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Purification and enzymological characterization of murine neurotrypsin

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ABSTRACT

An increasing number of studies indicate that serine proteases play an important role in structural plasticity associated with learning and memory formation. Neurotrypsin is a multidomain serine protease located at the presynaptic terminal of neurons. It is thought to be crucial for cognitive brain functions. A deletion in the neurotrypsin gene causes severe mental retardation in humans. For a biochemical characterization, we produced murine neurotrypsin recombinantly in a eukaryotic expression system using myeloma cells. From the culture medium we purified neurotrypsin using heparin-, hydrophobic interaction- and immobilized metal affinity chromatography. For an enzymological characterization two fragments of agrin containing the natural cleavages sites of neurotrypsin were used as substrates. The highest catalytic activity of neurotrypsin was observed in the pH range between 7.0 and 8.5. Calcium ions were required for neurotrypsin activity and an ionic strength exceeding 500 mM decreased substrate cleavage. Site-specific mutations of the amino acids flanking the scissile bonds showed that cleavage is highly specific and requires a basic amino acid preceded by a glutamate residue on the N-terminal side of the scissile bond. This sequence requirement argues for a unique substrate binding pocket of neurotrypsin. This observation was further substantiated by the fact that almost all tested serine protease inhibitors except dichloroisocoumarin and PMSF did not affect neurotrypsin activity.

Neurotrypsin is a member of the serine protease family S1A. The murine ortholog of neurotrypsin consists of 761 amino acids with a molecular mass of 84 kDa and a theoretical pI of 8.6. It consists of a signalpeptide (SP),[1] a proline-rich basic (PrB) region, a kringle (Kri) domain, three scavenger receptor cysteine rich (SRCR) domains, and a serine protease (Prot) domain. The protease domain comprises 244 amino acids and exhibits a 40% amino acid identity with trypsin. A zymogen activation site at the N-terminus of the protease domain bears the furin recognition sequence RRQKR. Murine neurotrypsin has three *N*-glycosylation site motives, one in the proline-rich basic domain and two in the protease domain [1]. In contrast to the rodent form of neurotrypsin, the human form exhibits an additional SRCR domain, which is conserved over all primates. The protease domain of murine and human neurotrypsin exhibit an amino acid identity of 89.9%. The overall amino acid sequence identity between murine and human neurotrypsin is 82.5% [2].

In mice, expression of neurotrypsin is predominantly found in the brain [1,3,4], the kidney [3], and the lung [1,3]. *In situ* mRNA hybridization on adult mouse brain sections revealed most prominent expression of neurotrypsin mRNA in the cerebral cortex, the subicular complex, the Ammon's horn, the dentate gyrus, and the lateral amygdala [4]. Upregulation of neurotrypsin RNA is found in many brain regions especially in the hippocampus, neocortex, and the midbrain during pre- and postnatal development of the central nervous system [4]. Immunoelectron microscopic studies showed an association of neurotrypsin with the presynaptic membrane and the presynaptic active zone [5].

Neurotrypsin plays an indispensible role for adaptive synaptic processes that are required for cognitive functions. A 4 base-pair deletion in the human neurotrypsin gene, which results in a truncated form of neurotrypsin lacking the protease domain, was identified as the cause of a severe form of mental retardation [6]. Recent studies identified the proteoglycan agrin as the so far unique proteolytic target of neurotrypsin, and demonstrated that neurotrypsin-dependent cleavage of agrin occurs at two homologous and highly conserved cleavage sites [3]. Live imaging studies demonstrated that both translocation of vesicular neurotrypsin to presynaptic nerve terminals and synaptic exocytosis of neurotrypsin are stimulated by neuronal activity [7]. Preparation of synaptosomes by subcellular fractionation and differential centrifugation and comparison of agrin and neurotrypsin-dependent agrin fragments in neurotrypsin-overexpressing and neurotrypsin-deficient

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[1] *Abbreviations used:* SP, signalpeptide; PrB, proline-rich basic; Kri, kringle; SRCR, scavenger receptor cysteine rich.

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mice indicated neurotrypsin-dependent cleavage of agrin as a process that is located at the synapse [7]. By local cleavage of the synapse-regulating proteoglycan agrin, neurotrypsin may be crucial for adaptive synaptic functions, such as the reorganization of synapses and neuronal circuits, which are required to establish and/or maintain higher cognitive functions.

This study describes the expression of full-length murine neurotrypsin in myeloma cells and its purification. It further provides an initial characterization of murine neurotrypsin that allowed to set-up an *in vitro* activity assay.

Materials and methods

Materials

The chromatography resins were all purchased from GE Healthcare. The secondary antibodies HRP-conjugated anti-rabbit and anti-goat were from Sigma–Aldrich. The 4–12% NuPAGE gels were from Invitrogen. SYPRO Ruby was from Molecular Probes, USA.

Generation of neurotrypsin-specific antibodies

Goat antiserum G87 was raised against the recombinant protease domain of human neurotrypsin (CAA04816), produced in *Escherichia coli*. The goat antiserum G86 was generated against the SRCR domains 1–4 of human neurotrypsin antigen, produced in *E. coli*. Goat antiserum G93 was raised against full-length murine neurotrypsin (CAA73646) produced in stably transfected mouse myeloma J558L cells. Rabbit antiserum SZ177 was raised against two synthetic peptides of the proline-rich basic segment corresponding to amino acids 22–40 and 45–57 of mouse neurotrypsin. Rabbit antiserum R89 was raised against the kringle domain of human and rat neurotrypsin (CAC35028), produced in *E. coli*.

Cloning of full-lengh neurotrypsin for expression in myeloma cells

For expression of neurotrypsin in myeloma cells, the coding region of full-length murine neurotrypsin (CAA73646) ranging from Met_1 to Ser_{761}, was inserted via the restriction sites SacI and HindIII into the pCD4-FvCD3-c vector [8]. This mammalian expression vector comprises an immunoglobuline V_κ promotor and an immunoglobuline κ enhancer. For positive selection the vector included a histidinol resistance gene [9].

Large-scale production of recombinant murine neurotrypsin in myeloma cells

The protoplast fusion of the neurotrypsin expression vector and the murine myeloma cells was done as described previously [10,11]. To achieve individual transfectants, the cells were diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biochrom AG, Germany) and plated in 96-well microtiter plates (PTT, Switzerland). Two days after transfection, cells with an insertion of neurotrypsin were selected by additional 5 mM L-histidinol to the growth medium (Sigma–Aldrich). Supernatants of surviving clones were analyzed for expression by Western blotting using the anti-neurotrypsin antibody SZ177. Clones with neurotrypsin expression were subcloned by limiting dilution for three rounds of single cell dilution. Briefly, cells were diluted and transferred into a 96-well microtiter plate to a calculated density of 0.5 cells per well. After 10–14 days in culture in presence of 5 mM L-histidinol the supernatants were again tested for neurotrypsin expression. Finally the best expressing clone was chosen for the large-scale production of murine neurotrypsin.

Adaptation of the neurotrypsin-expressing clones to protein-free medium

Starting from a medium composed of DMEM (Sigma–Aldrich) containing 4 mM L-glutamine and 10% FCS, the cells were adapted stepwise to grow in DMEM medium supplemented with 1% FCS. Adaptation was performed in 24-well plates (Nunc, Denmark) and the medium was exchanged every second day. When a density of about 1×10^6 cells/ml was reached, cells were transferred into a new well with a density of 3×10^5 cells/ml. Adapted cells growing in DMEM containing 1% FCS with a cell-doubling time of around 18 h were then transferred to the serum-free but protein-containing medium HL-1 (BioWhittaker Inc., USA) supplemented with 0.5% FCS. In HL-1 medium, the cells were then stepwise adapted to grow in HL-1 medium without FCS. Finally the HL-1 medium was stepwise exchanged from 0% to 50%, to 90%, to 98%, to 99.5% and finally to 100% by the protein-free medium TP-6 (Cell Culture Technology, Switzerland). The cells were maintained for neurotrypsin expression in roller bottles (Greiner Bio-One, Belgium) rotating at 2 rpm in humidified incubaters at 37 °C and 10% CO_2.

Chromatographic purification of murine neurotrypsin

For the purification of recombinant neurotrypsin, 14 L culture supernatant were concentrated using a Minisette tangential filtration cassette (Pall Corporation, USA). The retentate (500 ml) was adjusted to pH 8.0 by the addition of 20 mM Bis–Tris. Ammonium sulphate (Sigma–Aldrich) was added in small portions to a final saturation of 25% and afterwards stirred for 45 min at 4 °C. The solution was centrifuged for 30 min at 24,000*g*. The pellet was discarded, whereas the supernatant was subjected to another round of ammonium sulphate precipitation (a final saturation of 45%). After addition of the ammonium sulphate the pH of the solution was adjusted to 8.0 and once more stirred for 45 min at 4 °C. The solution was centrifuged and the pellet was resuspended in 200 ml loading buffer of the first chromatographic step. The resuspended protein was loaded onto a 60 ml heparin sepharose CL-B6 column equilibrated with the loading buffer (50 mM NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8). The column was washed with one column volume of loading buffer substituted with 100 mM NaCl and bound proteins were eluted in a gradient of three column volumes from 100 mM to 1 M NaCl in loading buffer. Fractions of the late peak shoulder of the elution at NaCl concentration of around 600 mM were pooled. For the HIC column 1 M NaCl was added to the neurotrypsin fractions. After dissolving, the sample was loaded onto a 12 ml butyl sepharose column equilibrated with 1 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8. Bound proteins were eluted in three column volumes with a gradient from 1.25 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 6.8–0.1% PEG6000, 20 mM MOPS in 30% ethylenglycol, pH 7.0. The neurotrypsin-containing fractions were pooled, supplemented with 10 mM imidazole, and loaded onto a 1 ml column of copper-labeled chelating sepharose column equilibrated with 0.25 M NaCl, 10 mM imidazole, 0.1% PEG6000 in 20 mM MOPS, pH 7.0. Proteins were eluted in eight column volumes with a gradient from 10 to 250 mM imidazole, 0.25 M NaCl, 0.1% PEG6000 in 20 mM MOPS, pH 7.0. All purification steps were performed on an Aekta Purifier Chromatography System (GE Healthcare).

The concentration of neurotrypsin in the pooled fractions was determined by measuring the absorption at 280 nm with a CARY 50 spectrometer and calculated using the specific extinction coefficient for murine neurotrypsin of $159280\,M^{-1}\,cm^{-1}$. Aliquots of 50 μl were frozen in liquid nitrogen and stored at −80 °C.

Immunodetection of neurotrypsin

Purified neurotrypsin (1.8 μg) was analyzed with 10% SDS–PAGE under reducing and non-reducing conditions. Electrotransfer was performed with semi-dry blotting apparatus. The immunodetection was done with the antibodies G93 (1:10,000 diluted), G86 (1:3000), G87 (1:5000) and SZ177 (1:2000).

Two in vitro substrates for neurotrypsin

α-Agrin bearing the α-cleavage site and β-agrin including the β-cleavage site served as substrates for cleavage assays with neurotrypsin. α-Agrin enclosed the rat agrin sequence (P25304 splice variant y4) from Lys_{51} to Val_{1752} with the signal sequence of human calsyntenin-1 (Met_1-Asn_{32} of NP_001009566) at the N-terminus, an N-terminal 8× His-tag and a fused EGFP at the C-terminus. β-Agrin encoded the region Leu_{1528}–Pro_{1959} of rat agrin including the signal sequence of human calsyntenin-1 and an N-terminal 8× His-tag. Both sequences were inserted into the PEAK8 vector (EdgeBio Systems, USA) and transiently transfected into HEK-293EBNA cells using PEI [12]. After 3–4 days of expression the culture supernatants were harvested. For purification of the recombinant protein the culture medium was replaced by loading buffer of the first chromatographic step by dialysis and cross-flow filtration, respectively. For the purification of α-agrin 2 L culture supernatant were concentrated and equilibrated with loading buffer by cross-flow filtration (Minimate TFF capsule, Pall corporation, USA). The retentate was loaded onto a HisSelect column (Sigma–Aldrich) in 400 mM NaCl, 20 mM imidazol, 0.1% PEG 6000 in 20 mM phosphate pH 8.0. After extensive washing the bound proteins were eluted in a gradient of seven column volumes from 20 up to 250 mM imidazol. The α-agrin positive fractions were pooled and loaded on a MonoQ column equilibrated with 400 mM NaCl, 0.1% PEG 6000 in 20 mM sodium phosphate pH 8.0. Proteins were eluted in a step gradient of 2 M NaCl. The final Superdex 200 gelfiltration carried out in 150 mM NaCl, 0.1% PEG in 20 mM MOPS, pH 7.0. For the purification of β-agrin about 500 ml culture supernatant were dialysed against the loading buffer of the HisSelect column. The single chromatography step, performed as for the α-agrin, yielded sufficiently pure β-agrin. The yield for α-agrin was up to 0.5 mg protein per 1 L culture supernatant, for β-agrin up to 5 mg protein per 1 L culture supernatant were obtained. All chromatographic steps were performed on an Aekta Purifier Chromatography System (GE Healthcare).

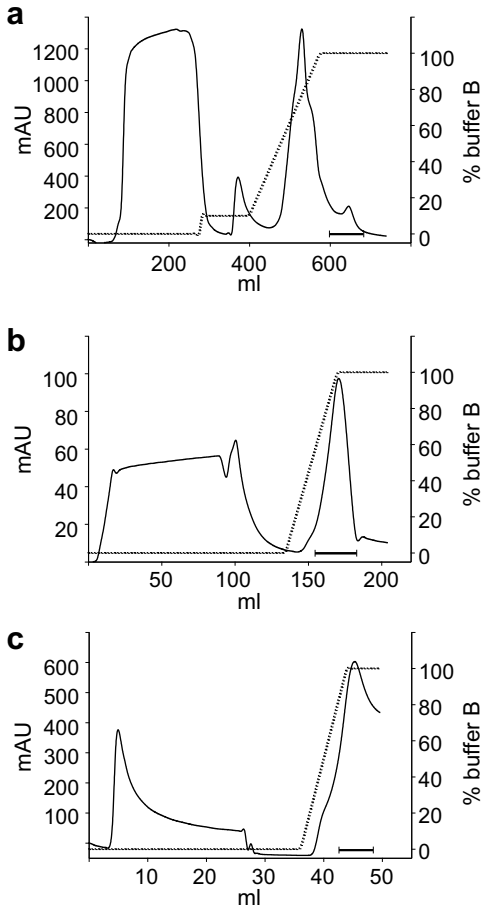

Fig. 1. Elution profiles of neurotrypsin chromatography: (a) heparin column, (b) HIC column, (c) IMAC column. Horizontal bars indicate collected fractions. Solid line indicates the absorption at 280 nm and the dotted line the percentage of buffer B.

In vitro activity assays for neurotrypsin

If not otherwise indicated, *in vitro* digestions were carried out as follows: neurotrypsin (0.025 μM) was incubated with either α-agrin (0.6 μM) or β-agrin (1 μM) for 1 h at 37 °C in a buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG6000 in 20 mM MOPS, pH 7.0. Assay samples were taken and the enzymatic reaction was stopped with 5× SDS–PAGE-sample-buffer containing EDTA (10% SDS, 30% glycerol, 0.6 M DTT, 100 mM EDTA, 0.35 M Tris, pH 6.8). The products of α-agrin were separated on 12% self-casted gels, the β-agrin product on 4–12% NuPAGE gels. The gels were stained with SYPRO Ruby (Molecular Probes, USA) and quantified by fluorescent densitometry using the imaging system LAS-3000 and the AIDA software (both Fujifilm, Japan).

Neurotrypsin broadband and selective inhibitor assay

The broadband serine protease inhibitors AEBSF, aprotinin, TLCK (all Roche Diagnostics, Switzerland), benzamidine (Acros Organics), 3,4-dichloroisocoumarin (DCI), leupeptin and PMSF (all Sigma–Aldrich) were tested at the concentrations indicated in Table 3. Neurotrypsin (0.025 μM) was preincubated with different inhibitors for 40 min before addition of β-agrin (1 μM). The reactions were stopped after 45 min, the proteins were separated on 4–12% NuPAGE gels and were quantified using densitometry. As control trypsin (5 nM) (Sigma–Aldrich) and the chromogenic substrate S-2222 (500 μM) (Chromogenix Instrumentation Laboratory S.p.A., USA) were used. The slope of the absorption of the increasing S-2222 product signal was measured for all inhibitors at 405 nm for 5 min using the $Victor_3$ plate reader (Perkin Elmer, USA) and compared to the measured slope of the not inhibited reaction.

Table 1
Purification profile of neurotrypsin

	Volume [ml]	Total protein [mg]	Protein [mg/ml]	Neurotrypsin [mg]	Neurotrypsin [mg/ml]	Purification factor
Conc. supernatant	200	240.0	1.22	7.5	0.038	–
Heparin column	90	7.5	0.08	2.1	0.023	48
Butyl column	24	1.3	0.06	1.3	0.055	867
IMAC column	4	0.5	0.13	0.6	0.15	2104

The specific inhibitors Pefablock PL, Pefablock uPA, Pefablock Try, Pefablock TH (all from Pentapharm, Switzerland) were dissolved in DMSO and tested at a concentration of 150 μM with 0.025 μM neurotrypsin and 1 μM β-agrin. The reactions were stopped after

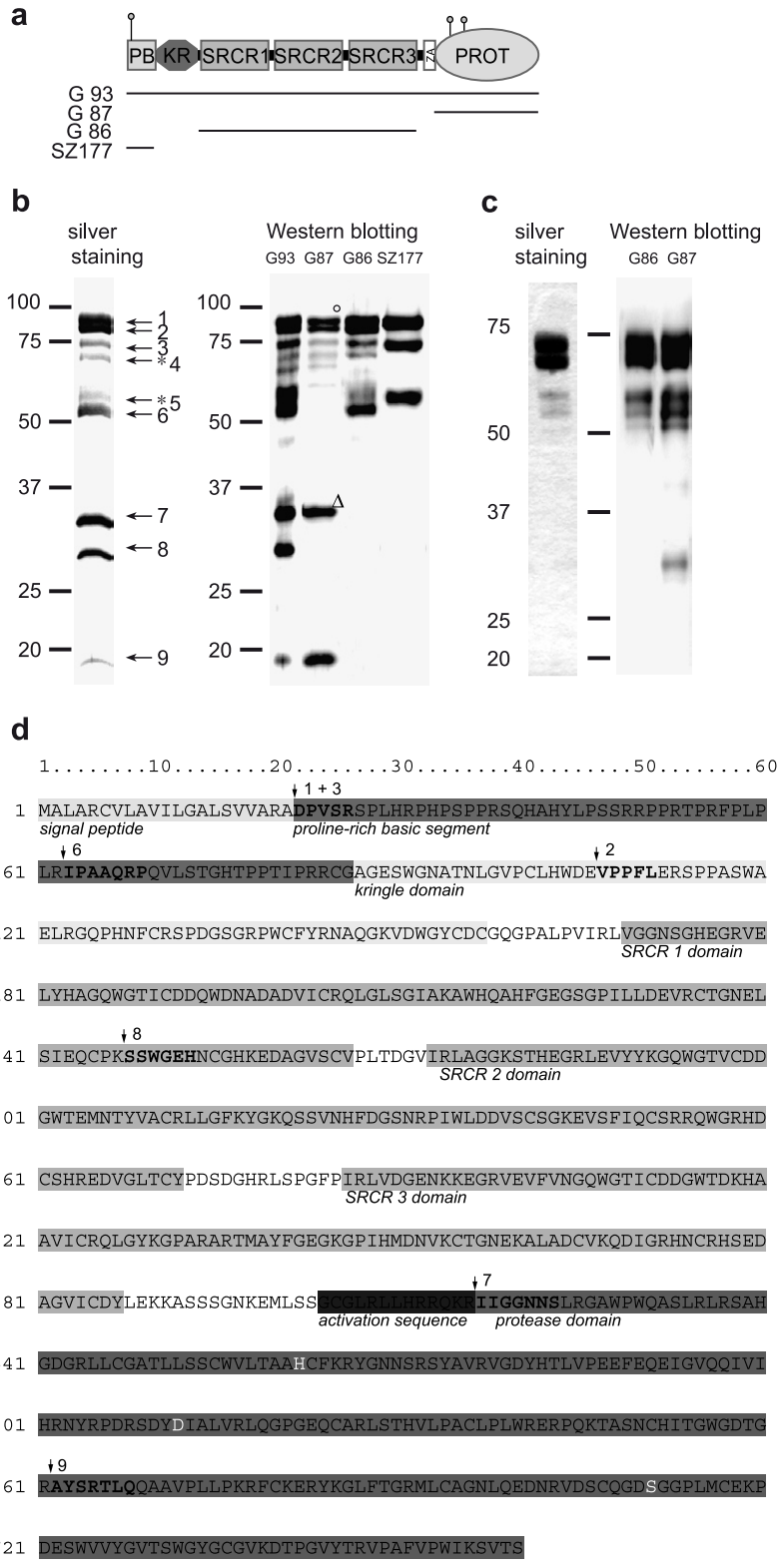

Fig. 2. Purity of recombinant neurotrypsin after chromatography. (a) Domain structure of murine neurotrypsin. PB, proline-rich basic domain; KR, kringle domain; SRCR1 to SRCR3, scavenger receptor cysteine-rich domains 1–3; ZA, zymogen activation region; PROT, serine protease domain; circles, putative *N*-glycosylation sites. Horizontal bars indicate the domains used as immunogenes for the production of the different antibodies. SDS–PAGE of 1.8 μg protein under reducing conditions (b) and under non-reducing conditions (c). Numbers in (b) refer to N-terminal sequencing in Fig. 3. The bands marked by a circle and a triangle on the Western blot shown in (b) represent the single-chain and the catalytic domain of the two-chain form, respectively. The signal intensities of these bands were used to determine the ratio of single-chain to two-chain neurotrypsin in our preparations. (d) Amino acid sequence of mouse neurotrypsin showing the N-termini of the purified enzyme. The vertical arrows indicate the N-terminal sites of fragments 1–3 and 6–9. Amino acids obtained by N-terminal sequencing are printed in bold type. Catalytic residues of the protease domain are depicted in white.

90 min. Product formation was investigated using SDS–PAGE and densitometry. The control experiment was performed on target enzymes with inhibitor concentrations 20 times higher than the corresponding K_i value. The slope of the absorption values of the increasing product was measured at 405 nm for 5 min using a CARY 50 spectrometer (VARIAN). Thrombin (750 nM) (Merck) was incubated with BzFVRpNA (17 μM) (Bachem), trypsin (20 nM) (Sigma–Aldrich) with 2 μM S-2222, factor Xa (70 μM) (Sigma–Aldrich) with 100 μM S-2288 (Chromogenix Instrumentation Laboratory S.p.A.), urokinase (120 nM) (Sigma–Aldrich) with BzVGRpNA (20 μM) (Sigma–Aldrich), and plasmin (90 nM) (Sigma–Aldrich) was incubated with 425 μM S-2288.

Assessment of the substrate preference of neurotrypsin with mutated agrin substrates

For activity measurements neurotrypsin (0.025 μM) was incubated with β-agrin (0.3 μM) or α-agrin (0.6 μM). At time points between 0 and 360 min for β-agrin and 0 and 120 min for α-agrin, samples were taken and the reaction was immediately stopped by adding SDS–PAGE-sample-buffer containing EDTA. The β-agrin cleavage products were separated in 4–12% NuPAGE gels and the α-agrin products in 12% self-casted SDS–PAGE gels. Afterwards the gels were stained with SyproRUBY and quantified by densitometry. Initial velocities were determined by linear regression in the early phase of the progress curve. Pseudo first-order rate constants were determined by fitting the integrated Michaelis–Menten equation ($[P_t] = [P_\infty]\ (1 - \exp^{(-kt)}) + c$, whereas P_t reflected the product concentration at timepoint t, P_∞ is the product concentration at complete turn-over, k reflects the rate constant and c is a correction factor) to the whole progress curve of product release. Calculations were performed with the SigmaPlot 9.0 software.

Results and discussion

Expression of recombinant murine neurotrypsin in myeloma cells

The full-length form of murine neurotrypsin was recombinantly expressed in a myeloma cell-based expression system, because several attempts for producing active neurotrypsin by other expression systems have failed. In brief, we expressed the protease domain as inclusion bodies in *E. coli* and refolded it. We also expressed the protease domain combined with the flanking SRCR domain into the periplasmatic space of *E. coli*. Both approaches did not result in active neurotrypsin. Likewise, the expression attempts of full-length neurotrypsin, as well as the isolated protease domain in HEK-293T, HEK-293EBNA cells, in *Pichia Pastoris*, and with the baculovirus expression system failed. For the stable expression, the coding region comprising the amino acids Met_1–Lys_{761} was engineered under the control of a κ light chain promotor into a vector that was specially designed for expression in myeloma cells [8]. This vector construct was introduced into J558L myeloma cells by protoplast fusion [11]. Positive selection for neurotrypsin gene integration into the myeloma cells was obtained with the aid of L-histidinol. Out of 5×10^6 cells of the protoplast fusion, 60 clones survived selection. These clones were analyzed for neurotrypsin expression by Western blotting with the neurotrypsin-specific antibody SZ177. Three of the surviving clones showed neurotrypsin expression and were subjected to three further rounds of subcloning. To facilitate the subsequent purification of neurotrypsin, the best expressing clone was then stepwise adapted to the protein free medium TP-6 and subsequently maintained in suspension culture in roller bottles. The concentration of recombinant neurotrypsin in the supernatant reached approximately 0.5 μg/ml, as determined on Western blots by comparison with a dilution series from a known concentration of purified neurotrypsin.

Purification of murine neurotrypsin

For the purification of recombinant neurotrypsin from the culture supernatant of myeloma cells a five-step protocol was established. Initially, 14 L culture supernatants were concentrated by cross-flow filtration. The retentate was precipitated at 45% saturation of ammonium sulphate. The highly basic nature of the N-terminal proline-rich basic segment suggested a binding of neurotrypsin to heparin. The obtained pellet was resuspended in the loading buffer of the heparin affinity column, the first chromatography step. Neurotrypsin was bound to the heparin matrix in a buffer containing 50 mM NaCl and eluted in a gradient to 1 M NaCl. Late elution fractions of the heparin affinity purification were collected (Fig. 1a). The heparin purification step separated 97% of the contaminating proteins. With the first step a purification factor of 48 and a yield of 28% was achieved. For the following hydrophobic interaction chromatography (HIC), 1 M NaCl was added to the pooled neurotrypsin-containing fractions and the sample was loaded onto the butyl sepharose column. Proteins were eluted from the HIC matrix with a buffer containing 30% ethylenglycol (Fig. 1b). The HIC purification increased the purification factor 18-fold, while the overall yield after this step was 17%. In the final step, an immobilized metal affinity chromatography (IMAC) column labelled with copper ions was used. Unspecific binding of proteins was suppressed by adding 10 mM imidazol to the loading buffer. Pure neurotrypsin was eluted in a gradient to 250 mM imidazol (Fig. 1c). Overall, 0.6 mg neurotrypsin were obtained from 14 L conditioned TP-6 culture supernatant with a purification factor of 2104 and a yield of 8% (Table 1). On the silver stained gel under reducing conditions 1.8 μg

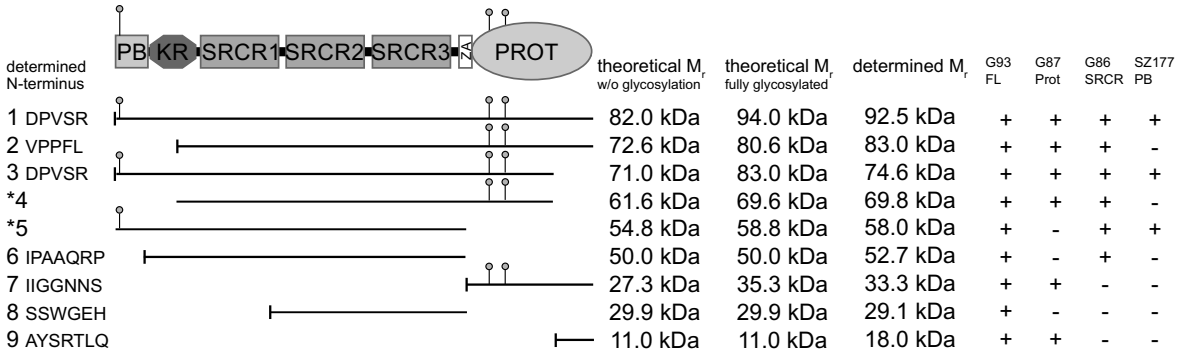

Fig. 3. Analysis of neurotrypsin fragments. The neurotrypsin fragments identified on the Western blots of purified neurotrypsin were mapped to the domain structure of murine neurotrypsin based on their N-terminal sequence, their apparent M_r, and their reactivity with different antibodies. Horizontal bars indicate the extensions of the neurotrypsin fragments. Abbreviations are as in Fig. 2.

purified neurotrypsin protein appeared as nine distinct bands as shown in Fig. 2b. These results indicated that expression of full-length neurotrypsin in our expression system was followed by several steps of partial proteolytic processing.

All nine fragments were subjected to Edman degradation. N-terminal sequences could be determined for seven fragments. The sequences identified all bands as components of murine neurotrypsin (Figs. 2b and 3). Sequencing of the faintest band migrating at 69.8 kDa (∗4) and of the blurred band at 58.0 kDa (∗5) failed. These bands were identified as neurotrypsin derivatives based on their immunoreactivity with three antibodies directed against different domains of neurotrypsin. Thus, all the nine fragments visible on the silver stained gel could be assigned to murine neurotrypsin (Fig. 2b). The apparent M_r of band ∗4 and its recognition by the antibodies G93 and G87 suggests that this fragment spans from Val_{107} (located in the kringle domain) to Arg_{662} (located in the protease domain) and is glycosylated at both predicted sites for N-linked oligosaccharides in the protease domain of typically ∼4 kDa (Fig. 3). The band ∗5 is detected by the antibodies G93, G86, and SZ177 and therefore most likely represents the non-catalytic domain of neur-

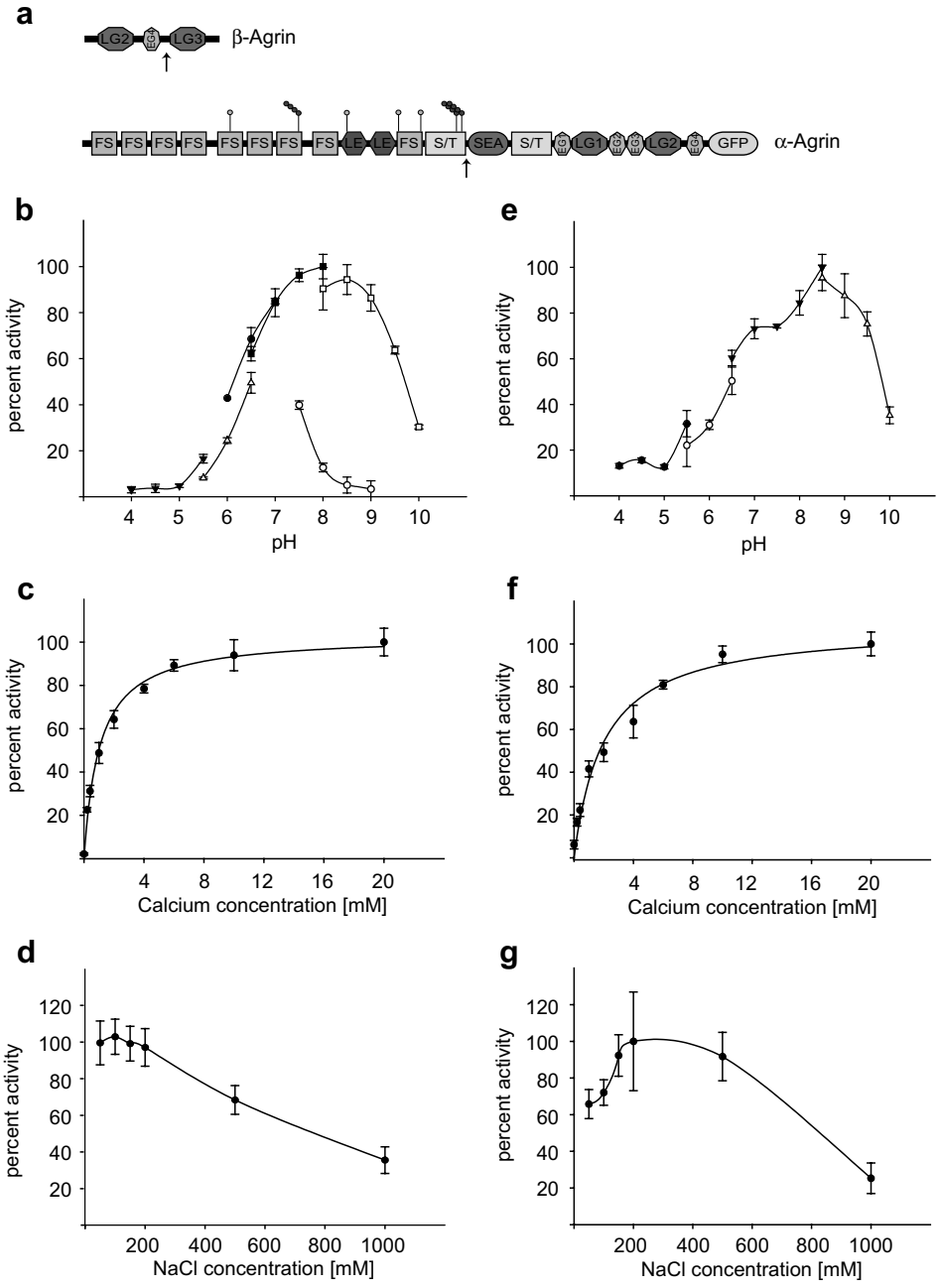

Fig. 4. Schematic drawings of the protein substrates β-agrin and α-agrin (a) each having a cleavage site for neurotrypsin, indicated by the arrow. Enzymological assays with purified β-agrin (b–d) and α-agrin (e–g) as substrate. (b) pH profile of neurotrypsin activity. Neurotrypsin showed highest activity between 7.0 and 8.5. All buffers were 100 mM: acetate for pH 4.0–5.5; MES for pH 5.5–6.5; PIPES pH 6.0–7.0; MOPS for pH 6.5–8.0; BICINE pH 7.5–9.0; AMPSO for pH 8.0–10.0. (c) Calcium dependence of neurotrypsin. (d) Influence of ionic strength. (e) pH profile of neurotrypsin activity. Neurotrypsin showed highest activity between 7.0 and 8.5. All buffers were 100 mM: acetate for pH 4.0–5.5; MES for pH 5.5–6.5; MOPS for pH 6.5–8.0; AMPSO for pH 8.0–10.0. (f) Calcium dependence of neurotrypsin. (g) Influence of ionic strength. Bars indicate standard deviation of at least three replicates. FS, cysteine-rich repeat similar to follistatin; LE, laminin EGF-like domain; S/T, serine/threonine-rich region; SEA, sperm protein, enterokinase, and agrin domain; EG, epidermal growth factor (EGF)-like domain; LG, laminin globular domain; single circles, sites of N-linked glycosylation; multiple circles, sites of glycan attachment.

otrypsin of theoretical 54.8 kDa bearing the predicted *N*-glyosylation in the proline-rich basic segment. Thus, this fragment ranges from the N-terminus of native neurotrypsin, Asp_{23}, to the zymogen activation site, Arg_{516} (Fig. 3). The discrepancy of the calculated molecular masses and the determined masses in the SDS–PAGE gel of fragment 3 might be due to an incomplete glycosylation. The activated protease domain, cleaved at the predicted zymogen activation site (between Arg_{516} and Ile_{517} as confirmed by N-terminal sequencing), migrates with an apparent molecular mass of 33.3 kDa on a 10% SDS–PAGE gel under reducing conditions. This band is recognized only by the neurotrypsin-specific antibody directed against the protease domain (G87) and by the antibody against the full-length neurotrypsin (G93). Furthermore, the N-terminal sequencing of the full-length band confirmed the predicted cleavage site of the signal peptide, which lies between Ala_{20} and Asp_{21}. SDS–PAGE analysis of the purified neurotrypsin under non-reducing conditions showed two major bands and three minor bands when silver stained. All these bands could be also detected with the two antibodies G86 and G87 directed against the neurotrypsin SRCR domains and the protease domains, respectively (Fig. 2c)

To measure the ratio between the zymogenic single- and the activated two-chain neurotrypsin, we used the protease domain-specific antibody G87 for Western blotting under reducing conditions (Fig. 2b-G87). This antibody was assumed to yield equivalent immunoreactivity signals for the isolated band of 33.5 kDa representing the two-chain form of neurotrypsin (marked by asterisk) and the catalytic domains associated with the single-chain forms of neurotrypsin (marked by circle). Based on densitometric quantifications of these bands on Western blots our preparations contained 50% of the neurotrypsin in single chain form and 50% in two-chain form.

Enzymological characterization of neurotrypsin

We used β-agrin as a substrate to assess the effects of pH, calcium, and ionic strength on neurotrypsin activity (Fig. 4). Six different buffer systems (sodium acetate, pK_a 4.7; MES, pK_a 6.1; PIPES, pK_a 6.8; MOPS, pK_a 7.2; BICIN, pK_a 8.3; and AMPSO, pK_a 9.0) covering the pH range between 4.0 and 10.0 were used for the determination of pH dependency of neurotrypsin. We found that neurotrypsin started to cleave its substrate at pH 5.5 and its activity increased continuously until maximal activity was reached at pH 8.5. At pH values higher than 9.0 the activity declined rapidly (Fig. 4b). In the cationic BICIN buffer neurotrypsin activity was considerably lower than in the other buffers. Calcium dependence was tested at concentrations between 0 and 20 mM. To ensure that no calcium was bound to enzyme or substrate, 10 mM EDTA was added to the 0 mM sample. We found that calcium is essential for neurotrypsin activity, with half maximal substrate turnover at a concentration of 0.45 mM. Maximal activity was found between 5 and 10 mM calcium (Fig. 4c). The ion strength-dependence of neurotrypsin was studied with a series of increasing concentrations of NaCl. In the range of 50–200 mM NaCl neurotrypsin activity was not affected. At NaCl concentrations above 500 mM the catalytic efficiency dropped significantly (Fig. 4d). Based on these results we composed the buffer for *in vitro* assays: 150 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG 6000 in 20 mM MOPS, pH 7.0.

The effects of pH, calcium, and NaCl on neurotrypsin activity were also assessed with the highly glycosylated α-agrin as substrate (Fig. 4e–g). For the determination of the pH dependence sodium acetate, MES, MOPS, and AMPSO were chosen. Neurotrypsin started to cleave α-agrin at pH 5.5 and activity was highest between pH 7.0 and 8.5. At basic pH values, higher than 9.0, the activity declined rapidly (Fig. 4e). A calcium concentration of 0.85 mM was required for half maximal substrate turnover, compared to 0.45 mM for β-agrin. Maximal activity was reached at approximately 10 mM $CaCl_2$ (Fig. 4f). Cleavage of α-agrin was less sensitive to ionic strength than cleavage of β-agrin (Fig. 4g). Maximal neurotrypsin activity was reached between 50 and 200 mM NaCl concentration, whereas higher concentrations resulted in a rapid decrease in activity.

A pseudo first-order rate constant for the cleavage of β-agrin of $4.7 \times 10^{-4}\,s^{-1}$ was determined by fitting the integrated Michaelis–Menten equation to the experimental data (Fig. 5). The pseudo first-order rate constant *k* obtained in this way corresponds to the V/K_m ratio, where *V* represents the limiting rate. Dividing

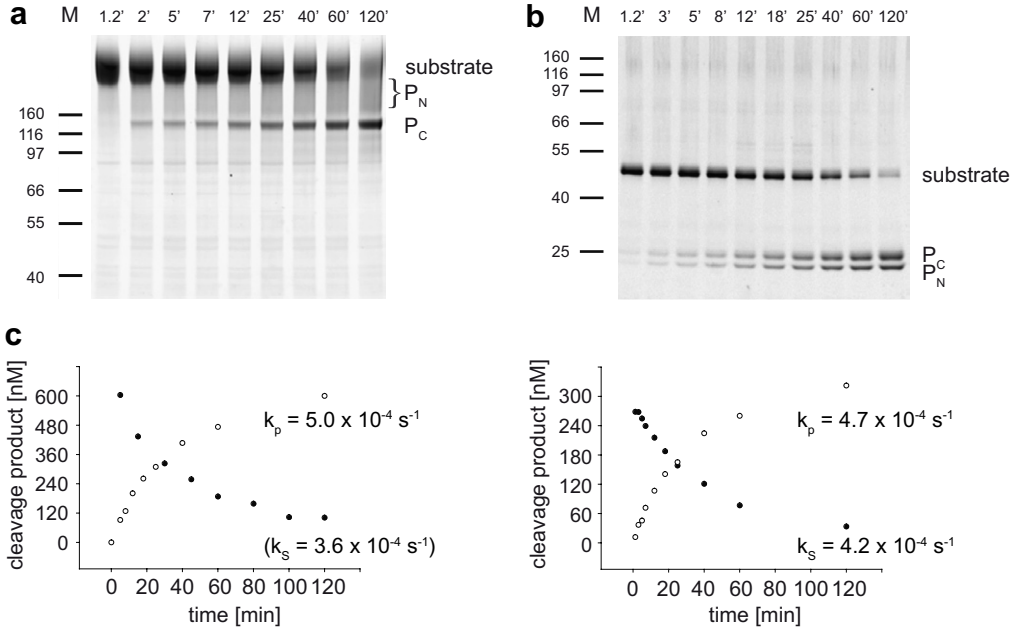

Fig. 5. Time course of neurotrypsin-dependent cleavage of agrin, (a) cleavage of α-agrin and (b) β-agrin visualized by SDS–PAGE using SYPRO Ruby. The signal intensities of product (open circles) and the substrate (black circles) were plotted and pseudo first-order rate constants were determined; k_s, rate constant for substrate consumption and k_p, rate constant for product formation.

this rate constant by the titrated enzyme concentration yields the kinetic constant k_{cat}/K_m, defining the catalytic efficiency. Unfortunately, an active site titrant for neurotrypsin was not available. Based on our gel analysis we estimated that 50% (25 nM) of the purified neurotrypsin was in the activated two-chain form, while 50% was in the zymogenic single-chain form (Fig. 2b-G87). Therefore, the catalytic efficiency k_{cat}/K_m was $1.9 \times 10^4 M^{-1} s^{-1}$. The discrepancy between the *k*-values of the substrate consumption and the product formation for the α-agrin in Fig. 5c is due to the quantification of the indistinct highly glycosylated N-terminal product signals in the gel. Because of the partial overlap of substrate and forming N-terminal product, we based our measurements on the formation of the C-terminal product fragment, which migrated well isolated on the SYPRO Ruby stained gels.

Inhibition of neurotrypsin activity by commercial serine protease inhibitors

The cleavage of β-agrin by neurotrypsin was used to evaluate the effect of four selective inhibitors of serine proteases and seven broadband serine protease inhibitors. Selective inhibitors against thrombin, trypsin, plasmin, and urokinase were used at a concentration of 150 μM, which was at least 180 times higher than the K_i determined for their cognate target protease. In control experiments with chromogenic substrates, at inhibitor concentrations 20 times higher than the indicated K_i, all the target proteases showed 10% or less residual activity. As shown in Table 2, none of the specific inhibitors had an effect on neurotrypsin activity.

The effects of seven broadband inhibitors of serine proteases were tested at two concentrations. The higher concentration used for the reversible inhibitors benzamidine, leupeptin, aprotinin, and for the initially reversible inhibitor TLCK was at least 20 times higher than their corresponding K_i determined for trypsin. The preincubation time for the irreversible inhibitors PMSF, AEBSF, and DCI was chosen at least 10 times longer than the half-life referred to $k_{obs}/[I]$ values for trypsin. In a control experiment the integrity of the inhibitors was verified for trypsin using a chromogenic substrate. As summarized in Table 3, the proteolytic activity of neurotrypsin was almost completely inhibited by the irreversible inhibitors PMSF (0.5 mM) and DCI (0.1 mM). Benzamidine a common competitive inhibitor for trypsin-like serine proteases had only a poor inhibitory effect. In contrast, leupeptin, aprotinin, AEBSF, and TLCK did not affect neurotrypsin activity at the tested concentrations at all.

Neurotrypsin exhibits high substrate specificity

Wild-type α- and β-agrin together with the mutants bearing single amino acid exchanges to alanine at P5–P1 of the α and at P4–P1 of the β cleavage sites were used to assess the specificity of murine neurotrypsin. We also analyzed the mutation from the P1 lysine to arginine and the mutation from P1 arginine to lysine in an α- and β-cleavage site, respectively. In addition, we swapped the recognition sequences between the cleavage sites. For both substrates the turnover of each mutant was analyzed in time course experiments. For quantification, the products were collected after increasing reaction times, separated by SDS–PAGE, and stained with SYPRO Ruby. Rate constants for β-agrin were determined by a first-order kinetic analysis. Fitting the exponential curve to the product data revealed a rate constant of $4.7 \times 10^{-4} s^{-1}$. The results are summarized in Fig. 6a. There was no substrate processing with an alanine at the position P1 and the catalytic efficiency was

Table 3
Effect of broadband serine protease inhibitors on the activity of purified neurotrypsin

Inhibitor	Concentration [mM]	Residual activity of neurotrypsin [%]	Residual activity of trypsin [%]
None	—	100 ± 13.3	100 ± 2.8
Benzamidine	1	96.4 ± 2.0	11.5 ± 0.3
	10	75.0 ± 3.6	
3,4-Dichloro-isocoumarin	0.1	15.7 ± 0.7	5.4 ± 0.6
	1	2.4 ± 0.5	
Leupeptin	0.01	98.8 ± 4.3	5.1 ± 0.5
	0.1	101.8 ± 3.9	
PMSF	0.05	41.0 ± 7.8	3.4 ± 1.3
	0.5	1.5 ± 3.3	
TLCK	0.3	102.8 ± 2.4	7.0 ± 0.3
	3	100.1 ± 0.9	
AEBSF	0.1	116.6 ± 3.3	<1.0
	1	110.2 ± 3.3	
Aprotinin	0.01	118.3 ± 0.1	<1.0
	0.1	127.1 ± 3.4	

Table 2
Effect of specific serine protease inhibitors on the activity of purified neurotrypsin

Inhibitor	Inhibited protease	K_i of spec. protease [μM]	Conc. [μM]	Residual activity of neurotrypsin [%]	Residual activity of targeted enzyme [%][a]
None	—	—	—	100 ± 2.8	—
Pefabloc Tyr1405	Trypsin	0.02	0.12	108 ± 7.8	10.5 ± 3.3
Pefabloc TH	Thrombin	0.006	0.12	113 ± 10.4	7.8 ± 0.1
Pefabloc uPA	uPA	0.54	10.8	115 ± 8.1	8.6 ± 1.5
Pefabloc Pl	Plasmin	0.14	2.8	108 ± 8.6	10.1 ± 3.4

[a] Obtained at an inhibitor concentration 20× larger than K_i.

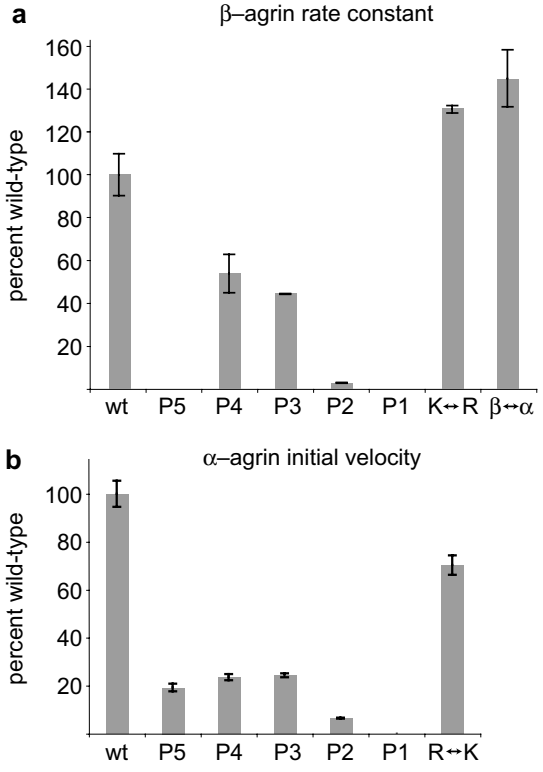

Fig. 6. Cleavage site mutagenesis reveals high dependence on a basic residue at P1 and a glutamic acid at P2. (a) Alanine scan of β-agrin from P4 to P1, Lys to Arg mutation at P1 (K/R), and the replacement of P3 to P2′ by the sequence of the α-cleavage site (α/β). (b) Alanine scan of α-agrin from P4 to P1, Lys to Arg mutation at P1 (K/R).

reduced 40-fold when the glutamate at the P2 site was mutated to alanine. Mutations of P3 and P4 reduced the substrate turnover to 44% and 54%, respectively. The P1 mutation from lysine to arginine and the replacement of the cognate recognition sequence of the β site by an α recognition sequence resulted in a minor increase of the catalytic efficiency to 130% and 144%, respectively, compared to wild-type. We also calculated the initial velocities of the P site mutants of β-agrin. We found the same substrate preference as with the rate constants (data not shown).

Due to uncertainties in the progress curves, it was not possible to determine single rate constants for the much larger and highly glycosylated α-agrin mutants. However, the determined initial velocities indicated the same kinetic changes after corresponding amino acid substitutions at both cleavage sites (Fig. 6b). α-Agrin with an alanine mutation at position P1 was not cleavable. An alanine replacing the glutamate at the P2 site resulted in a residual activity of 6%. Alanine mutations at P3, P4, and P5 in α-agrin had a similar impact on murine neurotrypsin activity and decreased activity to 19%, 23%, and 25%. Substitution of the arginine at the P1 site by lysine decreased neurotrypsin activity to 70%.

Conclusion

This work reports on the expression and the purification of active recombinant murine neurotrypsin. Based on enzymological characterizations, optimized conditions for *in vitro* assays were worked out. Highest neurotrypsin activity was found at a pH between 7.0 and 8.5, an ionic strength of around 150 mM and calcium ions at concentrations between 5 and 10 mM. The optimum conditions found for *in vitro* assays are in good accordance with the physiological working environment of the neurotrypsin in the extracellular space at the synapse [5,7].

Alanine-scanning of the P1–P5 amino acids of both cleavage sites confirmed the strict dependence of neurotrypsin activity on the basic P1 residue, as expected from a serine protease exhibiting a aspartate residue at position 189. A strong reduction of proteolyic activity was also observed by mutagenesis of the glutamic acid residue at P2. The relatively large effect of the glutamate to alanine mutation of this site is consistent with the strict evolutionary conservation of both neurotrypsin-dependent cleavage sites of agrin [3]. Only the irreversible serine protease inhibitors PMSF and DCI showed an inhibitory effect on neurotrypsin, while all of the other tested serine protease inhibitors were completely ineffective. These results indicate a narrow specificity of neurotrypsin.

The determined catalytic efficiency of neurotrypsin (k_{cat}/K_M $1.9 \times 10^4\,M^{-1}\,s^{-1}$) is comparable to that of other extracellular trypsin-like serine proteases with a regulatory function. For example thrombin cleaves protease-activated receptor 4 with a catalytic efficiency of $2.8 \times 10^5\,M^{-1}\,s^{-1}$ [13] or tissue-type plasminogen activator cleaves plasminogen with a k_{cat}/K_M of $9.5 \times 10^3\,M^{-1}\,s^{-1}$ [14].

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