FEBS Letters 372 (1995) 88–92 FEBS 16030

Somatostatinergic phenotype markers in the human neuroblastoma cell-line LA-N-2

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Received 3 August 1995

Abstract **We have characterized somatostatinergic phenotype markers of the human neuroblastoma, LA-N-2. A single mRNA-transcript (~850bp) and two cellular somatostatin immunoreactivity forms, a high molecular weight form (M_r 15,000) and a fragment corresponding to somatostatin-28 was found, while the somatostatin-14 peptide was absent. Saturation binding experiments demonstrated a single class of high-affinity somatostatin receptors with K_d and B_{max} of 0.27 ± 0.03 nM and 45 ± 1 fmol/mg protein. Partial G-protein uncoupling (30%) was demonstrated, using GTPγS, with an affinity of 9.7 nM. The LA-N-2 cell line, previously shown to be cholinergic, may serve as a simplified system to elucidate heterologous neurotransmittor interactions. Such studies are of interest since dysfunctions of the cholinergic basal forebrain neurons and somatostatin immunoreactive interneurons have been consistently observed in Alzheimer's disease.**

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

1. Introduction

Somatostatin (SS-14) is a tetradecapeptide originally identified in the hypothalamus as an inhibitory factor of growth hormone secretion [1]. Several SS-immunoreactive (SS-ir) peptides are derived from a common precursor molecule (proSS) through tissue-specific processing at dibasic or monobasic amino acids, in similarity to other neuropeptide systems. Prohormonal processing at the C-terminal segment generate two biologically active peptides, SS-14 and SS-28, as well as the non-somatostatin peptides pro-SS(1–76), pro-SS(1–64) and SS-28(1–12). More recently the peptide fragment antrin, proSS(1–10), derived through processing at the N-terminal portion of proSS has been described [2].

Although highest peptide concentrations are found in hypothalamic regions, such as the median eminence, later studies have revealed a much wider distribution of SS-ir neurons in the central nervous system (CNS) [3,4]. SS-containing neurons in the cerebral cortex and hippocampus has been suggested to influence cognitive functions, since this neuropeptide system is consistently affected in Alzheimer's disease [5]. Behavioral studies showing that transient depletion of central SS-ir with cysteamin impairs performance on passive avoidance retention testing have strengthened this reasoning [6,7]. Neuropeptidergic transmittors are generally classified as conveying slow signalling with a prolonged modulating response, as opposed to classic transmittors with a rapid mode of action. Thus impaired functional state of a neuropeptidergic neuronal population might affect pre- or postsynaptical responses mediated by fast-acting neurotransmittors. Iontophoretic application of SS-14 have been shown to enhance ACh-induced excitation in hippocampal and cortical cells [8]. SS-ir peptides have also been demonstrated to enhance K^+-evoked release of acetylcholine, when applied exogenously to hippocampal slice preparations [9].

Neuroblastoma cell lines may serve as a simplified model system to elucidate such molecular interactions with heterologous neurotransmittor systems. The neuroblastoma cells constitute a clonal homogenous populations of cells, as opposed to the mature central nervous system which is highly complex with a large number of terminally differentiated neuronal and glial cell types. Furthermore the cell cultures can be grown indefinitely in vitro, which is advantageous compared to dissociated primary cell cultures and brain tissue explants. The aim of the present study was to identify a human neuroblastoma cell line expressing somatostatinergic phenotype markers. The neuroblastoma, LA-N-2, was the only cell line found expressing preproSS-mRNA, SS-ir peptide fragments and [^{125}I]Tyr^{11}-SS-14 binding sites, among a variety of differentiated and non-differentiated cells lines initially screened for preproSS-mRNA expression.

2. Materials and methods

2.1. Cell culture

The neuroblastoma cell line LA-N-2 (passage 117–125) was grown at 37°C in RPMI-1640 culture medium (GibcoBRL, Life Technologies, Scotland) 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 exchanged 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. Pepstatin (1 μM; Boehringer) was supplemented to the PBS/EDTA solution when indicated. The cell pellet used for RNA preparation, radioimmunoassay and [^{125}I]Tyr^{11}-SS-14 receptor binding was obtained by centifugation at $1000 \times g$ for 5 min at 25°C.

2.2. Animals

Adult male Sprague–Dawley rats (Alab, Sollentuna, Sweden) were kept on a 12 h on/12 h off lighting schedule in a room thermostaticly maintained at 22 ± 1°C. Ad lib food (Lab Chow R36, Laktamin, Vadstena, Sweden) and water was available. The animals were decapitated

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SSDI 0014-5793(95)00958-2

and hypothalamic tissue, including the preoptic area, was rapidly dissected on ice and frozen on dry ice.

2.3. RNA preparation and Northern blot analysis

Total cellular RNA (~2×10^7 cells) were extracted [10] and checked for degradation by agarose gel electrophoresis and quantification of UV absorption at 260/280 nm. Total RNA (10 μg) was electrophoretically separated on a 1.2% (w/v) agarose gel containing 0.7% (v/v) formaldehyde gel in 1 × MOPS (1 × MOPS is 0.02M 3-*N*-morpholinopropanesulphonic acid, 8 mM sodium acetete, 1 mM EDTA, pH 7.0) followed by transfer onto Amersham Hybond-N$^+$-filter in 25 mM sodium phosphate buffer (pH 6.4). Prehybridization was carried out for 4 h at 65°C with 5 × SSPE (1 × SSPE in 0.15 M NaCl, 0.01 M $Na_2H_2P0_4$ 0.001 M EDTA, pH 7.4), 5 × Denhardt's solution, 0.5% sodium dodecylsulphate (SDS) and 20 μg/ml heat denatured salmon sperm DNA. Hybridization was performed over night at 55°C with 4 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.02 M PBS, 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 1% sarcosyl. The 48mer oligodeoxynucleotide probe, complementary to amino acids 96–111 (98% homology to human prepro-SS-mRNA), was 3'-end-labeled with ^{32}P-dCTP to a specific activity of ~0.50×10^9 cpm/μg probe. The nylon membrane was subsequently washed 4 times (15 min in each cycle) at 65°C with 1 × SSC, 0.1% SDS. The bands were visualised by exposure for 24 h at −70°C against Amersham MP films, using a Amersham intensifying screens.

2.4. Radioimmunoassay

Pelleted cells (2.5×10^6cells/ml 1 M HAc) and hypothalamic rat tissue (10 v/w of 1 M HAc) were dissolved and boiled in heated acetic acid for 10 min, cooled on ice and centrifuged at 17,600 × *g* for 20 min at 4°C. Pepstatin (0.15 mM; Boehringer) was supplemented to acetic acid when indicated. Aliquots of the supernatant were stored at −70°C. Hypothalamic tissue was homogenized with a teflon/glass homogenizer prior to centrifugation. The SS radioimmunoassay (RIA) was performed using a C-terminal-directed antisera (Amersham International plc, UK). [^{125}I]Tyr^{11}-SS-14 (3,500 cpm, 1.3 pg; Amersham Int. plc, UK) was used as tracer and somatostatin (Peninsula, Belmont, CA, USA) was used as standard. The samples and other substrates were diluted in 50 mM sodium phosphate buffer (pH 7.2) containing 10 mM EDTA and 0.5% bovine serum albumin. Sample or standard (25 μl) was mixed with 100 μl of tracer and 100 μl of antisera and incubated at 4°C for 24 h. Separation of bound and free peptide was achieved by adding 200 μl of a mixture containing 0.25% charcoal and 0.025% dextran T-70 in 50 mM sodium phosphate buffer (pH 7.2). The mixture was incubated on ice for 10 min and centrifuged at 9,500 × *g* for 1 min. A fixed volume (350 μl) of the supernatant was measured in a γ-counter. The detection limit (ED_{80}) was approximately 1.5 fmol/tube for the SS RIA.

2.5. Gel permeation chromatography

Extracted samples were applied to a Sephadex G-50 column (superfine, 110 × 1.5 cm) equilibrated with 0.1 M acetic acid at 4°C. The column was calibrated with the following molecular weight markers: dextran blue, cytochrome *c*, aprotinin, glucagon and sodium chloride. The samples were eluted with 0.1 M acetic acid at a flow rate of 8 ml/h, and collected in fractions of 2 ml for measurement of SS-ir.

2.6. [^{125}I]Tyr^{11}-somatostatin-14 receptor binding assay

Pelleted cells (~10^8cells) were dissolved in 5 ml ice-cold 50 mM Tris-HCl (pH 7.4), homogenized with a glass-teflon homogenizer and centrifuged at 700 × *g* for 10 min at 4°C. The supernatant was centrifuged at 14,000 × *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 Tris-HCl buffer (pH 7.4). The mixture was centrifuged at 11,000 × *g* for 30 min at 4°C and the pellet resuspended in fresh buffer to give a protein concentration of approximately 0.3 μg/μl. Dilutions of peptides and radioactive ligand were made in the incubation buffer which consisted of 50 mM Tris-HCl (pH 7.4) containing 0.5 mg/ml bovine serum albumin (BSA), 5 mM $MnCl_2$ and 0.03 mg/ml bacitracin. Binding assays were performed in precoated Eppendorf tubes (0.5 mg/ml BSA in redistilled water). Membrane preparation (~40 μg protein) and [^{125}I]Tyr^{11}-SS-14 (2000 Ci/mmol) was incubated for 60 min at 30°C with or without unlabeled SS-14 in a final volume of 200 μl. The incubation was terminated by centrifugation at 13,000 × *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 somatostatin. The effect of the guanidine nucleotide analogue, GTPγS, on [^{125}I]Tyr^{11}-SS-14 binding was studied as above at a ligand concentration of 30 pM with the inclusion of a range of GTPγS concentrations (1 nM–100 μM) in the assay buffer. All assays were performed in triplicates. Protein determinations were measured by the method of Lowry et al. [11].

3. Results

Various cell lines were examined in an initial screening procedure for expression of preproSS-mRNA by Northern blot analysis using a ^{32}P-labeled rat preproSS cRNA-probe. One human neuroblastoma, LA-N-2, among several differentiated and non-differentiated cell lines gave a positive hybridization signal. The following cell lines did not express detectable levels of preproSS-mRNA: SH-SY5Y, SK-N-MC, SK-EP (Fig. 1), LA-N-1, LA-N-5, IMR-32 and SK-N-BE2 (data not shown). The positive result of the screening procedure was confirmed by hybridization to a 48mer oligodeoxynucleotide probe. The analysis revealed the presence of a single transcript (~850 nucleotides) which migrated in parallel with preproSS-mRNA detected in total-mRNA prepared from rat brain tissue (Fig. 1). The LA-N-2 cell line was also found to contain SS-ir in pelleted cells at a concentration of ~2 fmol/μg protein. Characterization of the cellular SS-ir by gel permeation chromatography (Sephadex G-50 column, superfine, 110 × 1.5 cm) and measurements with a C-terminal directed antisera of the fractionated material showed presence of several immunoreactive peptide fragments. The elution profile was compared with SS-ir extracted from the rat hypothalamus. Hypothalamic tissue contained three major peaks of SS-ir. The predominant peak coeluted with SS-14 (M_r 1,600), the median peak with SS-28 (M_r 3,000) while the first peak had an apparent molecular weight of 15,000 (Fig. 2a). The elution profile of SS-ir extracted from the neuroblastoma LA-N-2 cell line was different (Fig. 2b). The

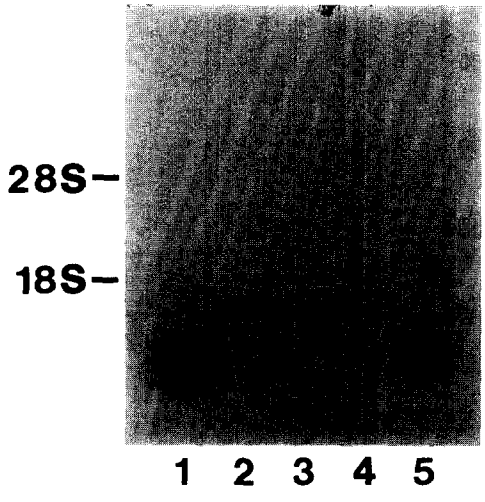

Fig. 1. Total mRNA (10 μg) prepared from various neuroblastoma cell lines (LA-N-2 (1), SK-N-MC (2), SH-SY5Y (3), SK-EP (4)) and rat brain tissue (5) hybridized with a ^{32}P-labeled preproSS-oligodeoxynucleotide probe. The RNA samples were separated on a denaturing 1.2% formaldehyde-agarose gel and blotted onto Hybond-N$^+$-filter. The positions of 28S and 18S rRNA are indicated in the figure.

last peak corresponded to synthetic SS-28, while the two larger peaks had apparent molecular weights of 6,000 and 15,000 Da. Pooled SS-ir material from both of these two peaks were measurable with a N-terminal directed antisera 2098 (recognition sequence aa 20–36 [12]). The first peak (15,000 Da) eluted at identical position to the prohormonal form detected in the rat hypothalamus. Presence of pepstatin during detachment of the cells (1 μM) and acetic acid extraction (0.15 mM) of pelleted cells abolished the 6 kDa peak. No immunoreactivity was detected in fractions corresponding to synthetic SS-14. The release of SS-ir into the cell medium was ~0.3 fmol/μg protein/h following a 4 h incubation period with fresh medium. Fresh cell medium was absent of SS-ir when checked with the C-terminal directed antisera. Specific binding of [^{125}I]Tyr^{11}-SS-14 to synaptosomal membrane preparations of LA-N-2 cells was observed with a rapid association kinetics, reaching half-maximal levels within 10 min (data not shown). [^{125}I]Tyr^{11}-SS-14 binding was studied in saturation binding experiments using different ligand

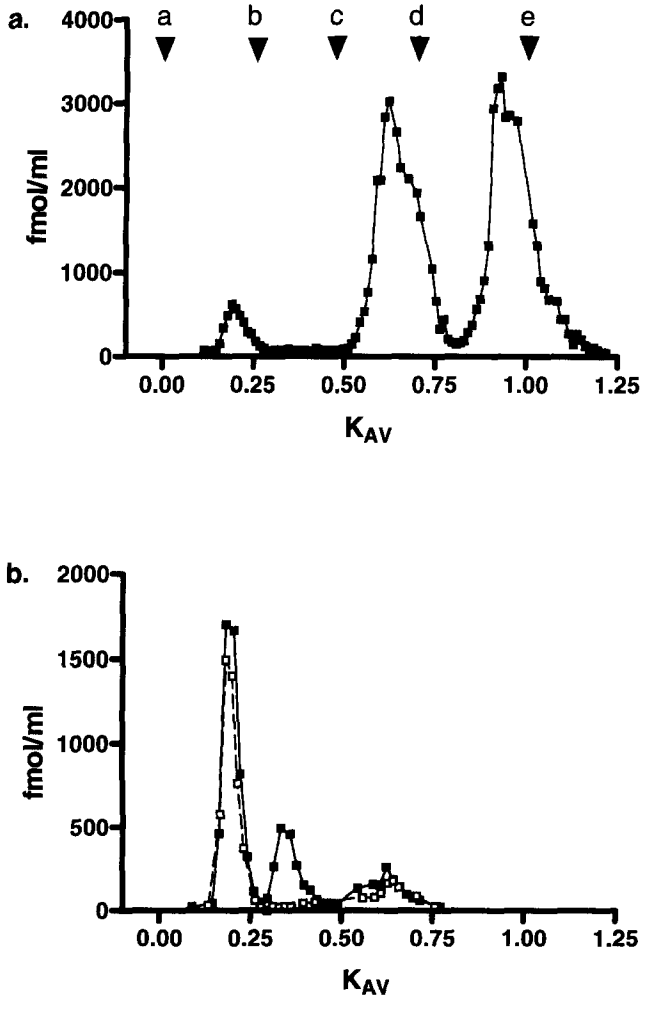

Fig. 2. Sephadex G-50 (superfine, 110 × 1.5 cm) gel permeation chromatography performed on rat hypothalamic tissue (a) (70 mg tissue) and pelleted LA-N-2 cells extracted in 1 M HAc, in absence (■) or presence (□) of 0.5 mM pepstatin (b) (~10^8 cells). The SS-immunoreactivity was eluted with 0.1 M acetic acid at a flow rate of ~8 ml/h and analyzed with a C-terminal directed antisera for SS-14 (Amersham, UK) in fractions of 2 ml. The arrows indicate different molecular weight markers: (a) blue dextran (M_r = 200,000), (b), cytochrome *c* (M_r = 12,400), (c) aprotinin (M_r = 6,500), (d) glucagon (M_r = 3,500), (e) sodium chloride (M_r = 58).

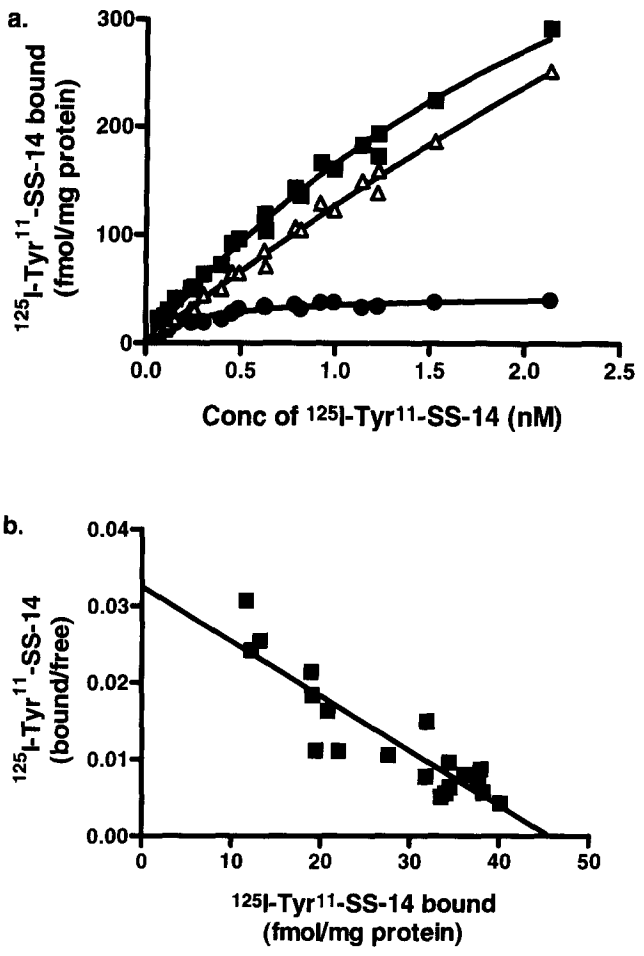

Fig. 3. (a) [^{125}I]Tyr^{11}-SS-14 binding to synaptosomal membrane preparations of LA-N-2 cells with increasing concentration of ligand. Total (■), specific (●) and non-specific (△) binding were measured as described in section 2. Values were obtained from three independent experiments. Each point represents the mean of three determinations. (b) Scatchard transformation of specific [^{125}I]Tyr^{11}-SS-14 binding data in (a).

concentrations. Specific binding was saturable, while non-specific binding increased linearly with increasing concentration of ligand (Fig. 3a). Scatchard analysis was linear and the Hill coefficient (0.93 ± 0.01 (n = 3)) was close to unity, indicating a single class of high-affinity binding sites. The data was fitted into a one-site model with K_d and B_{max} of 0.27 ± 0.03 nM and 45 ± 1 fmol/mg protein, respectively (n = 3, Fig. 3b). Furthermore the sensitivity of G-protein uncoupling of [^{125}I]Tyr^{11}-SS-14 specific binding was studied by adding different concentrations of GTPγS to the incubation mixture. The stable guanidine nucleotide analogue, GTPγS, partially inhibited specific binding in a concentration-dependent manner with an affinity of 9.7 nM and a maximum inhibition of 30% (Fig. 4).

4. Discussion

The present study describes the characterization of a neuroblastoma cell line with a somatostatinergic phenotype. Previous studies have shown that neuroblastomas may express somatostatin receptors [13–14], however to our knowledge there has been no reports on undifferentiated human neuroblastomas

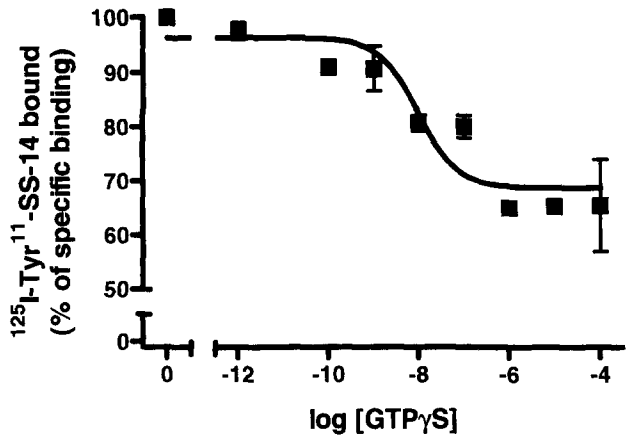

Fig. 4. % specific [^{125}I]Tyr^{11}-SS-14 binding to synaptosomal membrane preparations of LA-N-2 cells with increasing concentration of GTPγS. Total binding and non-specific binding in the absence of GTPγS was 3137 ± 73 cpm and 992 ± 52 cpm respectively (mean ± S.E.M.). Non-specific binding was defined as binding in the presence of 1μM unlabeled SS-14.

containing preproSS-mRNA and SS-ir peptides. Our findings suggest that this phenotypic feature is rare, since various non-differentiated and differentiated cell lines tested were negative for preproSS-mRNA expression. Northern blot analysis revealed the expression of a single transcript with a estimated size of ~850 bp, in agreement with previous human and rodent molecular cloning studies [15–16]. Gel permeation chromatography performed on cell pellet extracts from the neuroblastoma LA-N-2 resulted in one peak with an apparent molecular weight of 15 kDa using a C-terminal antisera. The elution position was identical with the first peak detected in hypothalamic tissue extracts, previuosly shown to represent a prohormonal form [17]. Furthermore pooled material of this peak was shown to have reactive epitopes against a N-terminal antisera 2098 (recognition sequence aa 20–36 [12]) suggesting identity with the prohormone, proSS(1–92), which is generated by removal of the N-terminal signal sequence. The estimated molecular weight of the plausible prohormone found in this study as well as other studies [17–18] was too high compared with the actual molecular weight deduced from sequence analysis (M_r 10,388). This variation is likely due to some interference of the prohormone with the Sephadex gel matrix. The second peak identified with the C-terminal directed antisera had an apparent molecular weight of 6 kDa and showed N-terminal immunoreactivity against the antisera 2098. A SS-ir fragment of similar size has been previously identified in the rat brain [19–20]. This fragment probably corresponds to proSS(33–92) generated through cleavage at the Leu_{32}–Leu_{33} bond, although the genuity of this position as a post-translational processing event has been questioned [21]. Inhibition of aspartate proteases with pepstatin during the extraction procedures eliminated the formation of this SS-ir form, consistent with previous findings [21]. The third peak eluted at a position identical to the peak of SS-28 detected in the hypothalamic tissue extract. The most striking difference compared to the rat hypothalamic tissue was the absence of the SS-14-ir fragment in the LA-N-2 cell extract. The most probable explanation is dissimilarities in activity of processing enzymes, which could be caused by different intravesicular milleu or basal expression of critical processing enzymes. The presence of independent processing pathways resulting in two separate end-products (SS-28 and SS-14) with possibly diverse transmittor function are evident [21, 22], although SS-28 may serve as a substrate for SS-14 through cleavage by converting enzyme at the dibasic amino acids (Arg_{13}–Lys_{14}) [23]. The subtilisin-like serine endoprotease, PC1, is likely to convert proSS(1–92) into SS-14, since expression of PC1 in COS-7 cells increased SS-14-ir in cells and medium [24]. Provided that e.g. differentiating conditions of the LA-N-2 cell line could activate the critical processing enzyme the current finding raises the possibility to identify additional genes by means of subtractive cDNA-cloning.

SS-ir peptides have been shown to regulate diverse signalling pathways such as adenylyl cyclase activity, Ca^{2+} and K^+-currents [25]. These cellular actions are mediated by membrane bound receptors frequently coupled to pertussis toxin sensitive G-proteins. The present data demonstrates expression of high-affinity [^{125}I]Tyr^{11}-SS-14 binding sites with sensitivity to G-protein uncoupling in the neuroblastoma LA-N-2, which is characteristic for SS-receptors. Partial inhibition of specific binding by GTPγS at similar concentrations have been previously observed in transfection systems [26] as well as in rat brain tissue [27]. Five different SS receptor subtypes, all belonging to the family of G-protein coupled receptors, have been cloned during the last years [28]. Signal transduction studies performed on the subtype receptors in transfection systems are confusing. A recent report illustrates that all SS receptor subtypes are capable of G-protein mediated adenylyl cyclase inhibition provided that the receptors are expressed in cellular strains expressing the proper inhibitory G-proteins [29]. Thus conclusion on particular SS-receptor subtype/subtypes expressed in the cell line requires further molecular biology studies or access to non-commercial ligands with subtype-specificity. The LA-N-2 cell line, originally derived from a primary neuroblastoma tumor, was established by Seeger et al. [30]. Later studies have demonstrated expression of cholinergic phenotype markers in this cell line, such as choline acetyltransferase (ChAT) activity [31], choline uptake, high levels of acetylcholine and potassium induced release of the transmittor [32]. Activation of phospholipase C and D by carbachol was inhibited by atropine indicating expression of cholinergic receptors by the LA-N-2 cell line [33]. Furthermore the cells responded to retinoic acid [31], ChAT developmental factor and basic fibroblast growth factor with altered ChAT activity [34].

Human neuroblastoma cell lines are frequently used in studies on neuronal functions. The homogenous populations of cells is particularly advantageous when elucidating regulatory mechanisms, such as transcriptional regulation [35], translation and prohormonal processing. The findings of attenuated maturation of peptide processing in transcriptionally active systems implies that these events might be separately regulated [36]. The identification of a cell line expressing somatostatinergic phenotype markers enables such regulatory studies on somatostatin biosynthesis. Furthermore the previous findings of various cholinergic markers in the LA-N-2 cell line renders possibilities to investigate interactions between the somatostatinergic and cholinergic systems. Such studies are of marked interest since dysfunctions of the cholinergic basal forebrain neurons as well as cortical and hippocampal SS-ir interneurons have been consistently observed in Alzheimer's disease.

Acknowledgements: The authors would like to thank Prof. Jens F. Rehfeld for valuable discussion and criticism of the manuscript. Prof. Jens J. Holst and Dr. Marianne Dryer for assistance with the N-terminal directed radioimmunoassay. This research was financed by grants from: Swedish Medical Research Council (12X-05664–11A), Ax:son Johnson Foundation, Gamla tjänarinnor Foundation, Åke Wibergs Foundation, Swedish Society for Medical Research, Loo och Hans Ostermans Foundation and the Swedish Alzheimer Foundation.

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