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Sialylation of Human Thyrotropin Receptor Improves and Prolonges Its Cell Surface Expression

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Running title: TSHR sialylation

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non-standard abbrevations

TSHR	thyrotropin receptor
GPCRs	G protein-coupled receptors
SIAT1	β-galactoside α(2,6)-sialyltransferase
SIAT4a	β-galactoside α(2,3)-sialyltransferase
AFTNs	autonomously functioning thyroid nodules
ST	normal surrounding thyroid tissue

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Abstract

Glycosylation of the thyrotropin receptor (TSHR) has been shown to be essential for correct protein folding and for cell surface targeting. In a recent study we detected increased expression of β-galactoside α(2,6)-sialyltransferase (SIAT1) in toxic thyroid adenomas where gain-of-function mutations of the TSHR have been invoked as one of the major causes. To investigate the physiological meaning of these findings we designed experiments to evaluate the consequences of sialylation for the expression of the TSHR. Hence we investigated the effect of co-expressing the TSHR and different sialyltransferases (SIAT1, SIAT4a and SIAT8a) for cell surface expression of the receptor. Coexpression of each of the three SIAT isoforms and the TSHR in COS-7 cells increased TSHR expression on the cell surface in the range of 50-100%. Moreover, western blot analysis with lectins specific for α(2,3) and α(2,6)-linked sialic acids and lectin-binding elisa support a direct effect on TSHR cell surface expression mediated by sialic acid transfer to the TSHR. Finally, we treated living COS-7 cells after cotransfection of TSHR and SIAT8a with neuraminidase for 30 min to remove covalently linked sialic acid. Subsequent loss of TSHR cell surface expression suggests that sialylation prolongs the resting time of the TSHR on the cell surface. Our data demonstrate for the first time that the transfer of sialic acid can improve and prolong cell surface expression of a transmembrane receptor.

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Introduction

Thyrotropin (TSH) signaling through its receptor mediates the paracrine control of thyroid function. The thyrotropin receptor (TSHR) belongs to a subgroup of G protein-coupled receptors (GPCRs) comprising the TSHR, the follicle stimulating hormone receptor (FSHR) and the Lutropin-choriogonadotropic hormone receptor (LH/CGR). These three GPCRs are characterized by large, heavily glycosylated extracellular domains that form the hormone-binding sites (Sanders *et al.*, 1997). In the human thyroid, the TSH-bound TSHR is first synthesized as a ~84 kDa polypeptide chain to which high mannose type carbohydrates are attached in the endoplasmatic reticulum (ER) (100 kDa-fragment). These are further processed to mature, complex type carbohydrates in the Golgi apparatus (120 kDa-fragment) (Nagayama *et al.*, 1998;Rapoport *et al.*, 1996). Whereas the 100 kDa-band in principle contains mannose type sugars, the 120 kDa-band in principle contains complex type sugars like fucose-linked [α(1-6)] N-acetylglucosamin, [α(1-3)] N-acetyllactosamin, [β(1-4)] N-acetylglucosamin and α(2,3) linked sialic acids (Oda *et al.*, 1999). Radioligand binding assays showed that 80% of radiolabeled TSH was bound to the 120 kDa-band (Oda *et al.*, 1999). Analysis of recombinant TSHR led to the conclusion that the TSHR-ectodomain contains six glycosylation sites (Asn-77, -99, -113, -177, -198 and -302), at least four of them have to be glycosylated to express a functional TSHR (Nagayama *et al.*, 2000).

An amino acid substitution of Asn-113 to Gln-113 disrupted TSH binding and TSH-stimulated cAMP synthesis, very likely because of altered TSHR conformation caused by impaired glycosylation (Nagayama *et al.*, 2000). However, deglycosylation of the native TSHR by PNGase F treatment does not affect autoantibody binding to the TSHR (Atger *et al.*, 1999). The importance of glycosylation has been also shown for the FSHR (Davis *et al.*, 1995). In contrast, acquisition of complex carbohydrates has not been found to be important for hormone binding in the case of the LH/CGR (Davis *et al.*, 1997;Dufau, 1998). Binding of calnexin (a lectin-like molecular chaperone, which facilitates correct protein folding (Helenius, 1994)) has been demonstrated for the immature forms (high mannose type carbohydrates) of all three glycoprotein hormone receptors (Rozell *et al.*, 1998;Mizrachi and Segaloff, 2004).

In addition to forming part of the binding site for the ligand, oligosaccharides synthesized and processed in the ER or the Golgi apparatus are crucial for protein folding and intracellular trafficking, respectively (Nagayama *et al.*, 1998). In case of the TSHR inhibition of N-linked glycosylation blocks cell surface expression (Nagayama *et al.*, 2000). Moreover,

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TSHR expressed in *E.coli* is not glycosylated and therefore not able to bind TSH (Huang *et al.*, 1992).

Sialic acid is a frequent component in complex carbohydrates that has been demonstrated to affect the biological activitiy or the metabolic half life of many proteins (Kaneko *et al.*, 2004;Otto *et al.*, 2004). This is also evident for the TSHR ligand and the thyroid hormone synthesis template and storage molecule thyreoglobulin (Tg) (Grollman *et al.*, 1993;Medeiros-Neto *et al.*, 1993;Persani *et al.*, 1998;Ronin *et al.*, 1986;Trojan *et al.*, 1998). An increased sialylation of TSH in hyperthyroid patients (Miura *et al.*, 1989) leads to a prolonged plasma half-life and prolonged bioactivity (Helton and Magner, 1995;Szkudlinski *et al.*, 1995). TSH not only demonstrates the ability to down-regulate steady state TSHR mRNA levels (Saji *et al.*, 1992) but also SIAT1 mRNA levels (Grollman *et al.*, 1993). Sialylation of membrane proteins, including the thyroid peroxidase, may participate in the TSH-regulated vectorial transport and maturation of Tg (Grollman *et al.*, 1993). After stimulation with TSH an increased sialylation of Tg was observed in porcine thyroid glands and follicles (Ronin *et al.*, 1986). In thyrothrophs of hypothyroid mice an increased expression of SIAT1 and SIAT4a was observed (Helton and Magner, 1995) whereas in liver cells, stimulated with thyroid hormones T_3 and T_4, expression of SIAT1 and SIAT4a was decreased (Feng *et al.*, 2000). The expression and activity of sialyltransferases are known to be regulated by thyroid hormone through negative feedback and hypothalamic thyrotropin releasing hormone (TRH), but also by sleep-related mechanisms (Persani *et al.*, 1998).

Recently, we could show a clear increase in mRNA expression of SIAT1 in autonomously functioning thyroid nodules (AFTNs) versus normal surrounding thyroid tissue (ST) (Eszlinger *et al.*, 2004). Because constitutive activation of the TSHR is an important step in the etiology of AFTNs (Krohn and Paschke, 2001) and since this increased SIAT1 expression in AFTNs might therefore be related to TSHR signaling, this findings prompted us to further evaluate the role of sialylation for TSHR function as well as SIAT expression in thyroid pathologies. We designed our experiments to study the consequences of SIAT action for the surface expression of the TSHR. Moreover we quantified SIAT1, 4a and 8a mRNA expression in thyroid nodular disease and primary cultures of thyrocytes.

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Material and Methods

Expression plasmids

A sequence-verified human TSHR cDNA cloned into pSVL (courtesy of Dr G. Vassart, Brussels) was used. Respectively for lectin binding ELISA we used a TSHR-GFP fusion protein (courtesy of Dr R. Latif, New York) where the human TSHR sequence lacking the stop codon was ligated into the mammalian expression vector pEGFP-N1 (Clontech). For SIAT8a we used the pcDNA3.1 GeneStorm Expression-ready Clone RG001497 from Invitrogen. The full-length cDNAs encoding the human SIAT1 and SIAT4a were subcloned into the eukaryotic expression vector pcDNA3.1/V5-His C (Invitrogen, Paisley, UK).

Tissue samples

All AFTNs were identified by ultrasound and scintigraphy. All preoperatively identified nodules were also identified at surgery and postoperatively characterized by histology according to the WHO criteria (Hedinger, 1988). Somatic TSH receptor mutations in the hot nodules were previously determined by denaturing gradient gel electrophoresis and subsequent direct sequencing of the positive PCR fragments (Trulzsch *et al.*, 2001). AFTNs without a TSHR mutation were screened for mutations in the exons 7 to 10 of the $G_s\alpha$ protein by direct sequencing (forward primer: 5‘-agt tgg caa att gat gtg agc–3‘, reverse primer: 5‘–tct cta taa aca gtg cag acc-3‘). However, no mutations in the $G_s\alpha$ protein were found. Informed consent for the analysis was given by the patients.

Primary thyrocyte cultures were obtained from specimens of non-nodular thyroid tissues of consecutive patients undergoing thyroid resection for treatment of their cold thyroid nodules (CTNs) were obtained at surgery. All patients with a CTN were euthyroid with normal TSH levels and negative thyroid antibodies.

RNA isolation

Total RNA was isolated from 15 AFTNs, 22 CTNs and 4 primary thyrocyte cultures using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Later, the total RNA was purified with an RNeasy kit (Qiagen, Hilden, Germany) according to the RNA clean-up protocol. The quality and quantity of the total RNAs was examined on agarose-gels and photometer (Ultrospec 3300 pro, Biochrom Ltd, Camebridge, UK).

Real time RT-PCR

One µg of total RNA was used to prepare double-stranded cDNA (M-MLV Reverse Transcriptase, Invitrogen, Karlsruhe, Germany) primed with oligo-dT. The quantification of

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three genes (SIAT 1, SIAT 4a, SIAT 8a) by real-time RT-PCR was performed using a Light Cycler (Roche, Mannheim, Germany) as previously described (Eszlinger *et al.*, 2001). The nucleotide sequences of the primer and PCR conditions are available on request. The determined ratios were normalized to the ratio of the housekeeping gene β-actin.

Cell culture and transfection

The influence of sialyltransferase expression on functional properties of the TSHR was investigated in cell culture. COS-7 cells were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. Cells were transiently transfected in 24-well plates (0,5 x 10^5 cells/well) with 0,5 µg of DNA/well (TSHR-pSVL (Libert *et al.*, 1989) and SIAT-pcDNA3.1/V5-His) for cAMP accumulation and TSH binding analysis. To determine inositol phosphate (IP) formation, cells were transiently transfected in 12-well plates (1 x 10^5 cells/well) with 1 µg of DNA/well using the FuGENE^{TM}6 reagent (Roche Applied Science).

Radioligand Binding Assay

Competitive binding studies were performed as previously described (Wonerow *et al.*, 1998). To determine the TSH radioligand binding at a similar level of TSHR cell surface expression, we balanced the effect of SIAT1 co-transfection on TSHR expression by co-transfecting equivalent amounts of the human V2 vasopressin receptor (Neumann *et al.*, 2001;Schoneberg *et al.*, 1996). Data were analyzed assuming a one-site binding model using the fitting module of Sigma Plot 2.0 for Windows (Swillens, 1995).

cAMP Accumulation Assay

Measurements of cyclic AMP (cAMP) accumulation were performed 48 h after transfection as previously described (Wonerow *et al.*, 1998).

Stimulation of IP Formation

Transfected COS-7 cells were incubated with 2 µCi/ml myo[^{3}H]inositol (Amersham Biosciences) for 8 h. Thereafter, cells were preincubated with serum-free DMEM containing 10 mM $LiCl_2$ for 30 min. Stimulation with bTSH for 1 h was performed with the same medium supplemented with 100 mU/ml bTSH. Evaluation of basal and TSH-induced increases of intracellular IP levels was performed by anion exchange chromatography as described (Berridge, 1983). IP values are expressed as the percentage of radioactivity incorporated from [H]inositol phosphates (IP1-3) over the sum of radioactivity incorporated in IPs and phosphatidylinositols.

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FACS analysis

Forty eight hours after transfection cells were detached from the dishes using 1 mM EDTA and 1 mM EGTA in PBS and transferred into Falcon 2054 tubes. Before incubation with the primary antibody, cells were washed once with PBS containing 0.1% BSA and 0.1% NaN_3. Afterwards, cells were incubated with a mouse anti-human TSHR antibody (2C11, Serotec, Oxford, U.K.; 10 µg/ml) in the same buffer for 1 h at 4°C. Tubes were washed twice and incubated for 1 h at 4°C in the dark with fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse IgG (Serotec, dilution 1:1000). Before FACS analysis (FACscan BD Biosciences), cells were washed twice and fixed with 1% paraformaldehyde. Receptor expression was determined by the fluorescence intensity, whereas the percentage of signal positive cells corresponds to the transfection efficiency.

Neuraminidase treatment

Thirty minutes, two and twelve hours before FACS-preparation, cells were treated with 0,1 U/ml neuraminidase from Clostridium perfringens (Sigma) in DMEM without FCS at 37°C and 5% CO_2.

ELISA

The cell surface expression of the TSHR determined by FACS analysis was confirmed by ELISA as previously described (Wonerow *et al.*, 2000).

Preparation of solubilized TSHR

COS-7 cells were transiently transfected in 145 cm^2 dishes (2 x 10^6 cells per dish) with 20 µg DNA/dish (1:1 pSVL-TSHR-Flag and pcDNA3.1/V5-His-SIAT) using the FuGENE TM6 reagent (Roche, Basel, Switzerland). Cells were grown to confluence for 2 days after transfection, and the cells were washed with PBS and scraped into 10 ml ice-cold buffer A (50 mM NaCl and 10 mM Tris/HCl, pH 7.5, containing 1 mmol/l phenylmethylsulfonyl-fluoride). The cells were centrifuged at 1000xg for 5 min at 4°C, the pellet was resuspended in 1 ml buffer A and homogenized with a glass homogenizer on ice. This homogenate was then centrifuged at 12000xg for 30 min at 4°C, resuspended in 0,5 ml ice-cold buffer A containing 1% Triton X-100, homogenized, and centrifuged at 90.000xg for 2 h at 4°C. The supernatant was aliquoted and stored at –80°C (Oda *et al.*, 1999).

SDS-PAGE followed by Western blotting

Aliquots of solubilized material were mixed with an equal volume of SDS-PAGE sample buffer (4% SDS, 20% glycerol, 100 mmol/l Tris/HCl (pH 6.8), and 0,002% bromphenolblau), heated to 37°C for 30 min, electrophoresed on 10% acrylamide gels (SDS-PAGE; Bio-Rad Laboratories Inc.) at 140 Volt for 110 min, and blotted onto nitrocellulose

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(Schleicher & Schuell, Inc., UK Ltd., London, UK) at 120 Volt for 120 min. The membranes were blocked using 5% low fat milk in TBST, incubated overnight at 4°C with a 1:2000 dilution of anti-FLAG M2 (Sigma) and developed using anti-mouse horseradish peroxidase conjugate (Cell Signaling) followed by SuperSignal West Pico chemiluminescence reagents (PIERCE, Rockford, USA).

Immunoprecipitation of TSHR

COS-7 cells were grown and transfected as decribed above (see preparation of solubilized TSHR). Cells were scraped into 10 ml ice-cold PBS and centrifuged at 1000xg for 5 min at 4°C. The pellet was resuspended in 50 µl lysisbuffer (25 mM Tris/HCl pH 7,4; 200 mM NaCl; 1 mM EDTA; 1% NP40; 1 mM NaF; 1 mM Na_3VO_4; 2 mM Imidazol; 1x Complete Proteaseinhibitor) and incubated 30 min at -80°C and then 1 h on ice with 5 times mixing. This homogenate was then centrifuged at 5000 rpm for 5 min at 4°C, and the supernatant was aliquoted and stored at -20°C. 30 µl mouse $IgG_1\kappa$ (1mg/ml; Sigma) were bound to 40 µl Protein G PLUS/Protein A Agarose (Oncogene) in an one hour incubation at 4°C on a welter blender. After rinsing two times with PBS, 0,4 µl mouse mAb anti-FLAG M2 (4,9 mg/ml; Sigma) diluted in 20 µl PBS was added followed by an one 1 hour incubation at 4°C. After rinsing two times with PBS, 25 µl cell lysate were added and incubated for 3 hours at 4°C. Afterwards the pellet was rinsed four times with PBS (Chazenbalk *et al.*, 2002). TSHR was eluted in 20 µl SDS-PAGE sample buffer, heated to 37°C for 30 min with shaking.

Lectin analysis

Ten-microliter samples of immunoprecipitated TSHR (described above) were electrophoresed on 10% SDS-PAGE gels, and blotted onto nitrocellulose membranes. The membranes were incubated for 1 h with 10 µg/ml biotin-labeled lectin (MALII or SNA; Vector Laboratories) in TBST and afterwards washed three times in TBST. Incubation with a 1:2000 dilution of anti-biotin horseradish peroxidase conjugate (Cell Signaling) in TBST was then carried out for 60 min, and the reaction was developed with SuperSignal West Pico chemiluminescence reagents (PIERCE, Rockford, USA).

Lectin binding ELISA

The lectin binding ELISA was performed on Reacti-Bind™ Anti-GFP Coated Plates (PIERCE, Rockford, USA). COS-7 cells cotransfected with TSHR-GFP and pcDNA3.1 or SIAT1 were harvested in NP40 lysis buffer and added to the wells. After 1 hour incubation at room temperature the plate was washed three times in PBST, incubated for 1 h with 10 µg/ml biotin-labeled lectin (SNA, Vector Laboratories) in PBST and afterwards washed three times

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in PBST. Incubation with a 1:2000 dilution of anti-biotin horseradish peroxidase conjugate (Cell Signaling) in PBST was then carried out for 60 min, and the reaction was developed with substrate (o-phenylendiamine, H_2O_2, citratbuffer pH 5,2) and measured at 492 nm.

Results

Real time RT-PCR

We detected differences in expression of SIAT1, 4a and 8a in autonomously functioning thyroid nodules (AFTNs) and cold thyroid nodules (CTNs) versus surrounding thyroid tissue (STs) (Eszlinger *et al.*, 2004) using light cycler quantification (Fig. 1). The mRNA expression of SIAT1 in AFTNs is increased compared with STs, whereas the expression in CTNs is decreased or shows no difference to STs respectively. In contrast, the expression of SIAT8a mRNA is decreased in AFTNs compared with STs and the expression in CTNs shows no difference to STs. The mRNA expression of SIAT4a in AFTNs and CTNs shows no significant differences to STs. The mRNA expression of this three genes (SIAT1, SIAT4a and SIAT8a) was also studied in primary thyrocyte cultures. Stimulation of primary thyrocyte cultures with TSH increased the SIAT1 and SIAT4a mRNA expression, whereas the mRNA expression of SIAT8a was decreased (Fig. 1).

Radioligand binding assay

The influence of TSHR-sialylation on its ability to bind TSH was determined by a competetive binding analyses. Cotransfection of SIAT1 markedly increased the binding of ^{125}I-labeled TSH by the receptor (B_{max}= 189,1 ± 13,3%) in comparison to cells cotransfected with TSHR and empty pcDNA-vector (100%). Similar data could be obtained for SIAT4a (B_{max}= 177 ± 19,8%) and SIAT8a (B_{max}= 162,3 ± 14,3%) in comparison to cotransfection of the empty pcDNA-vector (Fig. 2). To determine whether the increased TSH binding after SIAT1 cotransfection can be attributed to the increased cell surface expression of the TSHR, we reduced the cell surface expression of the TSHR in SIAT1 cotransfection experiments by additional cotransfection of the human V2 receptor (V2R) (Schoneberg *et al.*, 1996) as previously described (Arturi *et al.*, 1998). Thus, the TSHR cell surface expression was reduced below 100% (FACS: 76 ± 6%, ELISA: 85 ± 10%) of the reference experiment (transfection of wt TSHR with an empty pcDNA-vector). Concurrent with the reduced cell surface expression of the TSHR, the binding of labeled TSH by the TSH receptor decreased to B_{max}= 81 ± 5% (data not shown).

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cAMP Accumulation Assay

The consequence of sialylation on the ability of the TSHR to activate adenylatcyclase was determined by measurement of cAMP accumulation. Cotransfection of TSHR and SIAT1 markedly increased the accumulation of cAMP (basal 27,818 ± 6,941 pmol; stimulated 592,86 ± 37,5 pmol) in comparison to cells cotransfected with TSHR and empty pcDNA-vector (basal 14,452 ± 1,69 pmol; stimulated 397,65 ± 37,18 pmol). Similar data could be obtained for SIAT8a (basal 32,455 ± 6,983 pmol; stimulated 431,86 ± 56,67 pmol) in comparison to cotransfection with the empty pcDNA-vector.

Stimulation of IP Formation

In addition to cAMP accumulation the effect of sialylation on the ability of the TSHR to activate phospholipase C was determined by measurement of IP formation. Cotransfection of TSHR and SIAT1 markedly increased the formation of IP (basal 1,6 ± 0,4%; stimulated 32,7 ± 1,2%) in comparison to cells cotransfected with TSHR and empty pcDNA-vector (basal 2,4 ± 1,1%; stimulated 22,8 ± 1,7%). Similar data could be obtained for SIAT8a (basal 1,3 ± 0%; stimulated 28,3 ± 1,9%) in comparison to cotransfection with the empty pcDNA-vector. IP values are expressed as the percentage of radioactivity incorporated from [H]inositol phosphates (IP1-3) over the sum of radioactivity incorporated in IPs and phosphatidylinositols.

FACS analysis

We determined the TSHR expression by the fluorescence intensity and the transfection rate by the relation of signal-positive cells to the total number of cells. Cotransfection of TSHR with SIAT1, 4a or 8a results in an increased cell surface expression of the TSHR. Cotransfection with SIATs increased cell surface expression of the TSHR up to 151,6 ± 4,1% (SIAT1), up to 132 ± 4,3% (SIAT4a) or up to 185,7 ± 4,4% (SIAT8a)(Fig. 3).

Neuraminidase treatment

FACS analyses after treatment with neuraminidase for 30 min showed a decrease of cell surface expression of TSHR coexpressed with SIAT8a from 190% to 140% (Fig. 4). For coexpression of the TSHR and SIAT1 reduction of cell surface expression was most pronounced at 12 h (from 134% to the level of the TSHR cotransfected with empty pcDNA-vector, Fig. 4).

Elisa

The investigation of TSHR cell surface expression by ELISA confirmed the FACS data. The ELISA revealed an increase of TSHR cell surface expression to 144 ± 19% after cotransfection with SIAT1 (data not shown).

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Western Blot

Western blotting analysis of solubilized TSHR-FLAG from membrane-preparations is shown in Fig. 5A. The identification of the human TSHR as two bands of 100 and 120 kDa, was carried out using the anti-FLAG-M2 antibody (Sigma). After coexpression of the TSHR and sialyltransferases, especially SIAT8a (lane 3), TSHR protein level is increased in the membrane indicating a higher TSHR cell surface expression. In contrast, similar transfection/expression levels of TSHR are shown by Western blotting analysis of total cell lysates from COS-7 cells cotransfected with pSVL-TSHR-FLAG and different SIAT constructs (Fig. 5B).

Lectin analysis

The investigation of TSHR-sialylation was carried out using biotin-labeled lectins: Maackia amurensis lectin 2 (MALII), which is specific for carbohydrate structures containing α(2,3)-linked sialic acids, and Sambucus nigra (SNA), which is specific for carbohydrate structures containing α(2,6)-linked sialic acids. For this experiment we used immunopurified TSHR as described in *Materials and Methods*. Coexpression of TSHR with SIAT1 (lane 2), which specifically transfers carbohydrate structures containing α(2,6)-linked sialic acids, shows a clear reaction of the upper band with SNA, whereas coexpression with SIAT4a (lane 3), which specifically transfers carbohydrate structures containing α(2,3)-linked sialic acids, shows a clear reaction of the upper band with MALII (Fig. 6). We did not study coexpression of TSHR with SIAT8a, because a lectin specific for carbohydrate structures containing α(2,8)-linked sialic acids was not available. The stronger reaction of MALII compared to SNA, after cotransfection with SIAT1 or the empty pcDNA-vector, could be due to the basal expression of SIAT4a in COS-7 cells, which do not show basal expression of SIAT1 (data not shown).

Lectin binding ELISA

The investigation of TSHR-sialylation by ELISA confirmed the previous data obtained by western blot. The ELISA revealed an increase of SNA-binding to 146 ± 8,7% after cotransfection with SIAT1 compared to coexpression of TSHR and the empty pcDNA-vector (Fig. 6B).

Statistical analysis

We used oneway analysis of variance (ANOVA) to detected significant differences ($p<0,01$) for FACS analysis and Radioligand binding assay between TSHR cotransfected with different SIATs or with the empty pcDNA-vector. In a subsequent Student's *t* test we differentiated three levels of significance (*$p<0,05$, **$p<0,01$ and ***$p<0,001$) between experimental groups.

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Discussion

Expression of and signaling through the TSHR is an essential part of endocrine regulation that affects thyroid hormone metabolism. The TSHR is subject to a number of regulatory mechanisms that change the efficacy of TSH signaling (e.g. gene and protein expression, receptor phosphorylation, internalization). Our data add a new aspect of protein modification (i.e. sialylation) that has not been studied for functional consequences so far. Indeed, we demonstrate for the first time that the transfer of sialic acid to carbohydrate residues of the TSHR can improve and prolong cell surface expression of a transmembrane receptor thereby regulating the availability of the receptor for ligand signaling.

Motivation for our cotransfection studies came from GeneChip data that show increased SIAT1 mRNA expression in AFTNs versus surrounding tissue (Eszlinger et al Oncogene). To extend this study we determined SIAT1, 4a and 8a expression in autonomously functioning thyroid nodules (AFTNs) and cold thyroid nodules (CTNs), two frequent thyroid pathologies that most likely represent opposite pathophysiological states of thyrocyte activation and differentiation (Fig. 1). Because regulation of sialyltransferase expression was evident in AFTNs but not in CTNs we performed in vitro investigations with TSH stimulated primary cultures that very likely model the type of activation seen in AFTNs. Regulation of SIAT1 and SIAT8a in AFTNs is consistent with TSH stimulation in primary thyrocytes. This very likely indicates that functional activation of thyrocytes through signaling downstream of the TSH receptor affects SIAT1 and 8a expression in vivo and in vitro. Since mRNA expression of SIAT1 is upregulated and mRNA expression of SIAT8a is downregulated in AFTNs it is possible that the outcome for the total sialic acid load of the TSHR could be neutral. In contrast, SIAT4a expression is only altered after TSH stimulation of primary thyrocytes but not in thyroid nodules.

Cotransfection of each of the three sialyltransferases (i.e. 1, 4a and 8a) together with the TSHR increased cell surface expression of the TSHR, as shown by FACS analysis, ELISA and western blotting. Western blotting suggest a considerably higher TSHR cell surface expression after cotransfection of SIAT8a compared to SIAT1. However, for exact quantification chemiluminescence detection of western blots is inferior to FACS analysis. It is therefore more likely that the difference of the effect of SIAT1 and 8a on TSHR cell surface expression is rather minor. Moreover, no synergistic effect of SIAT1, 4a and 8a could be detected in FACS analysis and radioligand binding assay (data not shown), which further supports the notion that the type of glycosidic binding plays a minor role compared to the sialic acid load itself. As a practical consequence we did not use every SIAT isoform in all

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experiments, also because specific lectins were not available for every isoform. Consequently, increased surface expression of the TSHR induced by sialyltransferases increases the number of binding sites on the cell surface and thereby improves the binding and signaling of TSH. However, we did not detect an increased affinity of the TSHR for TSH which corresponds to the previous finding that carbohydrates do not directly affect TSH binding (Nagayama *et al.*, 2000;Atger *et al.*, 1999).

To investigate whether increased cAMP and IP accumulation as well as TSH binding are direct effects of the increased cell surface expression, we decreased the cell surface expression of the TSHR by cotransfection with SIAT1 to the level of the TSHR cotransfected with the empty pcDNA-vector, using the V2-Vasopressin receptor in a triple-transfection. Subsequent binding assays normalised for TSHR expression as described above showed no differences between TSH-receptors with different amounts of sialic acids. Therefore the increased B_{max}-values after cotransfection of the TSHR with sialyltransferases are most likely a direct effect of the increased cell surface expression than an effect caused by a higher affinity to TSH.

In order to investigate whether the improved cell surface TSHR expression after cotransfection with sialyltransferases is a direct effect of sialylation of the TSHR we studied Western blots of the TSHR after cotransfection in COS-7 cells. Western blotting of untreated human TSHR showed a characteristic doublet at 100 and 120 kDa (Oda *et al.*, 1999) whereas the peptide chain molecular weight of the TSHR is 84 kDa (Misrahi *et al.*, 1990;Frazier *et al.*, 1990;Parmentier *et al.*, 1989;Nagayama *et al.*, 1989;Libert *et al.*, 1989). Previous reports indicate that glycosylation of the TSHR is responsible for the higher molecular masses observed on Western blotting (Misrahi *et al.*, 1994;Rapoport *et al.*, 1996). In recent studies, Oda et al. showed that treatment of the receptor with neuraminidase, which removes sialic acids (Tarentino *et al.*, 1985), caused the 120 kDa TSHR band to run as a 110 kDa band. However, the 100 kDa band remained unaffected which suggests that only the 120 kDa band carries sialic acids. Selective binding to the upper TSHR band of the lectin MALII (Fig. 6A) (Oda *et al.*, 1999), which is known to bind to carbohydrate structures containing α(2,3)-linked sialic acids (Wang and Cummings, 1988) further confirms the 120 kDa band as the sialylated TSHR entity.

To clarify in detail which sialyltransferase modifies the TSHR we also carried out Western blotting using lectins specific for different carbohydrates. After cotransfection of the TSHR with SIAT1, the 120 kDa band of the TSHR was found to react with SNA, which is specific for carbohydrate structures containing α(2,6)-linked sialic acids (Wang and

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Cummings, 1988). A lectin binding ELISA was used to confirm this result. Indeed, we detected a stronger SNA-signal after cotransfection with SIAT1 compared to the empty pcDNA-vector. This result clearly confirmed that the TSHR is a substrate of the SIAT1. We also found that MALII, which is specific for carbohydrate structures containing α(2,3)-linked sialic acids (Wang and Cummings, 1988), binds to the 120 kDa band of TSHR after cotransfection of the TSHR with SIAT4a. This result confirmed that the TSHR is a substrate of both, the sialyltransferases 1 and 4a.

The transfection of sialyltransferases might have some impact on exit of sialyl-glycoproteins from trans-Golgi network to plasma membrane. To answer the question whether sialylation causes a more effective transfer of the TSHR to the cell surface or a longer resting time on the cell surface we neuraminidase-treated TSHR/SIAT1 and TSHR/SIAT8a cotransfected COS-7 cells to remove sialic acid residues. After the neuraminidase treatment the cell surface expression of TSHR decreased. This suggests that sialylation of the TSHR not only improves but also prolongs its resting time on the cell surface.

Our finding of increased SIAT1 mRNA expression in AFTNs and in primary thyrocyte cultures after TSH stimulation, together with an increased cell surface expression of the TSHR after SIAT1 cotransfection, which in turn increases TSH radioligand binding, underlines the importance of sialylation as one form of posttranslational TSHR modifications. Our results suggest, that sialylation is likely to be a new mode of regulating the receptor cell surface presence.

Recent reports controversially discussed the localisation of the TSHR to lipid rafts (Latif *et al.*, 2003;Costa *et al.*, 2004;Latrofa *et al.*, 2004). Moreover, a ganglioside component belonging to the G_{M1} family, found in lipid rafts, has been reported to interact with the TSHR α-subunits and is considered to be an integral part of the purified TSHR (Kielczynski *et al.*, 1991). Our finding that sialylation can modulate the surface expression of the TSHR could also impact on its lipid raft localisation. Our furture studies will therefore also focus on TSHR surface compartimentation as a response to sialylic acid load.

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Figure Legends

Figure 1. Comparison of the differential gene expression of SIAT1, 4a and 8a determined by real-time RT-PCR in AFTNs versus STs (white bars), CTNs versus STs (black bars) and in primary thyrocytes after stimulation with TSH versus unstimulated cells (4h light grey bars; 24h dark grey bars). The signal log ratio is the logarithm to the basis two of the mean fold change (n=15) for the expression in AFTNs versus STs.

Figure 2. Competitive Binding of ^{125}I-bTSH on COS-7 cells transfected with pSVL-TSHR and pcDNA3.1-SIAT1, 4a, 8a or empty pcDNA3.1-vector. To determine B_{max} values, transfected cells were subjected to displacement binding using 160.000-180.000 cpm ^{125}I-bTSH per well and increasing concentrations of unlabeled bTSH. TSH-binding is significantly higher after coexpression of the TSHR with any of the sialyltransferases in comparison to coexpression of TSHR with the empty pcDNA3.1-vector (*p<0,05; **p<0,01). All data are presented as means ± SEM of two independent experiments, each performed in duplicate.

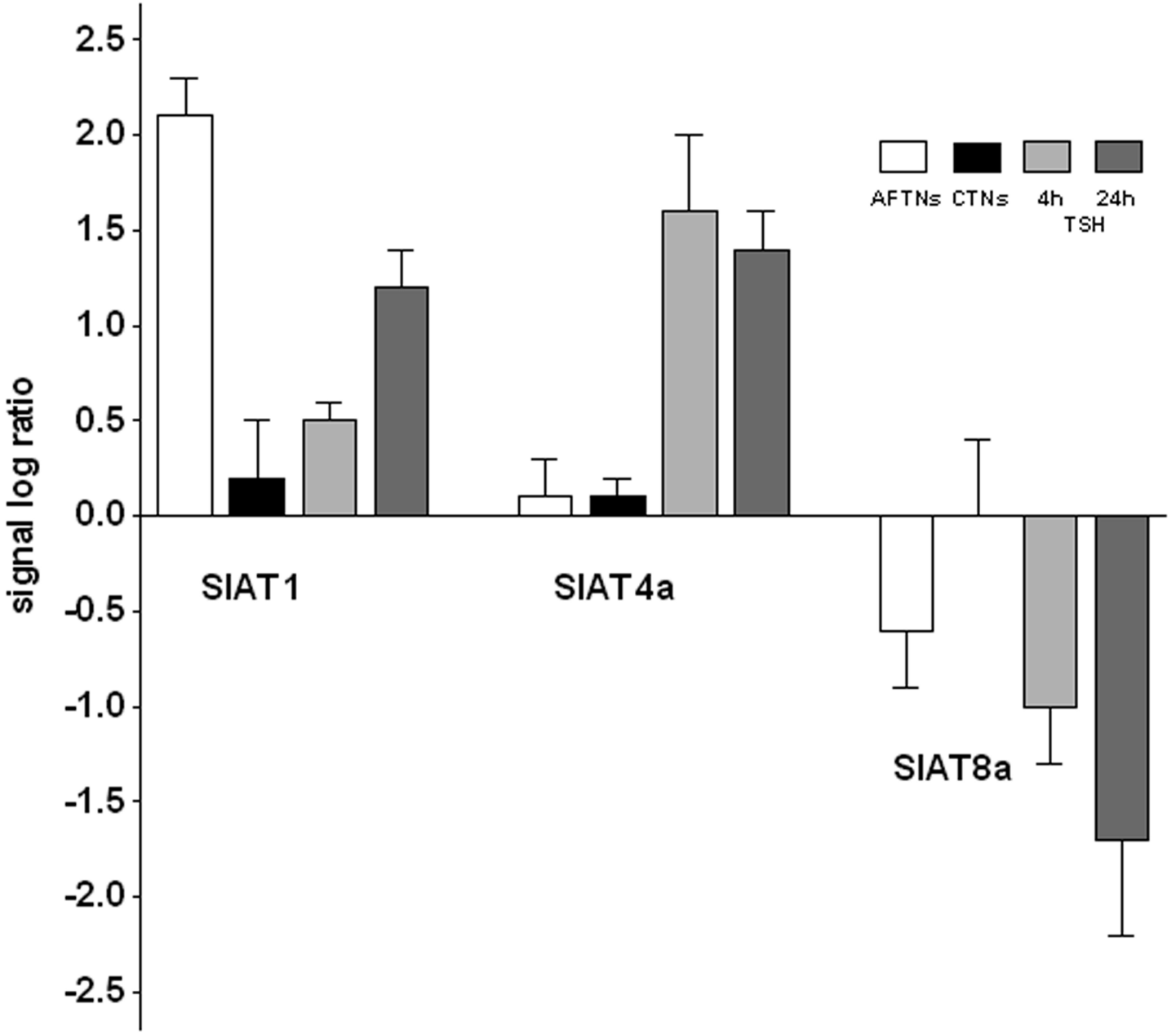
Figure 3. Cell surface expression of wt TSHR measured by Flow Immunocytometry using a mouse anti-human TSHR antibody (2C11, Serotec). pSVL-TSHR was cotransfected with empty pcDNA3.1-vector (lane 1), with pcDNA3.1-SIAT1 (lane 2), with pcDNA3.1-SIAT4a (lane 3) or with pcDNA3.1-SIAT8a (lane 4). Cell surface expression is significantly higher after coexpression of the TSHR with any of the sialyltransferases in comparison to coexpression of TSHR with the empty pcDNA3.1-vector (***p<0,001). All data are presented as mean ± SEM of two independent experiments, each performed in duplicate.

Figure 4. Cell surface expression of wt TSHR measured by Flow Immunocytometry using a mouse antihuman TSHR antibody. pSVL-TSHR cotransfected with empty pcDNA3.1-vector (lane 1-4), pcDNA3.1-SIAT1 (lane 5-8) or with pcDNA3.1-SIAT8a (lane 9-12). The white bars represent untreated COS-7 cells and the dark bars represent neuraminidase treated COS-7 cells (30 min – 12 h). Neuraminidase treatment significantly reduces cell surface expression of TSHR cotransfected with pcDNA3.1-SIAT8a compared to untreated (*p<0,05). Details are described

in *Material and Methods*. All data are presented as means ± SEM of two independent experiments, each performed in duplicate.

Figure 5. Western blotting analysis of pSVL-TSHR-FLAG cotransfected with the empty pcDNA3.1-vector (lane 1), with pcDNA3.1-SIAT1 (lane 2) or with pcDNA3.1-SIAT8a (lane 3) detected with anti-FLAG antibody. Protein extract containing membrane fraction (A) or total cell lysate (B). Western blotting analysis has been repeated twice with similar results.

Figure 6. A) Lectin blotting analysis of pSVL-TSHR-FLAG immunoprecipitate with anti-FLAG antibody bound to protein A/G agarose, cotransfected with the empty pcDNA3.1-vector (lane 1), with pcDNA3.1-SIAT1 (lane 2) or with pcDNA3.1-SIAT4a (lane 3) detected with MALII (upper panel) or SNA (lower panel). B) Lectin binding ELISA of TSHR-GFP cotransfected with the empty pcDNA3.1-vector or with pcDNA3.1-SIAT1 detected with SNA. Western blotting analysis and ELISA has been repeated twice with similar results.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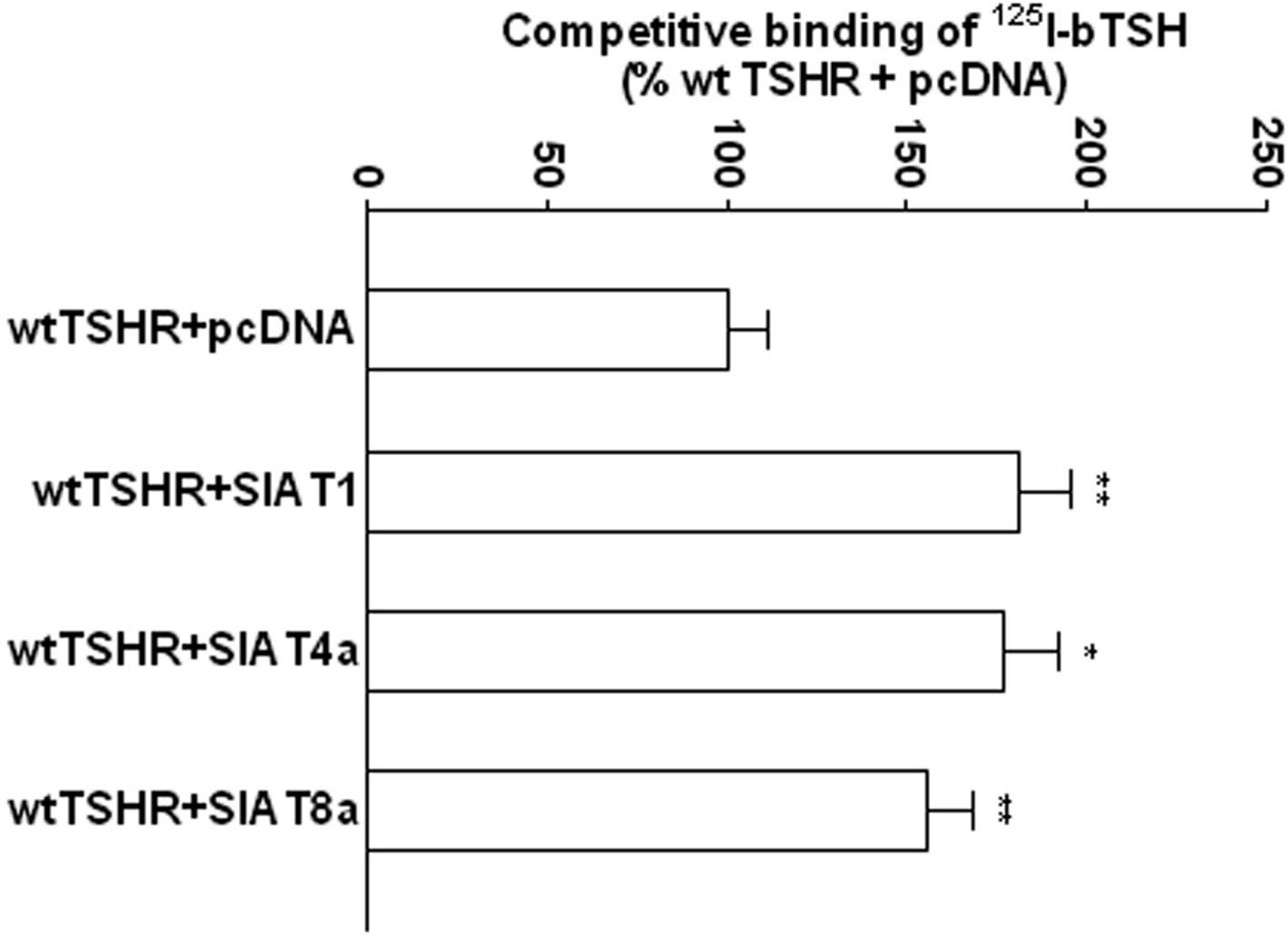
signal log ratio
AFTNs
CTNs
4h TSH
24h TSH
SIAT1
SIAT4a
SIAT8a


Competitive binding of 125I-bTSH
(% wt TSHR + pcDNA)
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50
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wtTSHR+pcDNA
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wtTSHR+SIA T4a
wtTSHR+SIA T8a
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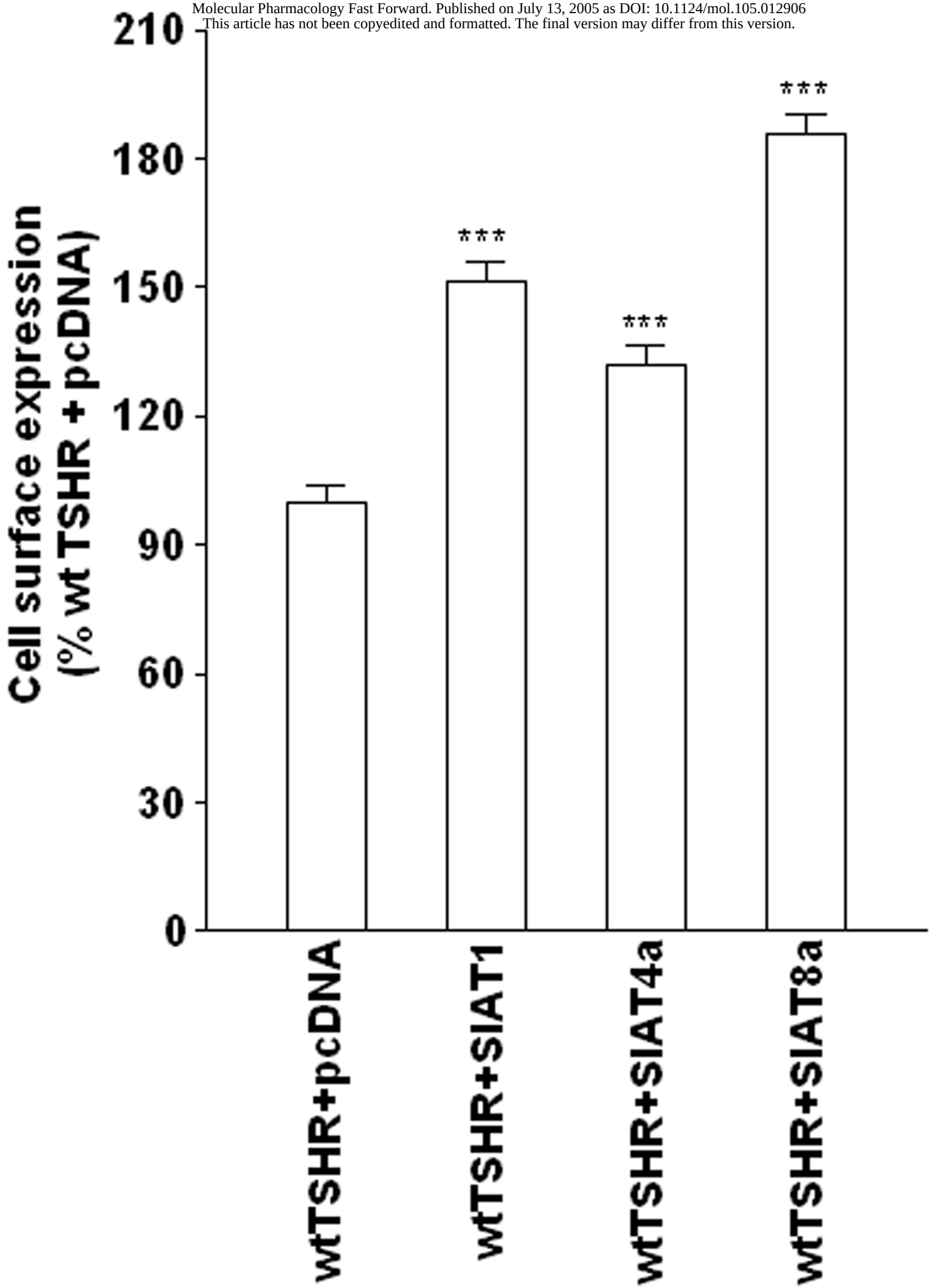
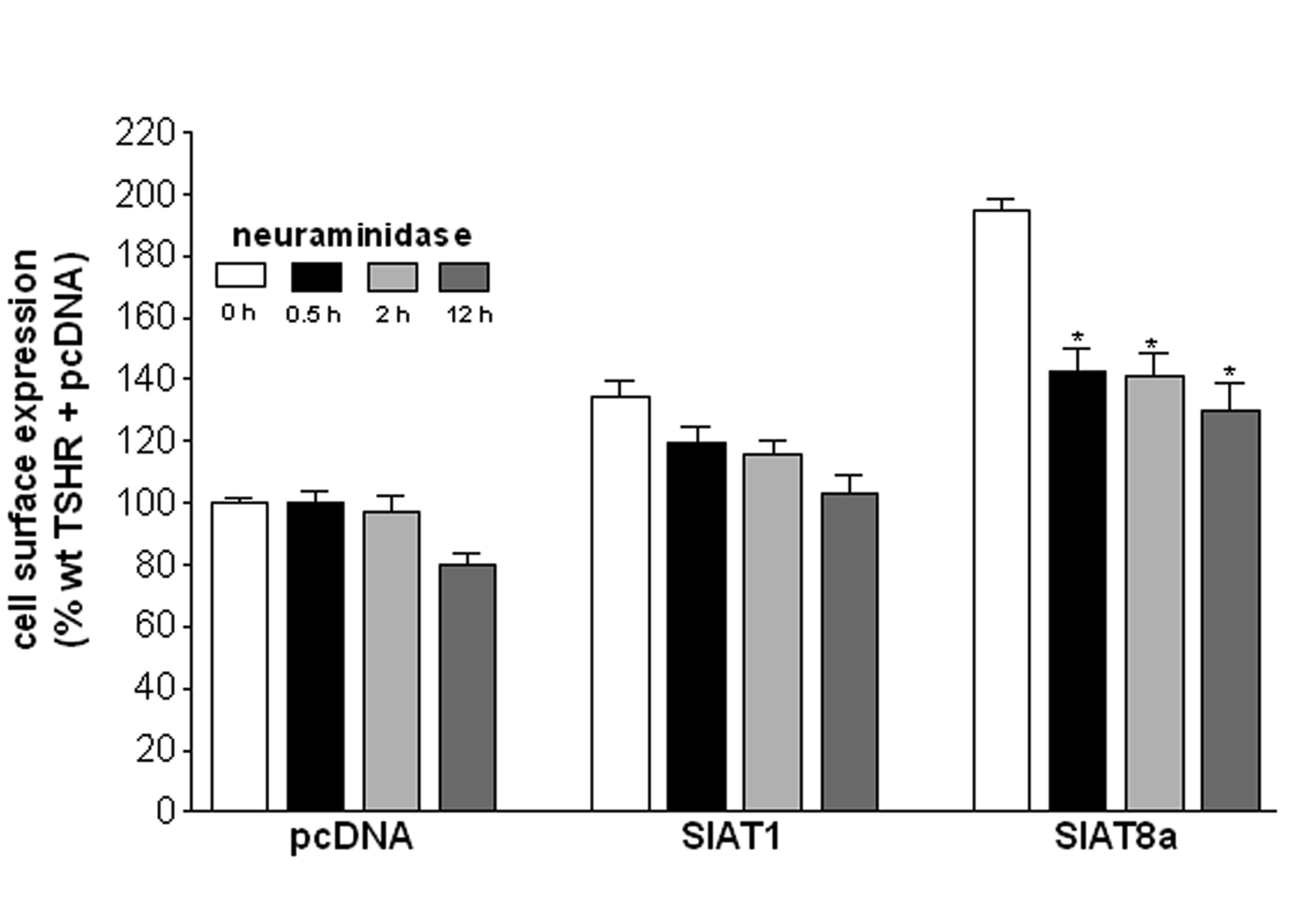
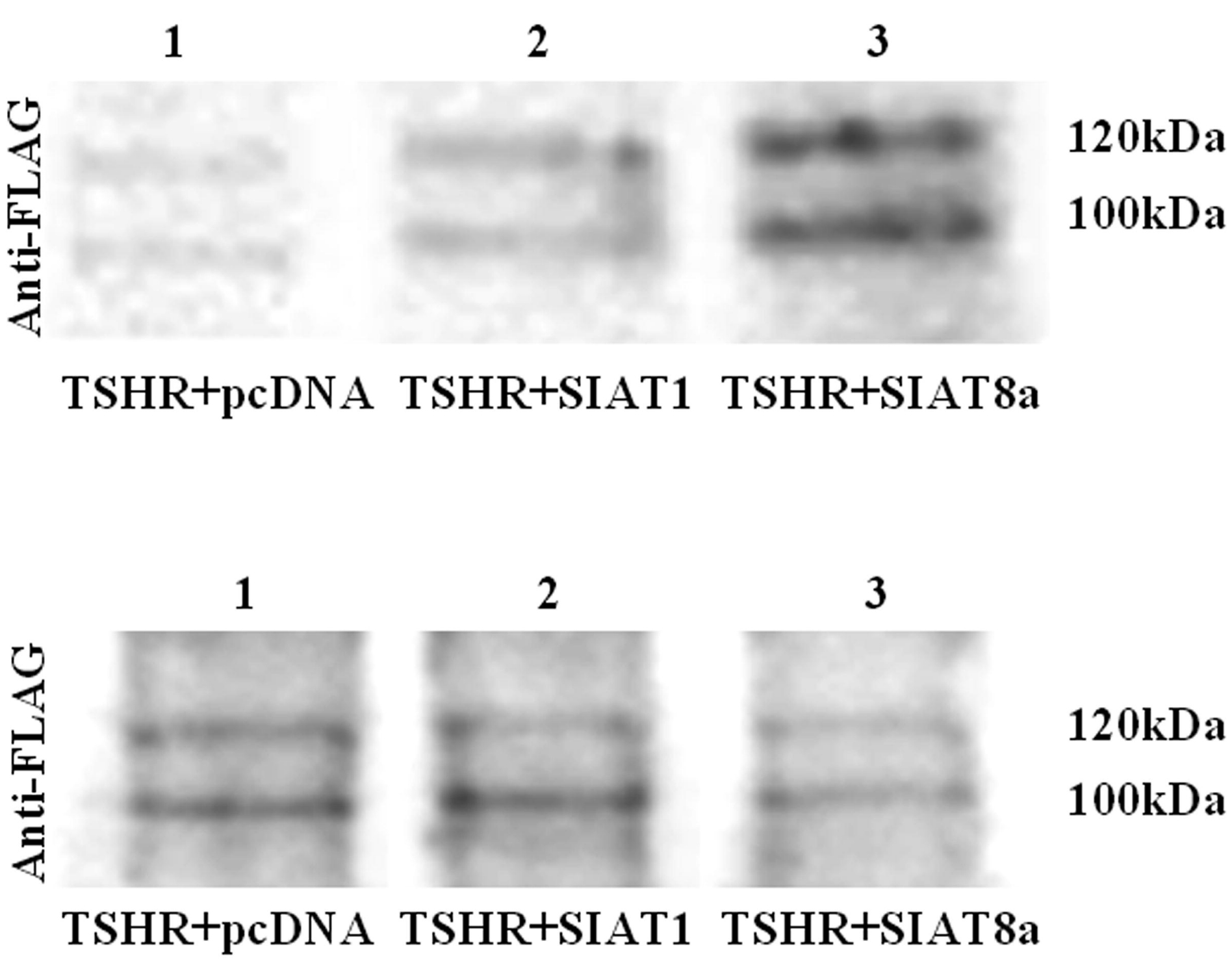



cell surface expression
(% wt TSHR + pcDNA)
neuraminidase
0 h
0.5 h
2 h
12 h
220
200
180
160
140
120
100
80
60
40
20
0
*
*
*
pcDNA
SIAT1
SIAT8a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2
3
Anti-FLAG
120kDa
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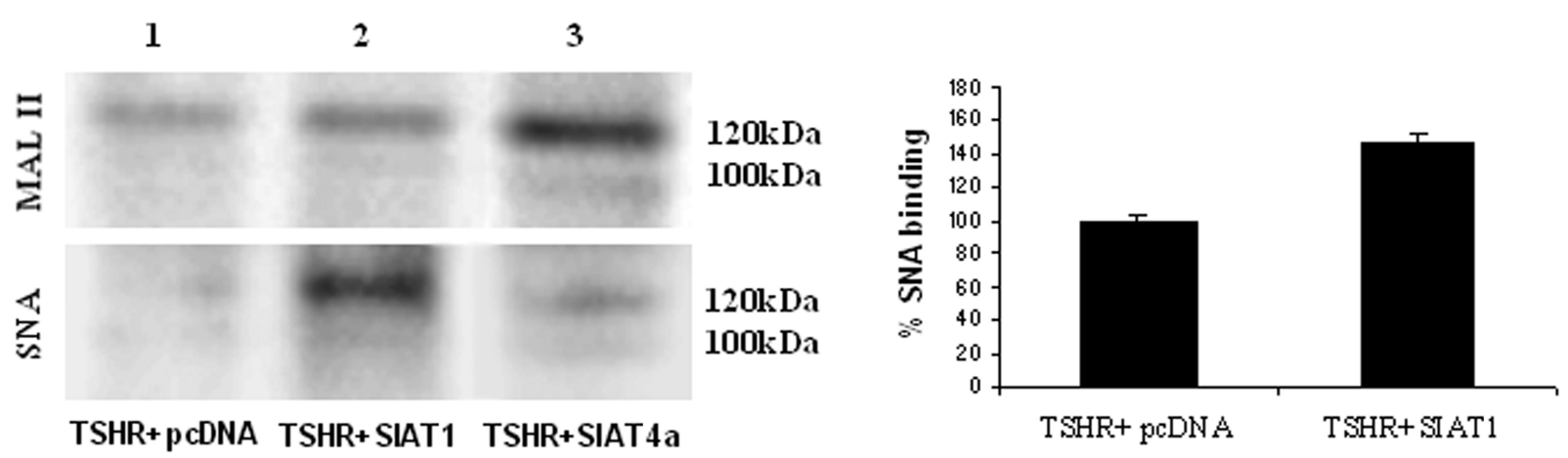
TSHR+SIAT8a


1
2
3
MAL II
SNA
120kDa
100kDa
120kDa
100kDa
TSHR+pcDNA
TSHR+SIAT1
TSHR+SIAT4a
% SNA binding
180
160
140
120
100
80
60
40
20
0
TSHR+ pcDNA
TSHR+SIAT1