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Alternative splicing of human and mouse NPFF2 receptor genes: Implications to receptor expression

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Abstract **Alternative splicing has an important role in the tissue-specific regulation of gene expression. Here we report that similar to the human NPFF2 receptor, the mouse NPFF2 receptor is alternatively spliced. In human the presence of three alternatively spliced receptor variants were verified, whereas two NPFF2 receptor variants were identified in mouse. The alternative splicing affected the 5′ untranslated region of the mouse receptor and the variants in mouse were differently distributed. The mouse NPFF system may also have species-specific features since the NPFF2 receptor mRNA expression differs from that reported for rat.**

Keywords: Neuropeptide FF2 receptor; Alternative splicing; Neuropeptide

1. Introduction

Neuropeptide FF (NPFF) was discovered 20 years ago [1] and it appears to participate in several physiological functions both in the CNS and non-neuronal tissues. In accordance with its known functions in pain transmission and endocrine functions, NPFF is found in the posterior pituitary, dorsal horn of the spinal cord, the hypothalamus and pons-medulla [2–4]. Besides the distinct distribution in the central nervous system (CNS), NPFF is found in the periphery [5], and it is secreted into blood [6].

The target molecules for NPFF and related peptides, NPFF1 and NPFF2 receptors, were identified recently [7–10]. The expression patterns of the two NPFF receptors are distinct [7,11], suggesting functional differences between them. In neuroblastoma cells NPFF signals through endogenous NPFF2 receptor [12] and currently, it seems that NPFF2 receptor is the receptor mostly responsible for the effects of NPFF peptide. NPFF2 receptor was first cloned as an orphan receptor [13,14], but a ligand was found soon after the initial discovery ([7,8]; Table S1). Interestingly, the reported NPFF2 receptor sequences are not identical and might represent alternative splice variants of the receptor. The gene for the mouse NPFF2 receptor has been cloned [11,14], but its mRNA distribution has not been described and the mouse NPFF2 receptor splice variants are unknown.

In this study, we show that human and mouse NPFF2 receptors are alternatively spliced. We cloned and analyzed the splice variants and investigated the tissue-specific expression of mouse NPFF2 receptor transcripts.

2. Materials and methods

2.1. Experimental animals

The permission for the mouse experiments was obtained from the Institutional Animal Care and Use committee of Abo Akademi University. All the experiments were conducted in accordance with the European community council directive 68/609/EEC guidelines. Adult male mice (C57B6/6ola or ICR wild type mice) were maintained at a 12-h dark–light cycle with food and water available ad libitum. The animals ($n = 3$) were sacrificed by the administration of CO_2 followed by decapitation. The brains were dissected and frozen in isopentane. The frozen tissues were embedded in Embedding Matrix^R (Shandon, Pittsburg, PA, USA) and sectioned at −20 °C. Twenty micrometer sections were thaw-mounted on poly-L-lysine slides (Menzel-Gläser, Braunschweig, Germany). For the RNA isolation the tissues were frozen in liquid nitrogen. The human placenta tissue sample was obtained from the Turku University Hospital (Turku, Finland) with the permission of the patient.

2.2. RNA isolation and cDNA synthesis

Total RNA from human placenta and mouse tissues was isolated with RNAwiz protocol (Ambion Inc., Austin, TX, USA). The total RNA was treated with DNaseI (Promega, Maddison, WI, USA) before the cDNA synthesis. cDNA from total RNA was produced with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA).

2.2.1. Rapid amplification of cDNA 5′ ends (5′ RACE). Five micrograms of human placental or mouse brain total RNA was used as template material for the GeneRacer kit (Invitrogen). A primary and nested PCR was performed with RNA-oligonucleotide specific forward primers provided by the kit and gene-specific reverse primers (Table S2). The PCR products were cloned into pGEM-T Easy vector (Promega) and several recombinant clones were isolated for sequencing analysis.

2.2.2. RT-PCR analysis of the expression of mouse NPFF2 receptor. Two sets of mouse NPFF2 receptor specific primers extending over introns were used for the RT-PCR analysis of the mouse NPFF2 receptor transcripts in non-neuronal tissues and different brain areas (Table S2). The samples were also analyzed for the expression of

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Abbreviations: 5′RACE, rapid amplification of cDNA 5′ ends; 5′UTR, 5′untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NPFF, neuropeptide FF; PBGD, phorphobilinogen deaminase

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glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the case of non-neuronal tissue samples and phorphobilinogen deaminase (PBGD) in the case of CNS samples. The samples were analyzed with agarose gel electrophoresis using ethidium bromide to visualize the PCR products.

2.3. In situ hybridization

The probes (Table S2) were 3′ end labeled with [^{33}P]- or [^{35}S]-deoxyadenosine 5-(-thio) triphosphate (Amersham Biosciences, Buckinghamshire, UK) using terminal deoxynucleotide transferase (Promega). The hybridization procedure has been described in detail before [5]. A 100-fold excess of unlabeled specific probe was used to inhibit binding of the labeled probe in control samples. The sections were exposed to Kodak BioMax X-ray films (Eastman Kodak, Rochester, NY, USA).

3. Results and discussion

3.1. The splice variants of human NPFF2 receptor

Since its initial discovery, several sequences for the human NPFF2 receptor have been published (Table S1). Apparently, these different NPFF2 receptor sequences represent alternative splice variants. First, we confirmed the identity of the human NPFF2 transcripts. After analyzing a number 5′RACE clones, three distinct receptor variants were verified (Fig. 1A and B). The shortest transcript, NPFF2 receptor variant1, corresponded to the GenBank AF257210 sequence. The variant2 differed from the variant1 at its 5′untranslated region (5′UTR) and it had a putative alternative start codon nine nucleotides upstream of the reported start codon. The variant2 corresponded to AF236083. The NPFF2 receptor variant3 had a long N-terminal extension that was not found in the other variants, and corresponded to AF119815. The exon–intron structures of the variants are shown in Fig. 1A and the exon–intron junctions in detail in Fig. 1B.

Of the three cloned NPFF2 receptor variants only the variant3 differs significantly in protein coding sequence. Laemmle et al. [15] showed that this isoform has much lower affinity to NPFF peptides than NPFF2 receptor. Thus, in human the NPFF2 receptor variant3 may have a distinct function from the other variants. Laemmle et al. [15] detected yet another NPFF2 receptor variant that is lacking exons1–3 and represents a truncated receptor. This truncated transcript was not detected by the primers used in this study. The exon2 found in the variant2 contains an alternative start codon. Whether this start codon upstream of the reported transcription start site is actually used, remains to be studied.

3.2. The splice variants of mouse NPFF2 receptor

Since mouse is an extensively used species to study the NPFF system, we next asked whether the mouse NPFF2 receptor is alternatively spliced. The 5′RACE analysis of mouse whole brain RNA revealed two NPFF2 receptor transcripts in mouse (Fig. 1A and C). The coding regions, but not the 5′UTRs, of the cloned mouse NPFF2 receptor splice variants corresponded to GenBank NM_133192. The 5′UTR reported for NM_133192 was not detected by 5′RACE or traditional cloning. The BLAST search for this sequence showed it to be a repetitive sequence and maybe a cloning artefact. Similar to human, the mouse NPFF2 receptor variants differed only slightly in their coding sequence but the additional exon in variant2 introduced a 5′UTR that is missing in the variant1. The mouse variant2 had a putative alternative start codon but it was followed by a termination codon and, thus, the protein coding sequence of the two mouse NPFF2 receptor variants appears to be the same. The strength of the putative splice sites was analyzed with Alternative Splice Site Predictor [16]. The exon1a and exon2 were found in both mouse variants and in accordance with this, donor splice site of the exon1a and the acceptor and donor sites of the exon2 showed very high scores for constitutive splice sites. The exon1b was found only in the receptor variant2 and it had splice site sequences typical for alternative splicing. In spite of the cloning efforts, a human variant3-like transcript was not detected in mouse. Accordingly, alternative splicing is reported to be more frequent in human than in mouse, both in terms of percentage of genes that are alternatively spliced and the number of alternative splicing events per gene [17]. Although the receptor variants1 and 2 hardly differ in their protein coding sequence, they do have a significantly different 5′UTR sequences. At least 25% of all alternative exons are predicted to regulate transcript abundance and the alternative splicing of the 5′UTR may have drastic effects on the gene expression [18–20]. Thereby, the alternatively spliced 5′UTR may have a role in the regulation of NPFF2 receptor expression.

3.3. Expression of NPFF2 receptor splice variants in mouse

Interestingly, the conserved alternative splicing did not change the reading frame of the transcript. Such conserved modular alternative splicing events seem to be associated with tissue-specific regulation of splicing [21]. Therefore, the expression pattern of the mouse receptor variants was analyzed by in situ hybridization and by RT-PCR. Furthermore, since there are significant differences in the NPFF binding between species [11] and even rodent strains [22], it was considered of importance to determine the NPFF2 receptor mRNA expression pattern in mouse. In addition, the differential expression of the receptor variants has not been studied.

The expression of NPFF2 receptor was detected at restricted sites in the mouse CNS (Fig. 2). The highest expression level was detected at the external plexiform layer of the olfactory bulb (EPl) and a strong signal in the nucleus of the solitary tract (Sol), dorsal tegmental nucleus (DTg) and reunions thalamic nucleus (Re). Moderate or low level of expression was detected also at other sites. Mouse NPFF2 receptor variant 2 mRNA was detected only in the olfactory bulb (Fig. 3).

The individual contribution of the receptor variant1 to the NPFF2 receptor expression could be revealed by RT-PCR. To get a more complete view of the contribution of the transcripts in mouse, the expression of the splice variants in peripheral organs was analyzed. Based on the RT-PCR analysis both variants were expressed in several brain regions (Fig. 4A) and non-neuronal tissues (Fig. 4B), but their relative abundance varied markedly between tissues. The analysis of brain regions with RT-PCR showed wider expression of NPFF2 receptor than was expected based on the in situ hybridization data (Data S1). In general, the variant2 was more abundant in the non-neuronal tissues and whole brain. In the CNS, the variant1 was more abundant than the variant2 only in the olfactory bulb. Similar to in situ hybridization analysis, the RT-PCR analysis of the CNS (Fig. 4A) indicated the highest NPFF2 receptor mRNA expression in the olfactory bulb. The role of NPFF in the olfactory system is not known and should be further investigated in detail. The strong mRNA sig-