate toxicity rather than when added *after* glutamate toxicity.

Trypan blue exclusion analyses were performed on GH-3 cells that had been treated with TRH-loaded nanoparticles for 24 hours. M/M = control cells in low serum media (LSM) for 32 hours, NP/M= Cells treated with 2% TRH-loaded nanoparticles for

24 hours, followed by 8 hours of LSM; T/M = Cells treated with TRH 10 μM for 24 hours, followed by LSM for 8 hours; BLANK/M = Cells treated with blank nanoparticles for 24 hours, followed by 8 hours in LSM. No apparent difference in cell viability was detected in cells treated with either TRH or TRH-loaded nanoparticles, vs. untreated control. (**Figure 30**)

LDH release analyses were performed on media from GH-3 cells, which had been treated with TRH-loaded nanoparticles for 24 hours. M/M = control cells in low serum media (LSM) for 32 hours, NP/M= Cells treated with 2% TRH-loaded nanoparticles for 24 hours, followed by 8 hours of LSM; T/M = Cells treated with TRH 10 μM for 24 hours, followed by LSM for 8 hours; BLANK/M = Cells treated with blank nanoparticles for 24 hours, followed by 8 hours in LSM. No apparent difference in LDH release was detected in cells treated with either TRH or TRH-loaded nanoparticles, vs. untreated control. (**Figure 31**)

Trypan blue exclusion analyses were performed on GH-3 cells that had been treated with TRH-loaded nanoparticles for 24 hours prior to 8 hours of glutamate toxicity. M/M = control cells in low serum media (LSM) for 32 hours, M/G = 24 hours of LSM followed by 8 hours of 100 nM glutamate exposure; NP/G= Cells treated with 2% TRH-loaded nanoparticles for 24 hours, followed by 8 hours of 100 nM glutamate exposure; T/G = Cells treated with TRH 10 μM for 24 hours, followed by 8 hours of 100 nM glutamate exposure. Cell viability was greater in cells pre-treated with either TRH or TRH-loaded nanoparticles prior to glutamate toxicity, vs. cells exposed to glutamate alone. (**Figure 32**)

LDH release analyses were performed on media from GH-3 cells that had been treated with TRH-loaded nanoparticles for 24 hours prior to 8 hours of glutamate toxicity. M/M = control cells in low serum media (LSM) for 32 hours, M/G = 24 hours of LSM followed by 8 hours of 100 nM glutamate exposure; NP/G= Cells treated with 2% TRH-loaded nanoparticles for 24 hours, followed by 8 hours of 100 nM glutamate exposure; T/G = Cells treated with TRH 10 μM for 24 hours, followed by 8 hours of 100 nM glutamate exposure. LDH release was lower in cells pre-treated with either TRH or TRH-loaded nanoparticles prior to glutamate toxicity, vs. cells exposed to glutamate alone. (**Figure 33**)

Trypan blue exclusion analyses were performed on GH-3 cells, which had been exposed to 8 hours of glutamate toxicity prior to 24 hours of treatment with TRH-loaded nanoparticles. M/M = control cells in low serum media (LSM) for 32 hours, M/G = 24 hours of LSM followed by 8 hours of 100 nM glutamate exposure; G/NP= Cells exposed to 8 hours of 100 nM glutamate exposure followed by treatment with 2% TRH-loaded nanoparticles for 24 hours; G/T = Cells exposed to 8 hours of 100 nM glutamate exposure followed by treatment with TRH 10 μM for 24 hours. Cell viability was greater in cells treated with either TRH or TRH-loaded nanoparticles after glutamate toxicity vs. cells exposed to glutamate alone. (**Figure 34**)

LDH release analyses were performed on media from GH-3 cells, which had been exposed to 8 hours of glutamate toxicity prior to 24 hours of treatment with TRH-loaded nanoparticles. M/M = control cells in low serum media (LSM) for 32 hours, M/G = 24 hours of LSM followed by 8 hours of 100 nM glutamate exposure; G/NP= Cells exposed to 8 hours of 100 nM glutamate exposure followed by treatment with 2% TRH-loaded

nanoparticles for 24 hours; G/T = Cells exposed to 8 hours of 100 nM glutamate exposure followed by treatment with TRH 10 μM for 24 hours. LDH release was lower in cells treated with either TRH or TRH-loaded nanoparticles after glutamate toxicity vs. cells exposed to glutamate alone. (**Figure 35**)

These results should hasten the development of polyanhydride-based sustained-release carriers for TRH as well as other neuropeptides *in vitro*.

VII. SUMMARY

The results of the cell study showed that TRH is protective against glutamate toxicity when added before, during, or after the toxic insult. This is important when one considers treatment for excitotoxic events such as epilepsy, stroke, and trauma.

The slice superfusion study increased the complexity of the environment in which the neuroprotective effect of TRH against excitotoxicty was investigated. This also allowed us to determine a possible mechanism for the neuroprotective effect of TRH; i.e., TRH modulates the effect of glutamate via heterologous receptor downregulation, as shown by the downregulation of $G_{\alpha q/11}$ when TRH or its stable analog, 3-Me-HTRH, is added during toxic exposure of hippocampal dentate gyri slices to NMDA + DHPG.

To further this investigation, the neuroprotecive effect of TRH against excitatory amino acid-induced toxicity in fetal neurons was also investigated.

The neuronal study results show the dose-dependent toxicity of dihydroxyphenylglycine (DHPG) on cell death and membrane damage in neurons when added either alone or in combination with N-methyl-D-aspartate (NMDA). Exposure to 30 μM NMDA or 10 μM DHPG, or to 10 μM or 100 μM DHPG + 30 μM NMDA resulted in significantly less cell viability and significantly more cell damage than exposure to 5 μM DHPG + 30 μM NMDA. The 5 μM DHPG appeared to block the NMDA effect.

Notably, the results of the neuronal study show that concurrent and continued treatment with 10 μM 3Me-HTRH (a stable analogue of TRH) resulted in significantly less neuronal death and damage when added to a toxic dose of 10 μM DHPG + 30 μM NMDA. Treatment with 1 μM 3Me-HTRH did not have a significant effect.

VIII. CONCLUSION

These results support and extend our ongoing research, as well as that of others showing a neuroprotective role for TRH against glutamate neurotoxicity (Jaworska-Feil et al., 2001).

This work has indicated that one mechanism of TRH protective action in hippocampal tissue may involve heterologous downregulation of the $G_{\alpha q/11}$ subunit of the heterotrimeric G-protein as a modulator of glutamate overexcitability (Milligan and Green, 1997); (Kim et al., 1994); (Pesanova et al., 1999). TRH receptors (TRH-R1) and group I glutamate receptors ($mGluR_{1,5}$) are colocalized in granule cells of the dentate gyrus and share this same signaling cascade ($G_{\alpha q/11}$) (Blumcke et al., 2000); (Heuer et al., 2000).

A role for group I metabotropic glutamate receptors in synaptic overexcitability involves pre-synaptic glutamate binding to group I receptors, resulting in indirect inhibition of both groups II and III metabotropic receptors. This causes increased calcium influx and excess pre-synaptic glutamate release. Post-synaptically, excess group I activation blocks groups II and III inhibition of receptor-mediated calcium influx and potassium efflux. PKC-mediated phosphorylation of NMDA receptors reduces the Mg^{++} block resulting in overexcitability (Mukhin et al., 1997); (Chen et al., 1999).

A proposed mechanism of TRH protection against excitatory amino-acid-induced excitotoxicity involves the TRH receptor complex internalizing upon binding, resulting in rapid $G_{\alpha q/11}$ removal from the plasma membrane initially, and heterologous

downregulation of the A subunit of the heterotrimeric G-protein of group I metabotropic glutamate receptors pre- and post-synaptically (Svoboda et al., 1996).

The data suggest that TRH may be a selective modulator of glutamate-induced toxicity. This is relevant to TRH neuropeptide treatment in post-traumatic and other neurodegenerative events, such as dementia (Alzheimer's disease), seizures, and stroke.

IX. FUTURE DIRECTIONS

A. Selectivity of the TRH Effect on Other G-Protein Subunits

Future studies should be developed to determine whether the neuroprotective effect of TRH extends to other G-protein coupled receptors, in which G_i, G_o, and G_s levels will be examined via Western blotting techniques in slice and neuronal systems.

B. Effect of TRH on Muscarinic Cholinergic Receptors in the Hippocampus

The effect of TRH on metabotropic acetylcholine receptors in the hippocampus should also be examined, as the neuroprotective effect of TRH may not be limited to heterologous receptor desensitization of metabotropic glutamate receptors. This has implications with treatment of Alzheimer's disease.

C. Effect of TRH on Tau Protein in the Hippocampus

The effect of exposure to TRH on *tau* protein development in hippocampal neurons should also be examined, as TRH may prove to be neuroprotective against the development or progression of Alzheimer's disease, through mechanisms as yet undiscovered.

X. APPENDICES

A. Protocol for Hippocampal/Dentate Gyri Slice Superfusion

1. Prepare SUPERFUSION Buffers 24 hours prior to Experiment, as follows:

(a) KRB STOCK A (Make in Duplicate, 2 liters, for each experiment)

NaH_2PO_4-H_2O	0.154 g
NaCl	5.747 g
$NaHCO_3$	1.867 g
Dextrose (10mM)	1.8 g
Na_2HPO_4	0.142 g

qs to 1 liter w/H_2O

pH to 7.4

(b) KRB STOCK B

NaCl	2.718 g
KCl (3.5 mM)	0.26 g
BSA	0.1 g
CaCl	0.147 g (LOW CALCIUM @ 1.0 mM)
	0.368 g (NORMAL CALCIUM @ 2.5 mM)

qs to 100 ml w/H_2O

2. Combine Stock A plus Stock B (low calcium) 2 hours prior to Experiment. Warm to room temperature in the SUPERFUSION Room for 1 hour. Reconfirm pH at 7.4. Oxygenate in 37°C water bath for 1 hour.

3. Combine Stock A plus Stock B (normal calcium) to make final volume of 2.2 liters normal calcium Buffer. Warm to room temperature and reconfirm pH at 7.4 prior to SUPERFUSION. Oxygenate in 37°C water bath for 1 hour prior to SUPERFUSION with normal calcium buffer.

4. Obtain beasts from LARC. Cover cart with cloth and transport animals to just outside the door of MS510. Only take 1 animal at a time into the Decapitation Room.

5. Weigh rat. Decapitate and remove brain. Place immediately in ice cold 0.9% NaCl.

6. Subdissect hippocampi/dentate gyri in accordance with dissection protocol in the SUPERFUSION Room under magnification, using an aluminum tray with a dry ice layer covered with regular ice (1:9) to prevent freezing and/or thawing. Keep the tissue wet with ice cold 0.9% NaCl at all times.

7. Once the desired tissue is obtained, slice to 500 μM thickness under magnification. Blot dry and weigh. Record DG/HIPP # by weight and date. Repeat for each hippocampus/dentate gyrus.

8. Place the tissue, by either animal R/L side or randomized sections, into SUPERFUSION wells. Superfuse with oxygenated low calcium buffer @ 0.5 ml/min for 2 hours. Keep oxygenation going throughout entire procedure.

9. After 2 hours, obtain 1 ml effluent from each well for LDH assay (T-0). Freeze at -20°C until assay is performed.

10. Without disrupting wells, switch to SUPERFUSION with normal calcium buffer with drug/dose of choice in wells 2 and 4, and normal calcium buffer with no drug (untreated controls) in wells 1 and 3.

11. Collect 1 ml effluent from each well at beginning (T-0), and every 4 hours until the end of the experiment (T endpoint). Label each tube with date/time of collection. Keep TOTAL effluent in labeled bottles. Place effluents into refrigerator for LDH/protein analyses. At endpoint, remove the superfused tissue from the wells, place in individually labeled 2ml Eppendorf tubes, and freeze at -70°C until ready to analyze.

12. When ready to analyze, remove the tissue from -70°C and place the superfused tissue in 500 µl ice cold IP Buffer (protease inhibitor). Homogenize with polytron homogenizer at 50% speed for 10 seconds. Cold centrifuge for 15 minutes. Separate tissue from effluent. Freeze pellets at -70°C for future analyses.

13. Perform Protein Estimation of cell lysate via Lowry Method. Calculate amount protein/wet weight of original tissue. Aliquot 15-30 µg protein from each sample. Add water and 4X Sample Buffer to obtain 40-50 µl total for each sample. Boil for 5 minutes.

14. Perform Lactic Dehydrogenase (LDH) Assay on cell lysate, using SIGMA LDH Assay kit. Record results.

15. Perform PAGE in 12% agarose gel. Run gel at 200V for 48 minutes. Perform western blot transfer to nitrocellulose membrane via running in transfer buffer at 110V for 60 minutes. Stain with Ponceau and visualize with acetic acid to verify serum protein transfer has occurred in equal amounts per well. Photograph blot in GDS system. Wash off Ponceau with TBST buffer for 10 minutes X3.

16. Block nonspecific binding with 3% gelatin incubation for 1-2 hours. Wash with TBST for 10 minutes X3.

17. Probe overnight with primary antibody of choice. Keep antibody and rinse blot with TBST for 10 minutes X3.

18. Hybridize with secondary antibody for 1-2 hours. Keep antibody and rinse blot with TBS buffer for 10 minutes X3.

19. Add biotinylated avidin (VECTASTAIN kit) for 1 hour. Rinse blot with TBS buffer for 10 minutes X3.

20. Develop blot with horseradish peroxidase.

21. Perform densitometric analysis of Western Blots with Scion Image program. Save blot in water or plastic-sealed bag for future probing.

22. Plot results on Excel. Calculate percent change of treated versus untreated cell lysates for protein of choice.

B. IMMUNOHISTOCHEMISTRY PROTOCOL (MORPHOLOGICAL ANALYSIS)

N.B. Use gelatin-coated slides for frozen sections.

Dilute antibody (Ab) in PBS. (e.g. Sheep kit: 1.5% Normal Serum + 0.3% Tx-100)

Day 1: Frozen sections (cryostat)

Day 2: <u>Immunohistochemistry Staining Protocol – Part 1</u>

1. <u>BLOCK</u>

   In graduated cylinder:

   90 ml PBS

   10 ml H_2O_2

2. Add PBS/ H_2O_2 solution to slides

   -rock for 10 minutes

   -air bubbles will form

3. <u>WASH</u> 3x (5-10 min each) with PBS

   -get rid of air bubbles

4. Circle tissue with <u>Liquid Blocker</u> (a liquid-repellant slide marker pen for staining)

   -dry for 5 minutes

5. Place wet paper towels in the bottom of the slide box, for humidification.

6. Incubate with 1° Ab (inside circle of liquid repellant) O/N.

   (N.B. Cover the slide box.)

Day 3: **Immunohistochemistry Staining Protocol – Part 2**

7. RINSE 3x for 10 minutes with PBS (0.1 μM)

8. 2° Ab (1:200) for 1 hour in TBS

9. RINSE 3x for 10 minutes with PBS

10. RINSE 2x for 10 minutes with TBS

11. Add ABC (2 drops A + 2 drops B in 5 ml TBS) and incubate for 1 hour.

12. Rinse 3x for 10 minutes with TBS.

13. Develop with DAB. (100 μl DAB + 900 μl Buffer)

C. FETAL NEURON HARVEST TECHNIQUE

1. Fetal rats were harvested from pregnant Sprague-Dawley dams at embryonic day 17, and placed in sterile Hank's buffered medium with pyruvate at pH 7.4.

2. Heads were removed, and then isolated under a dissecting scope on a black wax-coated sterile Petri dish (for ease of viewing).

3. Specimens were pinned to enable dissection from a superior view. The dura mater was peeled away, and the underlying arachnoid and pia mater were punctured and peeled away using sterile scissors and forceps.

4. The olfactory bulbs and cerebellum were removed, and the remaining cerebral lobes were bisected mid-sagittally. The midbrain was removed and the hippocampi were isolated.

5. Whole hippocampi were centrifuged for 2 minutes at low speed and the Hank's media was aspirated off the remaining pellet under a hood to maintain sterile conditions.

6. Neurobasal medium (10 ml) containing B27 (serum-free medium), L-Glutamine and Pen-Strep (with BDNF) was added to the pellet.

7. The hippocampal pellet was triturated until the mixture was cloudy and homogeneous.

8. Cells (10^6) were cultured in 2 ml neurobasal serum-free medium in eight-well dishes coated with 0.2 mg/ml poly-D-lysine as described (by Brewer, et. al., 1998). Neurons were maintained in a 5% CO_2 incubator at 37°C for 14 days, with media changes every 2 days.

9. Neurons were exposed for 30 minutes to the following: media control; DHPG (10 μM); NMDA (30 μM); DHPG (5, 10, or 100 μM) + NMDA (30 μM); and DHPG (10 μM) + NMDA (30 μM) + 3-Me-HTRH (1 μM OR 10 μM) in neurobasal medium.

10. This procedure was followed by 18 hours of treatment with 3-Me-HTRH (1 μM OR 10 μM) in neurobasal medium.

11. After 18 hours, 50 μl aliquots of conditioned media were collected for lactic dehydrogenase (LDH) analysis by a colorimetric tetrazolium dye method (Sigma) to assess cell membrane integrity. Cells were harvested, and cell counts were performed via trypan blue exclusion to determine cell viability.

XI. BIBLIOGRAPHY

Abe,T., Sugihara,H., Nawa,H., Shigemoto,R., Mizuno,N., Nakanishi,S., 1992. Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction. J Biol Chem 267, 13361-13368.

Albin,R.L., Greenamyre,J.T., 1992. Alternative excitotoxic hypotheses. Neurology 42, 733-738.

Allen,J.W., Eldadah,B.A., Faden,A.I., 1999. Beta-amyloid-induced apoptosis of cerebellar granule cells and cortical neurons: exacerbation by selective inhibition of group I metabotropic glutamate receptors. Neuropharm 38, 1243-1252.

Anwyl,R., 1999. Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Res Brain Res Rev 29, 83-120.

Aragay,A.M., Katz,A., Simon,M.I., 1992. The G alpha q and G alpha 11 proteins couple the thyrotropin- releasing hormone receptor to phospholipase C in GH3 rat pituitary cells. J Biol Chem 267, 24983-24988.

Arnold,P.S., Racine,R.J., Wise,R.A., 1973. Effects of atropine, reserpine, 6-hydroxydopamine and handling on seizure development in the rat. Exp Neurol 40, 457-470.

Arriza,J.L., Eliasof,S., Kavanaugh,M.P., Amara,S.G., 1997. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci U S A 94, 4155-4160.

Arriza,J.L., Fairman,W.A., Wadiche,J.I., Murdoch,G.H., Kavanaugh,M.P., Amara,S.G., 1994. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 14, 5559-5569.

Balcar,V.J., Roth-Schechter,B.F., Mandel,P., 1978. Effect of chronic pentobarbital treatment on high affinity uptake of L-glutamate by cultured glial cells. Biochem Pharmacol 27, 2955-2956.

Bardgett,M.E., Jackson,J.L., Taylor,G.T., Csernansky,J.G., 1995. Kainic acid decreases hippocampal neuronal number and increases dopamine receptor binding in the nucleus accumbens: an animal model of schizophrenia. Behav Brain Res 70, 153-164.

Bauer,K., Kleinkauf,H., 1980. Catabolism of thyroliberin by rat adenohypophyseal tissue extract. Eur J Biochem 106, 107-117.

Benes,F.M., Berretta,S., 2000. Amygdalo-entorhinal inputs to the hippocampal formation in relation to schizophrenia. Ann N Y Acad Sci 911, 293-304.

Bennett,G.W., Marsden,C.A., Fone,K.C.F., Johnson,J.V., Heal,D.J., 1989. TRH-catecholamine interactions in brain and spinal cord. Annals of the New York Academy of Sciences 553, 106-120.

Berridge,M.J., Irvine,R.F., 1984. Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315-321.

Blaabjerg,M., Baskys,A., Zimmer,J., Vawter,M.P., 2003. Changes in hippocampal gene expression after neuroprotective activation of group I metabotropic glutamate receptors. Brain Res Mol Brain Res 117, 196-205.

Blumcke,I., Becker,A.J., Klein,C., Scheiwe,C., Lie,A.A., Beck,H., Waha,A., Friedl,M.G., Kuhn,R., Emson,P., Elger,C., Wiestler,O.D., 2000. Temporal lobe epilepsy associated up-regulation of metabotropic glutamate receptors: correlated changes in mGluR1 mRNA and protein expression in experimental animals and human patients. J Neuropathol Exp Neurol 59, 1-10.

Blumcke,I., Behle,K., Malitschek,B., Kuhn,R., Knopfel,T., Wolf,H.K., Wiestler,O.D., 1996. Immunohistochemical distribution of metabotropic glutamate receptor subtypes mGluR1b, mGluR2/3, mGluR4a and mGluR5 in human hippocampus. Brain Res 736, 217-226.

Bonfoco,E., Krainc,D., Ankarcrona,M., Nicotera,P., Lipton,S.A., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A 92, 7162-7166.

Bordi,F., Ugolini,A., 1999. Group I metabotropic glutamate receptors: implications for brain diseases. Prog Neurobiol 59, 55-79.

Boudin,H., Doan,A., Xia,J., Shigemoto,R., Huganir,R.L., Worley,P., Craig,A.M., 2000. Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron 28, 485-497.

Bowers,C.Y., Friesen,H.G., Hwang,P., Guyda,H.J., Folkers,K., 1971. Prolactin and thyrotropin release in man by synthetic pyroglutamyl-histidyl-prolinamide. Biochem Biophys Res Commun 45, 1033-1041.

Bowers,C.Y., Schally,A.V., 1970. Assay of thyrotropin-releasing hormone. Meiter,J. (Ed.) Hypothalamic Hypophysiotropic Hormone. pp. 74-89. William and Wilkins, (Baltimore), p. 6.

Bradley,S.R., Levey,A.I., Hersch,S.M., Conn,P.J., 1996. Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J Neurosci 16, 2044-2056.

Bradley,S.R., Rees,H.D., Yi,H., Levey,A.I., Conn,P.J., 1998. Distribution and developmental regulation of metabotropic glutamate receptor 7a in rat brain. J Neurochem 71, 636-645.

Bradley,S.R., Standaert,D.G., Rhodes,K.J., Rees,H.D., Testa,C.M., Levey,A.I., Conn,P.J., 1999. Immunohistochemical localization of subtype 4a metabotropic glutamate receptors in the rat and mouse basal ganglia. J Comp Neurol 407, 33-46.

Brecht,S., Gelderblom,M., Srinivasan,A., Mielke,K., Dityateva,G., Herdegen,T., 2001. Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity. Brain Res Mol Brain Res 94, 25-34.

Bridges,R.J., Esslinger,C.S., 2005. The excitatory amino acid transporters: pharmacological insights on substrate and inhibitor specificity of the EAAT subtypes. Pharmacol Ther 107, 271-285.

Browne,P., O'Cuinn,G., 1983. An evaluation of the role of a pyroglutamyl peptidase, a post-proline cleaving enzyme and a post-proline dipeptidyl amino peptidase, each purified from the soluble fraction of guinea-pig brain, in the degradation of thyroliberin in vitro. Eur J Biochem 137, 75-87.

Bruno,V., Wroblewska,B., Wroblewski,J.T., Fiore,L., Nicoletti,F., 1998. Neuroprotective activity of N-acetylaspartylglutamate in cultured cortical cells. Neuroscience 85, 751-757.

Bullock,R., Zauner,A., Woodward,J., Young,H.F., 1995. Massive persistent release of excitatory amino acids following human occlusive stroke. Stroke 26, 2187-2189.

Burt,D.R., Snyder,S.H., 1975. Thyrotropin releasing hormone (TRH): apparent receptor binding in rat brain membranes. Brain Res 93, 309-328.

Calza, L., Giardino, L., Ceccatelli, S., Elde, R. P., and Hokfelt, T. Distribution of TRH-receptor mRNA in the rat brain: An in situ hybridization study. Society of Neuroscience - Abstract 18, 454. 1992.

Cao,J., O'Donnell,D., Vu,H., Payza,K., Pou,C., Godbout,C., Jakob,A., Pelletier,M., Lembo,P., Ahmad,S., Walker,P., 1998. Cloning and characterization of a cDNA encoding a novel subtype of rat thyrotropin-releasing hormone receptor. J Biol Chem 273, 32281-32287.

Cartmell,J., Schoepp,D.D., 2000. Regulation of neurotransmitter release by metabotropic glutamate receptors. J Neurochem 75, 889-907.

Chen,Q., Olney,J.W., Price,M.T., Romano,C., 1999. Biochemical and morphological analysis of non-NMDA receptor mediated excitotoxicity in chick embryo retina. Vis Neurosci 16, 131-139.

Choi,D.W., Mallet,J., 1999. Neurobiology of disease in the 21st century. Neurobiol Dis 6, 309.

Choi,D.W., 1992. Excitotoxic cell death. J Neurobiol 23, 1261-1276.

Choi,D.W., 1994. Calcium and excitotoxic neuronal injury. Ann N Y Acad Sci 747, 162-171.

Choi,D.W., 2003. Glutamate, cell death, and hats off to Carl Cotman. Neurochem Res 28, 1621-1624.

Choi,D.W., 1987a. Ionic dependence of glutamate neurotoxicity. J Neurosci 7, 369-379.

Choi,D.W., Hartley,D.M., 1993. Calcium and glutamate-induced cortical neuronal death. Res Publ Assoc Res Nerv Ment Dis 71, 23-34.

Choi,D.W., Maulucci-Gedde,M., Kriegstein,A.R., 1987b. Glutamate neurotoxicity in cortical cell culture. J Neurosci 7, 357-368.

Claing,A., Laporte,S.A., Caron,M.G., Lefkowitz,R.J., 2002. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. Prog Neurobiol 66, 61-79.

Clifford,D.B., Olney,J.W., Benz,A.M., Fuller,T.A., Zorumski,C.F., 1990. Ketamine, phencyclidine, and MK-801 protect against kainic acid-induced seizure-related brain damage. Epilepsia 31, 382-390.

Cohen,G.M., 1997. Caspases: the executioners of apoptosis. Biochem J 326 (Pt 1), 1-16.

Collins,R.C., Olney,J.W., 1982. Focal cortical seizures cause distant thalamic lesions. Science 218, 177-179.

Conn,P.J., 2003. Physiological roles and therapeutic potential of metabotropic glutamate receptors. Ann N Y Acad Sci 1003, 12-21.

Conn,P.J., Niswender,C.M., 2006. mGluR7's lucky number. Proc Natl Acad Sci U S A 103, 251-252.

Conn,P.J., Pin,J.P., 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37, 205-237.

Cotman,C.W., Foster,A., Lanthorn,T., 1981. An overview of glutamate as a neurotransmitter. Adv Biochem Psychopharmacol 27, 1-27.

Cotman,C.W., Monaghan,D.T., 1988. Excitatory amino acid neurotransmission: NMDA receptors and Hebb-type synaptic plasticity. Annu Rev Neurocsci 11, 61-80.

Coyle,J.T., Bird,S.J., Evans,R.H., Gulley,R.L., Nadler,J.V., Nicklas,W.J., Olney,J.W., 1981. Excitatory amino acid neurotoxins: selectivity, specificity, and mechanisms of action. Based on an NRP one-day conference held June 30, 1980. Neurosci Res Program Bull 19, 1-427.

Croucher,M.J., Bradford,H.F., 1989. Kindling of full limbic seizures by repeated microinjections of excitatory amino acids into the rat amygdala. Brain Res 501, 58-65.

Cruz,I.P., Nillni,E.A., 1996. Intracellular sites of prothyrotropin-releasing hormone processing. J Biol Chem 271, 22736-22745.

Csernansky,J.G., Bardgett,M.E., Sheline,Y.I., Morris,J.C., Olney,J.W., 1996. CSF excitatory amino acids and severity of illness in Alzheimer's disease. Neurology 46, 1715-1720.

Danik,M., Cassoly,E., Manseau,F., Sotty,F., Mouginot,D., Williams,S., 2005. Frequent coexpression of the vesicular glutamate transporter 1 and 2 genes, as well as coexpression with genes for choline acetyltransferase or glutamic acid decarboxylase in neurons of rat brain. J Neurosci Res 81, 506-521.

de la Pena,P., Delgado,L.M., del Camino,D., Barros,F., 1992. Cloning and expression of the thyrotropin-releasing hormone receptor from GH3 rat anterior pituitary cells. Biochem J 284, 891-899.

Deakin,J.F., Slater,P., Simpson,M.D., Gilchrist,A.C., Skan,W.J., Royston,M.C., Reynolds,G.P., Cross,A.J., 1989. Frontal cortical and left temporal glutamatergic dysfunction in schizophrenia. J Neurochem 52, 1781-1786.

Decker,T., Lohmann-Matthes,M.L., 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 115, 61-69.

Dingledine,R., 1983. Excitatory amino acids: modes of action on hippocampal pyramidal cells. Fed Proc 42, 2881-2885.

Drejer,J., Benveniste,H., Diemer,N.H., Schousboe,A., 1985. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo and in vitro. J Neurochem 45, 145-151.

Drmota,T., Gould,G.W., Milligan,G., 1998. Real time visualization of agonist-mediated redistribution and internalization of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor [In Process Citation]. J Biol Chem 273, 24000-24008.

Drummond,A.H., Hughes,P.J., Ruiz-Larrea,F., Joels,L.A., 1989. Use of receptor antagonists in elucidating the mechanism of action of TRH in GH_3 cells. Annals of the New York Academy of Sciences 553, 197-204.

Dubinsky,J.M., Oxford,G.S., 1985. Dual modulation of K channels by thyrotropin-releasing hormone in clonal pituitary cell. Proc Natl Acad Sci USA 82, 4282-4286.

Duthie,S.M., Taylor,P.L., Anderson,L., Cook,J., Eidne,K.A., 1993. Cloning and functional characterisation of the human TRH receptor. Mol Cell Endocrinol 95, R11-5.

Duvoisin,R.M., Zhang,C., Ramonell,K., 1995. A novel metabotropic glutamate receptor expressed in the retina and olfactory bulb. J Neurosci 15, 3075-3083.

Emerson,C.H., Vogel,W., Currie,B.L., 1980. Concentrations of thyrotropin-releasing hormone (TRH) and deamido-TRH, and TRH deamidase activity in brain. Endocrinology 107, 443-449.

Faden,A.I., 1993. Experimental neurobiology of central nervous system trauma. Crit Rev Neurobiol 7, 175-186.

Faden,A.I., Hallenbeck,J., Brown,C., 1982a. Treatment of experimental stroke: comparison of naloxone and thyrotropin releasing hormone. Neurol 32, 1083-1087.

Faden,A.I., Jacobs,T.P., Holaday,J.W., 1982b. Neuropeptides and spinal cord injury. Regulatory Peptides: From Molecular Biology to Function. Raven Press, New York, pp. 131-138.

Faden,A.I., Movsesyan,V.A., Knoblach,S.M., Ahmed,F., Cernak,I., 2005. Neuroprotective effects of novel small peptides in vitro and after brain injury. Neuropharm 49, 410-424.

Fairman,W.A., Vandenberg,R.J., Arriza,J.L., Kavanaugh,M.P., Amara,S.G., 1995. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375, 599-603.

Fariello,R.G., Garant,D.S., 1992. Neurotransmitter pharmacology of the epilepsies: discrepancies between animal models and human conditions. Epilepsy Res Suppl 8, 21-26.

Ferguson,S.S., 2001. Evolving concepts in G-protein coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 53, 1-24.

Ferguson,S.S., Barak,L.S., Zhang,J., Caron,M.G., 1996. G-protein coupled receptor regulation: role of G-protein coupled receptor kinases and arrestins. Can J Physiol Pharmacol 74, 1095-1110.

Fleckenstein-Grun,G., Frey,M., Thimm,F., Hofgartner,W., Fleckenstein,A., 1992. Calcium overload--an important cellular mechanism in hypertension and arteriosclerosis. Drugs 44 Suppl 1, 23-30.

Forloni,G., Blake,K., Hohmann,C.H., Coyle,J.T., 1989. The postnatal expression of acetylcholinesterase in somatostatin-positive cells of mouse hippocampus. Dev Brain Res 48, 73-85.

Frauli,M., Neuville,P., Vol,C., Pin,J.P., Prezeau,L., 2006. Among the twenty classical L-amino acids, only glutamate directly activates metabotropic glutamate receptors. Neuropharm 50, 245-253.

Friedman,T.C., Loh,Y.P., Cawley,N.X., Birch,N.P., Huang,S.S., Jackson,I.M., Nillni,E.A., 1995. Processing of prothyrotropin-releasing hormone (Pro-TRH) by bovine intermediate lobe secretory vesicle membrane PC1 and PC2 enzymes. Endocrinology 136, 4462-4472.

Gabellini,N., Manev,R.M., Manev,H., 1994. Is the heterologous expression of metabotropic glutamate receptors (mGluRs) an appropriate method to study the mGluR function? Experience with human embryonic kidney 293 cells transfected with mGluR1. Neurochem Int 24, 533-539.

Gary,K.A., Sevarino,K.A., Yarbrough,G.G., Prange,A.J., Jr., Winokur,A., 2003. The thyrotropin-releasing hormone (TRH) hypothesis of homeostatic regulation: implications for TRH-based therapeutics. J Pharmacol Exp Ther 305, 410-416.

Gerlai,R., Roder,J.C., Hampson,D.R., 1998. Altered spatial learning and memory in mice lacking the mGluR4 subtype of metabotropic glutamate receptor. Behav Neurosci 112, 525-532.

Gershengorn,M., Thaw,C., Gerry,R., 1983. Calcium influx is not required for thyrotropin-releasing hormone stimulation of prolactin release from GH3 cells. Cell Calcium 4, 117-124.

Gershengorn,M.C., 1989. Mechanism of signal transduction by TRH. Annals of the New York Academy of Sciences 553, 191-196.

Gershengorn,M.C., Osman,R., 1996. Molecular and cellular biology of thyrotropin-releasing hormone receptors. Physiol Rev 76, 175-191.

Griffiths,E.C., 1985. Thyrotrophin-releasing hormone: endocrine and central effects. Psychoneuroendocrinology 10, 225-235.

Griffiths,E.C., Kelly,J.A., Ashcroft,A., Ward,D.J., Robson,B., 1989. Comparative metabolism and conformation of TRH and its analogues. Ann N Y Acad Sci 553, 217-231.

Hall,R., Amos,J., Garry,R., Buxton,R.L., 1970. Thyroid-stimulating hormone response to synthetic thyrotrophin releasing hormone in man. Br Med J 2, 274-277.

Halpern,J., Hinkle,P.M., 1981. Direct visualization of receptors for thyrotropin-releasing hormone with a fluorescein-labeled analog. Proc Natl Acad Sci USA 78, 587-591.

Harris,A.R.C., Christianson,D., Smith,M.S., Fang,S.-L., Braverman,L.E., Vagenakis,A.G., 1978. The physiological role of thyrotropin-releasing hormone in the regulation of thyroid-stimulating hormone and prolactin secretion in the rat. J Clin Invest 61, 441-448.

Hartley,D.M., Choi,D.W., 1989. Delayed rescue of N-methyl-D-aspartate receptor-mediated neuronal injury in cortical culture. J Pharmacol Exp Ther 250, 752-758.

Hauser,W.A., Rich,S.S., Lee,J.R., Annegers,J.F., Anderson,V.E., 1998. Risk of recurrent seizures after two unprovoked seizures. N Engl J Med 338, 429-434.

Hedner,J., Hedner,T., Jonason,J., Lundberg,D., 1981. Central respiratory stimulant effect by thyrotropin in releasing hormone in the rat. Neurosci Lett 25, 317-320.

Heuer,H., Schafer,M.K., O'Donnell,D., Walker,P., Bauer,K., 2000. Expression of thyrotropin-releasing hormone receptor 2 (TRH-R2) in the central nervous system of rats. J Comp Neurol 428, 319-336.

Heuer,H., Schafer,M.K.H., Bauer,K., 1998. The Thyrotropin-Releasing Hormone-Degrading Ectoenzyme: The Third Element of the Thyrotropin-Releasing Hormone Signaling System. Thyroid 8, 915-920.

Hinkle,P.M., 1989. Pituitary TRH receptors. Annals of the New York Academy of Sciences 553, 176-187.

Hinkle,P.M., Tashjian,A.H., Jr., 1975. Thyrotropin-releasing hormone regulates the number of its own receptors in the GH3 strain of pituitary cells in culture. Biochemistry 14, 3845-3851.

Hokfelt,T., Fuxe,K., Johansson,O., Jeffcoate,S., White,N., 1975. Distribution of thyrotropin-releasing hormone (TRH) in the central nervous system as revealed with immunohistochemistry. Eur J Pharmacol 34, 389-392.

Hollmann,M., Heinemann,S., 1994. Cloned glutamate receptors. Annu Rev Neurosci 17, 31-108.

Houamed,K.M., Kuijper,J.L., Gilbert,T.L., Haldeman,B.A., O'Hara,P.J., Mulvihill,E.R., Almers,W., Hagen,F.S., 1991. Cloning, expression, and gene structure of a G-protein coupled glutamate receptor from rat brain. Science 252, 1318-1321.

Hwa,G.G., Avoli,M., 1991. The involvement of excitatory amino acids in neocortical epileptogenesis: NMDA and non-NMDA receptors. Exp Brain Res 86, 248-256.

Ikonomidou,C., Qin,Y., Labruyere,J., Kirby,C., Olney,J.W., 1996. Prevention of trauma-induced neurodegeneration in infant rat brain. Pediatr Res 39, 1020-1027.

Iriuchijima,T., Mori,M., 1989. Regional dissociation of cyclic AMP and inositol phosphate formation in response to thyrotropin-releasing hormone in the rat brain. J Neurochem 52, 1944-1946.

Ishimaru,M.J., Ikonomidou,C., Tenkova,T.I., Der,T.C., Dikranian,K., Sesma,M.A., Olney,J.W., 1999. Distinguishing excitotoxic from apoptotic neurodegeneration in the developing rat brain. J Comp Neurol 408, 461-476.

Itadani,H., Nakamura,T., Itoh,J., Iwaasa,H., Kanatani,A., Borkowski,J., Ihara,M., Ohta,M., 1998. Cloning and characterization of a new subtype of thyrotropin-releasing hormone receptors. Biochem Biophys Res Commun 250, 68-71.

Iversen,L., Mulvihill,E., Haldeman,B., Diemer,N.H., Kaiser,F., Sheardown,M., Kristensen,P., 1994. Changes in metabotropic glutamate receptor mRNA levels following global ischemia: increase of a putative presynaptic subtype (mGluR4) in highly vulnerable rat brain areas. J Neurochem 63, 625-633.

Jaworska-Feil,L., Kajta,M., Budziszewska,B., Leskiewicz,M., Lason,W., 2001. Protective effects of TRH and its stable analogue, RGH-2202, on kainate-induced seizures and neurotoxicity in rodents. Epilepsy Res 43, 67-73.

Joly,C., Gomeza,J., Brabet,I., Curry,K., Bockaert,J., Pin,J.P., 1995. Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. J Neurosci 15, 3970-3981.

Kamada,S., Kikkawa,U., Tsujimoto,Y., Hunter,T., 2005. A-kinase-anchoring protein 95 functions as a potential carrier for the nuclear translocation of active caspase 3 through an enzyme-substrate-like association. Mol Cell Biol 25, 9469-9477.

Kanai,Y., Hediger,M.A., 1992. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360, 467-471.

Kane,M.D., Vanden Heuvel,J.P., Isom,G.E., Schwarz,R.D., 1998. Differential expression of group I metabotropic glutamate receptors (mGluRs) in the rat pheochromocytoma cell line PC12: role of nerve growth factor and *ras*. Neurosci Lett 252, 1-4.

Kass,I.S., Lipton,P., 1986. Calcium and long-term transmission damage following anoxia in dentate gyrus and CA1 regions of the rat hippocampal slice. J Physiol 378, 313-334.

Keller,H.H., Bartholini,G., Pletscher,A., 1974. Enhancement of cerebral noradrenaline turnover by thyrotropin-releasing hormone. Nature 248, 528-529.

Kelly,J.A., 1995. Thyrotropin-releasing hormone: basis and potential for its therapeutic use. Essays Biochem 30, 133-149.

Kerwin,R.W., Patel,S., Meldrum,B.S., Czudek,C., Reynolds,G.P., 1988. Asymmetrical loss of glutamate receptor subtype in left hippocampus in schizophrenia. Lancet 1, 583-584.

Kew,J.N., Kemp,J.A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. Psychopharmacology (Berl) 179, 4-29.

Kim,G.D., Carr,I.C., Anderson,L.A., Zabavnik,J., Eidne,K.A., Milligan,G., 1994. The long isoform of the rat thyrotropin-releasing hormone receptor down-regulates G_q proteins. J Bio Chem 269, 19933-19940.

Knoblach, S. M., Durbin, S., and Kubek, M. J. Subregional changes in hippocampal thyrotropin-releasing hormone (TRH) after amygdaloid kindling in rats. Society for Neuroscience 15, 778. 1989.

Koenig,M.L., Yourick,D.L., Meyerhoff,J.L., 1996. Thyrotropin-releasing hormone (TRH) attenuates glutamate-stimulated increases in calcium in primary neuronal cultures. Brain Res 730, 143-149.

Koerner,J.F., Cotman,C.W., 1981. Micromolar L-2-amino-4-phosphonobutyric acid selectively inhibits perforant path synapses from lateral entorhinal cortex. Brain Res 216, 192-198.

Koh,J.Y., Choi,D.W., 1987. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 20, 83-90.

Koliatsos,V.E., Dawson,T.M., Kecojevic,A., Zhou,Y., Wang,Y.F., Huang,K.X., 2004. Cortical interneurons become activated by deafferentation and instruct the apoptosis of pyramidal neurons. Proc Natl Acad Sci U S A 101, 14264-14269.

Koliatsos,V.E., Kecojevic,A., Troncoso,J.C., Gastard,M.C., Bennett,D.A., Schneider,J.A., 2006. Early involvement of small inhibitory cortical interneurons in Alzheimer's disease. Acta Neuropathol (Berl).

Koliatsos,V.E., Price,D.L., Clatterbuck,R.E., Markowska,A.L., Olton,D.S., Wilcox,B.J., 1993. Neurotrophic strategies for treating Alzheimer's disease: lessons from basic neurobiology and animal models. Ann N Y Acad Sci 695, 292-299.

Kristian,T., Gido,G., Siesjo,B.K., 1993. Brain calcium metabolism in hypoglycemic coma. J Cereb Blood Flow Metab 13, 955-961.

Kristian,T., Siesjo,B.K., 1998. Calcium in ischemic cell death. Stroke 29, 705-718.

Krupnick,J.G., Benovic,J.L., 1998. The role of receptor kinases and arrestins in G-protein coupled receptor regulation. Annu Rev Pharmacol Toxicol 38, 289-319.

Kubek,M.J., Garg,B.P., 2002. Thyrotropin-releasing hormone (TRH) in the treatment of intractable epilepsy. Pediatr Neurol 26, 9-17.

Kubek,M.J., Knoblach,S.M., Sharif,N.A., Burt,D.R., Buterbaugh,G.G., Fuson,K.S., 1993. Thyrotropin-releasing hormone gene expression and receptors are differentially modified in limbic foci by seizures. Ann Neurol 33, 70-76.

Kubek,M.J., Lorincz,M.A., Wilber,J.F., 1977. The identification of thyrotropin-releasing hormone (TRH) in hypothalamic and extrahypothalamic loci of the human nervous system. Brain Res 126, 196-200.

Kubek,M.J., Low,W.C., Sattin,A., Morzorati,S.L., Meyerhoff,J.L., Larsen,S.H., 1989. Role of TRH in seizure modulation. Ann NY Acad Sci 553, 286-303.

Kullmann,D.M., 1999b. Synaptic and extrasynaptic roles of glutamate in the mammalian hippocampus. Acta Physiol Scand 166, 79-83.

Kullmann,D.M., Min,M.Y., Asztely,F., Rusakov,D.A., 1999a. Extracellular glutamate diffusion determines the occupancy of glutamate receptors at CA1 synapses in the hippocampus. Philos Trans R Soc Lond B Biol Sci 354, 395-402.

Laurie,D.J., Boddeke,H.W., Hiltscher,R., Sommer,B., 1996. HmGlu1d, a novel splice variant of the human type I metabotropic glutamate receptor. Eur J Pharmacol 296, R1-R3.

Lechan,R.M., 1987. Neuroendocrinology of pituitary hormone regulation. Endocrinol Metab Clin North Am 16, 475-501.

Lechan,R.M., Wu,P., Jackson,I., 1986a. Immunolocalization of the thyrotropin-releasing hormone prohormone in the rat central nervous system. Endocrin 119, 1210-1216.

Lechan,R.M., Wu,P., Jackson,I., Wolf,H., Cooperman,S., Mandel,G., Goodman,R.H., 1986b. Thyrotropin-releasing hormone precursor: Characterization in rat brain. Science 231, 159-161.

Lee,J.M., Grabb,M.C., Zipfel,G.J., Choi,D.W., 2000. Brain tissue responses to ischemia. J Clin Invest 106, 723-731.

Lee,J.M., Zipfel,G.J., Choi,D.W., 1999. The changing landscape of ischaemic brain injury mechanisms. Nature 399, A7-14.

Legrand,C., Bour,J.M., Jacob,C., Capiaumont,J., Martial,A., Marc,A., Wudtke,M., Kretzmer,G., Demangel,C., Duval,D., 1992. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. J Biotechnol 25, 231-243.

Leist,M., Nicotera,P., 1998a. Apoptosis versus necrosis: the shape of neuronal cell death. Results Probl Cell Differ 24, 105-135.

Leist,M., Nicotera,P., 1998b. Apoptosis, excitotoxicity, and neuropathology. Exp Cell Res 239, 183-201.

Leist,M., Single,B., Naumann,H., Fava,E., Simon,B., Kuhnle,S., Nicotera,P., 1999. Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. Exp Cell Res 249, 396-403.

Leist,M., Volbracht,C., Kuhnle,S., Fava,E., Ferrando-May,E., Nicotera,P., 1997. Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. Mol Med 3, 750-764.

Lipton,S.A., Nicotera,P., 1998. Calcium, free radicals and excitotoxins in neuronal apoptosis. Cell Calcium 23, 165-171.

Logan,W.J., Snyder,S.H., 1972. High affinity uptake systems for glycine, glutamic and aspaspartic acids in synaptosomes of rat central nervous tissues. Brain Res 42, 413-431.

Low,W.C., Farber,S.D., Hill,T.G., Sattin,A., Kubek,M.J., 1989a. Evidence for extrinsic and intrinsic sources of thyrotropin-releasing hormone (TRH) in the hippocampal formation as determined by radioimmunoassay and immunocytochemistry. Ann NY Acad Sci 553, 574-578.

Low,W.C., Roepke,J., Farber,S.D., Hill,T.G., Sattin,A., Kubek,M.J., 1989b. Distribution of thyrotropin-releasing hormone (TRH) in the hippocampal formation as determined by radioimmunoassay. Neurosci Lett 103, 314-319.

Lucas,D.O., Bajjalieh,S.M., Kowalchyk,J.A., Martin,T.F.J., 1985. Direct stimulation by thyrotropin-releasing hormone (TRH) of polyphosphoinositide hydrolysis in GH3 cell membranes by a guanine nucleotide-modulated mechanism. Biochem Biophys Res Commun 132, 721-728.

Lujan,R., Nusser,Z., Roberts,J.D.B., Shigemoto,R., Somogyi,P., 1996. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Euro J Neurosci 8, 1488-1500.

Mailleux,P., Mitchell,F., Vanderhaeghen,J.-J., Milligan,G., Erneux,C., 1992. Immunohistochemical distribution of neurons containing the G-proteins $G_{q\alpha}/G_{11}\alpha$ in the adult rat brain. Neurosci 51, 311-316.

Mailleux,P., Takazawa,K., Erneux,C., Vanderhaeghen,J.J., 1993. Distribution of the neurons containing inositol 1,4,5-trisphosphate 3-kinase and its messenger RNA in the developing rat brain. J Comp Neurol 327, 618-629.

Manaker,S., Rainbow,T.C., Winokur,A., 1986. Thyrotropin-releasing hormone (TRH) receptors: localization in rat and human central nervous system. Boast,C., Snowhill,E., Altar,C.A. (Eds.) Quantitative Receptor Autoradiography. Alan R. Liss Inc., pp. 103-135.

Manaker,S., Winokur,A., Rostene,W.H., Rainbow,T.C., 1985. Autoradiographic localization of thyrotropin-releasing hormone receptors in the rat central nervous system. J Neurosci 5, 167-174.

Mantyh,P.W., Hunt,S.P., 1985. Localization by light microscopic autoradiography in rat brain using (3H)(3-Me-His2)TRH as the radioligand. J Neurosci 5, 551-561.

Mao,L., Wang,J.Q., 2003. Group I metabotropic glutamate receptor-mediated calcium signaling and immediate early gene expression in cultured rat striatal neurons. Eur J Neurosci 17, 741-750.

Marcaida,G., Kosenko,E., Minana,M.D., Grisolia,S., Felipo,V., 1996. Glutamate induces a calcineurin-mediated dephosphorylation of Na^+/K^+-ATPase that results in its activation in cerebellar neurons in culture. J Neurochem 66, 99-104.

Marcaida,G., Minana,M.D., Grisolia,S., Felipo,V., 1997. Determination of intracellular ATP in primary cultures of neurons. Brain Res Brain Res Protoc 1, 75-78.

Marsden,C.A., Bennett,G.W., Fone,K.C.F., Johnson,J.V., 1989. Functional interactions between TRH and 5-hydroxytryptamine (5-HT) and proctolin in rat brain and spinal cord. Annals of the New York Academy of Sciences 553, 121-134.

Martin,T.F.J., Bajjalieh,S.M., Lucas,D.O., Kowalchyk,J.A., 1986. Thyrotropin-releasing hormone stimulation of polyphosphoinositide hydrolysis in GH3 cell membranes is GTP-dependent but insensitive to cholera or pertussis toxin. J Biol Chem 261, 10141-10149.

Martin,T.F.J., 1989. Effects of TRH on secondary messenger systems: Summary of section IV. Annals of the New York Academy of Sciences 553, 214-216.

Mason,W.T., Rawlings,S.R., Cobbett,P., Sikdar,S.K., Zorec,R., Ackerman,S.N., Benham,C.D., Berridge,M.J., Cheek,T., Moreton,R.B., 1988. Control of secretion of anterior pituitary cells linking ion channels, messengers and exocytosis. J Exp Biol 139, 287-316.

Masu,M., Tanabe,Y., Tsuchida,K., Shigemoto,R., Nakanishi,S., 1991. Sequence and expression of a metabotropic glutamate receptor. Nature 349, 760-765.

Matre,V., Karlsen,H.E., Wright,M.S., Lundell,I., Fjeldheim,A.K., Gabrielsen,O.S., Larhammar,D., Gautvik,K.M., 1993. Molecular cloning of a functional human thyrotropin-releasing hormone receptor. Biochem Biophys Res Comm 195, 179-185.

Mattson,M.P., 2006. Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders. Antioxid Redox Signal 8, 1997-2006.

McDonald,J.W., Gottlieb,D.I., Choi,D.W., 2000. Reply to "What is a functional recovery after spinal cord injury?". Nat Med 6, 358.

Meldrum,B., Garthwaite,J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol Sci 11, 379-387.

Meldrum,B.S., 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130, 1007S-1015S.

Merchenthaler,I., Meeker,M., Petrusz,P., Kizer,J.S., 1989. Identification and Immunocytochemical Localization of a New Thyrotropin-Releasing Hormone Precursor in Rat Brain. Endocrinology 124, 1888-1897.

Metcalf,G., 1974. TRH: a possible mediator of thermoregulation. Nature 252, 310-311.

Milligan,G., Green,A., 1997. Receptor-mediated down-regulation of cellular G-protein levels as a mechanism for the development of sustained heterologous desensitization. Sibley,D.R., Houslay,M.D. (Eds.) Regulation of cellular signal transduction pathways by desensitization and amplification. John Wiley & Sons, Ltd., New York, pp. 233-248.

Milligan,G., Mullaney,I., McCallum,J.F., 1993. Distribution and relative levels of expression of the phosphoinositidase-C-linked G-proteins $G_q$alpha and G_{11}alpha: absence of G_{11}alpha in human platelets and haemopoietically derived cell lines. Biochim Biophys Acta 1179, 208-212.

Minakami,R., Katsuki,F., Yamamoto,T., Nakamura,K., Sugiyama,H., 1994. Molecular cloning and the functional expression of two isoforms of human metabotropic glutamate receptor subtype 5. Biochem Biophys Res Commun 199, 1136-1143.

Monaghan,D.T., Cotman,C.W., 1985. Distribution of N-methyl-D-aspartate-sensitive L-[3H]glutamate-binding sites in rat brain. J Neurosci 5, 2909-2919.

Monaghan,D.T., Holets,V.R., Toy,D.W., Cotman,C.W., 1983. Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites. Nature 306, 176-179.

Mori,M., Iriuchijima,T., Yamada,M., Murakami,M., Kobayashi,S., 1991. A novel TRH analog, YM14673, stimulates intracellular signaling systems in the brain more potently than predicted by its pituitary actions. Res Commun Chem Pathol Pharmacol 71, 17-26.

Morley,J.E., 1981. Neuroendocrine control of thyrotropin secretion. Endocr Rev 2, 396-436.

Mouginot,D., Feltz,P., Schlichter,R., 1991. Modulation of GABA-gated chloride currents by intracellular Ca^{2+} in cultured porcine melanotrophs. J Physiol 437, 109-132.

Mukhin,A.G., Ivanova,S.A., Faden,A.I., 1997. mGluR modulation of post-traumatic neuronal death: role of NMDA receptors. Neuroreport 8, 2561-2566.

Mundell,S.J., Benovic,J.L., 2000. Selective regulation of endogenous G-protein coupled receptors by arrestins in HEK293 cells. J Biol Chem 275, 12900-12908.

Mundell,S.J., Pula,G., McIlhinney,R.A., Roberts,P.J., Kelly,E., 2004. Desensitization and internalization of metabotropic glutamate receptor 1a following activation of heterologous $G_{q/11}$-coupled receptors. Biochemistry 43, 7541-7551.

Nakajima,Y., Iwakabe,H., Akazawa,C., Nawa,H., Shigemoto,R., Mizuno,N., Nakanishi,S., 1993. Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. J Biol Chem 268, 11868-11873.

Nakanishi,S., 1992. Molecular diversity of glutamate receptors and implications for brain function. Science 258, 597-603.

Narumi,S., Nagai,Y., Miyamoto,M., Nagawa,Y., 1983. Thyrotropin-releasing hormone (TRH) and its analog (DN-1417): interaction with pentobarbital in choline uptake and acetylcholine synthesis of rat brain slices. Life Sci 32, 1637-1645.

Nelson,E.J., Hinkle,P.M., 1994. Thyrotropin-releasing hormone activates Ca^{++} efflux. J Biological Chemistry 269, 30854-30860.

Nicholson,D.W., Thornberry,N.A., 1997. Caspases: killer proteases. Trends Biochem Sci 22, 299-306.

Nicoletti,F., Meek,J.L., Iadarola,M.J., Chuang,D.M., Roth,B.L., Costa,E., 1986a. Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. J Neurochem 46, 40-46.

Nicoletti,F., Wroblewski,J.T., Novelli,A., Alho,H., Guidotti,A., Costa,E., 1986b. The activation of inositol phospholipid metabolism as a signal-transducing system for

excitatory amino acids in primary cultures of cerebellar granule cells. J Neurosci 6, 1905-1911.

Nicotera,P., Hartzell,P., Davis,G., Orrenius,S., 1986. The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system. FEBS Lett 209, 139-144.

Nicotera,P., Leist,M., 1997. Excitotoxicity. Cell Death Differ 4, 517-518.

Nicotera,P., Leist,M., Fava,E., Berliocchi,L., Volbracht,C., 2000. Energy requirement for caspase activation and neuronal cell death. Brain Pathol 10, 276-282.

Nie,Y., Schoepp,D.D., Klaunig,J.E., Yard,M., Lahiri,D.K., Kubek,M.J., 2005. Thyrotropin-releasing hormone (protirelin) inhibits potassium-stimulated glutamate and aspartate release from hippocampal slices in vitro. Brain Res 1054, 45-54.

Nillni,E.A., Friedman,T.C., Todd,R.B., Birch,N.P., Loh,Y.P., Jackson,I.M., 1995. Pro-thyrotropin-releasing hormone processing by recombinant PC1. J Neurochem 65, 2462-2472.

Nillni,E.A., Sevarino,K.A., 1999. The biology of pro-thyrotropin-releasing hormone-derived peptides. Endocrine Rev 20, 599-648.

Nillni,E.A., Sevarino,K.A., Jackson,I.M.D., 1993. Identification of thyrotropin-releasing hormone-prohormone and its posttranslational processing in a transfected AtT20 tumoral cell line. Endocrinology 132, 1260-1270.

Novelli,A., Tasker,R.A., 2000. On excitotoxicity. Amino Acids 19, 227-228.

O'Cuinn,G., O'Connor,B., Elmore,M., 1990. Degradation of thyrotropin-releasing hormone and luteinizing hormone-releasing hormone by enzymes of brain tissue. J Neurochem 54, 1-13.

O'Dowd,B.F., Lee,D.K., Huang,W., Nguyen,T., Cheng,R., Liu,Y., Wang,B., Gershengorn,M.C., George,S.R., 2000. TRH-R2 exhibits similar binding and acute signaling but distinct regulation and anatomic distribution compared with TRH-R1. Mol Endocrinol 14, 183-193.

Obrenovitch,T.P., 1999. High extracellular glutamate and neuronal death in neurological disorders. Cause, contribution or consequence? Ann N Y Acad Sci 890, 273-286.

Obrenovitch,T.P., Richards,D.A., 1995. Extracellular neurotransmitter changes in cerebral ischaemia. Cerebrovasc Brain Metab Rev 7, 1-54.

Obrenovitch,T.P., Urenjak,J., 1998. Glutamate release inhibitors: a critical assessment of their action mechanism. Amino Acids 14, 143-150.

Obrenovitch,T.P., Urenjak,J., Zilkha,E., Jay,T.M., 2000. Excitotoxicity in neurological disorders--the glutamate paradox. Int J Dev Neurosci 18, 281-287.

Okamoto,N., Hori,S., Akazawa,C., Hayashi,Y., Shigemoto,R., Mizuno,N., Nakanishi,S., 1994. Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. J Biol Chem 269, 1231-1236.

Olney,J.W., 2002. New insights and new issues in developmental neurotoxicology. Neurotoxicology 23, 659-668.

Olney,J.W., 1969. Glutamate-induced retinal degeneration in neonatal mice. Electron microscopy of the acutely evolving lesion. J Neuropathol Exp Neurol 28, 455-474.

Olney,J.W., 1989. Glutamate, a neurotoxic transmitter. J Child Neurol 4, 218-226.

Olney,J.W., 1982. The toxic effects of glutamate and related compounds in the retina and the brain. Retina 2, 341-359.

Olney,J.W., 1990a. Excitotoxic amino acids and neuropsychiatric disorders. Annu Rev Pharmacol Toxicol 30, 47-71.

Olney,J.W., 2003. Excitotoxicity, apoptosis and neuropsychiatric disorders. Curr Opin Pharmacol 3, 101-109.

Olney,J.W., 1990b. Excitotoxicity: an overview. Can Dis Wkly Rep 16 Suppl 1E, 47-57.

Olney,J.W., Adamo,N.J., Ratner,A., 1971. Monosodium glutamate effects. Science 172, 294.

Olney,J.W., Collins,R.C., Sloviter,R.S., 1986. Excitotoxic mechanisms of epileptic brain damage. Adv Neurol 44, 857-877.

Olney,J.W., Farber,N.B., 1995. NMDA antagonists as neurotherapeutic drugs, psychotogens, neurotoxins, and research tools for studying schizophrenia. Neuropsychopharmacology 13, 335-345.

Olney,J.W., Ishimaru,M.J., Bittigau,P., Ikonomidou,C., 2000. Ethanol-induced apoptotic neurodegeneration in the developing brain. Apoptosis 5, 515-521.

Olney,J.W., Newcomer,J.W., Farber,N.B., 1999. NMDA receptor hypofunction model of schizophrenia. J Psychiatr Res 33, 523-533.

Olney,J.W., Price,M.T., Samson,L., Labruyere,J., 1986b. The role of specific ions in glutamate neurotoxicity. Neurosci Lett 65, 65-71.

Olney,J.W., Sharpe,L.G., 1969. Brain lesions in an infant rhesus monkey treated with monsodium glutamate. Science 166, 386-388.

Olney,J.W., Teitelbaum,J., Pinsky,C., Debonnel,G., 1990. Domoic acid toxicity. Panel discussion: treatment. Can Dis Wkly Rep 16 Suppl 1E, 117-120.

Olney,J.W., Wozniak,D.F., Farber,N.B., 1997. Excitotoxic neurodegeneration in Alzheimer disease. New hypothesis and new therapeutic strategies. Arch Neurol 54, 1234-1240.

Olney,J.W., Wozniak,D.F., Jevtovic-Todorovic,V., Farber,N.B., Bittigau,P., Ikonomidou,C., 2002. Drug-induced apoptotic neurodegeneration in the developing brain. Brain Pathol 12, 488-498.

Oron,Y., Straub,R.E., Traktman,P., Gershengorn,M.C., 1987. Decreased TRH receptor mRNA activity precedes homologous downregulation: Assay in Oocytes. Science 238, 1406-1408.

Palucha,A., 2006. Are compounds acting at metabotropic glutamate receptors the answer to treating depression? Expert Opin Investig Drugs 15, 1545-1553.

Palucha,A., Pilc,A., 2002. On the role of metabotropic glutamate receptors in the mechanisms of action of antidepressants. Pol J Pharmacol 54, 581-586.

Palucha,A., Pilc,A., 2005. The involvement of glutamate in the pathophysiology of depression. Drug News Perspect 18, 262-268.

Patneau,D.K., Mayer,M.L., 1990. Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. J Neurosci 10, 2385-2399.

Pazos,A., Cortes,R., Palacios,J.M., 1985. Thyrotropin-releasing hormone receptor binding sites autoradiographic distribution in the rat and guinea pig brain. J Neurochem 45, 1448-1463.

Pesanova,Z., Novotny,J., Cerny,J., Milligan,G., Svoboda,P., 1999. Thyrotropin-releasing hormone-induced depletion of $G_q$alpha/G_{11}alpha proteins from detergent-insensitive membrane domains. FEBS Lett 464, 35-40.

Peterkofsky,A., Battaini,F., Koch,Y., Takahara,Y., Dannies,P., 1982. Histidyl-proline diketopiperazine: its biological role as a regulatory peptide. Mol Cell Biochem 42, 45-63.

Pilotte,N.S., Sharif,N.A., Burt,D.R., 1984. Characterization and autoradiographic localization of TRH receptors in sections of rat brain. Brain Res 293, 372-376.

Pin,J.P., Waeber,C., Prezeau,L., Bockaert,J., Heinemann,S.F., 1992. Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in Xenopus oocytes. Proc Natl Acad Sci U S A 89, 10331-10335.

Pin,J.P., Duvoisin,R., 1995. The metabotropic glutamate receptors: Structure and functions. Neuropharm 34, 1-26.

Pines,G., Danbolt,N.C., Bjoras,M., Zhang,Y., Bendahan,A., Eide,L., Koepsell,H., Storm-Mathisen,J., Seeberg,E., Kanner,B.I., 1992. Cloning and expression of a rat brain L-glutamate transporter. Nature 360, 464-467.

Porter,A.G., Janicke,R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6, 99-104.

Porter,A.G., Ng,P., Janicke,R.U., 1997. Death substrates come alive. Bioessays 19, 501-507.

Prasad,C., Mori,M., Wilber,J.F., Pierson,W., Pegues,J., Jayaraman,A., 1982. Distribution and metabolism of cyclo (His-Pro): a new member of the neuropeptide family. Peptides 3, 591-598.

Premont,R.T., Inglese,J., Lefkowitz,R.J., 1995. Protein kinases that phosphorylate activated G-protein coupled receptors. FASEB J 9, 175-182.

Pumain,R., Kurcewicz,I., Louvel,J., 1987. Ionic changes induced by excitatory amino acids in the rat cerebral cortex. Can J Physiol Pharmacol 65, 1067-1077.

Rastogi,R.B., Singhal,R.L., Lapierre,Y.D., 1981. Effects of MK-771, a novel TRH analogue, on dopaminergic and serotonergic systems. Eur J Pharmacol 73, 307-312.

Reid,K.H., Edmonds,H.L.Jr., Schurr,A., Tseng,M.T., West,C.A., 1988. Pitfalls in the use of brain slices. Prog Neurobiol 31, 1-18.

Renaud,L.P., Martin,J., 1975. Thyrotropin-releasing hormone (TRH): depressant action on central neuronal activity. Brain Res 86, 150-154.

Renaud,L.P., 1978. Peptides as neurotransmitters or neuromodulators. Lipton,M.A., DiMascio,A., Killam,K.F. (Eds.) Psypharmacology: A Generation of Progress. Raven Press, New York, pp. 423-430.

Renaud,L.P., Blume,H.W., Pittman,Q.J., Lamour,Y., Tan,A.T., 1979. Thyrotropin-releasing hormone selectively depresses glutamate excitation of cerebral cortical neurons. Science 205, 1275-1277.

Rivier,J., Vale,W., Monahan,M., Ling,N., Burgus,R., 1972. Synthetic thyrotropin-releasing factor analogs. 3. Effect of replacement or modification of histidine residue on biological activity. J Med Chem 15, 479-482.

Roberts,P.J., Watkins,J.C., 1975. Structural requirements for the inhibition for L-glutamate uptake by glia and nerve endings. Brain Res 85, 120-125.

Robinson,M.B., Sinor,J.D., Dowd,L.A., Kerwin,J.F., Jr., 1993. Subtypes of sodium-dependent high-affinity L-[3H]glutamate transport activity: pharmacologic specificity and regulation by sodium and potassium. J Neurochem 60, 167-179.

Romano,C., Price,M.T., Olney,J.W., 1995a. Delayed excitotoxic neurodegeneration induced by excitatory amino acid agonists in isolated retina. J Neurochem 65, 59-67.

Romano,C., Sesma,M.A., McDonald,C.T., O'Malley,K., van den Pol,A.N., Olney,J.W., 1995b. Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J Comp Neurol 355, 455-469.

Rothman,S.M., 1990. Glutamate and anoxic neuronal death *in vitro*. Epilepsia 687-694.

Rothman,S.M., Olney,J.W., 1986. Glutamate and the pathophysiology of hypoxic--ischemic brain damage. Ann Neurol 19, 105-111.

Rothman,S.M., Olney,J.W., 1995. Excitotoxicity and the NMDA receptor--still lethal after eight years. Trends Neurosci 18, 57-58.

Sattin,A., Kubek,M.J., Low,W.C., Staley,C.J., Simon,J.R., 1992. Some regional anatomical relationships of TRH to 5-HT in rat limbic forebrain. Neurochem Res 17, 469-474.

Saugstad,J.A., Kinzie,J.M., Shinohara,M.M., Segerson,T.P., Westbrook,G.L., 1997. Cloning and expression of rat metabotropic glutamate receptor 8 reveals a distinct pharmacological profile. Mol Pharmacol 51, 119-125.

Schally,A.V., Arimura,A., 1977. Physiology and Nature of Hypothalamic Regulatory Hormones. Martin,L., Besser,G.M. (Eds.) Clinical Neuroendocrinology. Academic Press, New York, pp. 1-42.

Schally,A.V., Bowers,C.Y., 1 A.D. The nature of thyrotropin-releasing hormone (TRH). Kenny,A.D., Anderson,R.R. (Eds.) Proceedings of 6th Midwest Conference on the Thyroid and Endocrinology. University of Missouri, Columbia, pp. 25-63.

Schaner,P., Todd,R.B., Seidah,N.G., Nillni,E.A., 1997. Processing of prothyrotropin-releasing hormone by the family of prohormone convertases. J Biol Chem 272, 19958-19968.

Schoepp,D., Bockaert,J., Sladeczek,F., 1990a. Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. Trends Pharmacol Sci 11, 508-515.

Schoepp,D.D., 2001. Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. J Pharmacol Exp Ther 299, 12-20.

Schoepp,D.D., Conn,P.J., 1993. Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol Sci 14, 13-20.

Schoepp,D.D., Desai,M.A., 1995. A neurochemical approach to elucidate metabotropic vs. ionotropic glutamate receptor activities in rat hippocampal slices. Schurr,A., Rigor,B.M. (Eds.) Brain Slices in Basic and Clinical Research. CRC Press, Boca Raton, pp. 171-186.

Schoepp,D.D., Gamble,A.Y., Salhoff,C.R., Johnson,B.G., Ornstein,P.L., 1990b. Excitatory amino acid-induced convulsions in neonatal rats mediated by distinct receptor subtypes. Eur J Pharmacol 182, 421-427.

Schoepp,D.D., Goldsworthy,J., Johnson,B.G., Salhoff,C.R., Baker,S.R., 1994. 3,5-Dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. J Neurochem 63, 769-772.

Schoepp,D.D., Jane,D.E., Monn,J.A., 1999. Pharmacological agents acting at subtypes of metabotropic glutamate receptors. Neuropharm 38, 1431-1476.

Schousboe,A., Waagepetersen,H.S., 2006. Glial modulation of GABAergic and glutamat ergic neurotransmission. Curr Top Med Chem 6, 929-934.

Schurr,A., Changaris,D.G., Rigor,B.M., 1987. Glutamine protects neuronal function against cerebral hypoxia: a study using the in vitro hippocampal slice preparation. Brain Res 412, 179-181.

Schurr,A., Payne,R.S., Heine,M.F., Rigor,B.M., 1995. Hypoxia, excitotoxicity, and neuroprotection in the hippocampal slice preparation. J Neurosci Methods 59, 129-138.

Schurr,A., Reid,K.H., Tseng,M.T., Edmonds,H.L.Jr., 1984. The stability of the hippocampal slice preparation: an electrophysiological and ultrastructural analysis. Brain Res 297, 357-362.

Schurr,A., Reid,K.H., Tseng,M.T., Edmonds,H.L.Jr., Rigor,B.M., 1985. A dual chamber for comparative studies using the brain slice preparation. Comp Biochem Physiol 82A, 701-704.

Seal,R.P., Amara,S.G., 1999. Excitatory amino acid transporters: a family in flux. Annu Rev Pharmacol Toxicol 39, 431-456.

Segerson,T.P., Hoefler,H., Childers,H., Wolfe,H.J., Wu,P., Jackson,I.M., Lechan,R.M., 1987. Localization of thyrotropin-releasing hormone prohormone messenger ribonucleic acid in rat brain in situ hybridization. Endocrinology 121, 98-107.

Sellar,R.E., Taylor,P.L., Lamb,R.F., Zabavnik,J., Anderson,L., Eidne,K.A., 1993. Functional expression and molecular characterization of the thyrotrophin-releasing hormone receptor from the rat anterior pituitary gland. J Mol Endocrinol 10, 199-206.

Sharif,N.A., 1989. Quantitative autoradiography of TRH receptors in discrete brain regions of different mammalian species. Ann NYAcad Sci 553, 147-175.

Sharif,N.A., Burt,D.R., 1985. Limbic, hypothalamic, cortical and spinal regions are enriched in receptors for thyrotropin-releasing hormone: evidence from (3H)ultrafilm autoradiography and correlation with central effects of the tripeptide in rat brain. Neurosci Lett 60, 337-342.

Sharif, N. A., To, Z. P., and Whiting, R. L. Analogs of thyrotropin-releasing hormone (TRH): receptor affinities in brains, spinal cords and pituitaries of different species. Neurochemical Research, 1990.

Shigemoto,R., Kinoshita,A., Wada,E., Nomura,S., Ohishi,H., Takada,M., Flor,P.J., Neki,A., Abe,T., Nakanishi,S., Mizuno,N., 1997. Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17, 7503-7522.

Shigemoto,R., Nomura,S., Ohishi,H., Sugihara,H., Nakanishi,S., Mizuno,N., 1993. Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. Neurosci Lett 163, 53-57.

Siesjo,B.K., 1989. Calcium and cell death. Magnesium 8, 223-237.

Siesjo,B.K., Bengtsson,F., Grampp,W., Theander,S., 1989. Calcium, excitotoxins, and neuronal death in the brain. Ann N Y Acad Sci 568, 234-251.

Simon,R.P., Griffiths,T., Evans,M.C., Swan,J.H., Meldrum,B.S., 1984a. Calcium overload in selectively vulnerable neurons of the hippocampus during and after ischemia: an electron microscopy study in the rat. J Cereb Blood Flow Metab 4, 350-361.

Simon,R.P., Swan,J.H., Griffiths,T., Meldrum,B.S., 1984b. Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science 226, 850-852.

Sladeczek,F., Pin,J.P., Recasens,M., Bockaert,J., Weiss,S., 1985. Glutamate stimulates inositol phosphate formation in striatal neurones. Nature 317, 717-719.

Slesinger,P.A., Patil,N., Liao,Y.J., Jan,Y.N., Jan,L.Y., Cox,D.R., 1996. Functional effects of the mouse weaver mutation on G-protein-gated inwardly rectifying K^+ channels. Neuron 16, 321-331.

Snyder,E.M., Philpot,B.D., Huber,K.M., Dong,X., Fallon,J.R., Bear,M.F., 2001. Internalization of ionotropic glutamate receptors in response to mGluR activation. Nat Neurosci 4, 1079-1085.

Sonsalla,P.K., Albers,D.S., Zeevalk,G.D., 1998. Role of glutamate in neurodegeneration of dopamine neurons in several animal models of parkinsonism. Amino Acids 14, 69-74.

Stewart,G.R., Zorumski,C.F., Price,M.T., Olney,J.W., 1990. Domoic acid: a dementia-inducing excitotoxic food poison with kainic acid receptor specificity. Exp Neurol 110, 127-138.

Storck,T., Schulte,S., Hofmann,K., Stoffel,W., 1992. Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci USA 89, 10955-10959.

Storm-Matheson,J., 1981. Glutamate in hippocampal pathways. DiChiara,G., Gessa,G. (Eds.) Glutamate as a Neurotransmitter. Raven Press, New York, pp. 43-55.

Storm-Mathisen,J., 1977. Localization of putative transmitters in the hippocampal formation: with a note on the connections to septum and hypothalamus. Ciba Found Symp 49-86.

Straub,R.E., Frech,G.C., Joho,R.H., Gershengorn,M.C., 1990. Expression cloning of a cDNA encoding the mouse pituitary thyrotropin-releasing hormone receptor. Proc Natl Acad Sci USA 87, 9514-9518.

Sucher,N.J., Aizenman,E., Lipton,S.A., 1991a. N-methyl-D-aspartate antagonists prevent kainate neurotoxicity in rat retinal ganglion cells in vitro. J Neurosci 11, 966-971.

Sucher,N.J., Lei,S.Z., Lipton,S.A., 1991b. Calcium channel antagonists attenuate NMDA receptor-mediated neurotoxicity of retinal ganglion cells in culture. Brain Res 551, 297-302.

Sugiyama,H., Ito,I., Hirono,C., 1987. A new type of glutamate receptor linked to inositol phospholipid metabolism. Nature 325, 531-533.

Sun,Y., Lu,X., Gershengorn,M.C., 2003. Thyrotropin-releasing hormone receptors - similarities and differences. J Mol Endocrinol 30, 87-97.

Sun,Y.M., Millar,R.P., Ho,H., Gershengorn,M.C., Illing,N., 1998. Cloning and characterization of the chicken thyrotropin-releasing hormone receptor. Endocrinology 139, 3390-3398.

Svoboda,P., Kim,G.D., Grassie,M.A., Eidne,K.A., Milligan,G., 1996. Thyrotropin-releasing hormone-induced subcellular redistribution and down-regulation of G_{11}alpha:

analysis of agonist regulation of coexpressed G_{11}alpha species variants. Mol Pharmacol 49, 646-655.

Svoboda,P., Milligan,G., 1994. Agonist-induced transfer of the alpha subunits of the guanine- nucleotide-binding regulatory proteins G_q and G_{11} and of muscarinic m1 acetylcholine receptors from plasma membranes to a light-vesicular membrane fraction. Eur J Biochem 224, 455-462.

Tache,Y., Stephens,R.L., Jr., Ishikawa,T., 1989. Central nervous system action of TRH to influence gastrointestinal function and ulceration. Ann N Y Acad Sci 553, 269-285.

Takahara,J., Arimura,A., Schally,A.V., 1974. Stimulation of prolactin and growth hormone release by TRH infused into a hypophyseal portal vessel. Proc Soc Exp Biol Med 146, 831-835.

Takamoto,A., Quiggin,L.B., Lieb,I., Shave,E., Balcar,V.J., Yoneda,Y., 2002. Differences between D- and L-aspartate binding to the Na^+-dependent binding sites on glutamate transporters in frozen sections of rat brain. Life Sci 70, 991-1001.

Takata,M., Shimada,Y., Ikeda,A., Sekikawa,K., 1998. Molecular cloning of bovine thyrotropin-releasing hormone receptor gene. J Vet Med Sci 60, 123-127.

Tamargo,R.J., Myseros,J.S., Epstein,J.I., Yang,M.B., Chasin,M., Brem,H., 1993. Interstitial chemotherapy of the 9L gliosarcoma: controlled release polymers for drug delivery in the brain. Cancer Research 53, 329-333.

Tanabe,Y., Masu,M., Ishii,T., Shigemoto,R., Nakanishi,S., 1992. A family of metabotropic glutamate receptors. Neuron 8, 169-179.

Terry,L.C., Craig,R., Hughes,T., Schatzle,J., Zorza,M., Ortolano,G.A., Willoughby,J.O., 1985. Hypothalamic monoaminergic activity and pituitary function in male rats with estrogen-induced pituitary hyperplasia. Neuroendocrinology 41, 269-275.

Teyler,T.J., DiScenna,P., 1984. The Topological Anatomy of the Hippocampus: A Clue to Its Function. Brain Research Bulletin 12, 711-719.

Thomsen,C., 1997. The L-AP4 receptor. Gen Pharmacol 29, 151-158.

Thomsen,C., Kristensen,P., Mulvihill,E., Haldeman,B., Suzdak,P.D., 1992. L-2-amino-4-phosphonobutyrate (L-AP4) is an agonist at the type IV metabotropic glutamate receptor, which is negatively coupled to adenylate cyclase [published erratum appears in Eur J Pharmacol 1993 Jan 15;244(2):187]. Eur J Pharmacol 227, 361-362.

Toide,K., Shinoda,M., Takase,M., Iwata,K., Yoshida,H., 1993. Effects of a novel thyrotropin-releasing hormone analogue, JTP-2942, on extracellular acetylcholine and

choline levels in the rat frontal cortex and hippocampus. Eur J Clin Pharmacol 233, 21-28.

Tong,Y., Zhao,H., Labrie,F., Pelletier,G., 1989. Ontogeny of prolactin mRNA in the rat pituitary gland as evaluated by in situ hybridization. Mol Cell Endocrinol 67, 11-16.

Trump,B.F., Berezesky,I.K., 1996. The mechanisms of calcium-mediated cell injury and cell death [correcgted]. New Horiz 4, 139-150.

Trump,B.F., Berezesky,I.K., Chang,S.H., Phelps,P.C., 1997. The pathways of cell death: oncosis, apoptosis, and necrosis. Toxicol Pathol 25, 82-88.

Turski,L., Meldrum,B.S., Cavalheiro,E.A., Calderazzo-Filho,L.S., Bortolotto,Z.A., Ikonomidou-Turski,C., Turski,W.A., 1987. Paradoxical anticonvulsant activity of the excitatory amino acid N-methyl-D-aspartate in the rat caudate-putamen. Proc Natl Acad Sci USA 84, 1689-1693.

Tymianski,M., Charlton,M.P., Carlen,P.L., Tator,C.H., 1994. Properties of neuroprotective cell-permeant Ca^{2+} chelators: effects on $[Ca^{2+}]_i$ and glutamate neurotoxicity in vitro. J Neurophysiol 72, 1973-1992.

Tymianski,M., Wallace,M.C., Spigelman,I., Uno,M., Carlen,P.L., Tator,C.H., Charlton,M.P., 1993. Cell-permeant Ca^{2+} chelators reduce early excitotoxic and ischemic neuronal injury in vitro and in vivo. Neuron 11, 221-235.

Uemura,K., Aki,T., Yamaguchi,K., Yoshida,K., 2003. Protein kinase C-epsilon protects PC12 cells against methamphetamine-induced death: possible involvement of suppression of glutamate receptor. Life Sci 72, 1595-1607.

Unnerstall,J.R., Wamsley,J.K., 1983. Autoradiographic localization of high-affinity [3H]kainic acid binding sites in the rat forebrain. Eur J Pharmacol 86, 361-371.

van Hooft,J.A., Giuffrida,R., Blatow,M., Monyer,H., 2000. Differential expression of group I metabotropic glutamate receptors in functionally distinct hippocampal interneurons. J Neurosci 20, 3544-3551.

Vignes,M., Clarke,V.R., Parry,M.J., Bleakman,D., Lodge,D., Ornstein,P.L., Collingridge,G.L., 1998. The GluR5 subtype of kainate receptor regulates excitatory synaptic transmission in areas CA1 and CA3 of the rat hippocampus. Neuropharmacology 37, 1269-1277.

Villalobos,C., Garcia-Sancho,J., 1995. Glutamate increases cytosolic calcium in GH3 pituitary cells acting via a high-affinity glutamate transporter. FASEB J 9, 815-819.

Villalobos,C., Nunez,L., Faught,W.J., Leaumont,D.C., Boockfor,F.R., Frawley,L.S., 2002. Calcium dynamics and resting transcriptional activity regulates prolactin gene expression. Endocrinology 143, 3548-3554.

Villalobos,C., Nunez,L., Garcia-Sancho,J., 1996. Functional glutamate receptors in a subpopulation of anterior pituitary cells. FASEB J 10, 654-660.

Volterra,A., 1994. Inhibition of high-affinity glutamate transport in neuronal and glial cells by arachidonic acid and oxygen-free radicals. Molecular mechanisms and neuropathological relevance. Ren Physiol Biochem 17, 165-167.

Vornov,J.J., Coyle,J.T., 1991. Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. J Neurochem 56, 996-1006.

Vornov,J.J., Park,J., Thomas,A.G., 1998. Regional vulnerability to endogenous and exogenous oxidative stress in organotypic hippocampal culture. Exp Neurol 149, 109-122.

Waelsch,H., 1951. Glutamic acid and cerebral function. Adv Protein Chem 6, 299-341.

Wang,W., Gershengorn,M.C., 1999. Rat TRH receptor type 2 exhibits higher basal signaling activity than TRH receptor type 1. Endocrinology 140, 4916-4919.

Watkins,J.C., 2000. l-glutamate as a central neurotransmitter: looking back. Biochem Soc Trans 28, 297-309.

Watkins,J.C., Jane,D.E., 2006. The glutamate story. Br J Pharmacol 147 Suppl 1, S100-S108.

Watkins,J.C., Krogsgaard-Larsen,P., Honore,T., 1990. Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. Trends Pharmacol Sci 11, 25-33.

White,W.F., Nadler,J.V., Hamberger,A., Cotman,C.W., Cummins,J.T., 1977. Glutamate as transmitter of hippocampal perforant path. Nature 270, 356-357.

Wieloch,T., Harris,R.J., Symon,L., Siesjo,B.K., 1984. Influence of severe hypoglycemia on brain extracellular calcium and potassium activities, energy, and phospholipid metabolism. J Neurochem 43, 160-168.

Wieronska,J.M., Branski,P., Szewczyk,B., Palucha,A., Papp,M., Gruca,P., Moryl,E., Pilc,A., 2001. Changes in the expression of metabotropic glutamate receptor 5 (mGluR5) in the rat hippocampus in an animal model of depression. Pol J Pharmacol 53, 659-662.

Wilber,J.F., Utiger,R.D., 1968. In vitro studies on mechanism of action of thyrotropin releasing factor. Proc Soc Exp Biol Med 127, 488-489.

Yamada,M., Monden,T., Satoh,T., Satoh,N., Murakami,M., Iriuchijima,T., Kakegawa,T., Mori,M., 1993. Pituitary adenomas of patients with acromegaly express thyrotropin-releasing hormone receptor messenger RNA: cloning and functional expression of the human thyrotropin-releasing hormone receptor gene. Biochem Biophys Res Commun 195, 737-745.

Yanagisawa,T., Prasad,C., Peterkofsky,A., 1980. The subcellular and organ distribution and natural form of histidyl-proline diketopiperazine in rat brain determined by a specific radioimmunoassay. J Biol Chem 255, 10290-10294.

Yokoi,M., Kobayashi,K., Manabe,T., Takahashi,T., Sakaguchi,I., Katsuura,G., Shigemoto,R., Ohishi,H., Nomura,S., Nakamura,K., Nakao,K., Katsuki,M., Nakanishi,S., 1996. Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. Science 273, 645-647.

Yu,R., Hinkle,P.M., 1998. Signal transduction, desensitization, and recovery of responses to thyrotropin-releasing hormone after inhibition of receptor internalization. Mol Endocrinol 12, 737-749.

Zhao,D., Yang,J., Jones,K.E., Gerald,C., Suzuki,Y., Hogan,P., Chin,W.W., Tashjian,A.H., Jr., 1993. Molecular cloning of a complementary deoxyribonucleic acid encoding the thyrotropin-releasing hormone receptor and regulation of its messenger ribonucleic acid in rat GH cells. Endocrinology 132, 2658.

CURRICULUM VITAE

Michael Yard

Education

Master of Science, Indiana University-Purdue University, Indianapolis, IN (2009)
Bachelor of Science, Purdue Univeristy, West Lafayetter, IN (1985)

United States Army Military Education

Military Intelligence Officer Advanced Course
Medical Officer Basic Course
Infantry Officer Advanced Course
Medical Officer Advanced Course
Armor Officer Advanced Course
Chemical Officer Advanced Course
Public Affairs Crisis Communication Course
Quartermaster Officer Advanced Course
Environmental Communications Course

United States Army Command and General Staff College

Strategic Studies Doctorate USACGSC (1993)
Command and General Staff Officer Course (1990)
Combined Arms and Services Staff School (1988)

Armed Forces Institute of Pathology

Essentials of Forensic Pathology (1991)
Practicum in Forensic Pathology and Forensic Science (**w/FBI Academy**) (1993)

Saint Louis University School of Medicine

Medicolegal Death Investigator Training Course (1996-97)

Honors, Awards, Fellowships

IUPUI School of Science "21 Club" 2007, 2008

IUPUI Favorite Professor Award 2006, 2008

Joseph Hingtgen Graduate Research Travel Award, Indiana University School of Medicine, 2002

Graduate Fellowship, Indiana University School of Medicine, Department of Psychiatry, 1998-2002

Charles M. Mallott Full Academic Scholarship, Purdue University, West Lafayette, IN 1978-1985

Research and Training Experience

Indiana University School of Medicine, Department of Psychiatry, Laboratory of Molecular Neurogenetics and Department of Anatomy (1998-2004): Graduate Assistant in Medical Neuroanatomy Lab, Graduate Neuroanatomy Lecture and Lab, and Molecular Biology Lab. Graduate Molecular Biology Training (1998-2004). Part-time Graduate Student (1992-1997).

Indiana University School of Medicine, Department of Pathology and Laboratory Medicine, Riley Hospital. Laboratory experience with DNA/RNA isolation and hybridization techniques, as well as cloning with bacterial and viral vectors, restriction mapping and nucleic acid sequencing (1995-2002).

Professional Experience

Indiana University Purdue University at Indianapolis, Department of Biology, Lecturer. Responsible for preparing, coordinating, and teaching undergraduate lecture and laboratory course in Human Anatomy, including Histology and Anatomical demonstrations to approximately 400 undergraduate medicine/nursing/dental/Allied Health majors each semester. (2002-present).

University of Indianapolis, Department of Biology, Associate Adjunct Instructor. Responsible for preparing, coordinating, and teaching undergraduate lecture and laboratory courses in Human Anatomy, Human Physiology, and Cell Biology, as well as graduate Gross Anatomy and Applied Neuroscience courses to a total of 700 undergraduate and graduate students. (2002-2005).

United States Army and United States Army Reserve Headquarters, 88th Regional Support Group, Served as Deputy Chief of Staff for Plans, Operations, and Training, for military units in Indiana, Ohio, Michigan, Illinois, Wisconsin, and Minnesota. Retired as promotable Lieutenant Colonel. Active in an international advisory role. (1976-2005)

Office of the Marion County Coroner, Indianapolis, IN. Served as Deputy Coroner. (1995-1997)

Indiana University School of Medicine, Department of Anatomical Pathology, Anatomic Pathology Technical Supervisor. (1979-1997).

Conferences Attended

Key Presenter, Lecture, video Anatomy Lecture Series II (Human Anatomy and Clinical Correlatives) for "Science in the News," Central Indiana Educational Services Center (2008).

Key Presenter, Lecture: *The Tapestry of Anatomy, Biology, Physics, Mathematics, and Chemistry, An Interdisciplinary Approach to Forensic Science,* for the Central Indiana Education Services Center (CIESC), at Butler University. (2008)

Attended *Project SEAM Workshop* for continuing coordination between High School and University faculty. Discussed Anatomy and Physiology preparation with local High School and University faculty, in an effort to better prepare our students for the rigors of undergraduate science education. (2008)

Attended and presented at *Meet the Expert*, Pike Township School Board Symposium. (2008-2009)

Consulted for U.S. Government agencies regarding forensic pathology issues, including crime scene analyses, gunshot and knife wound analyses, as well as emergency tactical preparation and hand-to-hand combat.

Office of the Marion County Coroner, Lecturer: presented programs to civic and youth groups regarding the importance of making the right choices; i.e., Don't Do Drugs, Wear Your Seatbelt and Home Safety. (1995-1997).

Expert witness in several forensic cases, Marion County Criminal Courts (1995-2001)

Publications

Thyrotropin-releasing hormone (TRH): Progress on its anticonvulsant mechanism and intranasal delivery to seizure foci. M. Kubek, M. Veronesi, D. Kubek, **M. Yard**, D. Lahiri, A. Domb (2007) Neuropeptides, Volume 40, Issue 6, Pages 439-440.

Characterization of Novel Intranasal Sustained-Release Nanoparticles for Delivery of Neuropeptides to the Brain, in Nanoparticles for Pharmaceutical Applications**,** edited by A. J. Domb, Y. Tabata, M. N. V. Ravi Kumar, and S. Farber. Michael J. Kubek, **M. Yard**, Debomoy K. Lahiri, and Abraham J. Domb (2007) American Scientific Publishers

An analog of thyrotropin-releasing hormone (TRH) is neuroprotective against glutamate-induced toxicity in fetal rat hippocampal neurons in vitro. Michael C Veronesi, **M. Yard**, James Jackson, Debomoy K Lahiri, Michael J Kubek. Brain Res. 2006 Nov 22.

Thyrotropin-releasing hormone (protirelin) inhibits potassium-stimulated glutamate and aspartate release from hippocampal slices in vitro. Y Nie, D D Schoepp, J E Klaunig, **M Yard**, D K Lahiri, M J Kubek. Brain Res. 2005 Aug 23:1054 (1):45-54.

Presented Abstract to Society for Neuroscience (2002). Effect of thyrotropin-releasing hormone (TRH) and its analogs on G_{α} levels in superfused hippocampal dentate gyrus. Paper pending.

Presented Abstract to Endocrine Society (2001). Protective effect of thyrotropin-releasing hormone (TRH) against glutamate-induced toxicity in cell culture.

IU Medical School Graduate Student Showcase Presentation (2000). TRH: A possible neuroprotective role?

Military Review (worldwide publication). April 1993. Author of article entitled Medical Officer Training Corps Addresses Physician Shortage.

Pathology Seminar, St. Louis, MO (1983). Presented research paper (with slides and photomicrographs), entitled Pancreatic Hyaline Membrane Disease

PROFESSIONAL MEMBERSHIPS

Member, American Tae Kwon Do Institute Black Belt Club
Member, Board of Directors of the Marion County Emergency Medical Services Council
Member, Purdue Alumni Association
Member, Indiana Medical History Museum
Member, Columbia Club, Indianapolis, IN
Member, Indiana State Coroners' Association
Member, Society for Neuroscience