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Pharmacologically distinct binding sites in rat brain for [^{3}H]thyrotropin-releasing hormone (TRH) and [^{3}H][3-methyl-histidine^2]TRH

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Abstract

We have used a directed peptide library, in which the histidyl residue of thyrotropin-releasing hormone (TRH) was systematically replaced by a series of 24 natural and unnatural amino acids, to characterise TRH binding sites in rat brain cortex. This was achieved by measuring the ability of library peptides to compete with [^{3}H][3-Me-His^2]TRH or [^{3}H]TRH binding to rat cortical homogenates. [^{3}H][3-Me-His^2]TRH was observed to bind to a single population of high-affinity, low-capacity sites (K_d: 4.54 ± 0.62 nM, N = 5; B_{max}: 4.38 ± 0.21 fmol/mg wet weight tissue, N = 5), consistent with them being central TRH receptors. Displacement studies showed TRH to bind to these sites with an apparent K_i of 22 nM. K_i values for the library peptides at [^{3}H][3-Me-His^2]TRH-labelled sites varied from 10^{-3} to 10^{-9} M; the potency order was: [3-Me-His^2] > His > Thi > Leu, Phe, Asn > Gln, Arg, Thr, Ala, HomoPhe. All other replacements had K_i values >10^{-4} M. [^{3}H]TRH was observed to label a single population of low-affinity, high-capacity sites (K_d: 7.55 ± 1.23 μM, N = 6; B_{max}: 3.40 ± 0.63 pmol/mg wet weight tissue, N = 6). The affinities of the synthetic peptides for [^{3}H]TRH-labelled sites did not correlate with their affinities for [^{3}H][3-Me-His^2]TRH-labelled sites ($r = 0.33$, N = 18, $P > 0.1$). They did, however, correlate significantly with previously reported binding affinities for TRH-degrading ectoenzyme ($r = 0.72$, N = 12, $P < 0.01$). These results strongly indicate that the identity of the low-affinity, [^{3}H]TRH-labelled site is the membrane-bound enzyme, TRH-degrading ectoenzyme, not a subpopulation of TRH receptors. They also provide the first comprehensive description of the influence of the histidyl residue in TRH on binding of TRH to brain receptors.

Keywords: Thyrotropin-releasing hormone (TRH); TRH receptors; TRH radioligand binding; TRH-degrading ectoenzyme (TRH-DE); [3-Methyl-histidine^2]TRH; TRH analogues

1. Introduction

TRH is a tripeptide with the amino acid sequence L-pyroglutamyl-L-histidyl-L-prolineamide (Glp-His-ProNH$_2$) (Fig. 1). It displays a broad spectrum of stimulatory central actions that are independent of the hypothalamic–pituitary–thyroid axis and is now believed to act as a neuromodulator and/or neurotransmitter within the central nervous system (CNS) [1–3].

The actions of peptides, such as TRH, are mediated by specific receptors [4]. In the case of TRH, early efforts to label receptors in the brain employed [^{3}H]TRH. These studies resulted in the detection of two TRH binding sites—a high-affinity site (dissociation constant, K_d = 50 nM) and a low-affinity site (K_d = 5 μM)—in a synaptic membrane fraction freshly-prepared from rat cerebral cortex [5]. The high-affinity site was considered to display characteristics consistent with a physiological receptor for TRH in the brain [5]. The low-affinity binding sites accounted for the majority of sites labelled by [^{3}H]TRH. The function of the low-affinity sites, to-date, is unknown, but their presence has rendered the detection of the small number of [^{3}H]TRH-labelled high-affinity sites difficult. The problems associated with using [^{3}H]TRH as ligand

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Abbreviations: TRH-DE, thyrotropin-releasing hormone-degrading ectoenzyme; TRH, thyrotropin-releasing hormone; TRHAMC, pyroglutamyl-histidyl-prolyl-7-amido-4-methyl coumarin; Glp, pyroglutamic acid; Nle, norleucine; Nva, norvaline; Thi, thienylalanine.

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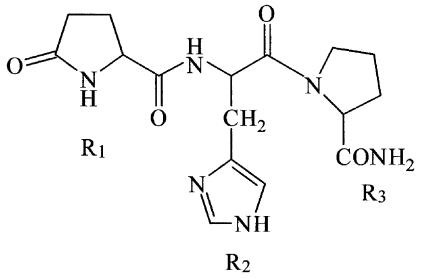


Fig. 1. The primary structure of TRH. TRH is a tripeptide with an N-terminal pyroglutamyl residue, a central imidazole (R_2) side-chain and an amidated proline residue at the C terminus.

were overcome in later studies with the use of [^{3}H][3-Me-His^2]TRH, which was found, under the assay conditions employed, to label only high-affinity sites (K_d approximately 5 nM) [6,7].

Vonhof *et al.* [8] raised the possibility that the low-affinity TRH binding sites observed in membrane fractions may represent enzyme sites. If low-affinity [^{3}H]TRH binding sites on tissue membranes were to correspond to enzyme binding sites, then it could be postulated that the most likely candidate would be TRH-degrading ectoenzyme (TRH-DE, EC 3.4.19.6), also known as pyroglutamyl peptidase II, which is appropriately located on neuronal cell membranes [9] and displays a similar affinity to that of low-affinity binding sites [8]. However, Vonhof *et al.* [8] concluded that the low-affinity TRH binding sites in brain were more likely to represent a sub-population of TRH receptors rather than enzyme sites.

It would seem, then, that both high- and low-affinity TRH binding sites might have physiological and/or pharmacological importance in mediating the actions of TRH in the CNS. Directed peptide libraries are powerful tools for identifying specific functional groups that are recognised by receptors and enzymes and are, thus, extremely valuable for characterising receptor subtypes and isoenzymes. Recently, we have used a directed peptide library, in which the central histidyl residue of TRH was systematically replaced by a series of amino acids, to define the specificity of TRH-DE for TRH-like peptides [10]. In the present study, we have employed this peptide library and other TRH derivatives to characterise both high- and low-affinity TRH binding sites in rat brain and to determine whether the pharmacological profiles of these two binding sites were similar. This was achieved by measuring the ability of the library peptides to displace [^{3}H][3-Me-His^2]TRH or [^{3}H]TRH binding to rat cortical membranes. Although data from a limited range of ligands indicate that the chemical nature of the central residue of TRH affects the binding of TRH to sites labelled with either [^{3}H][3-Me-His^2]TRH or [^{3}H]TRH in the brain [5,8,11,12], this is the first report of a directed peptide library being used systematically to probe the specificity of TRH binding sites in brain.

2. Materials and methods

2.1. Chemicals

All chemicals, except those specified below were of analytical grade and obtained from either Sigma–Aldrich or Merck. [^{3}H][3-Me-His^2]TRH (NET705, 64 Ci/mmol) and [^{3}H]TRH (NET577, 74 Ci/mmol) were purchased from NEN Life Science Products.

2.2. Peptide library

Glp-His-ProNH$_2$, Glp-Glu-ProNH$_2$ and Glp-His-ProOH were obtained from Sigma–Aldrich. Glp-Gln-ProNH$_2$ and Glp-Phe-ProNH$_2$ were purchased from Peninsula Laboratories, Inc. Glp-His-Pro-7-amido-4-methyl coumarin (Glp-His-ProAMC, TRHAMC) was obtained from Bachem UK Ltd. All other peptides used in this study were custom-synthesised by the American Peptide Company. All amino acids were in the L-configuration, unless otherwise stated.

2.3. Radioligand binding assays

Saturation and competition experiments were performed essentially as described by Vonhof *et al.* [8]. Briefly, male Sprague–Dawley rats were decapitated and their brains quickly removed and placed on ice. Cortex tissue was dissected out, weighed and homogenised in 30 vol. of ice-cold sodium phosphate buffer (0.02 M, pH 7.5). The homogenates were then centrifuged at 39,000 *g* at 4° for 30 min. The supernatant was discarded and the pellet was resuspended at 100 mg wet weight tissue/mL in sodium phosphate buffer containing 177 μM bacitracin. All assays contained 50 μL of membrane suspension (5 mg wet weight tissue) in a total volume of 100 μL and were carried out at 4° in 0.02 M sodium phosphate buffer (pH 7.5) containing 177 μM bacitracin and 0.1% bovine serum albumin. Non-specific binding (NSB) was determined in the presence of an excess concentration of unlabelled ligand (200–500 times K_d concentration).

2.3.1. [^{3}H][3-Me-His^2]TRH binding

In saturation binding experiments, membrane suspension was incubated, in duplicate, with increasing concentrations of [^{3}H][3-Me-His^2]TRH (0.6–20 nM) for 5 hr at 4°. NSB was determined in the presence of 10 μM TRH. Competition experiments were conducted under the same conditions by incubating membrane suspension with 6–8 nM [^{3}H][3-Me-His^2]TRH and increasing concentrations of library peptide (10^{-10} to 10^{-3} M). Separation of membrane bound from free ligand was achieved using a 24-well Brandel cell harvester with Whatman GF/B filters, followed by washing with 3 × 5 mL of ice-cold 0.9% NaCl solution. The radioactivity retained on the filters was measured by liquid scintillation spectrometry using an LKB 1217 Rackbeta liquid scintillation counter.

2.3.2. [^{3}H]TRH binding

K_d and B_{max} for TRH binding to rat cortical membranes were determined in homologous competitive binding experiments. The studies were carried out at constant radioactivity in which a fixed amount of [^{3}H]TRH (10^5 dpm) was incubated with increasing concentrations of non-radioactive TRH (0–14.4 μM). The total concentration of ligand in each tube was, therefore, equal to the sum of radiolabelled plus unlabelled ligand. NSB was determined in the presence of 1 mM TRH. The radioactivity bound at each total ligand concentration was converted to moles bound using a conversion factor based on the actual specific activity in those tubes. Following subtraction of non-specific binding, a standard saturation binding isotherm plotting specific ligand binding as a function of total amount (radiolabelled + unlabelled) added was constructed and data were fitted to one- and two-site models using non-linear regression analysis (Prism, Graph Pad Software, Inc.). Competition experiments were conducted by incubating the membrane suspensions, [^{3}H]TRH and increasing concentrations of library peptide (10^{-10} to 10^{-3} M) under the same conditions. Assays were performed in triplicate. Bound and free ligand were separated by vacuum filtration through GF/B filters, followed by washing with 3 × 5 mL of ice-cold sodium phosphate buffer. The radioactivity retained on the filters was measured by liquid scintillation as described earlier.

Z-Pro-Prolinal is a selective, slow-tight binding transition state inhibitor of prolyl endopeptidase (PO, EC 3.4.21.26) that has a K_i for PO in the low nanomolar range and has been shown to form a complex with the substrate binding pocket of PO [13–15]. To assess the influence of PO on [^{3}H]TRH binding to membrane suspensions, the ability of TRH to compete for [^{3}H]TRH-labelled binding sites was determined after pre-incubating membrane suspensions with Z-Pro-Prolinal (0.2 mM) for 10 min at 4°.

2.4. HPLC analysis of membrane suspensions

To test for the presence of TRH-degrading enzymes in the membrane preparation employed in the binding assays, a discontinuous HPLC assay was used as previously described [10,16,17]. Briefly, TRH (1 mM) was incubated with membrane suspensions for 4 hr at 37° in the absence and presence of the PO inhibitor, Z-Pro-Prolinal (0.1 mM). In addition, to test specifically for the presence of PAP-I, membrane suspensions were incubated with Glp-Asp-ProNH$_2$ in the presence of DTT (2 mM) and EDTA (2 mM) for 5 hr at 37°; PAP-I readily catalyses the removal of Glp from this peptide whereas TRH-DE does not [10,16]. DTT and EDTA were included for optimal expression of PAP-I activity [16]. Enzyme activity was terminated with the addition of 0.15% TFA and any degradation products formed were separated and identified using HPLC. To assess the stability of TRH and [3-Me-His^2]TRH during the binding assays, each peptide (1 mM) was incubated at 4° under identical conditions employed in their respective binding assays and incubates were analysed using HPLC as described earlier.

2.5. Data analyses

All binding data are presented as mean ± SEM and were analysed using non-linear curve fitting software (Prism, Graph Pad Software, Inc.). Saturation binding data were fitted to one- and two-site binding models. Data from N independent competition experiments were fitted simultaneously to one- and two-site models and an F-test was employed to determine which model fitted the data significantly better. IC_{50} values calculated from competition experiments were converted to K_i values using the Cheng–Prusoff equation $K_i = IC_{50}/(1 + L/K_d)$, [18], where L is the ligand concentration and K_d the apparent dissociation constant for [^{3}H][3-Me-His^2]TRH or [^{3}H]TRH, as appropriate. pK_i values ($-\log K_i$) were determined for comparative purposes. Statistical correlations were determined by non-weighted linear regression.

3. Results

3.1. Saturation binding of [^{3}H[3-Me-His^2]TRH and [^{3}H]TRH

Saturable binding was found for both [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH in the rat brain cortical homogenates used in this study (Figs. 2 and 3). Non-linear regression analysis revealed that [^{3}H][3-Me-His^2]TRH binding to rat cortical membranes comprises a single population of high-affinity, low-capacity sites; K_d was 4.54 ± 0.62 nM and the density of receptors (B_{max}) in this

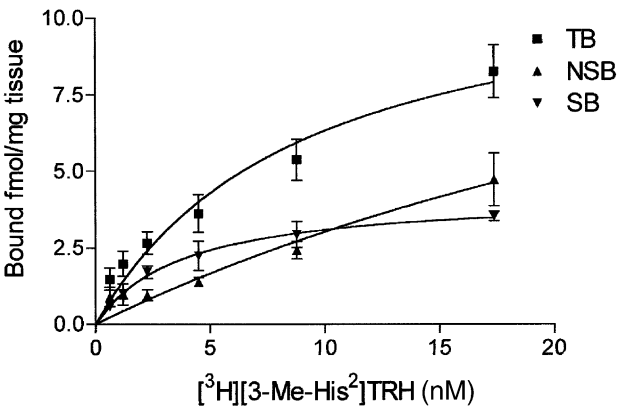


Fig. 2. Equilibrium binding of [^{3}H][3-Me-His^2]TRH to rat cortical membranes. Saturation studies were performed using increasing amounts of [^{3}H][3-Me-His^2]TRH. The plot shows saturation isotherms of the total (TB), non-specific (NSB) and specific (SB) binding of [^{3}H][3-Me-His^2]TRH binding. The specific binding isotherm was analysed using non-linear regression. Points are means ± SE of five independent experiments, each conducted in duplicate.

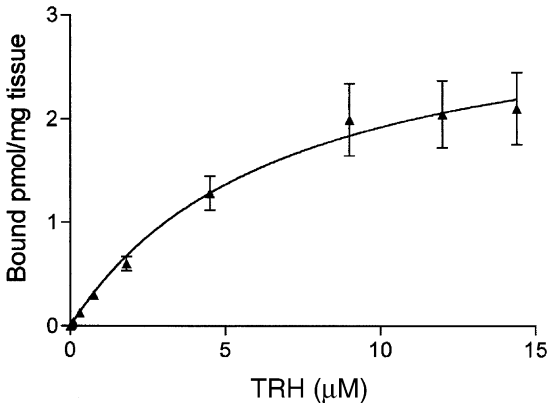


Fig. 3. Equilibrium binding of TRH to rat cortical membranes. Saturation studies were performed using a fixed amount of [^{3}H]TRH with increasing amounts of unlabelled TRH. The plot shows the specific binding (SB) isotherm, which was analysed using non-linear regression. Points are means ± SE of six independent experiments, each conducted in triplicate.

brain region was 4.38 ± 0.21 fmol/mg wet weight tissue (N = 5) (Fig. 2). Non-linear regression analysis of [^{3}H]TRH saturation binding data gave a significantly better fit to a one-site model, rather than a two-site model. Thus, [^{3}H]TRH was observed to label a single population of sites. These sites had low affinity and high capacity; the K_d was 7.55 ± 1.23 μM and the B_{max} was 3.40 ± 0.63 pmol/mg wet weight tissue (N = 6) (Fig. 3).

3.2. Competition studies—affinity of peptides for [^{3}H][3-Me-His^2]TRH-labelled sites

The ability of library peptides (0.1 nM to 1 mM) to compete with [^{3}H][3-Me-His^2]TRH binding to rat cortical homogenates is summarised in Table 1. The parent tripeptide, TRH, and its histidyl-methylated analogue, [3-Me-His^2]TRH, were found to exhibit the two highest affinities for [^{3}H][3-Me-His^2]TRH-labelled sites, displaying pK_i values of 7.64 ± 0.17 and 8.71 ± 0.16, respectively. As observed by several other groups [7,19,20], methylation of the imidazole group of the histidyl residue of TRH can be seen to produce an increase in affinity of approximately 10-fold (Fig. 4A). The most potent replacement of His was thienylalanine (Thi). The side-chain of Thi, like that of His, is a five-membered ring with aromatic character. In contrast to His, though, the side-chain of Thi does not contain either of the imidazole nitrogen atoms, but instead has a sulphur atom in place of the $N^{\delta 1}$ atom in the imidazole ring. The length of the central, R_2 amino acid side-chain can be seen to influence affinity of binding, as illustrated by comparison of competition curves for phenylglycine (Phg), Phe and HomoPhe (Fig. 4B). An increase in affinity for [^{3}H][3-Me-His^2]TRH-labelled sites was also observed when the side-chain of Val is increased by one methylene unit to give Leu (Table 1). L-Asn^2-TRH was >100-fold more potent than D-Asn^2-TRH at displacing [^{3}H][3-Me-His^2]TRH binding indicating that the interaction of the central residue of TRH with the receptor is stereospecific (Fig. 4C). Furthermore, substitution with Asp instead of Asn reduced affinity more than 100-fold, demonstrating that a negative charge in this position is unfavourable for binding (Fig. 4C). Similarly, incorporation of a positively charged amino acid, such as Arg, in this position reduced affinity (Table 1).

The ability of well-characterised ligands [7,21,22] to displace [^{3}H][3-Me-His^2]TRH in competition experiments revealed a potency order of [3-Me-His^2]TRH > TRH = Glp-His-Pro-GlyNH$_2$ ≫ TRH-OH (Fig. 5A, Tables 1 and 2). This potency order was identical to that previously reported for binding to TRH receptors [6,7,20–22].

3.3. Competition studies—affinity of library peptides for [^{3}H]TRH-labelled sites

Affinities for library peptides for [^{3}H]TRH-labelled sites are also shown in Table 1. All of the library peptides, including [3-Me-His^2]TRH, had lower affinities than TRH for [^{3}H]TRH-labelled sites. The rank order of potency for the well-characterised ligands described earlier to displace [^{3}H]TRH binding in competition experiments was found to be different from that observed for [^{3}H][3-Me-His^2]TRH and was as follows: TRH ≥ Glp-His-Pro-GlyNH$_2$ ≥ TRH-OH > [3-Me-His^2]TRH (Fig. 5B, Tables 1 and 2). The inclusion of 1 μM [3-Me-His^2]TRH to saturate any high-affinity sites present was not found to alter the pK_i value for TRH (5.09 ± 0.22, N = 3 vs. 5.45 ± 0.35, N = 6). Similarly, the pK_i value of 5.32 for TRH that was determined in the presence 0.2 mM Z-Pro-Prolinal was not found to be significantly different from that determined in its absence.

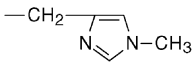
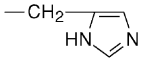
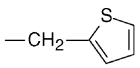
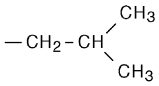
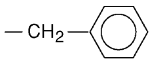
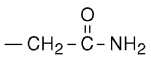
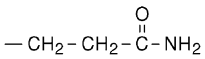
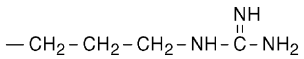
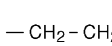
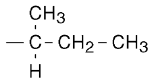
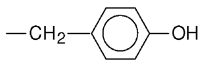
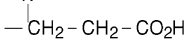
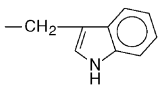
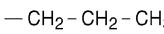
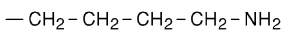
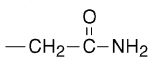
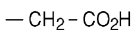
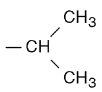
3.4. Affinity of other TRH analogues for [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH-labelled sites

During the course of this study the ability of several C-terminally modified TRH analogues to compete with [^{3}H][3-Me-His^2]TRH or [^{3}H]TRH binding in rat cortical homogenates was determined. Binding affinities for these peptides are shown in Table 2. Members of this series displayed very different binding affinities for [^{3}H][3-Me-His^2]TRH in comparison to [^{3}H]TRH-labelled sites. For example, Glp-His-Pro-GlyNH$_2$ was the most potent displacer of [^{3}H][3-Me-His^2]TRH binding and displayed 400-fold selectivity for [^{3}H][3-Me-His^2]TRH-labelled sites. Conversely, Glp-Asn-ProAMC was the most potent displacer of [^{3}H]TRH binding and displayed 100-fold selectivity for this site.

3.5. Determination of enzyme activities by HPLC analysis

When membrane suspensions were incubated with TRH at 37°, HPLC analysis revealed that Glp was produced. This would be consistent with the presence of TRH-DE

Table 1
Affinities of library peptides for [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH-labelled sites

No.	Library peptide—central amino acid		[^{3}H][3-Me-His^2] pK_i	[^{3}H]TRH pK_i	TRH-DE pK_m[a] or pK_i[b]
	Residue	R_2 side-chain			
1	3-Me-His		8.71 ± 0.16	4.46 ± 0.32	4.26[a]
2	His		7.64 ± 0.17	5.45 ± 0.35	4.46[a]
3	Thi		6.29 ± 0.16	4.52 ± 0.13	4.47[a]
4	Leu		5.40 ± 0.37	3.31 ± 0.81	ND
5	Phe		5.24 ± 0.30	4.61 ± 0.13	4.26[a]
6	Asn		5.15 ± 0.24	4.99 ± 0.31	4.76[b]
7	Gln		4.54 ± 0.18	4.50 ± 0.16	4.16[b]
8	Arg		4.35 ± 0.22	4.19 ± 0.17	ND
9	Thr		4.10 ± 0.52	3.25 ± 0.58	ND
10	Ala		4.10 ± 0.34	3.17 ± 0.96	ND
11	HomoPhe		4.09 ± 0.21	3.89 ± 0.15	ND
12	Isoleu		3.91 ± 0.54	<3	ND
13	Tyr		3.74 ± 0.44	4.10 ± 0.45	4.82[a]
14	Phg		3.74 ± 0.38	3.67 ± 0.28	3.45[b]
15	HomoPro		3.61[c]	<3	ND
16	Glu		3.54 ± 1.21	<3	<3[b]
17	Gly		3.48[c]	<3	<3[b]
18	Trp		<4	3.6[c]	3.64[b]
19	Norval		<4	3.47 ± 0.38	ND
20	Lys		<3	3.16 ± 1.41	ND
21	Pro		<3	<3	<3[b]
22	D-Asn		<3	<3	<3[b]
23	Asp		<3	<3	<3[b]
24	Ser		<3	<4	ND
25	Val		<3	<3	ND

Library peptides (10^{-10} to 10^{-3} M) were tested for their ability to compete with [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH binding to rat cortical homogenates. Data are the means of three to eight independent experiments ± SE, unless otherwise indicated. ND, not determined.

[a] Library peptide binding affinities are compared to pK_m values published previously for TRH-DE [10].

[b] Library peptide binding affinities are compared to pK_i values published previously for TRH-DE [10].

[c] Data are means of two.

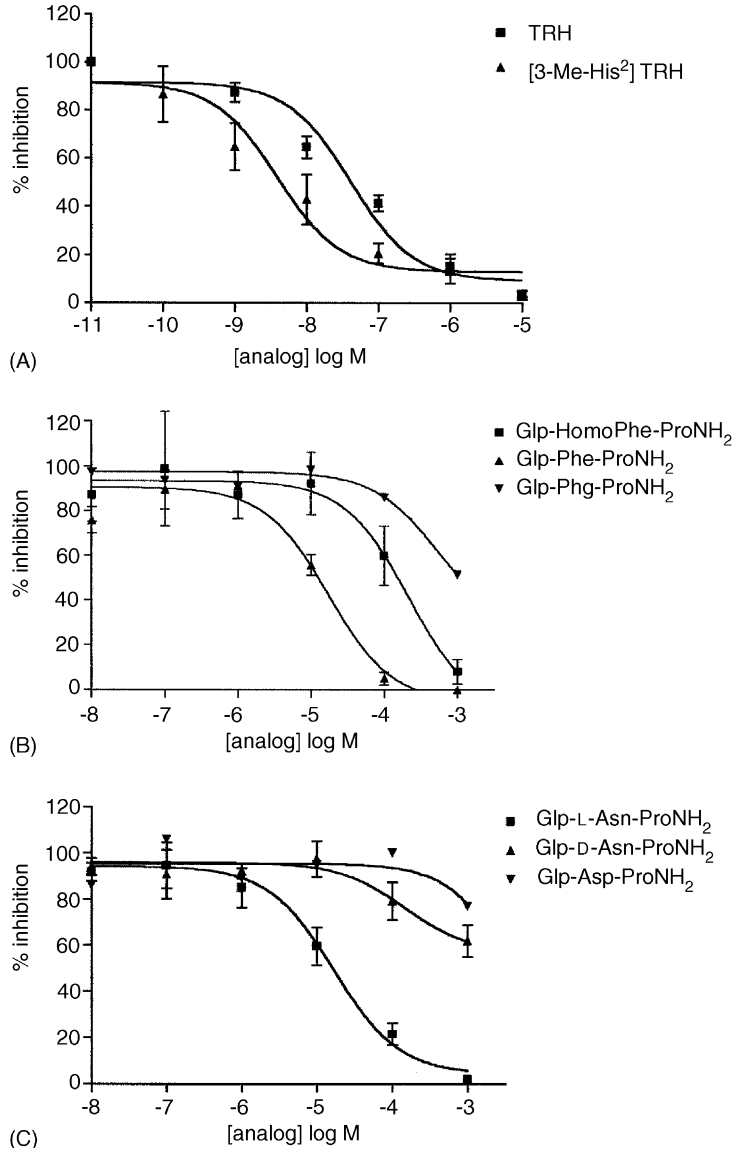


Fig. 4. Effects of structural modification of the central histidyl residue of TRH on affinity for rat cortical sites labelled with [^{3}H][3-Me-His^2]TRH. Competition curves illustrate (A) the effect of methylation of the histidyl residue: TRH vs. [3-Me-His^2]TRH, (B) the effect of increasing the length of the central amino acid side-chain: Glp-HomoPhe-ProNH$_2$, vs. Glp-Phe-ProNH$_2$ vs. Glp-Phg-ProNH$_2$ and (C) the effects of charge and stereospecificity: Glp-Asp-ProNH$_2$ vs. Glp-L-Asn-ProNH$_2$ vs. Glp-D-Asn-ProNH$_2$. Points are means ± SE of three to seven independent experiments or means of two independent experiments, each conducted in duplicate.

and/or PAP-I activity. No Glp could be detected, however, following the incubation of Glp-Asp-ProNH$_2$ with membrane suspensions under optimal conditions for PAP-I activity, indicating that PAP-I is not present in the membrane suspensions and that the removal of Glp from TRH is catalysed by TRH-DE. The rate of TRH hydrolysis was calculated to be 0.01 unit/mL of tissue homogenate, by measuring Glp production as previously described [10], where 1 U of enzyme activity was defined as that amount catalysing the formation of 1 μmol of product in 1 min under the conditions employed as outlined earlier. Deamido-TRH was also produced when membrane suspensions were incubated with TRH at 37°. The formation of this product was selectively inhibited by the presence of Z-Pro-Prolinal indicating that PO was present in the membrane preparation used in the binding assays, in addition to TRH-DE. HPLC analysis of samples of TRH and [3-Me-His^2]TRH incubated at 4° under identical conditions to those used in their respective binding assays showed that neither peptide was degraded by any detectable amount during the incubation conditions employed for binding.

3.6. Pharmacological characterisation of [^{3}H]TRH and [^{3}H][3-Me-His^2]TRH binding sites

The affinities of library peptides and TRH analogues for [^{3}H]TRH- and [^{3}H][3-Me-His^2]TRH-labelled sites were

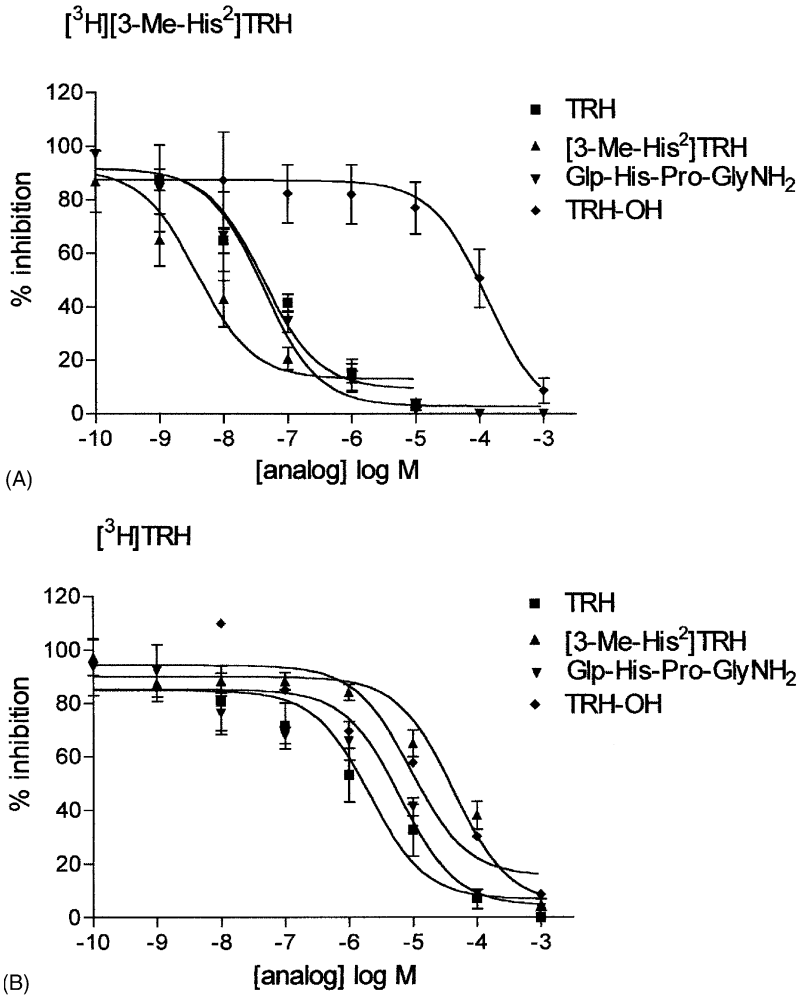


Fig. 5. Displacement of (A) [^{3}H][3-Me-His^2]TRH or (B) [^{3}H]TRH by TRH, [3-Me-His^2]TRH, Glp-His-Pro-GlyNH$_2$ and TRH-OH in rat cortical membranes. Points represent the means ± SE of three to eight independent experiments, except for TRH-OH vs. [^{3}H]TRH, where points are means, N = 2.

compared. No significant correlation ($r = 0.33$, N = 18, $P > 0.1$) was found between the relative affinities of the peptides for the two binding sites (Fig. 6A). Since it has been suggested that the site labelled with low affinity by [^{3}H]TRH may correspond to a TRH-degrading enzyme [8], we compared the affinities of the peptides determined in these radioligand binding experiments with their recently published affinities for TRH-DE [10]. There was no correlation between the affinities of peptides for the [^{3}H][3-Me-His^2]TRH-labelled site and for TRH-DE ($r = 0.009$, N = 11, $P > 0.9$) (Fig. 6B). In contrast, a highly significant correlation was observed between the affinities of peptides for the [^{3}H]TRH-labelled site and their affinities for TRH-DE ($r = 0.72$, N = 12, $P < 0.01$) (Fig. 6C). Exclusion of C-terminally modified TRH analogues from the analysis did not alter the outcome.

Table 2
Affinities of C-terminally modified TRH analogues for [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH-labelled sites

No.	C-terminally modified TRH analogues	[^{3}H][3-Me-His^2] pK_i	[^{3}H]TRH pK_i	TRH-DE pK_m[a] or pK_i[b]
26	Glp-Asn-ProAMC	4.32 ± 0.24	6.36 ± 0.55	6.01[b]
27	Glp-His-ProAMC	5.18 ± 0.15	5.16 ± 0.25	5.51[a]
28	Glp-His-ProOH	4.22 ± 0.43	5.04 ± 0.36	3.51[a]
29	Glp-His-Pro-GlyNH$_2$	7.82 ± 0.16	5.21 ± 0.19	ND
30	Glp-His-Pro-GlyOH	4.66 ± 0.33	4.72 ± 0.21	ND

C-terminally modified TRH analogues (10^{-10} to 10^{-3} M) were tested for their ability to compete with [^{3}H][3-Me-His^2]TRH and [^{3}H]TRH binding to rat cortical homogenates. Data are means of independent experiments ± SE (N = 3). ND, not determined.

[a] Binding affinities for library members are compared to pK_m values published previously for TRH-DE [10].

[b] Binding affinities for library members are compared to pK_i values published previously for TRH-DE [10].

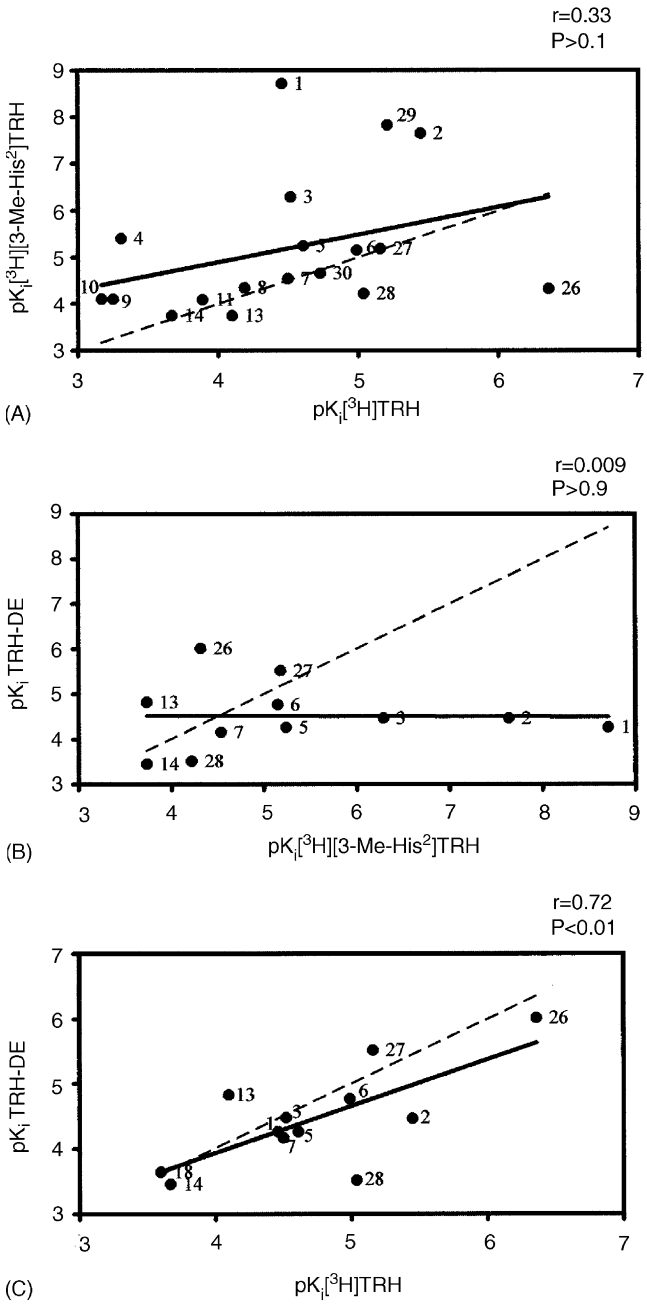


Fig. 6. Correlations between the affinities of various TRH-like peptides for rat cortical sites labelled with [^{3}H][3-Me-His^2]TRH or [^{3}H][TRH] and their affinities for TRH-DE isolated from porcine brain. pK_i values for TRH-DE were derived by taking −log of recently published K_i values for TRH-like peptides determined by non-linear regression analysis of kinetic data [10]. (A) pK_i [^{3}H]TRH vs. pK_i [^{3}H][3-Me-His^2]TRH, (B) pK_i [^{3}H][3-Me-His^2]TRH vs. pK_i TRH-DE and (C) pK_i [^{3}H]TRH vs. pK_i TRH-DE. The number beside each point refers to the identification number of the compounds listed in Tables 1 and 2. The regression line (solid line) and correlation coefficient (r) were determined by non-weighted linear regression analysis. The broken line represents the line of identity. For clarity, error bars have been omitted. Errors in reproducing the kinetic constants for TRH-DE were not >10% and are detailed in [10] and errors associated with the pK_i values reported herein are given in Tables 1 and 2.

4. Discussion

The results of this study show that the identity of the central residue in the TRH molecule has profound but different effects on the ability of TRH-like peptides to bind to [^{3}H]TRH and [^{3}H][3-Me-His^2]TRH-labelled sites in rat brain.

Data from this study show that [^{3}H][3-Me-His^2]TRH binds to a single population of high-affinity sites on rat brain cortical membranes with a K_d of 4.54 nM and that TRH competes for binding at these sites with K_i value of 22 nM. These results are consistent with those previously reported for high-affinity TRH receptors in rat brain homogenates [7,19,22]. In addition, the potency order for displacing [^{3}H][3-Me-His^2]TRH, which was [3-Me-His^2]TRH > TRH = Glp-His-Pro-GlyNH$_2$ ⩾ TRH-OH, also corresponded to that observed for binding to central high-affinity TRH receptors [6,7,20–22]. The density of the high-affinity TRH receptors in the tissue studied was found to be 4.38 fmol/mg wet weight.

The directed peptide library that was used in this study, in which the central histidyl residue of TRH was systematically replaced by a series of amino acids, provides critical, novel information with regard to the influence of specific functional groups on binding to TRH receptors labelled with [^{3}H][3-Me-His^2]TRH. Methylation of the 3-*N* of the imidazole ring of histidine increased the affinity of TRH, in agreement with previous reports [22]. This was found to be the only chemical change to enhance binding. Replacement of the histidyl residue with any other amino acid resulted in a decrease in affinity, highlighting the significance of this moiety within the TRH molecule for receptor binding.

The favourable influence of a neutral, hydrophobic side-chain in the R_2 position of the TRH molecule is suggested by the observation that substitution of His by Thi or Phe, the side-chains of which also possess aromatic character, resulted in peptides that bound to [^{3}H][3-Me-His^2]TRH-labelled sites with moderate affinity compared to TRH. Introduction of a negative or positive charge by incorporation of Asp, Glu, Arg or Lys had an unfavourable effect on binding. The imidazole ring of His has a pK_a of about 7.0 and, thus, can exist as a protonated, charged or unprotonated, uncharged species at physiological pH. From the data presented, however, it would appear that the unprotonated species is favoured for binding of TRH to its receptor. This is further supported by the observation that replacement of His with Thi, Leu or Phe, which are all uncharged, is relatively well tolerated.

A single methylene group separates the imidazole ring of His from the peptide backbone of TRH. In Glp-Phe-ProNH$_2$, the benzene ring of Phe is also linked by a single methylene group to the peptide backbone. Elongation of the side-chain of Phe by one methylene group (HomoPhe) caused a 10-fold loss of affinity. Likewise, reduction of the length of the side-chain by one methylene group (Phg)

resulted in a 30-fold loss in affinity. A similar pattern emerged when comparing Leu and Val. In fact, the R_2 side-chains of each of the six most potent library peptides have a functional group linked to the peptide backbone by one methylene group, indicating that the presence of a single $-CH_2-$ link may be preferential for enabling the R_2 side-chain to interact with the receptor.

Two TRH receptor subtypes have now been identified in rat brain, TRHR1 and TRHR2 [23,24]. These receptors have distinct amino acid sequences and distribution patterns, but both display a similar high affinity for [^{3}H][3-Me-His^2]TRH. Hence, [^{3}H][3-Me-His^2]TRH cannot be used to discriminate between these two known TRH receptor subtypes. Recently, *in situ* hybridisation studies have revealed that rat brain cortex expresses predominantly TRHR2 [25]. Thus, it might be speculated that the sites labelled by [^{3}H][3-Me-His^2]TRH in our study correspond to TRHR2. It cannot be ruled out, however, that [^{3}H][3-Me-His^2]TRH is binding to TRHR1 or a TRH receptor subtype in rat brain cortex that has yet to be identified.

The characteristics of the sites labelled by [^{3}H]TRH were different to those labelled by [^{3}H][3-Me-His^2]TRH. Non-linear regression analysis of [^{3}H]TRH saturation binding detected a single population of [^{3}H]TRH-labelled sites, which displayed a K_d of 7.55 μM and a B_{max} of 3.40 pmol/mg wet weight. The dissociation constant for this low-affinity [^{3}H]TRH binding site closely agrees with that published by others [5,8]. All of the data sets were best-fitted by a single-site model.

Previously, Burt and Snyder [5] and Vonhof *et al.* [8] also reported the presence of a small population of high-affinity [^{3}H]TRH binding sites in rat cortex, with K_d values of 47 and 65 nM, respectively. Both acknowledged, though, that the presence of the low-affinity sites made detection of these high-affinity sites difficult and that the low-affinity binding sites could account for the majority of saturable TRH binding sites depending on experimental conditions. Although the density of low-affinity [^{3}H]TRH-labelled sites was not stated by Vonhof *et al.* [8], it is noteworthy that the membrane fractions of rat brain cortex used by Burt and Snyder [5] contained a lower ratio of low:high-affinity sites ($B_{max,\ low}$:$B_{max,\ high}$) than those we used (277:1 vs. 775:1), thereby, facilitating detection of high-affinity sites. The relatively higher proportion of high-affinity sites in the preparation employed by Burt and Snyder [5] could possibly be attributable to their having used a synaptic membrane fraction to study [^{3}H]TRH binding, in contrast to the total particulate fraction that we used, and also, they used lighter, and therefore, presumably, younger animals that could potentially have a greater concentration of TRH receptors [26]. Under the assay conditions that we employed, the contribution of the high-affinity TRH receptor sites was so small relative to low-affinity binding sites, that it would not significantly perturb the single-site binding isotherm. Furthermore, it was found that blocking the high-affinity sites with 1 μM [3-Me-His^2]TRH did not affect the K_i value for TRH competing at [^{3}H]TRH-labelled sites. It is unlikely, therefore, that the presence of the small number of high-affinity sites would have any significant effect on the displacement of [^{3}H]TRH from low-affinity sites by the library peptides, since all of the peptides had a lower affinity for the high-affinity site than TRH itself.

The identity and function of the low-affinity sites has not been established, although it has been suggested that they might represent enzymes sites or a sub-population of TRH receptors, which mediate TRH actions within the CNS [8]. Comparison of the pharmacological profile of the [^{3}H]TRH-labelled site with that of the high-affinity [^{3}H][3-Me-His^2]TRH binding site shows that there is no statistical correlation between these two sites. This lack of correlation is particularly highlighted by the following observations: (i) methylation of the His residue increases affinity relative to TRH for the [^{3}H][3-Me-His^2]TRH-labelled site but decreases affinity for the [^{3}H]TRH-labelled site, (ii) deamidation of the C terminus causes a 1000-fold decrease in affinity for [^{3}H][3-Me-His^2]TRH-labelled site but only a 3-fold reduction for the [^{3}H]TRH-labelled site and (iii) addition of a large hydrophobic group to the C terminus causes a loss of affinity for [^{3}H][3-Me-His^2]TRH-labelled site but no change, or an increase in affinity for the [^{3}H]TRH-labelled site.

Comparison of the pharmacological profile of the [^{3}H]TRH-labelled, low-affinity site with previously published K_i values for the library peptides binding to TRH-DE [10] showed a significant correlation ($P < 0.01$) between binding of these peptides to these low-affinity sites and to TRH-DE purified from porcine brain [10]. The level of correlation is notable given the differences in methodologies and species employed. This correlation suggests that the identity of the low-affinity site is the cell-surface TRH-DE. Two other enzymes, PAP-I and PO, have been shown to recognise TRH [27,28]. If present in the membrane preparation, PAP-I and PO could potentially be labelled by TRH. However, PAP-I was not found to be present in the membrane preparation used for the binding studies and blocking PO sites with Z-Pro-Prolinal was not found to affect TRH binding significantly. Therefore, PAP-I and PO were not found to contribute to [^{3}H]TRH binding in the studies presented herein. Furthermore, both these enzymes have been shown to display broad specificity for TRH-like peptides [27,28], which clearly distinguishes them from low-affinity [^{3}H]TRH binding sites and TRH-DE. Thus, overall, the data strongly suggest that the identity of the low-affinity [^{3}H]TRH binding site is the cell-surface peptidase TRH-DE and not a TRH receptor or another TRH-degrading enzyme.

Of all the peptides tested, [Asn^2] TRHAMC was the only one to have a higher affinity for the [^{3}H]TRH binding site than for the [^{3}H][3-Me-His^2]TRH-labelled site. With a selectivity ratio of 100, this compound may prove useful for discriminating between effects at the TRH receptor vs. TRH-DE. Conversely, [3-Me-His^2]TRH is highly selective

for [^{3}H][3-Me-His^2]TRH-labelled sites (selectivity ratio >10,000) and, therefore, can be utilised to block receptor sites in radioligand binding assays employing [^{3}H]TRH as demonstrated by Vonhof *et al*. [8]. Such assays could provide a novel, efficient method for high-throughput screening of potential TRH-DE inhibitors, which have potential investigative and therapeutic potential [10].

In conclusion, using a directed peptide library it has been possible to show that [^{3}H]TRH and [^{3}H][3-Me-His^2]TRH label two pharmacological distinct sites in rat brain cortex. Our data show that there is a statistically significant correlation between the pharmacological profile of the site labelled with low affinity by [^{3}H]TRH and that of TRH-DE, supporting the hypothesis that the identity of the low-affinity site is TRH-DE. Conversely, there is no correlation between the pharmacological profile of this site and the site labelled with high affinity by [^{3}H][3-Me-His^2]TRH. The structure–activity relationships described in this study also provide the most comprehensive information to-date regarding the influence of the chemical nature of the central amino acid residue in the TRH molecule on binding to native central high-affinity TRH receptors in rat brain.

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