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Full Length Research Paper

Structural features in neuropsin as potential target for pharmacological inhibition in brain diseases

Reinaldo Barros Geraldo[1,4], Paula Campello Costa Lopes[2], Carlos Rangel Rodrigues[3], Russolina Benedeta Zingali[4], Helena C. Castro[1]

[1]Antibiotics Laboratory, Biochemistry, and Molecular Modeling Study (LABioMol), Department of Molecular Cell Biology, Institute of Biology, CEG - Universidade Federal Fluminense, Rio de Janeiro, Brazil.
[2]Neural Neuroplasticity Laboratory, Department of Neurobiology, Institute of Biology, CEG - Universidade Federal Fluminense, Rio de Janeiro, Brazil.
[3]Laboratory of Molecular Modeling and QSAR (ModmolQSAR), Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
[4]Institute of Medical Biochemistry, Structural Biology Program, CCS, UFRJ, CEP 21941-590, Rio de Janeiro, RJ, Brazil.

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Neuropsin is a serine-protease produced by neurons and glial cells that is directly involved in learning and memory processes. This enzyme presents an important role in brain diseases such as epilepsy and Alzheimer disease. In this work we reviewed several aspects about this enzyme including structure, biological activity and its role in the central nervous system. We also compared neuropsin with other serine-proteases such as trypsin, an enzyme found in digestive and brain systems, thrombin, a coagulation cascade enzyme, and Lm-TL of a thrombin-like enzyme present in *Lachesis muta* venom that affects the prey central nervous system, among others, using bioinformatic tools. The analysis of the structural features of neuropsin and of these enzymes that present different and specific biological activities despite their high homology degree may help on designing selective inhibitor for treating several diseases including brain disorders.

Key words: Neuropsin, serine proteases, plasticity, homology, docking.

INTRODUCTION

Serine-proteases are hydrolyses that use the serine 195 hydroxyl group to cleave amidic linkage in peptides and proteins (Barrett, 1995). These enzymes belong to a large family composed by different sub-families, classified in accord to their crystal structure (Rao et al., 1998; Krem and Di Cera, 2000). Among them, the subfamily that is represented by trypsin, a pancreatic digestive enzyme (Perona and Craik, 1995; Perona and Craik, 1997), and includes mesotrypsin/trypsin IV, a neurogenic inflammation and pain modulator (Knecht and Adamesi, 2007); thrombin, a coagulation cascade enzyme (Carter, 2005); Lm-TL, a thrombin-like enzyme from Lachesis muta muta venom (Castro, 2001; Castro, 2004); urokinase plasminogen activator (u-PA); an enzyme involved in cancer dissemination and metastasis (Andreasen et al., 2000; Zeslawska et al., 2000); neurosin, a protease involved in neurodegenerative disease (i.e. Alzheimer disease, Parkinson disease and Multiple sclerosis) (Wang et al., 2008) and neuropsin, expressed in neuronal and glial cells and involved in learning and memory processes and multiple sclerosis (Terayama et al., 2005). Neuropsin has been described as participating on different processes, such cell adhesion, migration, proliferation and motility (Chen et al., 1995; Underwood et al., 1999; Shiosaka, 2004; Kishi et al., 1999).

In this work, we briefly reviewed important aspects about neuropsin including its role in central nervous system, molecular structure and biologic activity. We also evaluated the activity-structure relationship of this enzyme with a substrate by performing a theoretical docking analysis with the tri-peptide Val-Pro-Arg

*Corresponding author: E-mail:hcastrorangel@yahoo.com.br. 55-21-26299954

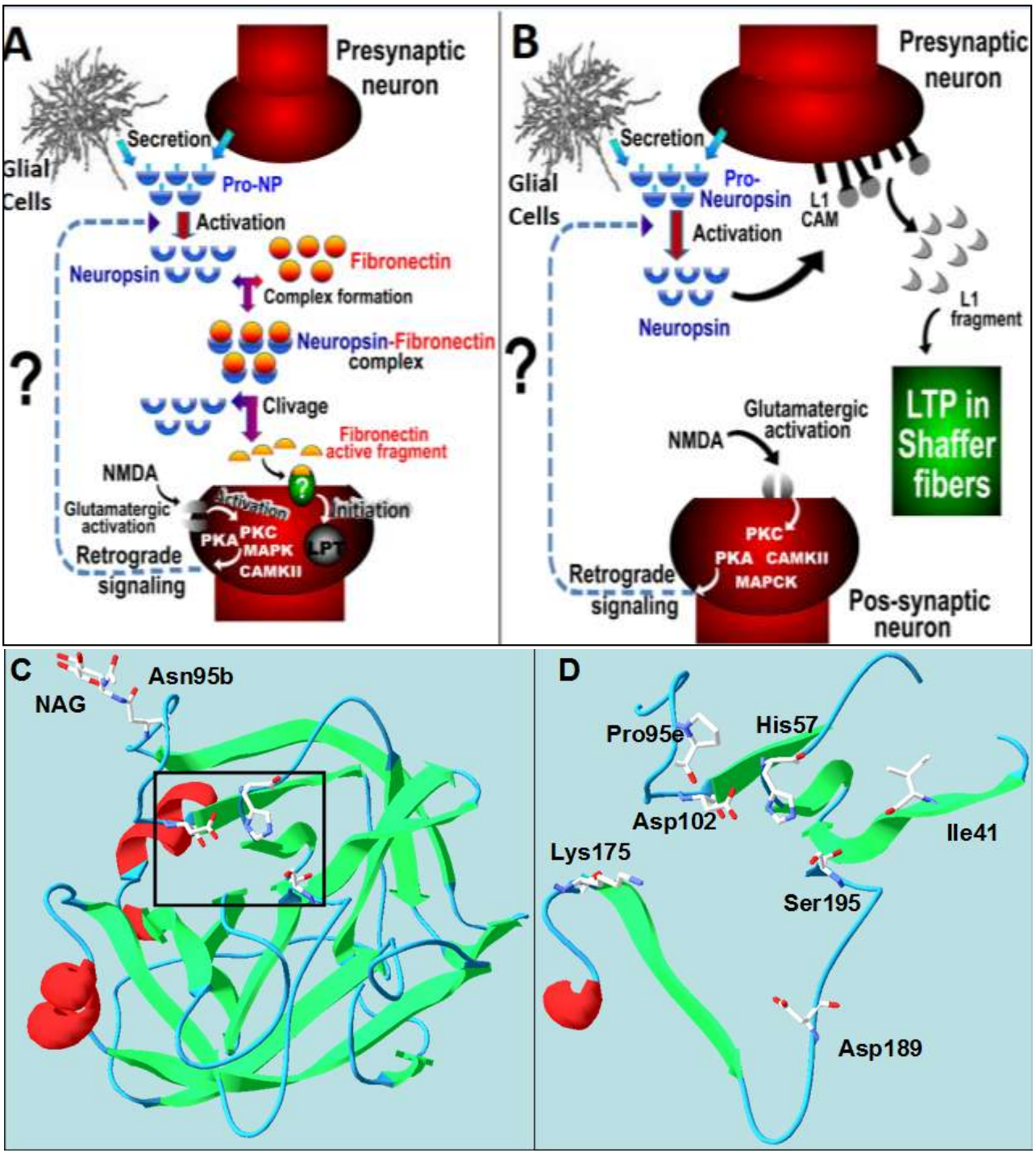


Figure 1. Theoretical mechanism scheme of neuropsin action. Pro-neuropsin is secreted and activated by neuronal NMDA receptor stimuli, the neuropsin may act in two ways: A)fibronectin degradation, and/or B) L1 cleavage in cell adhesion molecule (CAM) into a presynaptic neuron. (C) Neuropsin 3D-structure highlights the handle glycosylation and position of catalytic site, typical of the trypsin and kalicrein family. D) The neuropsin catalytic site showing the catalytic triad, Asp189 (S1), Pro95 (S2), the Lys175 (S3) and Ile41 (S1').

(Shimizu et al., 1998). Finally, to identify some characteristics related to its distinct biological functions we compared this enzyme with, six homologous serine-proteases that are important in some physiological and pathological processes in the organism, including trypsin, trypsin IV, thrombin, neurosin, Lm-TL and u-PA.

METHODOLOGY

The literature search since 1990 was performed in the Pubmed (http://www.ncbi.nlm.nih.gov/pubmed), and ScienceDirect (http://www. sciencedirect. com/) database using thas keywords neuropsin and K8K, with the subheadings expression, function, synthesis and activation.

RESULTS

Neuropsin: origin and function

Neuropsin, also called kallikrein 8 (KLK8) (Magklara et al., 2001; Kishi et al., 2003; Yousef et al., 2003), brain serine-protease 1 (BSP1) (Davies et al., 2001), or tumor-associated differentially expressed gene-14 (TAG-14) (Cane et al., 2004) is highly expressed in hippocampal pyramidal neurons, in the lateral and basolateral nucleus of the amygdala, and in other limbic system areas (Chen et al., 1995; Davies et al., 1998), suggesting their role in neuroplasticity.

Ontogeny study on mice pointed to the involvement of the neuropsin in embryonic cell differentiation, migration, axonal outgrowth and synaptogenesis (Suzuki et al., 1995). Indeed, during early stages of development, this enzyme hydrolyze extracellular matrix components and regulates neural circuits formation. In adults, its importance includes not only the regulation of survival and cellular proliferation, but also the activation and processing of neuropeptide, hormones and growth factors. (Davies et al., 2001). At synaptic level, neuropsin has been implicated in Schaffer-collateral long term potentiation, since studies using recombinant neuropsin showed a potential impact for NP. In LTP it might be a major effecter situated upstream of a signaling cascade for synaptic plasticity. r-NP alone can occlude TBS-induced LTP and induce LTP-associated phosphorylation of the AMPA receptor. In particular, the role of NP in LTP is critical in its early phase, since loss of NP or inhibition of NP completely abolished E-LTP induced by a single tetanus. (Komai et al., 2000; Tamura et al., 2006). The proteolysis contributes to the remodeling of the neural contacts during learn and consolidation of the memory (Bailey and Kandel, 1993).

It has also been shown that neuropsin could regulate neuronal plasticity through cleavage of fibronectin, a protein of the extracellular matrix (Figure 1) (Shimizu et al., 1998; Tani et al., 2001) or through cellular adhesion L1 (L1-CAM), located in the pre-synaptic neurons of rats hippocampus (Figure 1) (Matsumoto-Miyai et al., 2003). Studies using neuropsin-deficient mice revealed a decrease in synaptic transmission in the *stratum radiatum* of hippocampus (Hirata et al., 2001), whereas patients with Alzheimer disease present an increase in mRNA levels for this enzyme compared to normal individuals. In addition, models for epilepsy and neuronal plasticity present an increase in neuropsin expression and a progressive variation in the electric activity and behavior of animals (kindling epilepsy) (Chen et al., 1995; Okabe et al., 1996; Tomizawa et al., 1999). Interestingly, literature suggests that the inhibition of neuropsin may be useful in the epilepsy treatment, since it may affect the episodes in development (Momota et al., 1998; Oka et al., 2002).

In addition to the central nervous system, the mRNA that codifies neuropsin was found in normal and pathological mouse skin (*i.e.* psoriasis vulgaris and squamous cell carcinoma). Although the mechanism of neuropsin action is still unknown, it has been suggested that this enzyme could participate in cellular differentiation. Assays using neuropsin-deficient mice showed that this enzyme is involved in skin differentiation, but not in migration and desquamation. These studies using morphological comparison between neuropsin -/- and +/+ mice revealed an irregular cells alignment in the thickened epidermis obvious on day 2 after irradiation in the wild-type mice. Differently, it was prolonged for at least 2 days in the neuropsin-deficient mice (Suzuki et al., 1995; Matsumoto-Miyai et al., 2002; Kirihara et al., 2003). Its profile involves the process of epidermis keratinizing in mucous membrane and hair (Chen et al., 1998; Kuwae et al., 1998). Moreover, the presence of the neuropsin mRNA in pregnant uterus, suggests its participation in the decidual process, where occurs the proteolysis of some proteins of cellular adhesion, such as fibronectin (Chen et al., 1998; Kitagawa et al., 2002).

Neuropsin: synthesis and activation

The neuropsin is released in the extracellular matrix, as an inactive precursor (pro-neuropsin), which is activated by removal of four amino acids residues in the amino-terminal region identified as Glu-Gly-Ser-Lys in mouse and Glu-Gln-Asp-Lys in humans (Shimizu et al., 1998). According to the literature, neuropsin secretion mechanism involves potassium and calcium ions, which induces its expression in Neuro2a cells. The intracellular neuropsin signals were localized as punctate granules in neuronal cell bodies and neurites. After secretion, the extracellular periplasmic localization of neuropsin is probably due to binding of the enzyme to the cell surface molecules including L1 and fibronectin bound to integrin (Nakamura et al., 2006; Oka et al., 2002). Using an *in vivo* LTP approach, literature suggests that the mechanism of neuropsin activation involves N-methyl-D-aspartate receptor (NMDAr) activation, since this process is inhibited by DL-2-amino-5-phosphonovaleric acid (AP-5), a NMDA receptor antagonist. Likewise, NMDAr activation or inhibition seems to respectively activate or blockade the

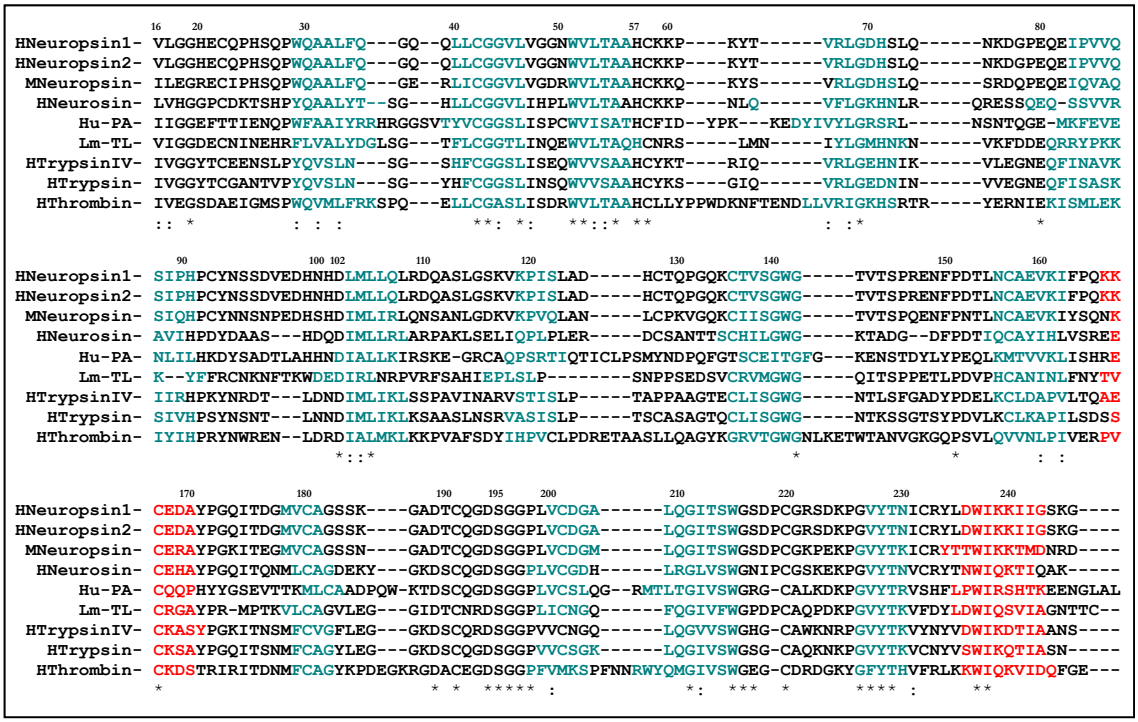


Figure 2. Primary and secondary alignments of the mouse (M) or Human (H) Type I and type II neuropsin, Neurosin, Lachesis muta - Thrombin like (Lm-TL), Trypsin,thrombin, urokinase plasminogen activator (Hu-PA) and trypsin IV. sequences. Identical amino acids are characterized by asterisks (*), the similar conservative by (:) and similar non-conservative (.). We also can notice the catalytic triad amino acids (H57, D102, S195), S1 subsite (D189), S2 and S3 subsites common to the three neuropsins (S2-S3, Pro95-Lys173) as well as the S1 subsite '(Ile41) of mouse neuropsin and the subsites S2 (Gly216) and S3 (Gly226) of Lm-TL and trypsin. The secondary structure of a-helix structures in red, ß-sheets in blue and random in black were predicted using the program Jpred (http://www.compbio.dundee.ac.uk/www-jpred/submit.html). Numbering is in accordance with trypsin.

conversion of the pro-neuropsin in active enzyme. The activation of NMDA receptor involves the protein kinase activation as calmodulin-dependent kinase II (CaMK II), mitogen-activated protein kinase (MAPK), proteins kinase A and C (PKA and the PKC), suggesting that the activation of the neuropsin could also be related to these proteins activity (Matsumoto-Miyai et al., 2003).

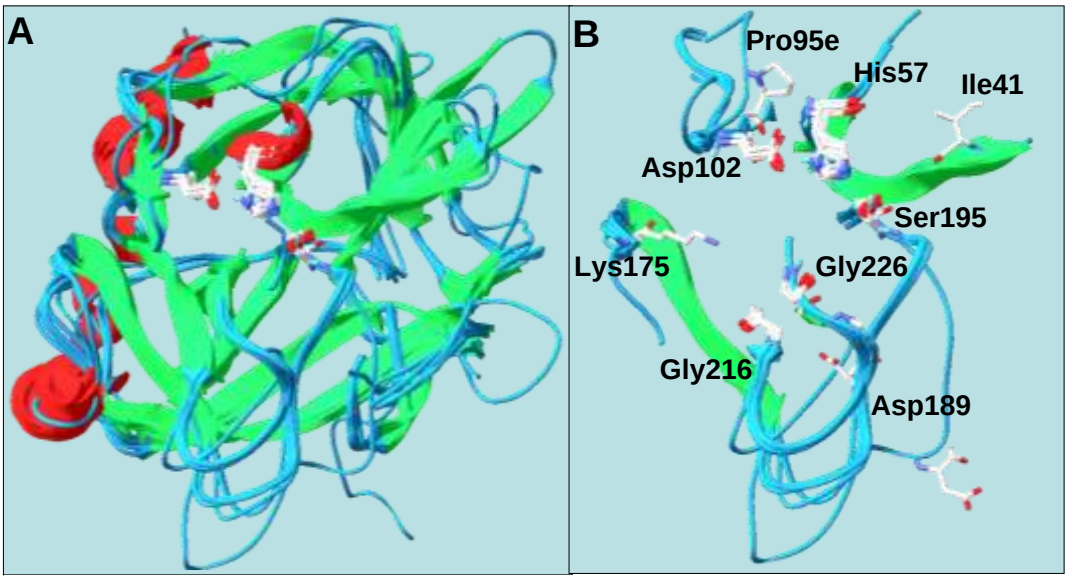
Neuropsin: structure-activity relationship

Currently literature describes two human neuropsin isoforms, homologous to mouse neuropsin called human neuropsin type I (NH-1) and one exclusively expressed in humans, called neuropsin human type II (NH-2) (Figure 2). The NH-2 is more expressed in adults and presents 44 amino acids more than NH-1 (Figure 2) (Li et al., 2004). The comparison of the primary sequence of the NH-1 with the mouse neuropsin shows 72% of similarity (Figure 2), including the presence of a small N-glycosylated region (Mitsui et al 1999; Yoshida et al., 1998). Studies that compared the structure of the NH-2 with the neuropsin of others primates than human point out the origin of this enzyme as a relatively recent event of approximately 18 million years (Li et al., 2004).

The gene that codifies human neuropsin presents six exons and five introns, similarly to the serine-proteases of trypsin subfamily (Yoshida et al., 1998, Yoshida et al., 2000). Neuropsin is also called KLK8 as in human, this gene is codified amongst a set of genes of the kallikrein multigens family (Gan et al., 2000; Diamandis et al., 2000; Clements et al., 2001; Yousef and Diamandis, 2001).

Neuropsin presents 260 amino acids (30 kDa) (Figure 2) and six disulfides bonds, which are conserved in protease family (C_{22}-C_{157}, C_{42}-C_{58}, C_{128}-C_{232}, C_{136}-C_{201}, C_{168}-C_{182}, C_{191}-C_{220}). Neuropsin secondary structures are composed by two α-helices and fourteen β-strands, which form two groups of seven-β-strand. The neuropsin antiparallel β-sheet fold is a typical trypsin-like structural arrangement where the catalytic site geometry guarantee the catalytic triad (H_{57}, D_{102} and S_{195}) (Figure 1) (Dodson and Wlodawer, 1998). This enzyme still presents a N-glycosylated loop, called the kallikrein loop, which an Asn-X-Ser sequence that binds to N-acetylglucosamine (NAG) (Figure 1 and 2) (Kishi et al., 2003; Katz et al., 1998; Takahashi et al., 1999).

In this work we performed a comparative study among neuropsin and others six serine-proteases that present



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	R.M.S.D. values (Å)						
	Neuropsin	Neurosin	Trypsin	TrypsinIV	Thrombin	u-PA	Lm-TL
Neuropsin	-	0.86	0.81	0.83	0.92	1.04	0.77
Neurosin	0.86	-	0.77	0.80	1.10	0.94	0.87
Trypsin	0.81	0.77	-	0.40	0.89	0.94	0.58
TrypsinIV	0.83	0.80	0.40	-	0.92	0.97	0.64
Thrombin	0.92	1.10	0.89	0.92	-	0.95	0.83
u-PA	1.04	0.94	0.94	0.97	0.95	-	0.94
Lm-TL	0.77	0.87	0.58	0.64	0.83	0.94	-

Figure 3. Comparison of Neuropsin structural features. (A) 3D-alignment of the whole structure and (B) the catalytic site of neuropsin, neurosin, trypsin IV, trypsin , thrombin, u-PA and Lm-TL. (C) Comparison of the root mean square deviation (RMSD) value of each structural alignment.

diverse functions in the human organism, including, trypsin, involved in digestion process; trypsin IV, responsible for an edema and inflammatory cell recruitment (Knecht et al., 2007); neurosin, acting in the maintenance of myelination (Bando et al., 2006); thrombin, from coagulation cascade; urokinase-type plasminogen activator (u-PA), involved in cell adhesion, migration, proliferation, motility and metastasis process, and Lm-TL a snake venom thrombin-like enzyme. Since these proteases belong to the same protease subfamily, literature suggests a high degree of structural similarity among neuropsin, u-PA, trypsin and the Lm-TL, although they participate in different biological processes (Perona and Craik 1997; Castro et al., 2001; Castro et al., 2004; Kishi et al., 1999, Perona et al., 1995). Moreover, we investigated the structural characteristics of these seven enzymes (neuropsin, trypsin, trypsin IV, neurosin, thrombin, u-PA and Lm-TL), which is important for establishing its intrinsic specificities. On that purpose we used the crystals structures of neuropsin (PDB1npm), trypsin (PDB1pph), trypsin IV (PDB1h4w), neurosin (PDB1gvl) thrombin (PDB1hxe), and u-PA (PDB1f5l), obtained from Protein Databank (www.pdb.org), and the theoretical model of the Lm-TL, constructed using program SWISS-MODEL (www.swissmodel.expasy.org) (Castro et al., 2001; Guex and Peitsch, 1997).

The alignment of the primary sequences of mouse neuropsin, human neuropsin type 1 and type 2, human neurosin, human trypsin and human trypsin IV, human thrombin, human u-PA and Lm-TL, using the program CLUSTALW (www.ebi.ac.uk/clustalw) and swissprt spdb-viewer respectively, showed as expected the conservation of the catalytic triad (His_{57}, Asp_{102} and Ser_{195}) and the S1 site (S1=Asp_{189}) confirming their experimental preference for basic residues in the chromogenic and protein substrates (arginine or lysine) in the P1 position (R.M.S.D. = 0,2-0,24Å). However, the S2 and S3 positions vary in neuropsin (P_{95} and K_{175}) compared to the other enzymes (G_{216} and G_{226}) (Figure 2 and 3). These features, which probably orientate for their different biological activities observed in these enzymes and their different protein targets. Interestingly, the mouse neuropsin primary structure presents a significant homology degree with the others structures (34-73%).

	POSITIVES AMINOACIDS	NEGATIVES AMINOACIDS
Neuropsin	R_{20}, R_{39}, R_{50}, K_{59}, K_{60}, K_{62}, R_{107}, K_{117}, K_{119}, K_{130}, K_{135}, K_{161}, K_{167}, R_{170}, K_{221}, K_{224}, R_{233}, K_{239} and K_{240}	E_{18}, E_{21}, E_{38}, D_{43}, D_{70}, E_{80}, E_{82}, E_{97}, D_{98}, D_{102}, D_{116}, E_{149}, E_{169}, E_{178}, D_{189}, D_{194}, D_{202}, D_{218}, E_{223} and D_{243}
Neurosin	K_{15}, K_{18}, K_{24}, H_{48}, K_{59}, K_{60}, K_{70}, R_{74}, R_{87}, R_{107}, R_{110}, K_{113}, R_{125}, K_{143}, K_{189}, R_{233} and K_{240}	E_{91}, D_{93}, D_{95}, D_{100}, D_{102}, E_{116}, E_{124}, D_{128}, D_{148}, D_{150}, D_{153}, E_{166}, E_{167}, E_{169}, D_{185}, E_{186}, D_{194}, D_{207} and E_{223}
u-PA	R_{35}, R_{36}, H_{37}, K_{61}, K_{62}, K_{70}, K_{72}, K_{92}, K_{107}, R_{109}, K_{110b}, R_{110e}, R_{116}, K_{143}, K_{156}, K_{161}, R_{166}, K_{179}, K_{187}, R_{206}, R_{217}, K_{223a} and K_{224}	E_{20}, E_{25}, D_{60}, D_{93}, D_{97}, D_{102}, E_{110c}, E_{144}, E_{148}, E_{153}, E_{167}, E_{175}, D_{189}, D_{194} and D_{223b}
Lm-TL	R_{28}, H_{57}, H_{70}, K_{72}, K_{75}, R_{81}, R_{82}, K_{85}, K_{86}, K_{87}, R_{90}, K_{94}, K_{98}, R_{104}, R_{107}, R_{110}, R_{137}, R_{169}, R_{174}, R_{179}, R_{193}, K_{225} and K_{230}.	D_{20}, E_{21}, E_{26}, D_{35}, D_{77}, D_{78}, D_{79}, D_{100}, E_{101}, D_{102}, E_{116}, E_{126}, D_{128}, E_{187}, D_{189}, D_{194}, D_{218}, D_{224}, D_{233} and D_{236}
Thrombin	R_{35}, K_{36}, R_{50}, R_{57}, K_{60}, R_{67}, K_{70}, H_{71}, R_{73}, R_{75}, K_{81}, K_{87}, H_{91}, R_{97}, R_{101}, K_{107}, K_{109}, K_{110}, H_{119}, R_{126}, K_{135}, K_{137}, K_{145}, R_{165}, K_{169}, R_{173}, R_{175}, K_{185}, K_{186}, R_{187}, K_{202}, R_{206}, R_{221}, K_{224}, H_{230}, R_{233}, K_{235}, K_{236} and K_{240}	E_{18}, E_{21}, E_{23}, E_{39}, D_{60}, D_{63}, D_{116}, D_{125}, E_{127}, E_{146}, E_{164}, D_{170}, D_{186b}, E_{186c}, D_{189}, E_{192}, D_{194}, D_{221} and D_{243}.
Trypsin	K_{60}, R_{66}, K_{87}, H_{91}, K_{107}, K_{109}, R_{117}, K_{145}, K_{156}, K_{159}, K_{169}, K_{188}, K_{204}, K_{222}, K_{224}, K_{230} and K_{239}	E_{70}, D_{76}, E_{77}, E_{80}, D_{102} D_{153}, E_{186} and D_{194}
Trypsin IV	H_{40}, H_{57}, K_{60}, R_{66}, K_{74}, K_{87}, R_{90}, H_{91}, K_{93}, R_{96}, K_{107}, K_{156}, K_{169}, K_{175}, K_{188}, R_{193}, H_{217}, K_{222}, R_{224}, K_{230} and K_{239}	E_{48}, E_{70}, E_{77}, E_{80}, D_{97}, E_{135}, D_{150}, D_{153}, D_{159}, E_{186}, D_{189}, D_{194}, D_{236} and D_{240}

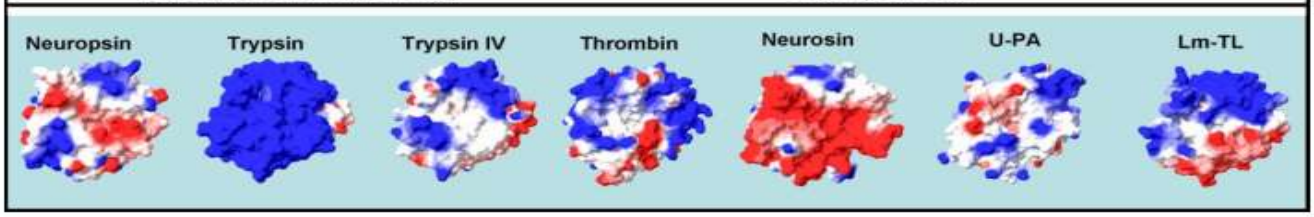

Figure 4. Comparison of the charged regions of neuropsin with other known serine proteases. Several residues (R =Arginine, K = Lysine, H = Histidine, D = Aspartic acid and E = Glutamic Acid) form the positive and negative regions of these proteases (up) that results in different 3D electrostatic potential maps that influence their enzymatic specificity (positive - blue and negative regions - red).

The analysis of the secondary structure of these enzymes revealed the conservation of the two α-helices, and the two groups of β-sheet (Figure 2 and 3). Three disulfides bonds are conserved in all of these enzymes including Cys_{42} - Cys_{58}, Cys_{168} - Cys_{182} and Cys_{191} - Cys_{220}. However they present exclusive bonds such as Lm-TL with Cys_{22} - Cys_{157}, Cys_{136} - Cys_{201}, similar neuropsin, neurosin and trypsin and Cys_{91} - Cys_{250} in the carboxyl-terminal region that is characteristic of snake venom serine-proteases (Castro et al., 2004). Interestingly, neuropsin, neurosin and trypsin have the same disulfide bonds, Cys_{22} - Cys_{157}, Cys_{136} - Cys_{201} and Cys_{128} - Cys_{232}, whereas thrombin presents C_1 - C_{122} and u-PA, Cys_{50} - Cys_{111} and Cys_{136} - Cys_{201}. Trypsin IV presents only five disulfides bonds (Cys_{22} - Cys_{157}, Cys_{42} - Cys_{58}, Cys_{136} - Cys_{201}, Cys_{168} - Cys_{182} and Cys_{191} - Cys_{220}), because the amino acids Cys_{128} and Cys_{232} that are substituted by Pro_{128} and Tyr_{232}.

As expected, the alignment of the three-dimensional structures of trypsin, trypsin IV, thrombin, u-PA, neurosin and Lm-TL, with neuropsin shows low R.M.S.D. values (0,81Å; 0,83Å; 0,92Å; 1,04Å; 0,86Å and 0,73Å respectively) (Figure 3). However the surfaces of these molecules significantly vary as expect since the electrostatic effects are important factors in determining not only the conformation and stability of proteins in solution, but also its binding to that ligands (Sharp & Honig, 1990; Bajorath *et al.*, 1991). So, the electrostatic potential maps analysis may reveal positive regions, due to presence of basic amino acids as histidine (H), lysine (K) and arginine (R), or with negative regions, due to acidic amino acids (acid aspartic - D and acid glutamic - E). The electrostatic potential map of the seven enzymes studies herein showed that neuropsin, thrombin, u-PA and Lm-TL present different distribution of the negative and positive regions in their surface explaining once more their different protein target despite their homology (Figure 3). In neuropsin, the positive (*i.e.* K_{59}, K_{60}, K_{62}, K_{161}, K_{167} and R_{170},) and negative (*i.e.* E18, E21, E_{38}, E_{169}, E_{178}, D_{189}, D_{194},) regions are distributed forming positives and negatives patches in protein surface. The neurosin MEPs posses a the negative portion near from the catalytic site (i.e. D185, E_{186}, D_{194} and D_{207}) and the positive in small regions (K_{15}, K_{18}, K_{24}, K_{60}, K_{70}, R_{74}, R_{233} and K_{240}). In u-PA, the electrostatic potential map are (E_{20}, E_{25}, D_{60}, D_{93}, D_{97}, D_{102}, E_{110c}, E_{144}, E_{148}, E_{153}, E_{167}, E_{175}, D_{189}, D_{194} and D_{223b}) and positive regions (R_{35}, R_{36}, H_{37}).In Lm-TL, two great distinct regions, one negative (D20, E21, E26, D35, D77, D78, D79, D100, E101, D102, E116, E126, D128, E187, D189, D194, D218, D224, D233 and D236) and another one positive (R28, H57, H70, K72, K75, R81, R82, K85, K86, K87, R90, K94, K98, R104, R107, R110, R137, R169, R174, R179, R193, K225 and K230) Thrombin presents a positive sites formed in N-terminal (i.e. R35, K36, R50, R57, K60, R67 and K70) and in C-Terminal (i.e. K202, R206, R221, K224, H230, R233, K235, K236 and K240) and the negative loads mainly in the catalytic sites (i.e. D170,D186b, E186c, D189, E192, D194). Trypsin posses an electropositive map, presenting still small regions negative, next to the catalytic site formed by E70, D76, E77,

E80, D102 D153, E186 and D194 (Figure 2). However, in trypsin IV posses a large electronegative region that posses (E49, E70, E77, E80, D97, E135, D150, D153, D159, E186, D189, D194, D236, D240) and presenting still two regions positive composed by (H40, H57, K60, R66, K74, K87, R90, H91, K93, R96, K107, K156, K169, K175, K188, R193, H217, K222, R224, K230 and K239) (Figure 4). These structural electronic differences suggested that these regions must guide the more complex maps, needing a perfect alignment and complementarities with the macromolecular substrate, different from trypsin and thrombin (Figure 3). The determination of the amino acids of these regions may be important for the development of a more specific inhibitor for these enzymes to identify the interactions of the neuropsin with a small substrate.

CONCLUSION

Neuropsin plays a crucial role in the synaptic plasticity, involved in learning and memory process and pathologies such as epilepsy and Alzheimer´s disease. Interesting, currently literature presents neuropsin as a clinical target for the diagnosis or prognostic of diseases such as ovarian cancer.

Therefore, this enzyme could be useful as target for therapeutically action and design of new drugs for treating related pathologies (i.e. multiple sclerosis).

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