Coexistence of somatostatin receptor subtypes in the human neuroblastoma cell line LA-N-2

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Received 7 November 1996

Abstract Five distinct human somatostatin receptor subtypes have recently been cloned and characterized. Previous studies have suggested that these receptor subtypes might display coexistent localization, based on in situ hybridization or immunoblockage experiments. Here we provide evidence for coexistence of somatostatin receptor subtypes 2 and 5 in the human neuroblastoma cell line LA-N-2, using a combined approach with RT-PCR and receptor binding studies with somatostatin analogues. Somatostatin receptor subtypes simultaneously localized to a single cell might serve distinct functions in terms of targeting to different intraneuronal compartments or subtype specificity against so far unidentified somatostatin-related peptides.

Key words: Somatostatin; Neuroblastoma; Human; LA-N-2; Coexistence; Receptor; Subtype

1. Introduction

Somatostatin (SS) is a neuropeptide with a wide distribution in the central nervous system (CNS) and peripheral tissues such as the pancreas and gastrointestinal tract [1,2]. It exerts various hormonal actions like inhibition of growth hormone (GH), thyroid stimulating hormone (TSH), insulin and glucagon secretion [3]. Furthermore, this neuropeptide has been demonstrated to influence complex behavioral functions such as locomotor activity and cognition [4,5]. Thus heterogeneity of the SS receptor has for a long time been suggested and supported by experimental data such as the biphasic manner of ligand displacement [6] and divergent functional effects of SS-immunoreactive forms [7]. The definitive evidence for multiple receptors was provided by the molecular cloning of five different receptor subtypes (for review [8]).

We have recently identified a human neuroblastoma cell line with expression of a single SS transcript, the prohormonal form ($proSS_{1-92}$), the processed form SS-28 as well as high-affinity receptors with sensitivity to G-protein uncoupling [9]. The aim of the present study was to investigate which SS receptor subtypes were expressed in this cell line. Several authors have previously shown coexistent expression of SS receptor subtype transcripts and suggested simultaneous occurrence of multiple SS receptor subtypes in a single cell [10,11]. Here we provide evidence for multiple SS receptor subtypes in the clonal neuroblastoma cell line LA-N-2 using a combined approach with RT-PCR methodology and competitive radioligand binding with subtype-specific analogues.

2. Materials and methods

2.1. Cell culture

The neuroblastoma cell line LA-N-2 was grown at 37°C in RPMI-1640 culture medium (Gibco BRL, Life Technologies) supplemented with 10% fetal calf serum, L-glutamine 0.29 mg/ml, penicillin 100 IU/ml and streptomycin 50 μg/ml in humidified air containing 5% CO_2. The medium was changed every third day. Confluent cells (2×10^5 cells/cm^2) were washed once in ice-cold phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, pH 7.4) supplemented with 10 mM EDTA and harvested in ice-cold PBS buffer. The cell pellet used for RNA preparation and [^{125}I]LTT-SS-28 receptor binding was obtained by centrifugation at $1000\times g$ for 5 min at 25°C.

2.2. Human postmortem brain

Brain tissue was obtained at autopsy from two female subjects at 64 and 79 years of age (both with a postmortem delay of 5 h) without previous signs of neurological or psychiatric disorders (Huddinge Brain Bank). The left hemisphere was dissected into discrete regions and slowly frozen at −70°C, while the right hemisphere was fixed in 10% buffered neutral formalin. Paraffin-embedded material was stained in various ways to exclude neuropathological changes at macroscopic or microscopic levels. Synaptosomal membrane preparation was prepared from tissue material that had been slowly thawed on ice and then homogenized in 10 volumes (w/v) of ice-cold 0.32 M sucrose using a glass-teflon homogenizer. Subsequent centrifugation steps were carried out in a similar way as for the neuroblastoma cells.

2.3. RNA preparation and RT-PCR

Total RNA (250 μg) was extracted [12] and treated with DNase I, amplification grade (5 U; Life Technologies) according to the manufacturer's instructions in the presence of RNasin (1 U/μl; Promega) to eliminate remaining genomic DNA. The RNA extraction procedure was repeated once to remove possible remaining DNase. Single stranded cDNA was synthesized from total RNA (~30 μg) using oligo(dT)-primed and reverse-primed AMV reverse transcriptase (1 U/μg total RNA; Promega). The cDNA was amplified with Taq DNA polymerase (1.5 U; Boehringer) in 50 μl reaction assays with cycles of 95°C for 1 min, 65°C for 1 min, 72°C for 1 min and a final period at 72°C for 10 min. The primers were designed with a similar GC content and checked for other sequences with similarity in the EMBL gene database (FASTA; Table 1). The primer pairs for the human SS gene were located in separate exons and in the intron to serve as a positive control as well as to reveal possible contamination with genomic DNA. Following 40 cycles the amplification reaction (15 μl) was electrophoretically separated on an 3.0% agarose gel. Genomic DNA was purified with a QIAamp tissue kit (Qiagen, Germany). The amplified DNA bands were excised, purified with QIAquick (Qiagen, Germany) and further investigated by restriction endonuclease mapping.

2.4. [^{125}I]LTT-SS-28 receptor binding assay

Pelleted cells (~10^8 cells) were dissolved in 5 ml ice-cold 50 mM Tris-HCl (pH 7.4), homogenized with a glass-teflon homogenizer and centrifuged at $700\times g$ for 10 min at 4°C. The supernatant was centrifuged at $14000\times g$ for 30 min at 4°C and the pellet obtained resuspended in 1 ml 50 mM Tris buffer (pH 7.4) and frozen at −20°C until use in binding assays. Membrane suspensions were thawed and diluted in 5 ml ice-cold 50 mM HEPES-KOH buffer (pH 7.5). The mixture was centrifuged at $11000\times g$ for 30 min at 4°C and the pellet resuspended in fresh buffer to give a protein concentration of approxi-

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PII S0014-5793(96)01441-X

mately 1.5 μg/μl. The ligand, LTT-SS-28 (3 μg; Sigma), was iodinated with ^{125}I (1 mCi, 556 mCi/ml; Amersham) using the chloramine-T (1 μg) method in a total volume of 27 μl and separated on a 5 μm C_{18} reversed-phase HPLC column (LKB, Stockholm) [17]. The column was eluted at room temperature at 0.5 ml/min with 10–40% CH_3CN and 0.1% trifluoroacetic acid. Dilutions of peptides and radioactive ligand were made in the incubation buffer which consisted of 50 mM HEPES-KOH buffer (pH 7.5) containing 0.2 mg/ml bovine serum albumin (BSA), 5 mM $MgCl_2$, 0.03 mg/ml bacitracin, 200 kIU/ml aprotinin and 0.02 μg/ml PMSF. Binding assays were performed in precoated Eppendorf tubes (0.5 mg/ml BSA in redistilled water). Membrane preparation (~120 μg protein) and [^{125}I]LTT-SS-28 (1050 Ci/mmol) was incubated for 60 min at 30°C in the absence or presence of increasing concentrations of peptide analogues in a final volume of 200 μl. The incubation was terminated by centrifugation at 13000×g for 1 min, the supernatant was aspirated and the pellet washed with 1.25 ml ice-cold incubation buffer. Tissue-bound radioactivity was determined following a second centrifugation and aspiration. Non-specific binding was defined as binding in the presence of 1 μM unlabeled SS-14. All assays were performed in triplicate. Protein determinations were measured by the method of Lowry et al. [18]. The following peptides and analogues were used: Octreotide (Sandostatin, Sandoz) and SS-14 (Peninsula). The CGPIII analogue was synthesized and checked with mass spectroscopy by Dr. Gunnar Holmberg, Immunology Department, Uppsala, Sweden. DC23-60 was kindly provided by Dr. W. Murphy and Dr. D. Coy, Tulane University, New Orleans, LA, USA.

3. Results

A RT-PCR methodology was initially developed to screen for presence of SS receptor subtype transcripts due to the high sensitivity of this technique and limited need of mRNA. The human SS mRNA was used as a positive control transcript, since gene expression has been previously demonstrated in the LA-N-2 cell line with Northern blot [9]. The transcript was amplified with primers located in separate exons at various annealing temperatures to evaluate the stringency of the PCR conditions. A single band was obtained at annealing temperatures between 55°C and 70°C, although the yield was lower at 70°C (Fig. 1A). Control experiments using exon primers of the SS gene revealed a single band (~1100 bp) in the genomic DNA and one band (~200–250 bp) in the cDNA preparation (Fig. 1B), while the intron primers produced a single band (~250–300 bp) in the genomic DNA from the cell line as expected (Fig. 1C). In contrast, no signs of contaminating genomic DNA were evident either in the cDNA preparation, the RNA preparation not subjected to reverse transcription or in the reagents supplied with the reverse transcriptase kit (Fig. 1B,C). Primers for the different SS receptor subtypes were used at an annealing temperature of 65°C. The subtypes SSTR2 and SSTR5 were found to be expressed in the cell line, while the SSTR1, SSTR3 and SSTR4 transcripts were not detected. No bands were present when the cDNA was replaced by RNA preparation not subjected to reverse transcription in the PCR reactions (Fig. 1D,E). The genuineness of the amplified DNA bands was verified by the presence of two nondegenerated hexamer restriction endonuclease motifs.

In order to investigate whether these subtypes were translated into receptor proteins competitive binding experiments with [^{125}I]LTT-SS-28 as ligand and various subtype-specific peptide analogues were used [17]. The iodinated ligand, [^{125}I]LTT-SS-28, was checked by saturation binding experiments. Specific binding to membrane preparations from human medial frontal cortex was found to consist of a single population of high-affinity binding sites (B_{max} = 19.46 ± 1.94 fmol/mg protein; K_d = 0.46 ± 0.08 nM; Hill coefficient = 0.97 ± 0.12; n = 3; Fig. 2A,B). The assay conditions were the same as those of Srikant and Patel except that a higher concentration of bacitracin was used in previous experiments (0.03 mg/ml instead of 0.02 μg/ml; [9]). The higher concentration was used since initial experiments showed that the specific binding to cellular membrane preparations was unstable at equilibrium using low concentrations of bacitracin. The high-affinity interaction between the ligand and the binding site was unaffected by this change as determined by association kinetics and displacement studies (data not shown).

The presence of SS receptor encoded proteins in synaptosomal membrane preparations was investigated using competitive radioligand binding (Fig. 3A–C; Table 2). The SS analogues SMS201-995 (selective for SSTR2, SSTR3 and SSTR5) and DC23-60 (selective for SSTR2 and SSTR5) as well as CGPIII (selective for SSTR5) were used together with the nonselective ligand SS-14. The SS analogue CGPIII displayed a biphasic manner of ligand displacement against synaptosomal membrane preparations from the neuroblastoma cell line LA-N-2. The competitive binding curve fitted significantly better to a two-site model compared to a one-site model suggesting two classes of receptor subtypes ($F_{2,6}$ = 5.35, P = 0.046, F-test). The inhibition constants (pK_i) were 10.3 ± 0.9 and 5.8 ± 0.2 (mean ± S.E.M.) for the high-affinity and low-affinity

Table 1
Primers used in the study

Gene	Primer (forward/reverse)	Codon	Size (bp)
SSTR1	5′-TGCTGAGCAGGTCGACGCCA-3′	987–1006	192
[13]	5′-CTCTAGAGCGCGGTGGCGTA-3′	1159–1178	
SSTR2	5′-CCTCTAGAACCTGAGTGGGC-3′	792–811	374
[13]	5′-GAGGAGGATCCTCTGGGTCT-3′	1146–1165	
SSTR3	5′-ATCGTCGACGTGGTGTGCCC-3′	929–948	196
[14]	5′-CCCACAGTGGGATCCTGGTC-3′	1106–1125	
SSTR4	5′-GGTCGTGGTCGACTTTGTGC-3′	839–858	250
[15]	5′-AGTAGTCCAGGGGATCCTCC-3′	1070–1089	
SSTR5	5′-CAACATCGTCGACCTGGCCG-3′	851–870	215
[15]	5′-TGTCTGGACGCGGATCCGTG-3′	1046–1065	
SOM-EXON	5′-GCTCCCTCGGATCCCAGACT-3′	1303–1322	233/1110
	5′-TTGCGTTCCCGGGGTGCCAT-3′	2393–2412	
SOM-INTRON	5′-CCCTAAGCCTTGCTCCTGCC-3′	1410–1429	278
[16]	5′-CTTCCCAGGGTCAGCACCAG-3′	1668–1687	

binding sites. The fraction of high-affinity binding sites represented 15 ± 5% (mean ± S.E.M.) of total binding. Human postmortem pituitary tissue showed a one-site displacement curve ($pK_i = 8.8 \pm 0.3$) representing 60 ± 6% of total binding, while a single low-affinity site was found in the cerebral cortex ($pK_i = 6.1 \pm 0.1$). Competitive binding curves by the DC23-60 ligand fitted to a one-site model with similar inhibition constants for the LA-N-2 cell line ($pK_i = 8.8 \pm 0.1$, $n = 4$) as well as the pituitary ($pK_i = 8.5 \pm 0.2$, $n = 3$) and the cerebral cortex ($pK_i = 8.4 \pm 0.1$, $n = 4$). The fraction of total binding displaced represented 94 ± 3%, 59 ± 3% and 79 ± 2% respectively. The SS analogues SMS201-995 also fitted to a one-site model for the LA-N-2 cell line ($pK_i = 9.7 \pm 0.1$, $n = 4$), the pituitary ($pK_i = 8.6 \pm 0.3$, $n = 4$) and the cerebral cortex ($pK_i = 9.2 \pm 0.1$, $n = 3$). The fraction of total binding displaced represented 88 ± 4%, 66 ± 6% and 89 ± 3% respectively. The SS-14 ligand which binds to all SS receptor subtypes with comparable affinity produced monophasic manner of ligand displacement in the neuroblastoma cell line ($pK_i = 9.5 \pm 0.1$, $n = 3$), the pituitary ($pK_i = 8.9 \pm 0.2$, $n = 3$) as well as the cerebral cortex ($pK_i = 9.5 \pm 0.1$, $n = 3$) as expected.

4. Discussion

The present study demonstrates expression of multiple SS receptor transcripts (SSTR2 and SSTR5) in the human neuroblastoma cell line LA-N-2. The receptor binding data suggest that both of these transcript are translated into receptor proteins. To our knowledge this is the first report where experimental evidence for coexistent SS receptors has been provided by detection of receptor subtype transcripts as well as radioligand binding studies. Furthermore this is one of few studies presenting experimental data on which SS receptor subtypes are present at high densities in human cerebral cortex and pituitary.

Four of the SS receptor genes are unspliced, while the SSTR2 gene has an intron generating the SSTR2A and SSTR2B transcripts [20]. Thus we were unable to locate our primers in different exons, but used primer pairs of the human SS gene located in separate exons as well as the intron to reveal possible contamination with genomic DNA. The inclusion of a RT-PCR adapted RNase-free DNase as well as repeated RNA extraction was found necessary to eliminate trace amounts of genomic DNA as well as DNase with remaining stability of mRNA and nascent cDNA. The PCR-amplified bands were of expected size on the agarose gel and the authenticity of both receptor transcripts was verified by two restriction endonucleases.

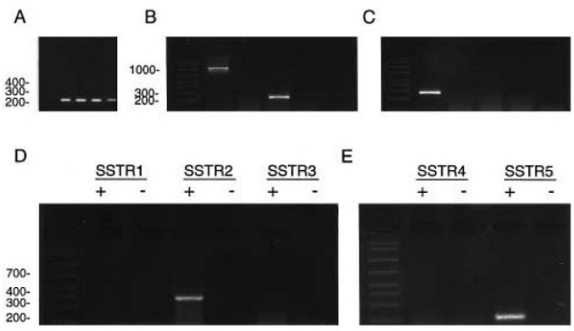

Fig. 1. PCR amplification of cDNA from the human neuroblastoma cell line LA-N-2. Amplification reactions (15 μl) were electrophoretically separated on a 3.0% agarose gel. The stringency of primer annealing was evaluated using the somatostatin transcript as a positive control (lanes 1–4: 55°C, 60°C, 65°C and 70°C: A). Absence of genomic DNA was demonstrated in control experiments with primers located in separate exons (B) as well as the intron (C) (lanes 1–5: genomic DNA, total RNA, cDNA, mixture of RT reagents and H_2O). Various primer pairs were used to reveal expression of somatostatin receptor transcripts in total RNA preparation assayed in the presence (+) or absence (−) of reverse transcriptase (D and E).

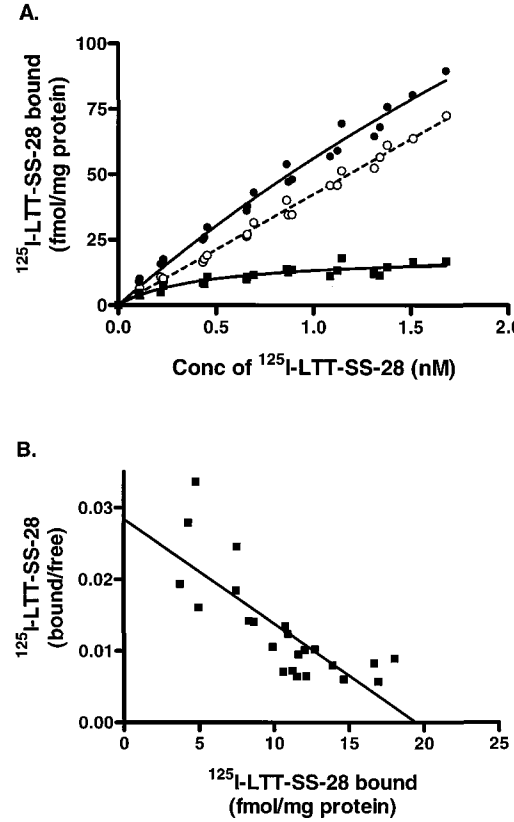

Fig. 2. [^{125}I]LTT-SS28 binding to synaptosomal membrane preparations of human medial frontal cortex with increasing concentration of ligand. Total (●), specific (■) and non-specific (○) binding were measured as described in Section 2 (A). Scatchard transformation of specific [^{125}I]LTT-SS28 binding data (B). Each point represents the mean of binding data obtained from three separate experiments.

A number of SS analogues with partial subtype specificity have been designed and pharmacologically characterized [21,22]. Recently several of these SS analogues were screened for their subtype selectivity against all five human receptor subtypes under identical experimental conditions [17]. We have adapted these assay conditions in our experiments, since the LA-N-2 cell line is of human origin. The ligand, [^{125}I]LTT-SS-28, displayed expected binding characteristics when tested in saturation binding experiments against human cerebral cortical tissue [23,24].

In the current experiments only those SS analogues with pronounced selectivity for a particular receptor subtype/subtypes were used. At low concentrations the cyclic ligand CGPIII partially displaced [^{125}I]LTT-SS-28 binding to the neuroblastoma cell line suggesting the presence of SSTR5 receptor protein. The DC23-60 ligand, with high affinity for SSTR2 and SSTR5 subtypes, displayed an almost complete

displacement of [^{125}I]LTT-SS-28 binding to the neuroblastoma cell line. Thus the SSTR2 receptor subtype appears to be the predominant one in the neuroblastoma cell line LA-N-2, with low densities of the SSTR5 receptor subtype. The prominence of the SSTR2 subtype in neuroblastoma tumors is in agreement with other studies [25,26]. Interestingly both the SSTR2 and SSTR5 subtypes have been demonstrated to inhibit cell proliferation, although by distinct intracellular mechanisms [27].

The SSTR5-selective analogue, CGPIII, displaced [^{125}I]LTT-SS-28 binding to pituitary tissue membranes with high affinity suggesting that this receptor subtype is frequent in the pituitary. In contrast the density of the SSTR2 receptor subtype seemed to be negligible, since low concentrations of DC23-60 had no additional effect on binding displacement. The SMS201-995 ligand displaced [^{125}I]LTT-SS-28 binding to a slightly higher degree in human pituitary membranes suggesting low densities of the SSTR3 receptor. Thus the SSTR5 subtype and an additional receptor subtype are predominant in the human pituitary. The SSTR5 subtype is the only subtype which preferentially binds SS-28 [28]. Interestingly others have demonstrated a binding selectivity for SS-28 as compared to SS-14 in pituitary membranes [29], as well as increased potency for inhibition of GH and TSH secretion [30,31]. However, our findings of low SSTR2 receptor density are rather surprising since the SSTR2 subtype has been considered crucial for hypothalamic-pituitary functions [8,21,22]. It should, however, be considered that the intrinsic activity for most of the SS analogues has been less well studied and that many SS analogues simultaneously display high affinity for SSTR2 and SSTR5 [17]. Furthermore, species differences might puzzle studies since the amino acid residues involved in receptor interaction with artificial ligands are likely less evolutionarily conserved [32].

In the human medial frontal cerebral cortex the density of the SSTR5 subtype appeared to be low, since the CGPIII ligand was unable to displace [^{125}I]LTT-SS-28 binding at low concentrations. This finding is supported by other studies showing low levels of the SSTR5 mRNA in the cerebral cortex [28]. The DC23-60 ligand displaced most of the [^{125}I]LTT-SS-28 binding with high affinity in the cerebral cortex. We conclude that the SSTR2 subtype is the major SS receptor subtype in the human cerebral cortex with low concentrations of SSTR3, since the SMS201-995 ligand additionally displaced [^{125}I]LTT-SS-28 binding to a slight degree. This finding is also supported by others showing a predominance of SSTR2 in the cerebral cortex of monkey [33]. Thus SSTR2-selective analogues, particularly of nonpeptidergic character, appear to be of interest in order to evaluate possible influence of SS on higher cognitive functions [5,34].

The affinities found for SS-14 and the high-affinity interaction for the SS analogues DC23-60 and SMS201-995 closely resemble those presented by Patel and Srikant [17]. The inhibition constant for high-affinity binding of the CGPIII analogue to human pituitary membranes is similar to that of SSTR5, while the low-affinity binding to receptor subtypes SSTR2, SSTR3 and SSTR4 was not exactly determined [17]. The only discrepancy was the affinity of the CGPIII analogue for neuroblastoma membranes which was higher than found following transient transfection of SSTR5 into CHO-K1 cells [17]. We suggest that this difference might be due either to inability to accurately determine the inhibition constant since the binding density was relatively low or to cell type-specific differences in post-translational modification of the SSTR5 receptor subtype. We are aware that cell type-specific post-translational modification in general might puzzle the binding data, thus the optimal control would have been transfected LA-N-2 cell cultures run in parallel with native cell cultures [35]. However, these differences probably exert a minor influence on the radioligand binding studies since the ligands chosen differ in their affinity for the receptor subtypes by several orders of magnitude.

The purpose for neuronal coexistence of neuropeptide receptor subtypes might seem elusive since SS-14 displays almost identical affinity for all of the human SS receptor subtypes, although the fifth SS receptor subtype preferentially binds SS-28 [28]. However, intracellular targeting mechanisms could regulate the trafficking of receptor subtypes differentially resulting in distinct cellular localization. Thereby the SS receptor subtypes could fulfill separate aims of SS signaling in terms of both compartmentalization and signal transduction. An alternative theory could be that other yet undiscovered neuropeptides display high affinity for certain SS receptor subtypes as has been shown for the tachykinin family of neuropeptides [36]. Recently a brain-derived cDNA clone with a high degree of structural similarity to the preproSS gene at the carboxy-terminus was found [37]. The putative neuropeptide derived from the prohormone, termed cortistatin-14, exerted pharmacological actions via SS receptors. Interestingly the electrophysiological effects differed from those of SS-14. An appealing hypothesis is that cortistatin is a SS-related peptide which acts via certain SS receptor subtypes and perhaps even via some of the phylogenetically related opioid receptor subtypes [38]. The mechanisms and functional consequences by which SS-immunoreactive and putative SS-related peptides mediate signaling via coexistent SS receptors remain to be established.

Acknowledgements: We thank Prof. Bengt Winblad for access to human postmortem brain tissue from the Huddinge Brain Bank; Dr.

Table 2
Inhibition constants (pK_i) for SS-14 and SS analogues to LA-N-2 cell line and human postmortem brain material

	SS-14	SMS201-995	DC23-60	CGPIII
LA-N-2 cell line	9.5 ± 0.1 (3)	9.7 ± 0.1 (4)	8.8 ± 0.1 (4)	10.3 ± 0.9 (5) 5.8 ± 0.2 (5)
Medial frontal cortex	9.5 ± 0.1 (3)	9.2 ± 0.1 (3)	8.4 ± 0.1 (4)	6.1 ± 0.1 (3)
Pituitary	8.9 ± 0.2 (3)	8.6 ± 0.3 (4)	8.5 ± 0.2 (3)	8.8 ± 0.3 (4)

Results are the mean ± S.E.M. Numbers in parentheses denote numbers of separate experiments.
Binding data were fitted to one-site and two-site models and IC_{50} values determined by nonlinear regression analysis using the GraphPad Prism 2.0 software (GraphPad, San Diego, CA). Values of the inhibition constants (pK_i, $-\log K_i$) were estimated from competitive binding using the equation $K_i = IC_{50}/(1+L/K_d)$ where L is the concentration of the radioligand [19].

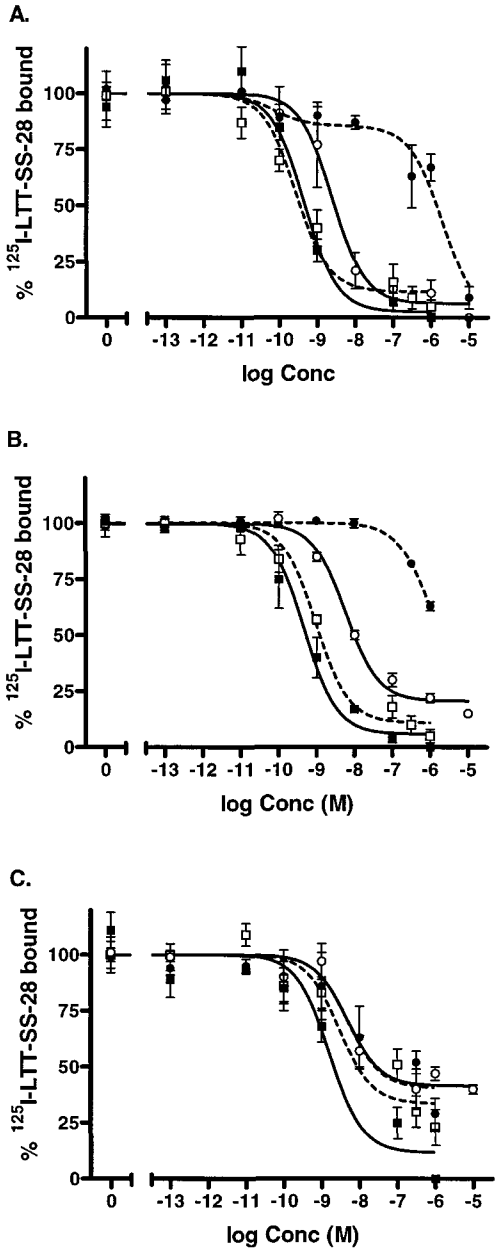

Fig. 3. Competitive inhibition of [^{125}I]LTT-SS28 binding to membrane preparations from the neuroblastoma cell line LA-N-2 (A), the human medial frontal cerebral cortex (B) and human pituitary (C). SS14 (■) as well as the SS analogues (SMS201-995 (□), CGPIII (●) and D23-60 (○)) with varying subtype selectivity were used as displacers. Results are the mean ± S.E.M. of 3–5 separate experiments. Total binding and non-specific binding was 4547 ± 331 and 1593 ± 69 cpm (human medial frontal cortex), 1509 ± 155 and 647 ± 116 cpm (human pituitary), 1126 ± 110 and 673 ± 67 cpm (the LA-N-2 cell line).

Nenad Bogdanovic for neuropathological examination and Mrs. Inga Volkmann for technical assistance; Prof. Jens F. Rehfeld for valuable discussion and criticism of the manuscript; Dr. William Murphy and Dr. D. Coy for providing us with DC23-60; Mr. Liu Zhurong for assistance with iodination and HPLC purification of LTT-SS-28. This research was financed by grants from: Swedish Medical Research Council (12X-05664-16C), European brainbank network program, Gamla tjänarinnor Foundation, Sigurd and Elsa Golje Memorial Foundation, Åke Wibergs Foundation, Martin Rinds Foundation and Loo och Hans Ostermans Foundation. L.N. was supported by postdoctoral fellowships from the STINT Foundation and Riksbankens Jubileumsfond.

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