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Prolyl endopeptidase mRNA expression in the central nervous system during rat development

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ABSTRACT

Prolyl endopeptidase (PEP) is a serine protease that cleaves small peptides at the carboxyl side of L-proline. PEP has been reported to have important functions in the brain being implicated in learning and memory processes, psychological disorders and neurodegenerative diseases. Several PEP substrates have been shown to play a role during brain development and this observation led us to investigate the expression of PEP mRNA in the rat brain and spinal cord, from embryo to adult stages. *In situ* hybridization revealed that PEP mRNA is expressed early, from embryonic day 15, notably in germinative areas including the neocortical, hippocampal, pallidal, thalamic, anterior hypothalamic, tectal, cerebellar, pontine and medullary neuroepithelia. PEP mRNA was also found in the differentiating fields of the olfactory bulb, the orbital and cingulate cortex, the hippocampal formation, the cortical plate and the subventricular zone of the cortex. Quantitative RT-PCR analysis in various brain areas and the spinal cord showed that PEP mRNA levels are more abundant during the perinatal stages, coinciding with a period of neuronal migration and differentiation. From then on, PEP mRNA expression decreased, reaching its lowest levels at adulthood. Overall, the present data support the possibility that PEP exerts specific functions related to neurodevelopment besides those proposed to date.

1. Introduction

Prolyl endopeptidase (PEP; EC 3. 4. 21. 26), also known as prolyl oligopeptidase, is a serine peptidase that hydrolyzes small peptides (less than 30 amino acids) at the carboxyl side of L-proline (Cunningham and O'Connor, 1997). This enzyme is present in all organisms including bacteria, fungi, plants and animals (Orlowski et al., 1979; Taylor et al., 1980; Yoshimoto et al., 1980; Faivre-Bauman et al., 1981; Yoshimoto et al., 1991; Kuwabara and Suzuki, 1994; Kabashima et al., 1998; Agirregoitia et al., 2005; Brandt et al., 2005). In mammals, PEP is detected in different organs notably in brain, liver, heart, kidney, spleen, pancreas, lung, testis (Yoshimoto et al., 1979; Taylor and Dixon, 1980; Agirregoitia et al., 2007), in fluids, such as blood, cerebrospinal fluid (Momeni et al., 2003) and seminal fluid (Valdivia et al., 2004). The highest concentrations of PEP are found in the brain, as demonstrated by enzyme activity measurement (Kato et al., 1980; Fuse et al., 1990; Irazusta et al., 2002). In addition, several studies have described a wide distribution of PEP mRNA and protein in the brain (e.g., Bellemère et al., 2004; Myöhänen et al., 2007). Consistent with its high cerebral expression, it has been proposed that PEP may play a role in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Mantle et al., 1996; Rossner et al., 2005), in various affective disorders, i.e., amnesia, depression, mania, schizophrenia, bipolar disorders, bulimia and anorexia nervosa (Yoshimoto et al., 1987; Maes et al., 1994, 1995, 2001; Breen et al., 2004), and in cognitive processes such as memory and learning (Huston and Hasenohrl, 1995; Brandt et al., 2007).

The mechanisms by which PEP exerts its biological effects in the brain remain obscure. PEP is involved in the degradation of a number of neuropeptides including thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), substance P, α-melanocyte-stimulating hormone (α-MSH), bradykinin, angiotensin, neurotensin, oxytocin and arginine–vasopressin (Taylor et al., 1980; Wilk, 1983; Bellemère et al., 2003, 2005; Garcia-Horsman et al., 2007). Several of these peptides are implicated in memory, learning or mood disorders such as depression (Jégou et al., 2006;

Abbreviations: PEP, prolyl endopeptidase; TRH, thyrotropin-releasing hormone; GnRH, gonadotropin-releasing hormone; α-MSH, α-melanocyte-stimulating hormone; IP3, 1,4,5-triphosphate; Dig, digoxigenin; SSC, standard saline citrate; B2M, beta2-microglobulin; EGL, external granule cell layer.

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Rosenkranz, 2007; Bao et al., 2008). It has also been reported that, in the brain, PEP participates in the inositol 1,4,5-triphosphate (IP3) signaling pathway and hence could represent a possible target for mood stabilizing drugs (Schulz et al., 2002; Williams et al., 2002; Cheng et al., 2005). Furthermore, in neuroblastoma and glial cell lines, PEP is associated with the cytoskeletal component, tubulin, and may thus contribute to the transport and secretion of neuropeptides (Schulz et al., 2005). More recently, it has been demonstrated that both native and catalytically inactive PEP bind to the synaptic protein GAP-43, indicating that PEP can regulate synaptic function in a non-enzymatic manner (Di Daniel et al., 2009).

Several studies have shown that PEP activity and expression in different organisms fluctuate during development (Kato et al., 1980; Faivre-Bauman et al., 1981; Martins et al., 1987; Fuse et al., 1990; Jiang et al., 2001; Agirregoitia et al., 2003a,b, 2007). Some of these studies have shown a high level of PEP activity in the rat brain early during ontogenesis (Kato et al., 1980; Fuse et al., 1990; Agirregoitia et al., 2003a). Concurrently, PEP has been found to be implicated in neuronal proliferation and differentiation (Ohtsuki et al., 1994; Szappanos et al., 1994; Moreno-Baylach et al., 2008). These observations indicate that PEP may play important functions during brain development. As a matter of fact, many PEP substrates have been implicated in ontogenesis processes (Eberle, 1988; Yamanaka et al., 1999; Shibusawa et al., 2000). Surprisingly, however, the developmental pattern of central PEP mRNA expression is currently unknown. Therefore, in the present study, we have investigated the distribution of PEP mRNA, by using *in situ* hybridization (ISH), in the rat central nervous system from embryo to adult stages. In addition, using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), we have determined the variations of PEP mRNA expression in the rat CNS and pituitary during development. The spatio-temporal developmental pattern of PEP could provide new insights into the role of PEP in brain development and neuronal organization.

2. Materials and methods

2.1. Animals

Wistar rats were housed in a temperature-controlled room (22 ± 1 °C), under a 12-h light/12-h dark schedule with free access to food and water. The day on the morning of which spermatozoids were found in vaginal smear was considered to be day 1 of pregnancy. The pregnant rats were anesthetized with thiopental (25 mg/kg, Abott, Rungis, France) and the fetuses were removed from the mothers on embryonic days 15 (E15), 18 (E18) and 21 (E21). Rats of both sexes were studied on E15, E18, E21 and parturition day (P0). On postnatal days 2 (P2), 5 (P5), 11 (P11), 15 (P15), 20 (P20), 30 (P30) and in postnatal months 3 (PM3), 8 (PM8) and 13 (PM13), only male rats were used. At each stage, 6–10 animals from 3 different litters were killed by decapitation between 9:00 AM and 11.00 AM. Animal manipulations were performed according to the recommendations of the French Ethical Committee guidelines and were conducted by authorized investigators.

2.2. Collection of tissues

For ISH, the entire fetuses (E15), whole heads (E18) or dissected brains were collected, frozen immediately in isopentane (−30 °C) and stored at −80 °C until use. For qRT-PCR, the brains were promptly removed and sliced by means of an ice-chilled plexiglass holder. The main olfactory bulb, cortex, caudate putamen, septal area, hippocampus, thalamus, hypothalamus, amygdala, cerebellum, medulla oblongata, cervical spinal cord and pituitary gland were dissected out and immediately frozen on dry ice and stored at −80 °C until homogenization. For the earlier stages (E18, E21, P0, P2 and P5), pools of tissue collected from 3 to 5 rats were prepared; 6 pools were analyzed per developmental stage. For later stages 5–7 tissue samples were collected.

2.3. In situ hybridization

Total RNAs from amygdala were reverse transcribed to generate a 480-bp DNA fragment of PEP. The reverse transcription product was amplified by PCR using the following primers.

Forward primer, 5′-CAGACAGTGAACAGACAAAGG-3′ and reverse primer, 5′-CCTGCTGTGGGTATGAGTTGT-3′ (Proligo). The amplified 480-bp PCR product was subcloned into the pGEM-T vector, and the resulting plasmid was linearized and transcribed with T7 or SP6 RNA polymerase to generate antisense or sense probes, respectively. The probes were labeled by incorporation of [^{35}S]UTP (Amersham Biosciences Europe, Les Ulis, France) or digoxigenin (Dig)-11-UTP (Roche Diagnostics, Meylan, France).

Sagittal sections for E15 and frontal sections for the other stages (18 μm thick) were cut in a cryomicrotome (2800 Frigocut; Leica, Heidelberg, Germany), collected on 0.5% gelatin/0.05% chrome alum/0.01% polylysine-coated glass slides and hybridized as previously described (Bellemère et al., 2004). Sections were fixed in 4% paraformaldehyde, incubated in 0.1 M, pH 8.0, triethanolamine for 5 min, treated with Triton X-100 (0.2%), rinsed in 2× standard saline citrate (SSC), and covered with prehybridization buffer (50% formamide/0.6 M NaCl/0.01 M Tris–HCl/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/1 mM EDTA/550 μg/ml salmon sperm DNA, and 50 μg/ml yeast tRNA, pH 7.5). Hybridization was performed overnight at 55 °C in the same buffer (except for the salmon sperm concentration which was reduced to 60 μg/ml) supplemented with 0.01 mM dithiothreitol and 10% dextran sulfate, using heat-denatured ^{35}S-labeled PEP probes (10^7 cpm/ml) or Dig-labeled probes (1%, v/v). Slices were washed in 2× SSC at 60 °C and treated with RNase A (50 μg/ml) for 60 min at 37 °C. Five high-stringency washes were performed in 0.1× SSC containing 14 mM 2-mercaptoethanol and 0.05% sodium pyrophosphate at 60 °C. For radioactive ISH, brain slices were dehydrated in ethanol and exposed onto Hyperfilm-β-max (Amersham Biosciences Europe) for 2 weeks. The autoradiograms were analyzed with a SAMBA image analyzer (SAMBA Autoradio 4.10; SAMBA Technologies, Meylan, France).

For non-radioactive ISH, brain sections were washed in a Tris–NaCl buffer (0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5; buffer 1) and incubated for 30 min in a buffer containing 2% blocking agent (Roche Diagnostics) in NaCl/maleic acid buffer (0.15 M NaCl, 0.1 M maleic acid, pH 7.5). The sections were incubated overnight at 4 °C in buffer 1 containing anti-Dig Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics) diluted 1:200 and 1% normal sheep serum. The sections were rinsed in buffer 1 for 10 min and in a 0.1 M Tris–HCl buffer supplemented with 50 mM $MgCl_2$ and 0.1 M NaCl, pH 9.5, for 10 min. The sections were then incubated in the chromogen solution containing 4-nitroblue tetrazolium-chloride and 5-bromo-4-chloro-3-indolyl phosphate supplemented with levamisole (2.4 mg/10 ml) for 3 h. The reaction was stopped by two successive washes in a 10-mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA. Brain sections were dehydrated in ethanol and mounted in Glycergel (Dako, Trappes, France). Sections were observed with a Nikon Eclipse E660 microscope (Champigny-sur-Marne, France) interfaced with the Mercator software (ExploraNova, La Rochelle, France). Images were imported into Adobe Photoshop software (Adobe Systems Inc, San José, CA) and only minor adjustments in brightness and contrast were made. Single photomicrographs were then compiled into figures using PowerPoint (Microsoft, Redmond, WA). The nomenclature used to describe the anatomical regions of the rat brain follows that proposed in Paxinos et al. (1991) and Paxinos and Watson (1998).

2.4. Quantitative reverse transcription-polymerase chain reaction analysis

Total RNAs were extracted by the acid guanidinium–thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987) using Tri-Reagent (Sigma–Aldrich, Saint-Quentin Fallavier, France). The concentration of total RNA was determined by measuring the optical density at 260 nm. Contaminating DNA was removed by treatment with RNase-free DNase I (Promega, Charbonnières, France), and cDNA was synthesized with an ImProm-II Reverse Transcription System (Promega) from 1 μg of total RNA. Parallel reactions for each RNA sample were run in the absence of reverse transcriptase to assess any genomic DNA contamination of the RNA. Quantitative RT-PCR was carried out in an ABI 7700 sequence detector system (Applied Biosystems, Courtaboeuf, France) using a SYBR Green PCR Mastermix (Applied Biosystems). Each sample was analyzed in duplicate along with standard and no-template controls. The reaction contained 25 ng of cDNA in 1× Mastermix with pre-set concentrations of dNTPs, $MgCl_2$, AmpliTaq Gold DNA polymerase and buffers, along with 300 mM forward and reverse primers (Proligo, Paris, France), and the SYBR Green reporter dye. PCR parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. PCR amplification efficiency was assessed for each primer set from the slope of a standard curve generated with serial dilutions of hypothalamic cDNA. In all cases, the slope of the standard curves was close to −3.3 indicating maximal PCR amplification efficiency. The purity of the PCR product was verified by dissociation curves and agarose gel analysis. The amount of cDNA in each sample was determined by the $2^{-\Delta\Delta Ct}$ method as outlined in User Bulletin # 2 (Applied Biosystems), using beta2-microglobulin (B2 M) as an internal control. The average ΔCt of the youngest animals was used as the calibrator. Expression of B2M mRNA did not change during the different stages of development and the ratio of the ΔCt value did not vary with the amount of cDNA.

Gene-specific forward and reverse primers were chosen using the Primer Express 2 software (Applied Biosystems) as follows: 5′-TCCTGGATCCCAACACTCTGT-3′ and 5′-ATCTTCGCTGAAGGCATAACCT-3′ for PEP (GenBank, accession number. AB012759); 5′-CATCCTGGCTCACACTGAATTC-3′ and 5′-TGACGTGTTTAACTCTG-CAAGCA-3′ for B2M (GenBank, accession number NM012512.1).

2.5. Statistical analysis

Data were analyzed statistically using SPSS® 14. The results, recorded as means ± SEM, were analyzed by one-way analysis of variance (ANOVA) followed by the *post hoc* multiple comparison Scheffé test. Statistically significant differences were considered at $p \leq 0.05$, $p \leq 0.005$ and $p \leq 0.001$.