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Opioid precursor protein isoform is targeted to the cell nuclei in the human brain

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ABSTRACT

Background: Neuropeptide precursors are traditionally viewed as proteins giving rise to small neuropeptide molecules. Prodynorphin (PDYN) is the precursor protein to dynorphins, endogenous ligands for the κ-opioid receptor. Alternative mRNA splicing of neuropeptide genes may regulate cell- and tissue-specific neuropeptide expression and produce novel protein isoforms. We here searched for novel *PDYN* mRNA and their protein product in the human brain.
Methods: Novel *PDYN* transcripts were identified using nested PCR amplification of oligo(dT) selected full-length capped mRNA. Gene expression was analyzed by qRT-PCR, PDYN protein by western blotting and confocal imaging, dynorphin peptides by radioimmunoassay. Neuronal nuclei were isolated using fluorescence-activated nuclei sorting (FANS) from postmortem human striatal tissue. Immunofluorescence staining and confocal microscopy was performed for human caudate nucleus.
Results: Two novel human *PDYN* mRNA splicing variants were identified. Expression of one of them was confined to the striatum where its levels constituted up to 30% of total *PDYN* mRNA. This transcript may be translated into ΔSP-PDYN protein lacking 13 N-terminal amino acids, a fragment of signal peptide (SP). ΔSP-PDYN was not processed to mature dynorphins and surprisingly, was targeted to the cell nuclei in a model cellular system. The endogenous PDYN protein was identified in the cell nuclei in human striatum by western blotting of isolated neuronal nuclei, and by confocal imaging.
Conclusions and general significance: High levels of alternatively spliced ΔSP-*PDYN* mRNA and nuclear localization of PDYN protein suggests a nuclear function for this isoform of the opioid peptide precursor in human striatum.

1. Introduction

A function of neuropeptide precursor proteins is to give rise to neuropeptides, small peptide molecules that serve as neurotransmitters, neuromodulators or neurohormones acting through the G-protein coupled receptors. The precursor molecules translocate into the endoplasmic reticulum where they fold and undergo posttranslational modifications, then transported to the Golgi apparatus and targeted to the regulated secretory pathway [1–4]. In endocrine cells, precursor proteins are cleaved to mature neuropeptides in the trans-Golgi network and secretory vesicles [1–6]. In neurons, the neuropeptide precursors are processed during transport from the perikaryon to axon terminals, or at these terminals and dendrites where they are stored in unprocessed form [7].

Splicing of precursor mRNA is essential for eukaryotic gene expression especially in the brain in which the most complex pattern of

Abbreviations: Dyn A, dynorphin A; Dyn B, dynorphin B; CTF, C-terminal fragment; FANS, fluorescence-activated nuclei sorting; LER, leucine-enkephalin-arginine; NLS, nuclear localization signal; PDYN, prodynorphin; PENK, proenkephalin; RIA, radioimmunoassay; SP, signal peptide; SS, splice site; TSS, transcription start site.

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alternative splicing contributes to cell differentiation, neuronal migration and synaptogenesis [8]. For example, over 28 alternatively spliced variants of the *OPRM1* gene have been identified, which most produce changes in the C-terminal protein sequence. The respective receptor proteins bind μ-opioids with similar affinities while markedly differ in their potency and efficacy for the agonist-induced G protein coupling [9]. Little is known about alternative mRNA splicing of neuropeptide genes that may regulate cell- and tissue-specific neuropeptide expression, subcellular localization of precursor proteins and neuronal plasticity, or produce novel protein isoforms.

The prodynorphin gene (*PDYN*) gives rise to dynorphins, the endogenous opioid peptides that are ligands for the κ-opioid receptor [10]. Dynorphins have a wide distribution in the brain including basal ganglia (caudate nucleus, putamen, nucleus accumbens and globus pallidus), amygdala, hippocampus, cerebral cortex and hypothalamus [11] and are essential for regulation of sensory processes [1,5,6], modulation of reward [2,3], motor control [4] and stress-induced behavioral responses [12]. The canonical form of *PDYN* pre-mRNA consists of three introns, and four exons of which the 3rd and 4th encode the full-length protein (FL-*PDYN* mRNA) [13,14] (Fig. 1A). Altogether, eight human *PDYN* transcripts *FL1*, *FL2*, splicing variants *Sp1* and *Sp2*, and 5′-truncated *Taf1*, *Taf2*, *T1*, and *T2* mRNAs were described in previous studies [14,15]. Three variants (*GTEx 1-3*) of dominant FL-*PDYN* transcripts that differ in the length of exon 1, the presence of exon 2 and distribution in human brain were identified by RNA-Seq analysis (GTEx Portal, http://www.gtexportal.org/home/) (Fig. 1A). Searching for novel *PDYN* mRNA, we here identified two *PDYN* splice variants, and characterized the expression levels and distribution in the human brain of one of them, i.e. ΔSP-*PDYN*. This transcript gives rise to a protein that lacks the 13 N-terminal aa, a fragment of the signal peptide. Surprisingly, ΔSP-PDYN ectopically expressed in a model cell line and the endogenous protein in human striatum, is located in the cell nuclei suggesting a nuclear function for this neuropeptide precursor protein isoform.

2. Materials and methods

2.1. Postmortem human brain tissue

Brain tissues were collected from ten deceased subjects with no evidence of neuropathology and no known history of neuropsychiatric disease by KI Donatum at Forensic Medicine, Department of Oncology and Pathology, Karolinska Institute, Stockholm, Sweden, by qualified pathologists under the full ethical clearance from the Stockholm Ethical Review Board. Donation was performed after an informed consent by the next of kin or by a registration as a donor by the deceased. Tissue samples of cerebellum, striatum (caudate nucleus, putamen and nucleus accumbens), dorsolateral prefrontal cortex and orbitofrontal cortex were obtained from seven subjects and used for gene expression validation by qRT-PCR (Table 1, subjects 1–7). Samples from cerebellum and striatum were used for fluorescence-activated nuclei sorting followed by western blot and radioimmunoassay (Table 1, subjects 8–10).

Sections of caudate nucleus used for analysis of ΔSP-PDYN intracellular localization by confocal microscopy were obtained from four subjects from the Postmortem Brain Core, Center for Psychiatric Neuroscience, University of Mississippi Medical Center, Jackson, MS, USA. The protocol for recruitment, tissue collection, and interviews was approved by the Institutional Review Boards of University Hospitals Case Medical Center, Cleveland, OH, and University of Mississippi Medical Center. Written informed consent was obtained from legally-defined next of kin for tissue collection and informant-based retrospective diagnostic interviews (Table 1, subjects 11–14). No subjects had evidence of head trauma, neurologic, neuropathological, or psychiatric disease.

2.2. RNA purification and cDNA synthesis

Total RNA was purified using RNeasy Lipid Tissue Mini Kit (QIAGEN, Maryland, USA), treated with RNase–free DNase I on-column for 30 min at room temperature and stored at −80 °C for further use. Total RNA amount was quantified by Nanodrop® (Nanodrop Technologies, Inc., USA). RNA Quality Indicator was analyzed by Eukaryote Total RNA StdSens assay using a Bio-Rad Experion instrument (Bio-Rad Laboratories, Hercules, CA, USA). For cDNA synthesis, total mRNA was reverse transcribed with *PDYN*-specific primer (Table 2, Fig. S1) using iScript Select kit (Bio-Rad Laboratories, Hercules, CA, USA). *PDYN*-specific 3′ primer is complementary to the exon 4 sequence that was spliced out as an intron in ΔSP/NLS-*PDYN* mRNA, thus allowing specific detection of ΔSP- but not ΔSP/NLS-*PDYN* mRNA.

2.3. Nested PCR

cDNA from human brain (FirstChoice® RACE-Ready cDNA Kit, Ambion) which is synthesized from high quality total RNA enriched for mRNA content by three rounds of oligo(dT) selection and amplified only from full-length, capped mRNA was used as a source. First round amplification was performed with F1 and R1 primers (Table 2; Fig. 1B, C) on a Mastercycler (Eppendorf, Berlin, Germany) followed by digestion with *Bgl*II to eliminate the dominant *FL1*- and *FL2*-*PDYN* transcripts. In the second round amplification was performed with F2 and R2 primers (Table 2), the resulting PCR products were purified using a Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK Limited, Little Chalfont, UK), digested with *Bgl*II and subjected to a third round of amplification with F3 and R3 primers (Table 2). Two PCR products of 850 bp size (ΔSP-*PDYN*) and 560 bp size (ΔSP/NLS-*PDYN*), were excised from the gel, purified using an Illustra GFX™ PCR DNA and Gel Band Purification Kit and sequenced in both directions. The following conditions were applied for the endpoint PCR; 95 °C for 3 min followed by 40 cycles at 95.0 °C for 30 s, 60.0 °C for 45 s and 72.0 °C for 1 min, followed by final extension at 72.0 °C for 7 min.

2.4. Quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed using Vector NTI advance 11 software (Table 2, Fig. S1). The following conditions were applied for the two steps RT-qPCR reaction; 95.0 °C for 3 min followed by 40 cycles at 95.0 °C for 10 s, annealing temperature for 61.5 °C for 30 s. A relative quantification analysis was performed, based on an external calibration curve [16], constructed from purified ΔSP- and FL-*PDYN* PCR products. The level of transcripts expressed shown in fmol/μg of total RNA. Melting curve analysis was performed to confirm the specificity of amplification and lack of primer dimers. To ensure correct amplification, PCR products were separated on agarose gel and sequenced in both directions.

2.5. Plasmid construction

cDNAs encoding FL- and ΔSP-*PDYN* were amplified by PCR using FL-pCMV4 [15] and ΔSP-pCMV4 primers (see Table 2), generating a *Bgl*II site at the 5′ end and a *Hind*III site at the 3′ end. This facilitated cloning into the *Bgl*II/*Hind*III sites of pCMV4 vector (Clontech, San Diego, CA, USA) to construct pCMV-FL-PDYN and pCMV-ΔSP-PDYN. pCMV-FL-PDYN contains coding part of exons 3 and 4 and encodes FL-PDYN (254 aa), while pCMV-ΔSP-PDYN with a 5′-truncated exon 3 and intact exon 4 gives rise to ΔSP-PDYN (241 aa). Sequencing was performed to check the correctness of both plasmids.