Thyrotropin-Releasing Hormone Analogs

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Abstract: Thyrotropin releasing hormone (TRH: pyroglutamic acid-histidine-prolineamide) regulates the activity of cells in the anterior pituitary and within the central and peripheral nervous systems. TRH, which has been the subject of much research over the past three decades, exerts its effects by acting through class A G-protein coupled receptors. The recent discovery of a second receptor subtype has generated an interest in the discovery of receptor subtype-selective TRH analogs. In this review, we describe advances in the development of TRH analogs and in the understanding of their mechanism of interaction with TRH receptors. We also describe the recent breakthrough in the identification of analogs that bind selectively at TRH-R2.

Keywords: TRH; TRH receptor; hormone; neuropeptide; analogs.

INTRODUCTION

Thyrotropin-releasing hormone (TRH: pyroglutamic acid – histidine - proline amide) (Fig. **1**) has been the subject of much research over the past 30 years (for review see [1-5]), for it was the first peptide shown to display a dual role as a hormone and as a neuropeptide. As a hypothalamic regulatory hormone, TRH stimulates the release of thyrotropin (thyroid-stimulating hormone, TSH) and prolactin from the anterior pituitary. As a modulatory neuropeptide in the CNS, it is involved in the augmentation of various neurotransmitter systems mainly involving cholinergic neurons [6,7], and it exerts a variety of extrahypothalamic effects. When administered to animals, low doses of the hormone have been shown to be active in stimulating the release of pituitary hormones, while at higher concentrations, TRH produces a variety of effects that do not depend on pituitary function including effects on behavior and thermoregulation. TRH has been tested in preclinical studies where it appears to exhibit neuroprotective actions and in the clinic where it may hold promise in the treatment of spinal cord trauma [8] and Alzheimer's disease [9].

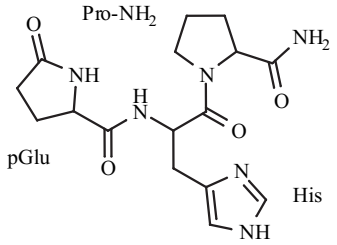

Fig. (1). Structure of TRH.

TRH signals via specific cell surface membrane receptors that belong to the class A family of G-protein coupled receptors (GPCRs). GPCRs constitute the richest receptor target for drug discovery, and are involved with nearly 60 percent of all prescription drugs on the market today. There are two subtypes of TRH receptors, TRH-R1 [10] and TRH-R2 [11], both of which were cloned originally from mammals. Of note, only a single subtype, which is more homologous to TRH-R1 than TRH-R2, has been found in humans [12,13]. A three dimensional model of TRH-R1 was developed earlier in our laboratory [14], and, along with TRH-R2, is currently the subject of further investigation in a system that encompasses the lipid bilayer and the explicit solvent (Fig. **2**). Many of the animal studies of TRH and TRH analogs in which biological responses were monitored were performed before both receptor subtypes were identified, and hence involved experimental systems in which both TRH-R1 and TRH-R2 may have been present. It can now be appreciated that the effects on anterior pituitary function in animals, *via* so-called "endocrine receptors", are primarily if not exclusively mediated by TRH-R1. (In this review, TRH-R is used when it is unclear whether TRH-R1 and/or TRH-R2 is being considered). Ligands selective for TRH-R1 or TRH-R2 would be important probes to study the roles of these receptors in normal physiology in animals. Moreover, since expression of TRH-R1 and TRH-R2 exhibit distinct distributions, findings with subtype-selective ligands in animals may provide insight into the distinct roles of the single TRH receptor type in humans when it is expressed in different locations.

Given the vulnerability of TRH to enzymatic degradation, numerous analogs with replacements at all three moieties have been synthesized and evaluated for their binding and activating capabilities. This has led to the discovery of TRH analogs with varying pharmacological and brain penetration properties. The aim of this review is to report on the advances in the development of these analogs and in the understanding of their mechanism of action.

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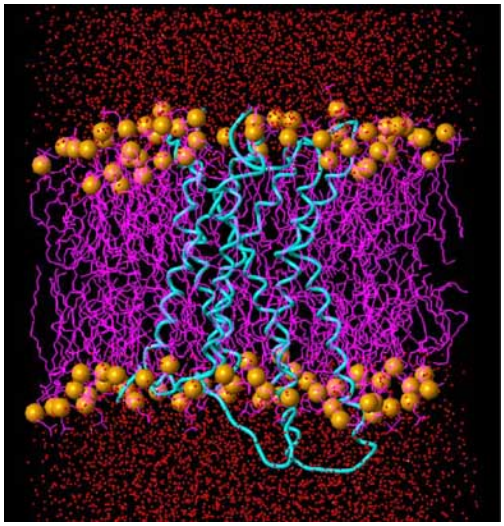

Fig. (2). 3D model of the TRH-R1 (cyan tubes) imbedded in a lipid bilayer (magenta tails-orange phosphorus), solvated on the intra-cellular (bottom of picture) and extra-cellular (top of picture) with explicit waters (oxygens shown in red).

HORMONAL ACTIVITY

Pyroglutamic Acid

The pyroglutamic acid residue (pGlu) is a frequent structural determinant in hormones and neuropeptides where it originates at the N-terminus from the post translational cyclization of glutamyl or glutaminyl residues [15]. Physiological functions of pGlu are suggested by the existence of three forms of pyroglutamyl cyclases involved in its formation [16-19], two of which show substrate specificity towards TRH and TRH-like peptides. In order to overcome susceptibility to enzyme degradation, numerous substitutions of pyroglutamic acid have been performed and the resulting compounds subsequently tested. For instance, Hinkle *et al.* [20] showed that compounds in which pGlu was replaced with an oxazolidinone or a pyrrolidine moiety had a 10-100 fold decreased affinity for TRH-R1 when compared to TRH in rat pituitary cells expressing TRH-R1s. Additional substitutions with acyclic moieties also demonstrated the critical requirement for the five-membered ring of pGlu for activity. More recently, pGlu was replaced with its sulphonamido counterpart (**1**) (Fig. **3**) which appears to significantly stabilize the ligand towards hydrolysis by pyroglutamyl peptidases. This modification resulted in a compound whose prolactin releasing activity was preserved, albeit to a lesser extent, hence suggesting that TRH-R1 binding properties were maintained [15]. In a separate study, it was shown that restricting the pyroglutamate region of TRH with a spirocyclic peptide analog (**2**) (Fig. (**3**)) did not stop the molecule from binding and activating TRH-R [21] which indicates that introducing bridges into the pyroglutamate region of TRH analogs could lead to compounds that bind and activate TRH-R1.

Perlman *et al.* [22] found that retention of the carbonyl but replacement of the ring NH by a methylene group provides an analog whose binding affinity and signal transducing potency are approximately 100-fold less than those of TRH itself. In a subsequent study, Jain *et al.* [23] replaced the N-terminal pyroglutamic acid with various carboxylic acids and showed that other hydrogen-bond donating moieties in the peptide do not stop the analog from binding and activating TRH-R1.

Histidine

It is important to extend the knowledge on the significance of the His-moiety for the structure-release relationships of TRH. Because of the imidazole moiety, TRH may exist at physiological pH in a protonated charged form or as an unprotonated uncharged species. In a detailed analysis of the pH dependency of TRH binding, Perlman *et al.* [24] demonstrated that the protonated form of TRH does not bind as well as the unprotonated form and concluded

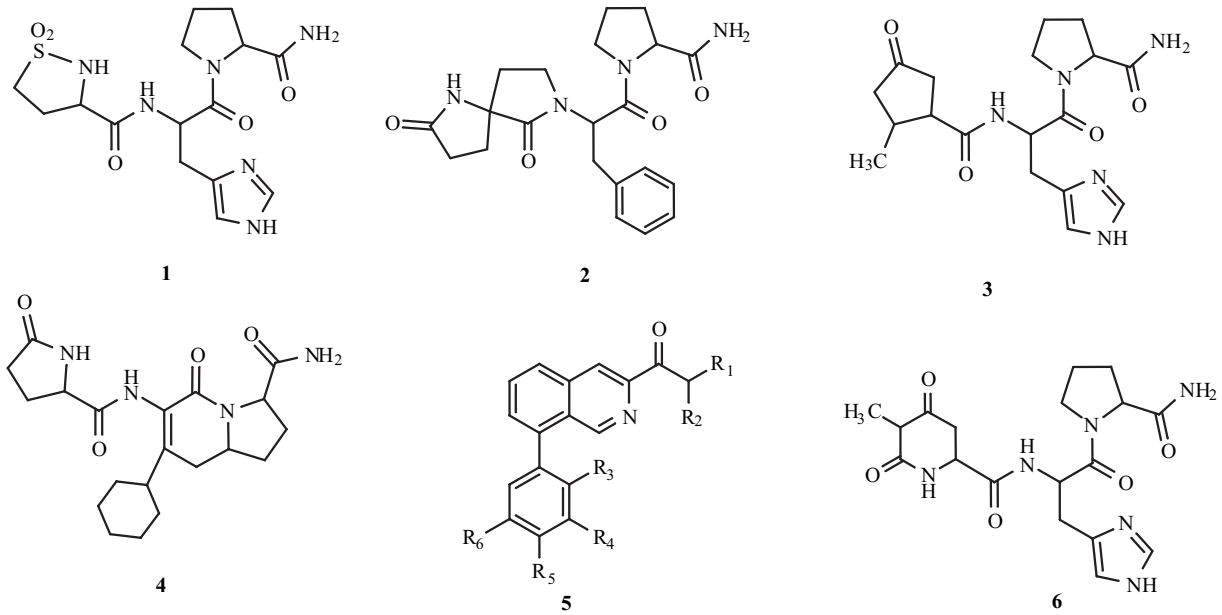

Fig. (3). Structures of TRH analogs.

that TRH binds as an unprotonated uncharged species.

In earlier studies [25,26], His was replaced with Phe, Trp and Tyr. Hormonal activity was only observed for Phe which had up to 10% of the activity of TRH. This suggested that both the pi electrons and the basicity of His were functional for ultimate release of TSH. The importance of the basicity of His had been suggested earlier by the 5-10 fold higher affinity of Me-TRH to TRH-R in comparison to TRH [27] which could be due to the inductive effect of the methyl group and hence the basicity of His. To date, Me-TRH remains the only TRH analog with higher affinity for TRH-R than the cognate ligand.

Interestingly, although the presence of the specific basic, aromatic and steric properties of the imidazole side chain of the central histidine is regarded as a decisive factor in the binding of TRH to TRH-R1, and hence for the full thyrotropin releasing activity [28], the imidazole NH appears not to be essential for cognitive effects in the Morris water maze model as mentioned below [29].

Prolineamide

TRH-R appears to have stringent requirements for the carboxy-terminal position of the ligand. Indeed, Hinkle *et al*. [20] showed that of 14 compounds with various substitutions for the prolineamide, only the proline methyl and ethyl amides retained as much as 1% of the binding activity of the parent molecule.

Subsequent structure-hormonal activity studies of TRH analogs appear to show that the pyroglutamic acid and prolineamide residues are fundamentally responsible for the full thyrotropin-releasing activity of the natural hormone [28], with replacement of the pyrrolidine ring of proline by other five or six membered heterocycles resulting in analogs with decreased hormonal activity [30].

CNS ACTIVITY

In the brain, the highest concentration of TRH (~30% of the total) is found in the hypothalamus, with the remainder being found widely distributed throughout extrahypothalamic brain [31]. Preliminary clinical reports suggested that intravenous injections of TRH produced an antidepressant effect in patients which was immediate in onset and lasted for up to 3 days [32,33]. These reports attracted widespread attention because the effect was rapid, apparently unrelated to the endocrine activity of the peptide and also unrelated to mechanisms by which conventional antidepressant drugs were considered to act. Since then, there has been growing evidence from animal studies that CNS-activating pharmacological effects of TRH are mediated through various neurotransmitters, most prominently catecholamines, serotonin and acetylcholine [3;6;34;35]. This suggests that the peptide could function as a facilitatory neuromodulator at a number of different synapses by increasing the quantity of primary transmitter released or by enhancing transmitter efficacy. The overall effect of such neuromodulation would be to increase the effectiveness of trans-synaptic communication regardless which primary transmitter is involved [36]. Brunetti *et al.* [37] have shown that TRH is able to inhibit hypothalamic dopamine release , suggesting TRH involvement either in central mechanisms controlling anorectic behavior or in the modulation of prolactin release via an indirect pathway. Additional CNS effects of TRH/TRH analogs include their arousal property, their ability to reverse cognitive deficits produced by experimental procedures, and to reverse motor dysfunctions resulting from spinal cord injury and spinocerebellar degeneration. While much research has gone into defining the properties of the CNS TRH receptor, little has been accomplished as to understanding its role in mediating possible central functions of TRH [3]. This is mainly because of the absence of selective and potent isosteric antagonists of the TRH receptor. It is now known that both TRH-R1 and TRH-R2 are expressed in the CNS with distinct distributions [38]. In the brain, the extensive distribution of TRH-R2 over TRH-R1 suggests that it mediates many of the known functions of TRH that are not transduced by TRH-R1. Whether there are other receptors that mediate the CNS actions, especially at pharmacological doses of TRH and TRH analogs, is not yet known.

The use of TRH as a CNS-active agent is hampered by several factors: a poor blood brain barrier penetration, an endocrine effect that is usually manifested at doses lower than those needed for its CNS effects, and a short half life [39]. Furthermore, TRH is not biologically stable, and can be deaminated by pyroglutamate aminopeptidase to form histidyl-prolineamide which readily cyclizes to histidyl-proline diketopiperazine (cyclo(His-Pro), CHP). Because both CHP and TRH share some similarities in pharmacological activity and because exogeneous administration of TRH can increase CHP, it has been hypothesized that at least some of the effects of TRH administration may occur through CHP [40]. This peptide was shown to have many effects on the brain where it is present in much higher concentrations than TRH and to have several properties both related and unrelated to TRH [3,6,35,41,42]. Prakash *et al.* [43] have synthesized two isomeric compounds based on the structure of CHP in which His was replaced by 3,5-di-tert-butyltyrosine. The resulting diketopiperazines prevented neuronal death in an model of traumatic injury suggesting that they may be useful as treatments for neuronal degeneration . To date however, no specific binding sites have been discovered for these TRH metabolites; CHP does not bind to either TRH-R1 or TRH-R2.

Improvement of analog half life is critical; in this respect, preliminary experiments to explore the biological stability of compounds that exhibit enhanced potency in neuropharmacological screening tests have confirmed that such analogs possess improved resistance to enzymatic degradation [36]. In an effort to improve biological stability, all three moieties of TRH are being targeted.

Pyroglutamic Acid

Earlier studies revealed that replacement of the terminal amino acids in TRH resulted in remarkable enhancement of the CNS activity [30,44], most likely as a consequence of increased bioavailability [34,36]. For instance, replacement of the pyroglutamyl moiety of TRH by a cyclopentanone structure showed increased potency and longer duration of action on the CNS compared to TRH [45-48]. This

compound (**3**) (Fig. **3**) was later shown by Urayama *et al.* [48] to exert fairly potent and sustained occupation of brain TRH-Rs under condition, making it a potentially useful clinical candidate for the treatment of CNS disorders.

As mentioned above, further work by Brunetti *et al.* [15] has revealed that replacement of pyroglutamic acid with its sulphonamido counterpart (**1**) (Fig. **3**) slightly decreases the prolactin releasing activity of the analog, while suppressing its ability to inhibit dopamine release through activation of hypothalamic TRH-Rs. Furthermore, Nutt *et al.* [30] showed that ring expansion of pyroglutamic acid as well as replacement of one methylene group of the prolineamide by a sulfur enhances the CNS activity of TRH 35-fold, but not the hormonal potency. These results supports the hypothesis that the endocrine and CNS effects of TRH may be mediated through different TRH receptor subtypes.

Histidine

Szirtes *et al.* [28] showed that a 3- or 4-membered straight or branched alkyl side chain in the position of His had a 2.5 to 10 times stronger anticataleptic effect than TRH. This result demonstrated that the presence of histidine is not essential for the CNS activity while, as mentioned above, the steric properties of the amino acid at position 2 are critical in the ligand's hormonal activity. Subsequently, it was shown [49] that further replacement of pyroglutamic acid by pyro-2-aminoadipic acid in addition to replacement of His by Leu or Nval resulted in analogs with prominent CNS activity with little or no hormonal potency.

Hinkle *et al.* [50] tested a number of TRH-like peptides and determined that Val^2-TRH and Leu^2-TRH are analeptics following intracisternal injection while Phe^2-TRH and Tyr^2-TRH are not. This further indicated that the imidazole ring is not necessary for the analeptic activity and that a hydrophobic residue at position 2 is not sufficient. Interestingly, they observed that these TRH-like peptides that exert important effects in the CNS and peripheral tissues do not exert their antisedative activities through the two known TRH receptors, hence suggesting that novel receptors mediate some of the neuronal actions of TRH-like peptides. The authors further suggested that the TRH-like peptides that occur abundantly in the CNS and peripheral tissues either have their own, yet to be identified receptor, or act via a common but still unidentified signal pathway.

Following Szirtes [28] demonstration of the non-criticality of the central histidine, Prokai *et al.* replaced it with various substituted pyridinium moieties [39]. While no binding to TRH-R was observed, analeptic and acetylcholine-releasing actions were observed upon intravenous administration of these analogs. The authors concluded that there was increased brain uptake of pyridinium-containing analogs and suggested that these compounds constitute novel leads for centrally acting TRH analogs.

It is interesting to note that although [3-MeHis]TRH has a very high affinity for TRH-R in the CNS and that it is the most potent TRH analog known to date at the TRH-R1 receptor, it is experimentally less active in the brain than TRH [5]. Ward *et al.* suggested that it is likely due to its difficulty in adopting the (Y2,3)-conformation calculated as preferred for central effects [51]. More recently, Asai *et al.* [52] measured inhibition of [^{3}H]MeTRH binding by TRH and its analog taltirelin and showed that it was monophasic in the anterior pituitary, hypothalamus and brain stem but biphasic in the cerebral cortex and cerebellum.

These observations not only support the presence of distinct high and low affinity TRH-Rs in the CNS in contrast to the pituitary, but also further support the existence of different receptor subtypes involved in the CNS and hormonal activity of TRH. Interestingly, Cao and colleagues presented data that methyl-TRH, which had been shown to display higher affinity than TRH at TRH-R1, also displays higher affinity at the rat TRH-R2 [11]. Replacement of His by Val and cyclohexAla, did not contribute to significant differences in affinity between TRH-R1 and TRH-R2 [38].

Prolineamide

In an effort to improve resistance to enzymatic degradation, Brewster *et al.* [53] introduced several modifications at the prolineamide moiety. In particular, they incorporated a trans-3 methyl group into the proline residue of TRH which produced an analog with increased potency at TRH-R in the CNS but not in endocrine tests. As observed previously with other moieties, it is worth noting that greater stability to enzymatic degradation once again improved the analog's biological activity in the CNS. More recently, TRH analogs with a modified prolineamide moiety (i.e. Pyr, Pro-Gly) were tested at both TRH-R1 and TRH-R2 but showed no apparent difference in affinity of binding at either receptor [38]. Interestingly, the results of Cao *et al.* suggested that TRH-R2 may be less susceptible than TRH-R1 to NH_2 terminal modification of the ligand [11].

Backbone

As mentioned above, when injected intravenously TRH has poor access to the CNS, hence the requirement for much larger doses to produce neuropharmacological rather than peripheral effects [54]. Furthermore, intravenously injected TRH is rapidly metabolized with a half life of only 4-5 min [55,56]. For this reason, in addition to the various modifications performed at the three individual moieties pGlu, His and $ProNH_2$, backbone modifications have also been performed. Laakkonen *et al.* [57] studied the structures of conformationally restricted TRH analogs using a Monte Carlo biased sampling technique. These restricted cyclohexyl/Ala^2-TRH analogs, which use a lactam ring to restrict two of the six free torsional angles of TRH, were synthesized and tested in competitive binding and signaling assays. The data revealed that one of the diastereomeric analogs (**4**) (Fig. **3**) exhibited higher affinity and potency than the unrestricted analog, and that the conformation of this compound could be superimposed onto that of the bound conformation of trans-TRH found in a model of the TRH-TRH-R1 complex. Interestingly, these analogs showed no difference in TRH-R1 and TRH-R2 binding or signaling [38]. Further testing to evaluate their CNS activity would be of interest. In a separate study, Olson *et al.* [29] designed analogs in which the peptide backbone was entirely replaced by a cyclohexane framework. These mimetics were potent

and orally active in a behavioral model of cognition in which TRH is active. However, they did not exhibit binding to TRH receptors nor did they increase release of TSH, i.e. they did not exhibit endocrine activity. These studies, therefore, suggested that the conformation is the determinant of cognitive activity and that binding to the micromolar affinity binding site in rat brain slices is distinct from, and probably unrelated to, binding to TRH-R [29].

ANTAGONISTS

To date, the use of nonpeptidic, small molecule ligands to modulate the activities of TRH receptors has been largely ignored. Fully constrained tetracyclic peptidomimetics have been shown to exhibit partial agonism for TRH-R1 [58], which suggests that it is possible to develop peptidic antagonists for TRH-Rs. Several nonpeptide benzodiazepine drugs are low affinity antagonists of TRH-Rs by competing with TRH for binding to the receptor and inhibiting its stimulation; these have also been shown to exhibit inverse agonist activity [59]. There are many interactions between TRH and benzodiazepines. Indeed, not only do these compounds decrease the secretion of thyrotropin and prolactin in various experimental models [60-63], but they also antagonize the pressor, narcoleptic and ulcerogenic effects of TRH [64-66]. In addition, benzodiazepines displace TRH from its binding sites in the brain and pituitary. The studies of benzodiazepine interaction with TRH have included drugs affecting the central type benzodiazepine receptor only (e.g. chlordiazepoxide, clonazepam), drugs binding both to the central and peripheral type receptors (e.g. diazepam) [67], as well as drugs binding the peripheral benzodiazepine receptors (e.g. 4'-chlorodiazepam/Ro 5-4864) [68]. It was shown that all three types of ligands competitively displaced [^{3}H]MeTRH from its binding sites in the brain and anterior pituitary, and that the duodenal smooth muscle effect of TRH was reversed by the central and peripheral type benzodiazepine agonists, antagonists and inverse agonists [69]. However, utilization of benzodiazepines as TRH-R modulators would be accompanied by CNS depression, thereby limiting their usefulness as reagents to study TRH receptor biology. It is of interest to note that more recently, TRH has also been found to antagonize the inhibition of glucose transport by barbiturates, diazepam, melatonin and galanin in human erythrocytes [70].

SUBTYPE-SELECTIVE LIGANDS

To date, a limited although diverse set of TRH analogs have shown no differences in TRH-R1 and TRH-R2 binding or in acute stimulation of signaling [38]. Additional testing of peptide analogs is needed to further our understanding of selectivity mechanism between the two subtype receptors. It is noteworthy that all six residues that constitute the binding site in TRH-R1 which interact directly with TRH [1,71] are conserved in TRH-R2. This suggests that although differences may exist, there will be significant similarities between the binding sites of the two receptors and hence significant challenges in identifying receptor specific ligands. Recently, Jiang *et al.* [72] reported the synthesis of several 1-(phenyl)isoquinoline carboxamide analogs (**5**) (Fig. **3**). These are the first ligands reported that show selective binding to TRH-Rs. This is a significant finding as these low molecular weight peptidomimetic compounds are likely to cross the blood brain barrier more readily. These results will allow subsequent studies to better probe the function of the TRH receptors .

CONCLUSIONS

TRH cellular signaling is mediated through at least two GPCR subtypes. Beyond this level of understanding, much has yet to be learned about the molecular basis that differentiates the hormonal from the CNS actions of TRH. One proposal by Yarbrough [73] is that enhancement of the acetylcholine effects by TRH on cerebral cortical neurons results from a blockade of neuronal K^+ channels by TRH. Recent studies reveal that TRH can indeed block both inwardly rectifying background K^+ channel, and block open rectifying leak K^+ channels that are opened by volatile anesthetic [74]. The blockage of these metabotropic ion-conducting proteins in neurons by TRH could possibly underly all of the CNS effects of TRH [73].

Identifying the factors involved in selectivity between TRH-R1 and TRH-R2 can also be critical in further understanding the hormonal and CNS mechanisms of action of TRH. Therefore, it is important that additional low molecular weight selective compounds [72] be synthesized to further help delineate the physiological roles and pharmacological characteristics of the TRH receptors.

To date, only one TRH analog compound (TA-0910) (**6**) (Fig. **3**) is marketed in Japan for the treatment of spinocerebellar degeneration. If the proposal by Gary *et al.* [75] that TRH functions in the CNS to integrate the regulation of several interrelated systems, that is to regulate "homeostasis" in the nervous system, the promise of TRH analogs for therapeutic application in a variety of disorders represents an area of opportunities that has gone largely unrealized.

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