# Specific binding sites for rat prepro-TRH-(160–169) on C6 glioma and BN1010 clonal neural cells

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**Abstract A connecting decapeptide corresponding to rat prepro-TRH-(160–169) (Ps4) displays several biological activities that are related or unrelated to TRH. We have previously characterized pituitary binding sites for this connecting peptide and elucidated structural determinants for high peptide binding affinity. In the current study, a series of cell lines was screened for the presence of specific binding sites with a highly potent derivative of Ps4, the monoiodinated radioligand [$^{125}$I-Tyr$^0$]Ps4. Neuroblastoma×glioma hybrid NG108-15, glioma C6 and neuroblastoma BN1010 cell lines were found to have high-affinity [$^{125}$I-Tyr$^0$]Ps4 binding sites containing 600, 9700 and 130 000 sites/cell, respectively. The specific binding of [$^{125}$I-Tyr$^0$]Ps4 was rapid, time-dependent, reversible and proportional to the amount of C6 and BN1010 membrane preparation. Furthermore, Scatchard or Hill analysis revealed that [$^{125}$I-Tyr$^0$]Ps4 was bound by a single population of non-interacting sites with dissociation constants in the subnanomolar range. Competition studies made with Ps4 analogues indicated that [$^{125}$I-Tyr$^0$]Ps4 binding sites on C6 and BN1010 cells were similar to those previously described on rat pituitary membranes. It is concluded that C6 and BN1010 cells are suited for studies on the intracellular events following binding of the Ps4 and for the molecular characterization of the Ps4 binding sites.**



*Key words:* Thyrotropin-releasing hormone; Connecting peptide; Neural cell; Receptor binding assay

## 1. Introduction

Rat prepro-TRH is a 255-amino-acid polypeptide containing five copies of the TRH progenitor sequence, Gln–His–Pro–Gly, each flanked by paired basic amino acids (Lys–Arg or Arg–Arg) and linked together by connecting peptide sequences sharing no identity with TRH [1]. Like other plurifunctional prohormone proteins, pro-TRH undergoes differential proteolytic processing in various tissues to generate, in addition to authentic TRH, several other novel peptides corresponding to C-terminally extended forms of TRH and connecting fragments [2–4].

Although the physiological significance of TRH in the regulation of the pituitary–thyroid axis has been well established (for a recent review, see reference [5]), the role of pro-TRH connecting peptides is still not clear. In that context, we have reported a specific potentiation of TRH-induced thyrotropin (TSH) release by pituitary fragments in vitro by the connecting peptide prepro-TRH-(160–169) (Ps4) [6]. Interestingly, it was demonstrated that Ps4, like TRH, induces a dose-dependent increase of β-TSH mRNA content in intact animals, primary pituitary cell cultures and in a heterologous TSH expression assay [7,8]. Recently, another group has revealed the biological importance of Ps4 in the dorsal motor nucleus of the vagus, a region where TRH plays a pivotal role to activate directly neurons leading to the stimulation of vagal outflow to the stomach and gastric function. Thus, Ps4 was shown to potentiate the action of TRH in the rat CNS to stimulate gastric acid secretion [9].

Using [$^{125}$I-Tyr$^0$]Ps4 as a metabolically stable radioligand derivative, specific binding sites have been detected in the distal lobe of the pituitary by autoradiography [6]. The existence of a single homogeneous population of high-affinity ($K_d$ = 0.22 nM) binding sites for [$^{125}$I-Tyr$^0$]Ps4 was demonstrated in the homogenates of rat pituitary membrane. We have shown that the Ps4 binding sites were distinct from the TRH receptors and that there was no interaction between Ps4 and TRH for receptor binding [10]. We have also shown that Ps4 and its binding sites were largely distributed in the rat brain and peripheral tissues [11].

Significant levels of [$^{125}$I-Tyr$^0$]Ps4 binding sites were detected in the neuronal tissues containing an important amount of Ps4, such as the hypothalamus, spinal cord, and olfactory lobes. These findings reinforce the suggestion that Ps4 is not only a hypophysiotropic hormone but may also act as a neuromodulator or neurotransmitter in the CNS. Whereas binding characteristics of the Ps4 pituitary binding sites and molecular determinants for binding affinity have been clearly defined, little is known about the structure of the Ps4 binding sites and the molecular mechanism(s) by which Ps4 elicits its physiological effects. Since this gap in knowledge is due in large part to the lack of Ps4-responsive cellular models, we have screened cell lines of various origin for Ps4 binding sites that could be used as a starting material to clone the Ps4 receptor.

## 2. Materials and methods

### 2.1. Solid-phase synthesis of prepro-TRH-(160–169) and analogs

Prepro-TRH-(160–169) (Ps4), [Tyr$^0$]prepro-TRH-(160–169) ([Tyr$^0$]Ps4), prepro-TRH-(158–169) ([Arg$^{-1}$,Arg$^0$]Ps4), and prepro-TRH-(159–169) ([Arg$^0$]Ps4) were prepared by stepwise solid phase synthesis using Fmoc polyamide active ester chemistry on a Milligen 9050 Pepsynthesizer as described previously [10].

### 2.2. Cell culture

The BN1010 cells which are capable of synthesizing TRH were derived from an ethylnitrosourea-induced rat CNS tumor [12] and C6 cells from a rat glioma [13]. Both BN1010 and C6 cell lines were cultured on 10-cm-diameter petri dishes in RPMI-1640 medium

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*Abbreviations:* TRH, thyrotropin-releasing hormone; pro-TRH, thyrotropin-releasing hormone prohormone; Ps4, prepro-TRH-(160–169); CNS, central nervous system; TFA, trifluoroacetic acid


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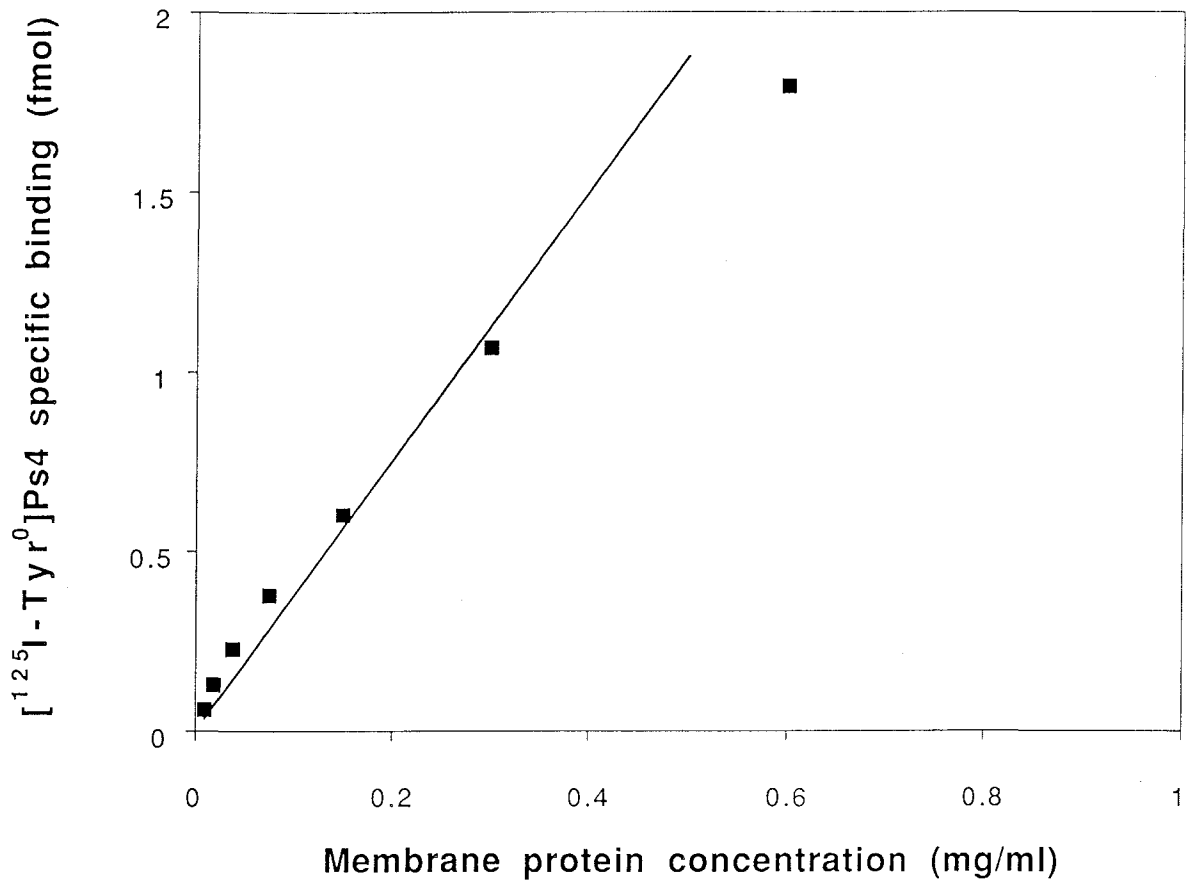


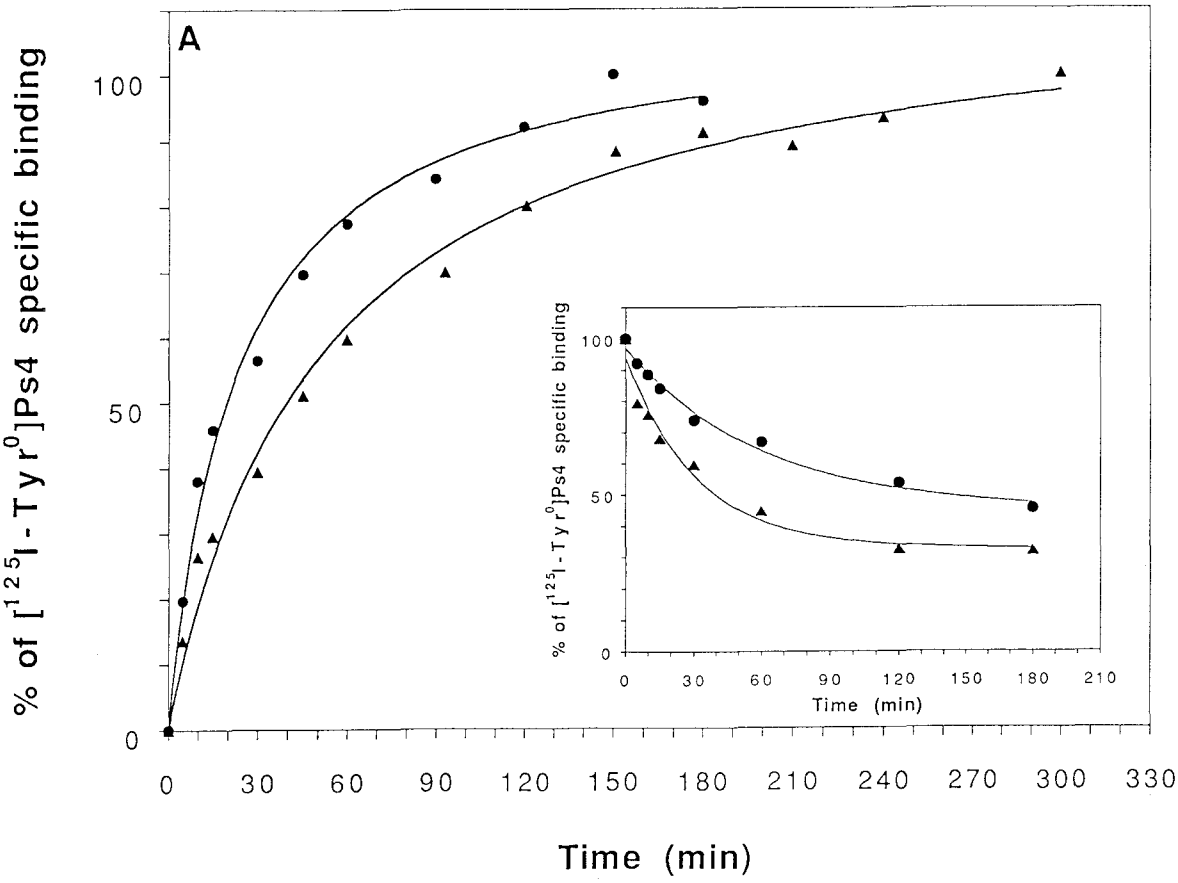

Fig. 1. Specific binding of [$^{25}$I-Tyr$^0$]Ps4 to rat C6 membrane homogenates as a function of increasing protein concentrations. [$^{125}$I-Tyr$^0$]Ps4 (0.2 nM) were incubated for 120 min with various concentrations of C6 membrane tissues (9.4 μg/ml to 0.6 mg/ml of membrane proteins) and specific binding was measured as the difference between femtomole bound in the absence and presence of 1 μM unlabeled [Tyr$^0$]Ps4. Results are expressed as femtomole of specific [$^{125}$I-Tyr$^0$]Ps4 bound and represent the mean of duplicated determinations.

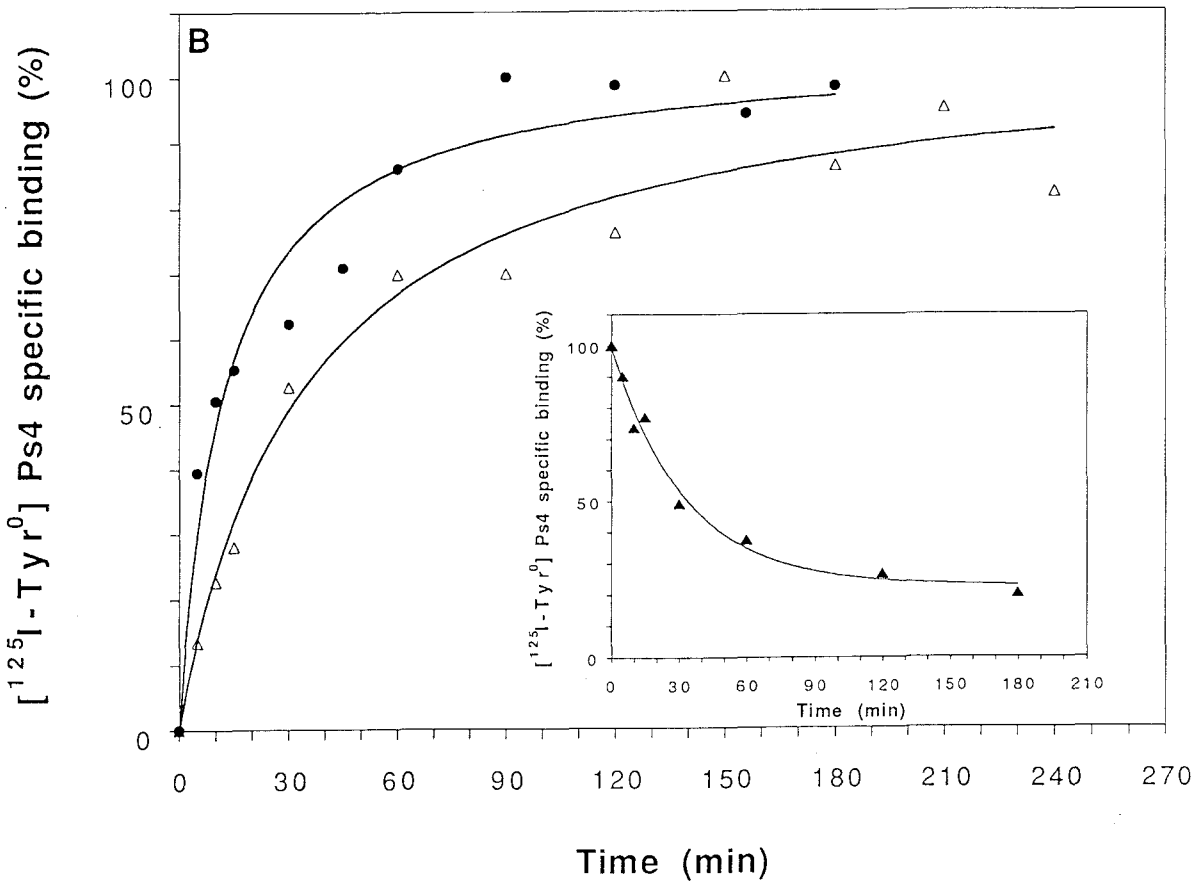
supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin, in a humidified atmosphere (5% $CO_2$/95% air) at 37°C.

*2.3. Receptor binding assays*

After reaching confluency, cells were detached by incubation in ($Ca^{2+}$, $Mg^{2+}$)-free phosphate-buffered saline (PBS) containing 1 mM EDTA for 5 min at 37°C. Cells were then centrifuged and homogenized in 50 mM Tris-HCl, pH 7.4, by four cycles of Polytron and centrifugation (20 min, 10000×*g*) at 4°C. The resulting pellet was resuspended in the same buffer containing 20% glycerol and stored at −80°C. The final protein concentration of the C6 and BN1010 extract was 2.15 mg/ml and 1.36 mg/ml, respectively, as determined by the method of Lowry [14] using bovine serum albumin (BSA) as standard. Binding assays were performed as described [10] in 50 mM Tris-HCl, pH 7.4, plus 0.1% BSA and 0.01% bacitracin using monoiodinated [Tyr$^0$]Ps4 as primary ligand. [Tyr$^0$]Ps4 was iodinated by using chloramine-T. Purification of monoiodinated [$^{125}$I-Tyr$^0$]Ps4 was performed by reverse-phase HPLC on a Lichrosorb RP-18 column (5 mm; 25×0.4 cm). The specific activity of the peptide was 2000 Ci/mmol. Briefly, each assay contained, in a final volume of 300 μl, the membrane preparation (90 μg of C6 membrane proteins and 1.13 μg of BN1010 membrane proteins) and the iodinated ligand at the desired concentration with or without unlabeled ligand. Binding experiments were also performed on viable cells using the same procedure with PBS containing 1 mM HEPES, 5 mg/ml BSA, and 1 mg/ml glucose as binding and washing buffers. Specific binding was considered as the difference in radioactivity trapped on the filters either in the absence or presence of 1 μM of the unlabeled primary ligand. All determinations were performed in duplicate. The 50% inhibitory concentration values ($IC_{50}$) were obtained from non-linear least-squares regression to a two-parameters logistic equation of the percent specific binding versus log(dose) curves. The inhibitory constant ($K_i$, nM) of the various unlabeled ligands was calculated from the relation $K_i = IC_{50}/(1+[L]/K_d)$ [15] where $L$ is the labeled ligand and $K_d$ its equilibrium dissociation constant determined by saturation binding analysis.

Fig. 2. Kinetics of specific association and dissociation of [$^{125}$I-Tyr$^0$]Ps4. A: C6 membranes (90 μg proteins) were incubated with 0.3 nM [$^{125}$I-Tyr$^0$]Ps4 for increasing periods of time to determine the association kinetics at 24°C (●) and 4°C (▲). Inset: Dissociation kinetics, at 24°C (●) and 4°C (▲), were initiated by the addition of 1 μM of unlabeled [Tyr$^0$]Ps4 to the C6 membrane preparations at equilibrium (180 min incubation). Specific binding was obtained by substracting non-specific binding from total binding. Each point is the average of triplicate determinations obtained from three similar experiments. B: BN1010 membranes (1.13 μg proteins) were incubated with [$^{125}$I-Tyr$^0$]Ps4 (0.2 nM) for the indicated time intervals and specific binding (●) was measured at 24°C. Similar experiments were performed on BN1010 whole cells (1.35 $10^6$ cells) at 4°C (△). Inset: Dissociation experiments: after 120 min incubation at 4°C (▲), 1 μM of unlabeled [Tyr$^0$]Ps4 was added to the BN1010 membranes and radioactivity specifically bound was determined. Results were obtained as described in (A).


A
% of [125I-Tyr0]Ps4 specific binding
100
50
0
0 30 60 90 120 150 180 210 240 270 300 330
Time (min)
% of [125I-Tyr0]Ps4 specific binding
100
50
0
0 30 60 90 120 150 180 210
Time (min)


B
[125I-Tyr0] Ps4 specific binding (%)
100
50
0
0 30 60 90 120 150 180 210 240 270
Time (min)
[125I-Tyr0] Ps4 specific binding (%)
100
50
0
0 30 60 90 120 150 180 210
Time (min)

## 3. Results and discussion

### 3.1. Screening of cell lines for [$^{125}$I-Tyr$^0$]Ps4 binding sites

Since Ps4 binding sites are widely distributed in the CNS and peripheral tissues [11], we screened a series of neural and endocrine cell lines for [$^{125}$I-Tyr$^0$]Ps4 binding sites. Incubations were carried out at room temperature for 60 min with 0.2 nM [$^{125}$I-Tyr$^0$] Ps4 in the absence or the presence of 1 μM [Tyr$^0$] Ps4 to determine non-specific binding. Using membrane homogenates, we demonstrated that out of the 10 cell lines studied, three types of neural cells, i.e. C6 (rat glioma), BN1010 (rat neuroblastoma) and NG 108-15 (neuroblastoma × glioma hybrid), exhibited specific binding sites of [$^{125}$I-Tyr$^0$]Ps4 (Table 1). No binding sites were found on the endocrine pancreas tumor cell lines RINm5F, INS-1 and αTC and on the pituitary tumor cell lines GH3, SUG, SUF and GC. Rat C6 glial cell line is a classical cell line that was established by alternate culture and animal passage after tumor induction by *N*-nitrosomethylurea. The cell line retained the ability to produce the brain-specific protein S-100 [13]. The BN1010 cell line, which derived from an ethylnitrosourea-induced rat CNS tumor, was reported to synthesize TRH and to be devoid of TRH receptors [12]. Moreover, these cells responded to isoproterenol by elevating their intracellular cAMP and exhibit sodium flux responses to certain neurotoxin in a manner characteristic of action potential sodium channels. NG108-15 hybrids were obtained by fusion of neuroblastoma clone N18TG-2 and C6Bu-1 a clone of the rat glioma C6 [16].

### 3.2. Kinetic characteristics of [$^{125}$I-Tyr$^0$]Ps4 specific binding to C6 rat glioma and BN1010 cell lines

The specific [$^{125}$I-Tyr$^0$]Ps4 binding increased linearly with the membrane protein concentration up to 0.3 mg/ml (correlation coefficient = 0.98) (Fig. 1). Higher protein concentrations tended to lower the amount of [$^{125}$I-Tyr$^0$]Ps4 specifically bound because of the increase of proteases concentration and viscosity. The time course of [$^{125}$I-Tyr$^0$]Ps4 specific binding to C6 membranes (4°C and 24°C) is shown in Fig. 2A. At 4°C, the near maximum specific binding, $B_{max}$, was reached in 270 min and the half-time of binding was approximately 40 min. Non-specific binding was determined in the presence of 1 μM unlabeled [Tyr$^0$]Ps4 and represented 7.5% of the total binding. When similar experiments were performed at 24°C, binding equilibrium was reached in 150 min and the half-time of binding was approximately 20 min (Fig. 2A). Addition of 1 μM unlabeled [Tyr$^0$]Ps4 at equilibrium produced dissociation of the bound radioligand, demonstrating the reversibility of [$^{125}$I-Tyr$^0$]Ps4 binding (Fig. 2A, inset). The dissociation kinetic obtained at 4°C followed a first-order rate law with a half-time of dissociation of approximately 38 min. After 180 min, almost 70% of the radioiodinated peptide was dissociated. However, the dissociation of the receptor–ligand complex showed a rather slow decrease at 24°C, with a half-time of approximately 138 min (Fig. 2A, inset). After 3 h, only 53% of [$^{125}$I-Tyr$^0$]Ps4 was dissociated. This phenomenon had already been observed for Ps4 pituitary binding sites and could be due to a conformational change of the receptor after peptide binding [10]. At 24°C, the rate constants for association ($k_1$) and dissociation ($k_{-1}$) were 4.15 $10^7$ $M^{-1}$ $min^{-1}$ and 7.72 $10^{-3}$ $min^{-1}$, respectively.

The specific binding of [$^{125}$I-Tyr$^0$]Ps4 to BN1010 membrane homogenates reached equilibrium within the 120 min incubation at 24°C with a half-time binding value of 11.5 min (Fig. 2B). Since incomplete dissociation was previously observed for pituitary and C6 membranes at room temperature, dissociation experiments were realized on BN1010 membranes at 4°C. The results obtained revealed that [$^{125}$I-Tyr$^0$]Ps4 specific binding was reversible (Fig. 2B, inset). The half-time of dissociation was 34.4 min; approximately 77% of the radioiodinated peptide was dissociated after 180 min incubation. Specific binding was also detected in BN1010 whole cells at 24°C, suggesting a plasma membrane localization for the [$^{125}$I-Tyr$^0$]Ps4 binding sites. However, probably due to peptide internalization or degradation, binding association was very slow and $B_{max}$ was still not reached after 5 h incubation. Similar experiments performed on BN1010 whole cells at 4°C indicated that $B_{max}$ is reached after 180 min incubation with a half-time of binding of 31.2 min (Fig. 2B).

### 3.3. Saturation studies

Ligand saturation experiments were performed by incubating C6 membrane preparations with increasing concentrations of [$^{125}$I-Tyr$^0$]Ps4 (16.4 pM to 1.9 nM). Over this concentration range, specific binding of [$^{125}$I-Tyr$^0$]Ps4 was saturable reaching a maximum at approximately 1.9 nM (Fig. 3A, lower inset). The non-specific binding, on the contrary, increased linearly. At the highest [$^{125}$I-Tyr$^0$]Ps4 concentration (1.9 nM) studied, specific binding represented 61% of the total bound ligand while non-specific binding accounted for 38%. Scatchard plot of the binding data gave a straight line (correlation coefficient = 0.95) (Fig. 3A), indicating the presence of a single class of binding sites for [$^{125}$I-Tyr$^0$]Ps4 with a $K_d$ value of 0.77 nM and a $B_{max}$ value of 122 fmol/mg of membrane protein. Hill plot (Fig. 3A, upper inset) yielded a $n_H$ of 1.07, excluding a cooperativity in binding of [$^{125}$I-Tyr$^0$]Ps4.

When BN1010 membranes (final protein concentration = 3.77 μg/ml) were incubated with increasing concentrations of [$^{125}$I-Tyr$^0$]Ps4 (1.58 pM to 0.75 nM), non-specific binding was linear (Fig. 3B, lower inset). In contrast, the specific binding of [$^{125}$I-Tyr$^0$]Ps4 was saturable within the range of concentrations studied. Specific binding accounted for 78% at 0.75 nM [$^{125}$I-Tyr$^0$]Ps4. Scatchard representation of [$^{125}$I-Tyr$^0$]Ps4 saturation binding isotherm was linear (correlation coefficient = 0.99) (Fig. 3B). These results, together with Hill analysis ($n_H$ = 1.08) (Fig. 3B, upper inset) demonstrated that [$^{125}$I-Tyr$^0$]Ps4 bound specifically to a single population of non-

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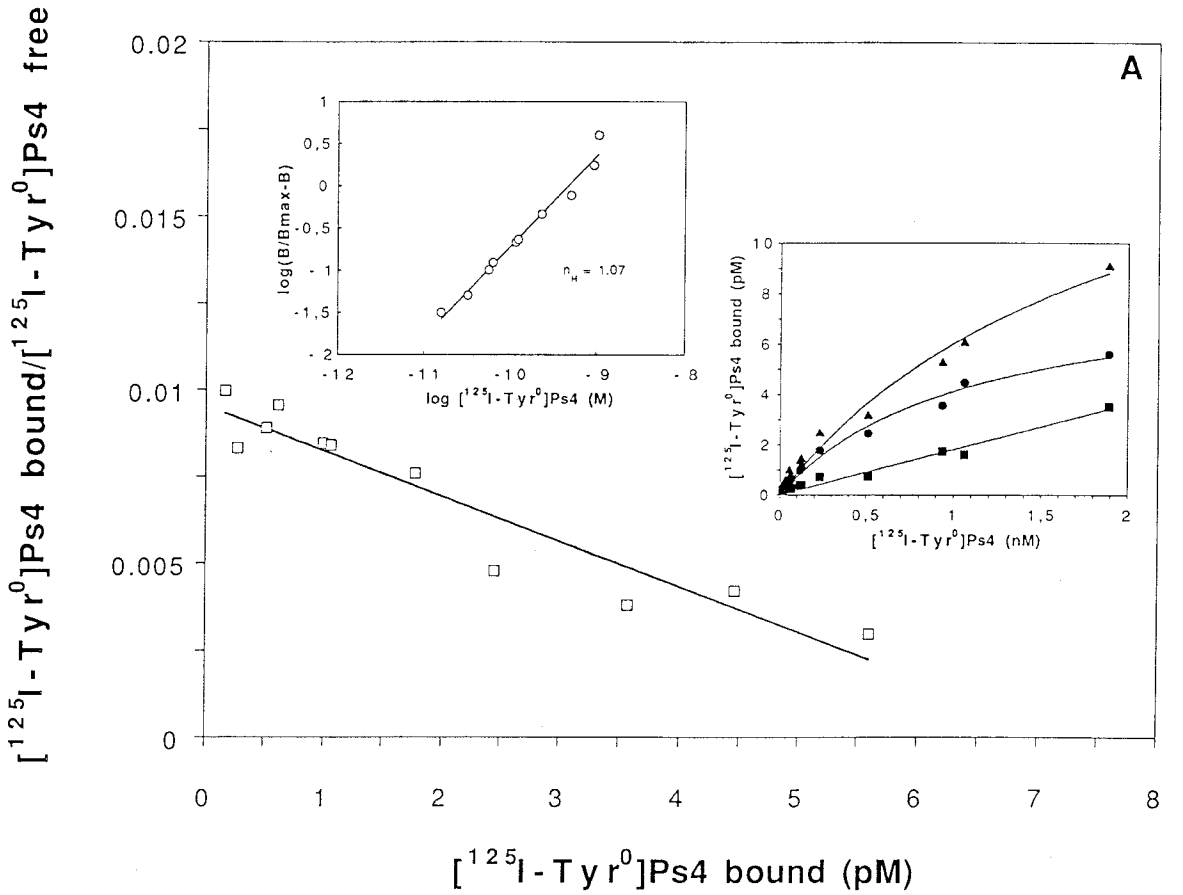
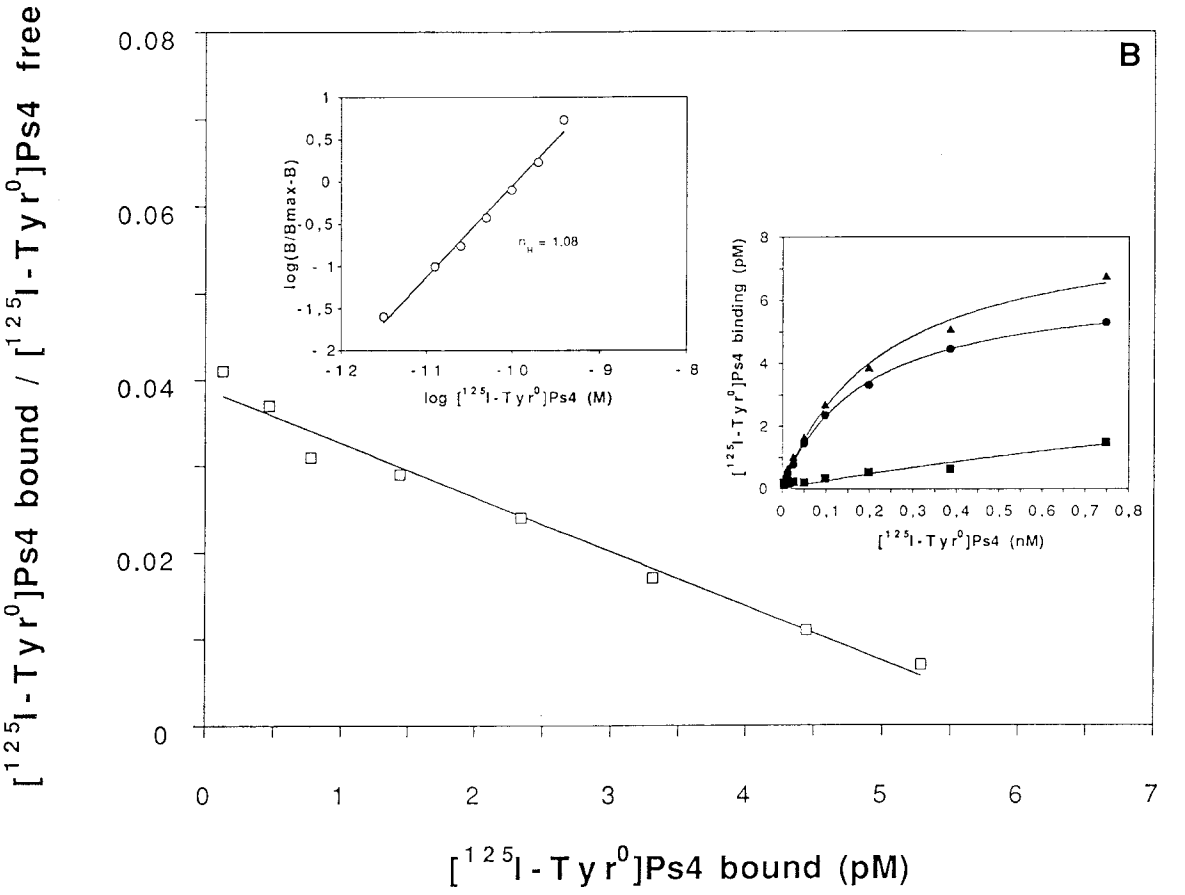
Fig. 3. Saturation binding of [$^{125}$I-Tyr$^0$]Ps4 to C6 and BN1010 membranes. A: Scatchard plot of [$^{125}$I-Tyr$^0$]Ps4 binding to C6 membranes. The slope of the line ($-1/K_d$) was determined by linear regression analysis. Lower inset: Binding of [$^{125}$I-Tyr$^0$]Ps4 to C6 membrane preparations as a function of [$^{125}$I-Tyr$^0$]Ps4 concentration. Total (▲), non-specific (■), and specific (●) binding were estimated as described in Section 2. Values are the mean of duplicate incubations. Upper inset: Hill plot of specific binding. The $n_H$ determined by linear regression analysis was 1.07. B: Scatchard analysis of [$^{125}$I-Tyr$^0$]Ps4 binding to BN1010 membranes. Lower inset: Binding of [$^{125}$I-Tyr$^0$]Ps4 to BN1010 membrane preparations as a function of [$^{125}$I-Tyr$^0$]Ps4 concentration. Total (▲), non-specific (■), and specific (●) binding. Upper inset: Hill plot ($n_H$ = 1.08). Results were expressed as described in (A).


A
[125I-Tyr0]Ps4 bound/[125I-Tyr0]Ps4 free
[125I-Tyr0]Ps4 bound (pM)
log(B/Bmax-B)
nH = 1.07
log [125I-Tyr0]Ps4 (M)
[125I-Tyr0]Ps4 (nM)


B
[125I-Tyr0]Ps4 bound / [125I-Tyr0]Ps4 free
[125I-Tyr0]Ps4 bound (pM)
log(B/Bmax-B)
nH = 1.08
log [125I-Tyr0]Ps4 (M)
[125I-Tyr0]Ps4 binding (pM)
[125I-Tyr0]Ps4 (nM)

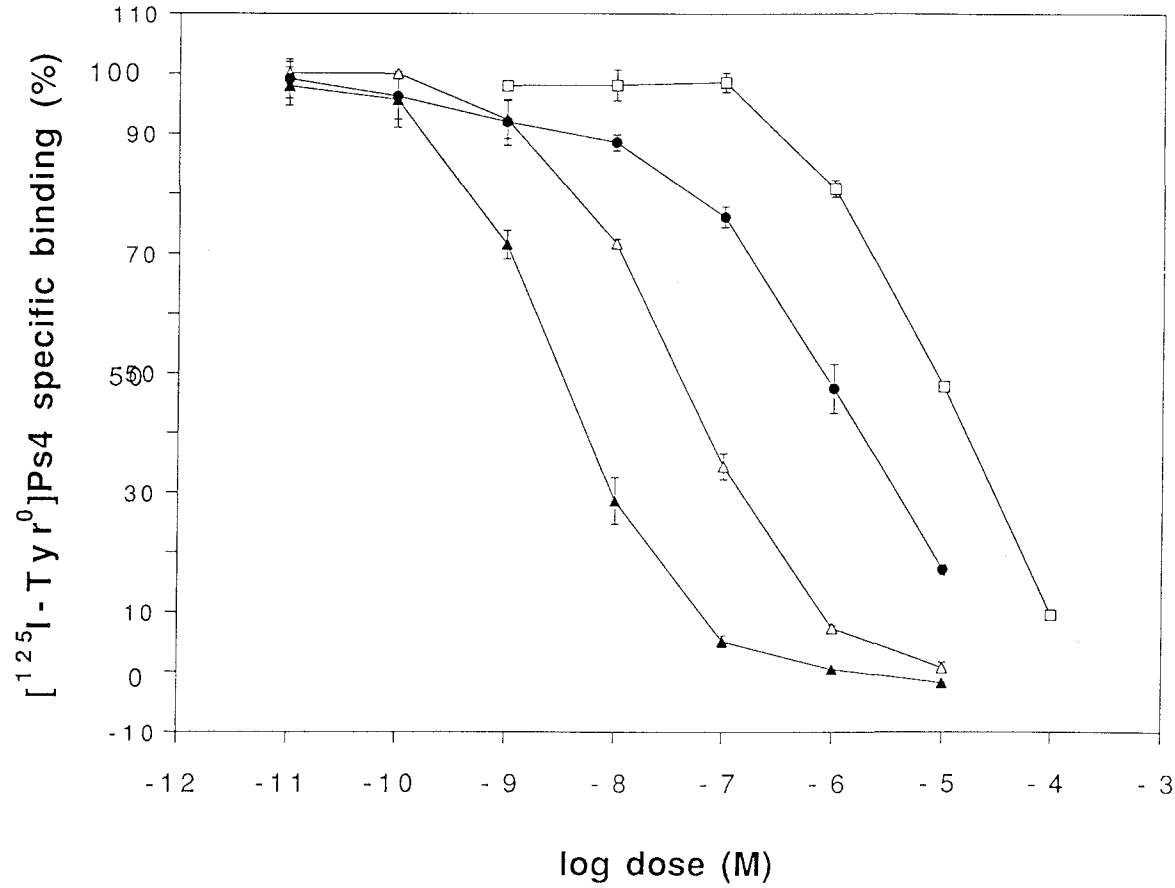



Fig. 4. Displacement of [$^{125}$I−Tyr$^0$]Ps4 (0.2 nM) specific binding by Ps4 analogs on C6 membrane homogenates. C6 membranes (90 μg proteins) were incubated for 180 min at 25°C with 0.2 nM [$^{125}$I−Tyr$^0$]Ps4 and increasing concentrations of the following peptides: ▲, [Tyr$^0$]Ps4; △, [Arg$^0$]Ps4; ●, [Arg$^{-1}$,Arg$^0$]Ps4; □, Ps4. The percentage of specific binding was calculated as $100\times(B_s-B_n)/B_0-B_n)$ in which $B_s$ and $B_0$ are the amount bound in the presence and in the absence of competing compound, respectively. $B_n$ is the non-specific binding. The data shown are for a single representative experiment. Value for $IC_{50}$ were determined by regression analysis based upon three independent experiments carried out in duplicate.

interacting high-affinity binding sites. Values of $K_d$ and $B_{max}$ were 0.16 nM and 1643 fmol/mg of membrane protein, respectively.

The Ps4 binding sites are abundantly expressed in BN1010 cells since more than $10^5$ copies are present per cell. This level is comparable to the expression of epidermal growth factor receptor [17]. On the other hand, saturation experiments performed on NG108-15 neuroblastoma×glioma hybrid cell membranes revealed the presence of a very low amount of [$^{125}$I-Tyr$^0$]Ps4 binding sites (Table 1). In contrast to the parent cell C6 which expressed the binding sites at an intermediate level ($10^4$ copies per cell), less than $10^3$ copies of Ps4 binding sites are present in NG108-15. Thus, it seems that the gene(s) encoding the TRH-potentiating peptide receptor is partially deactivated by hybrid formation.

### 3.4. Competition studies

Competition experiments were carried out by incubating membrane preparations with increasing concentrations ($10^{-11}$ to $10^{-4}$ M) of Ps4 analogues in the presence of 0.2 nM [$^{125}$I-Tyr$^0$]Ps4 (Fig. 4). The displacement curves obtained gave pseudo-Hill coefficients close to unity and could be best

Table 1
Equilibrium binding parameters of [$^{125}$I-Tyr$^0$]Ps4 binding to different membrane preparations at 24°C

| Cell line | Total (cpm) | Nonspecific (cpm) | $K_d$ (nM) | $B_{max}$ (binding sites/cell) |
|---|---|---|---|---|
| C6 | 2170 | 634 | 0.77 | 9700 |
| BN1010 | 3328 | 464 | 0.16 | 130000 |
| NG108-15 | 776 | 568 | 0.2 | <600 |
| RINm5F | 899 | 702 | – | – |
| INS−1 | 883 | 864 | – | – |
| αTC | 615 | 534 | – | – |
| GH3 | 444 | 409 | – | – |
| SUG | 648 | 686 | – | – |
| SUF | 299 | 252 | – | – |
| GC | 305 | 294 | – | – |

Values reported are the mean of duplicate determinations of two independent experiments.

described by a single-binding site model. The rank order of potency of competitors for [$^{125}$I-Tyr$^0$]Ps4 binding on membranes was [Tyr$^0$]Ps4 > [Arg$^0$]Ps4 > [Arg$^{-1}$,Arg$^0$]Ps4 > Ps4 > TRH-Ps4. $K_i$ values obtained for [Tyr$^0$]Ps4 (2.5 nM) and Ps4 (6.6 μM) were in the same range than those reported previously for the pituitary binding sites (4.96 nM and 5.7 μM, respectively). The two N-terminally extended forms of Ps4, prepro-TRH-(159–169) ([Arg$^0$]Ps4) and prepro-TRH-(158–169) ([Arg−1,Arg0]Ps4), which may be generated by an incomplete processing of the pro-TRH, kept the ability to inhibit [$^{125}$I-Tyr$^0$]Ps4 binding. Interestingly [Arg$^0$]Ps4 ($K_i$ = 31.1 nM) was only 12-fold less potent than [Tyr$^0$]Ps4 ($K_i$ = 2.5 nM). Moreover, the addition of a Arg residue in the N-terminal position of [Arg$^0$]Ps4 resulted in a 23-fold reduction in receptor binding affinity ([Arg$^{-1}$,Arg$^0$]Ps4, $K_i$ = 712 nM). A more important extension in the N-terminal end of Ps4 caused a profound decrease in binding potency, as it was demonstrated with prepro-TRH-(154–169) (TRH-Ps4) which exhibited a much lower affinity ($K_i$ = 12000.0 nM) than Ps4 itself. Of particular interest is the fact that two intermediate products of TRH-Ps4 to Ps4 conversion exhibit higher binding potency than TRH-Ps4 and Ps4 themself. An activation–deactivation phenomenon of the hormonal message could be considered in that case.

In conclusion, we have identified glial and neural cell line that markedly express the high-affinity binding sites for the TRH-potentiating peptide. Our data suggest that C6 and BN1010 cells represent a Ps4-responsive model for studying the mechanism of action of Ps4 at the molecular level. Furthermore, the high number of TRH-potentiating peptide binding sites in BN1010 cells and the high affinity of the radioligand [$^{125}$I-Tyr$^0$]Ps4 should enable the molecular characterization of the receptor molecule by expression cloning.

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