Identification of five mouse \( \mu \)-opioid receptor (MOR) gene (\( \text{Oprm1} \)) splice variants containing a newly identified alternatively spliced exon

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Abstract

The mouse \( \mu \)-opioid receptor gene, \( \text{Oprm1} \), currently contains 18 recognized alternatively spliced exons [Doyle, G.A., Sheng, X.R., Lin, S.S.J., Press, D.M., Grice, D.E., Buono, R.J., Ferraro, T.N., Berrettini, W.H., 2007. Identification of three mouse \( \mu \)-opioid receptor (MOR) gene (\( \text{Oprm1} \)) splice variants containing a newly identified alternatively spliced exon. Gene 388 (1–2) 135–147, in press (doi:10.1016/j.gene.2006.10.017). Electronic publication 2006 November 1] that generate 27 splice variants encoding at least 11 morphine-binding isoforms of the receptor. Here, we identify five MOR variants that contain an as yet undescribed exon (exon 19) of the gene, and we provide evidence that these MOR splice variants are expressed in mouse and rat. The fourth splice variant encodes a novel \( \mu \)-opioid receptor isoform, MOR-1U, and contains exon 19 in its coding region. The cytoplasmic tail of the putative MOR-1U isoform contains a putative nuclear localization signal encoded by the sequence of exon 19. Exon 19 appears to be conserved in the rat, but not in humans. In mouse and rat \( \text{Oprm1} \), exon 19 is located between described exons 7 and 8. We also report the cloning of the “full-length” MOR-1T splice variant [Kvam, T.-M., Baar, C., Rakvag, T.T., Kausa, S., Krokan, H.E., Skorpen, F., 2004. Genetic analysis of the murine \( \mu \)-opioid receptor gene has increased complexity of \( \text{Oprm1} \) gene splicing, J. Mol. Med. 82 (4) 250–255] that encodes MOR-1 and contains the newly identified exon in its 3′ UTR. RT-PCR analysis suggests that splice variants MOR-1Eii, MOR-1Eii, MOR-1Eiv, MOR-1T and MOR-1U are expressed in all brain regions analyzed (cortex, cerebellum, hypothalamus, thalamus and striatum). These exon 19-containing splice variants add to the growing complexity of the mouse \( \text{Oprm1} \) gene.

1. Introduction

There are three major receptors for morphine and related compounds: the \( \mu \), \( \delta \) and \( \kappa \)-opioid receptors. Of these, the \( \mu \)-opioid receptor (MOR) is the main site of morphine binding in the brain (Pasternak, 2001; Wood and Iyengar, 1988). Since its initial discovery, the increasingly complex nature of the mouse \( \text{Oprm1} \) gene has been appreciated. Initially, it was thought to contain one promoter and four exons that encoded one protein, MOR-1 (Kaufman et al., 1995; Min et al., 1994). It is now known that the mouse \( \text{Oprm1} \) gene has two promoters (Liang et al., 1995; Oprm1) and eighteen exons (Pan et al., 2001) and that the mouse \( \text{Oprm1} \) gene has been appreciated. Initially, it was thought to contain one promoter and four exons that encoded one protein, MOR-1 (Kaufman et al., 1995; Min et al., 1994). It is now known that the mouse \( \text{Oprm1} \) gene has two promoters (Liang et al., 1995; Min et al., 1994; Pan, 2002; Pan et al., 2001) and eighteen exons (Doyle et al., 2007), encompassing 230–250 kbp of proximal chromosome 10 (Giros et al., 1995; Kozak et al., 1994; Kvam et al., 2004; Pan et al., 2005). To complicate matters, exons 1–18
are alternatively spliced to generate at least 27 splice variants that encode at least 11 morphine-binding MOR isoforms (Doyle et al., 2007; Kvam et al., 2004; Pan et al., 1999; Pan et al., 2000; Pan et al., 2001, Pan et al., 2005).

Most of the MOR splice variants originate at the downstream Oprml dual promoter region (5′ of exon 1). At least eleven of these MOR isoforms are capable of binding morphine strongly in vitro and are expressed throughout the brain and central nervous system (Abbadie et al., 2000; Pan et al., 1999; Pan et al., 2000; Pan et al., 2005). These isoforms include splice variants containing exons 1, 2 and 3 followed by exon 4 (MOR-1) or some other exon (MOR-1A through MOR-1F; Pan et al., 2001; Pan et al., 2005). This leads to a number of receptors differing in their carboxy termini that bind morphine, and other mu class opiates (for extensive review of the μ-opioid receptor gene in mouse, rat and humans, see Pan, 2005). Recently, Pan et al. (2001) reported 8 splice variants originating at an upstream promoter (5′ of exon 11). Three of these new variants (MOR-1H, MOR-1I, MOR-1J) encode the MOR-1 isoform in addition to novel peptides encoded by upstream open reading frames (Pan et al., 2001). Most of these exon 11-containing variants encode proteins that bind morphine very weakly in vitro (Pan et al., 2001).

In a previous paper (Doyle et al., 2007), we described the identification of three MOR splice variants containing a newly identified, alternatively spliced exon, exon 18, that encode two new putative MOR isoforms, MOR-1V and MOR-1W. Here, we identify four additional MOR splice variants that contain another as yet undescribed Oprml exon, exon 19. We have localized exon 19 within the mouse and rat Oprml genes. Theoretically, three of the novel splice variants encode MOR-1E with novel exonic sequence from exon 19 in their 3′ untranslated regions (UTR). Kvam et al. (2004) previously reported the partial cDNA of splice variant MOR-1T that theoretically encodes MOR-1. We now report that the “full-length” MOR-1T encodes the MOR-1 isoform, but contains the novel exon 19 in its 3′ UTR. The final novel splice variant reported here, MOR-1U, contains exon 19 in its coding region. These findings add to the increasing complexity of the murine Oprml gene and the endogenous opioid system in mice and rats. We discuss the implications of these findings as they may relate to mRNA stability, localization and/or translation of the variants containing exon 19 in their 3′ UTR. In addition, we discuss how a putative nuclear localization signal (NLS) in the carboxy terminus of the MOR-1U isoform may relate to receptor internalization and nuclear translocation.

2. Materials and methods

2.1. Isolation of RNA and amplification of partial cDNAs

Brains from male C57BL/6 (B6) or DBA/2 (D2) mice were dissected after anesthesia with isofluorane and euthanasia by cervical dislocation. Total RNA derived from B6 or D2 mouse brain (either whole brain, gross brain sections or dissected brain regions) was purified using the Ultraspec™ (Biotecx Laboratories, Houston, TX) or TRIZol® reagent (Invitrogen, Carlsbad, CA) RNA purification kit as per the manufacturers’ instructions. One to five micrograms of total brain RNA was converted to cDNA with oligomer (dT)12–18 as primer using the Superscript™ II reverse transcription kit (Invitrogen) as per the manufacturer’s instructions. A partial cDNA, MOR-1Eii, containing exon 19, was amplified by polymerase chain reaction (PCR) of reverse transcribed mRNA using the forward primer 3fi (5′-ATC CCA ACT TCC TCC ACA ATC G-3′) and reverse primer 9r2 (5′-GCT TTC TTC CTC TTC TTC GTG GAC-3′) that annealed to Oprml exons 3 and 9, respectively. PCR cycle conditions were 94 °C for 5 min followed by 30 cycles of 94°C for 30 s, 58 °C for 30 s, 72 °C for 90 s, then 72 °C for 5 min.

2.2. Amplification and cloning of “full-length” cDNAs

Five micrograms of whole brain total RNA from either B6 or D2 mouse was converted to cDNAs as described (Doyle et al., 2007). To avoid amplification of genomic DNA, all PCR primers were designed such that the forward and reverse primers were separated by multiple large introns. Materials and methods for identification of “full-length” transcripts by RT-PCR and Southern blotting were as described (Doyle et al., 2007) with the exception that radiolabeled Oprml exon 19 was used as probe in the Southern blots rather than exon 18.

“Full-length” cDNA amplicons were subcloned from the secondary PCR reactions into the pCR®-2.1-TOPO® vector using the TOPO TA-cloning kit (Invitrogen). Colonies containing MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U were identified by colony hybridization with radiolabeled exon 19 as probe under Southern blotting conditions as described (Doyle et al., 2007). Inserts were fully sequenced at the Nucleic Acid/Protein Research Core Facility at the Children’s Hospital of Philadelphia using cycle sequencing with ABI Prism® BigDye™ chemistry on an Applied Biosystems, Inc. 3730 DNA analyzer.

2.3. Analysis of MOR mRNA expression in brain regions

To analyze expression of variants MOR-1Eii and MOR-1U in different brain regions, PCR was done as follows. Approximately 50 ng of oligo(dT)12–18-primed cDNAs were amplified in a primary PCR using a forward primer in Oprml exon 3, 3fi (see above) paired with a reverse primer at the exon 19-exon 7 junction found in MOR-1Eii and MOR-1U, 19/7 (5′-TGTCAGCTAGTCACTTGCCA TG-3′). Secondary PCR was unnecessary as bands were readily apparent after the primary reactions. For primary PCRs, conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 70 °C for 30 s, then 70 °C for 5 min, to complete the extension of all amplicons, under standard salt, dNTP and primer concentrations, but with the addition of 1 M betaine and 0.8 μg/μl bovine serum albumin to the reaction mixes. Amplicons from the primary PCR reactions were subcloned into pCR®-2.1-TOPO® and sequenced to confirm their identities as MOR-1Eii and MOR-1U.

To analyze expression of MOR-1Eiii and MOR-1Eiv, approximately 50 ng of oligo(dT)12–18-primed cDNAs were amplified in a primary PCR using a forward primer that annealed to the junction of Oprml exons 7 and 19, found in MOR-1Eiii/iV, but not in MOR-1Eii or MOR-1U, 7/19f (5′-TTG CAT GGA
CAG ACT GCT AC-3') paired with a reverse primer that annealed in exon 8, 8r (5'-CTG TCT TGG GCC ATC ATC AG-3'). PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min then 72 °C for 5 min, to complete extension of all amplicons, under the same reaction conditions stated above. Weak gel bands of numerous sizes were seen after the primary PCR step, but most were of the incorrect sizes to be MOR-1Eiiii or MOR-1Eiv. Therefore, half-nested secondary PCR was done using about one tenth (2.5 μl) of the primary PCR as template with forward primer 7/19f (see above) and the nested reverse primer 8+ Cr (5'-GAA GAA GGT TCC TCA TTC CTC-3'). To prevent carryover of primers, nucleotides and incorrect extension products from the primary PCR reaction, prior to use in the secondary PCR, the primary template (2.5 μl) was treated with 1 μl of ExoSAP-IT® (USB Corporation, Cleveland, OH) at 37 °C for 20 min, then 80 °C for 20 min to inactivate the enzymes. Secondary PCR conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min then 72 °C for 5 min, to complete extension of all amplicons, under the same reaction conditions stated above. Some of the secondary reactions (those that gave single bands) were treated with ExoSAP-IT® (USB Corporation) prior to sequencing in both directions to confirm their identities as MOR-1Eiiii and MOR-1Eiv.

To analyze expression of MOR-1T, approximately 50 ng of oligo(dT)12–18-primed cDNAs was amplified in a primary PCR using a forward primer in Oprm1 exon 3, 3f1 (see above) paired with a reverse primer at the exon 6-exon 4 junction, 6/4r (5'-CAG CTT TTC ACA ACA GAG TAT TG-3') found only in MOR-1T (Kvam et al., 2004). PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min then 72 °C for 5 min, to complete extension of all amplicons, under the same reaction conditions stated above. MOR-1T reactions were treated with ExoSAP-IT® (USB Corporation) according to the manufacturer's instructions. Reactions were then sequenced in both directions with primer 3f1 or 6/4r, confirming that the bands were in fact MOR-1T.

To ensure the quality of the cDNA and determine the relative abundance of template used in the reactions, we amplified G6PDX using the forward primer g6.888 (5'-CAG GTT CAG ATG ATG TCC-3') and the reverse primer g6.1908 (5'-GTG ATT CAG GTC TTG TGG-3') in a separate standard 50 μl reaction. For G6PDX, PCR conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s, 70 °C for 1 min, 20 s then 70 °C for 5 min to complete the extension of all amplicons.

2.4. Sequence comparisons and analyses

Sequences were compared against the non-redundant and mouse EST GenBank databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and against the mouse and other genomes using BLAT (Kent, 2002) at the Human Genome Browser at UCSC (genome.ucsc.

Fig. 1. Schematic of Oprm1 gene with novel MOR cDNAs. Shown are the exon and intron structure of the Oprm1 gene and the novel splice variants described in this paper. The newly identified exon 19 is shaded. Transcription start sites are indicated as bent arrows. Intron sizes (in kilobases) are based on the C57BL/6 genomic sequence deposited in Ensembl. Double slanted lines within introns indicate a large distance. Thick black vertical lines in exons represent areas of exons that are alternatively spliced. The splicing and structure of the partial MOR-1Eiiii (DQ363374) cDNA and of the “full-length” MOR-1Eiiii (EF105311), MOR-1Eiv (EF105312), MOR-1T (EF105313) and MOR-1U (EF105314) cDNAs are shown. Question marks indicate areas of uncertain structure. Not drawn to scale.

3. Results

3.1. Isolation of initial, partial MOR splice variant MOR-1Eii

RT-PCR of mouse brain mRNAs using primers in exon 3 and exon 9 initially yielded a new partial MOR cDNA from the B6 strain (Figs. 1 and 2, MOR-1Eii). The structure and sequence of this cDNA revealed an as yet undescribed exon, exon 19, in the Oprml gene (Fig. 1). The sequence and theoretical translation of this cDNA has been deposited into the GenBank database under accession number DQ363374 and is shown in Fig. 2.

3.2. Identification of additional “full-length” exon 19-containing MOR splice variants

In order to clone “full-length” MOR-1Eii, we performed long distance RT-PCR on B6 and D2 whole brain cDNAs, followed by Southern blotting with an Oprml exon 19-specific probe. Using primer pairs in exon 1 and exon 9, Southern analysis with exon 19 detected a major band, possibly a doublet, of approximately 2.3 kb and minor bands of approximately 1.8, 2.0 and 2.5 kb (Fig. 3). We did not clone “full-length” MOR-1Eii, however, we cloned four other exon-19-containing variants; MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U (Figs. 4 and 5). The 2.0 kb band is close to the size of the “full-length” MOR-1Eiii variant which is 1.93 kb long (Fig. S1, GenBank Accession No. EF105311). The doublet at 2.3 kb is consistent with the size of “full-length” MOR-1Eiv (Fig. S2, GenBank accession no. EF105312) and with the predicted size of MOR-1Eii assuming a structure similar to the other MOR-1E splice variants. The 2.5 kb band was only detected in whole brain cDNA derived from D2 mouse polyA+ mRNA, while all other bands were detected in both B6 and D2 whole brain cDNA pools. The size of the 2.5 kb minor band is consistent with the predicted size (∼2.6 kb) of the “full-length” MOR-1T variant that was cloned from D2 mouse brain (Fig. S3, GenBank accession no. EF105313). The minor band of approximately 1.8 kb is consistent with the size of the “full-length” MOR-1U variant (Fig. S4, GenBank accession no. EF105314).

To determine whether exon 19-containing splice variants originate from the downstream dual (5′ of exon 1) or upstream (5′ of exon 11) Oprml promoter, we performed RT-PCR with primers in exon 1 or 11 paired with primers in either exon 4 or 9, followed by Southern analysis. No bands were detected with the exon 19 probe in Southern blots of RT-PCR products using exon 11 primers paired with either exon 4 or exon 9 (data not shown). However, ethidium bromide staining of the gel revealed amplicons prior to Southern blotting of nested secondary PCR using primers in exon 11 and exon 4 indicating that these primer pairs could in fact amplify MOR cDNA.

Fig. 2. Partial MOR-1Eii cDNA sequence. The C57BL/6 sequence of the partial cDNA containing exon 19 (underlined) is shown. Exon 19 is spliced between exons 7 and 8 in the partial cDNA that theoretically encodes the MOR-1E isoform. Exons are indicated by numbers and splice junctions by downward arrows. The full-lengths of exons 3 and 9 are not included due to the primers used in the PCR step; however the polyadenylation site in exon 9 in the genomic DNA is located 43 nucleotides downstream of the primer used (Pan et al., 1999). This sequence has been deposited in GenBank under accession number DQ363374.

Fig. 3. Southern blot of whole brain mRNA RT-PCR. RT-PCR was done in duplicate on whole brain mRNA from either C57BL/6 (B6) or DBA/2 (D2) mice using primers that annealed to mouse Oprml exons 1 and 9. Secondary nested PCR products were separated on a 1% agarose gel, transferred to Hybond N’ membrane and then probed with radiolabeled mouse Oprml exon 19. Molecular weight markers (in kb) are indicated on the left. Based on the sizes of the cloned cDNAs, arrows indicate the positions of bands putatively identified as MOR-1Eii, MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U.
variants. The fact that no bands were detected by Southern blotting with exon 19 in RT-PCR samples indicates that they lack exon 19. We do not observe exon 19-containing amplicons from RT-PCRs using primers in exon 11 suggesting that MOR-1Eii, MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U, as well as other possible exon 19-containing variants, do not contain exon 1 spliced within an exon 11-originating transcript as is the case with the MOR-1H, MOR-1I and MOR-1J splice variants (Pan et al., 2001). Thus, MOR-1Eii, MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U most likely originate solely from the dual downstream promoter upstream of exon 1, not the promoter upstream of exon 11 (Pan 2002).

3.3. Comparison of exon 19 to the databases

Comparison of the MOR-1Eii cDNA to genome databases using BLAT revealed that the BLAST revealed over 100 hits to the same sequence in multiple database sequences suggesting a repetitive element was present in exon 19. Indeed, RepeatMasker analysis revealed this repetitive element to be a B2 short interspersed element (data not shown). RepeatMasker also revealed a mammalian apparent LTR retrotransposon (MaLR) in exon 19 (data not shown).

Comparative genomic analysis using BLAT revealed that chromosome 1 of the rat genome contains the newly identified Oprml exon 19 (Fig. 6A). The rat conserves the splice acceptor site at the 5′ end of exon 19, but the splice donor site is not conserved (Fig. 6A). While exon 19 is non-coding in MOR-1Eii, MOR-1Eiii, MOR-1Eiv and MOR-1T, it is within the coding region of MOR-1U. Therefore, we translated the nucleotide
sequence of rat \textit{Oprm1} exon 19 to determine if its amino acid sequence, in particular the NLS found in MOR-1U, was conserved between mouse and rat (Fig. 6B). While there is conservation of the amino side of frame 2 between mouse and rat in exon 19, it does not conserve the NLS located in MOR-1U. This suggests that if exon 19 is employed similarly in the rat, it does not generate an analogous MOR-1U isoform with a putative NLS, as in the mouse.

All other available vertebrate genomes, including human, showed no significant or weak matches to the newly identified mouse \textit{Oprm1} exon 19 when BLAT comparison was done.

### 3.4. RT-PCR analysis of MOR variants in mouse brain regions

To determine the distribution of MOR-1Eii and MOR-1U in different brain regions, we performed RT-PCR on total RNA derived from various B6 and D2 mouse brain regions. Primers directed at exon 3 and at the junction between exon 7 and 19 yielded amplicons after a single round of PCR. With the

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**Fig. 6.** Nucleotide and amino acid alignments of mouse and rat exon 19. (A) The nucleotide BLAST alignment of mouse versus rat exon 19 is shown. Downward arrows indicate the positions of the splice sites for MOR-1Eii, MOR-1Eiii, MOR-1Eiv, MOR-1T and MOR-1U. The frame 2 stop codon of the rat sequence is bolded and underlined. (B) The frame 2 amino acid alignment of mouse versus rat exon 19 is shown. Vertical bars between amino acids indicate conservation of translation. Note the lack of conservation of the putative NLS in the mouse translation of exon 19.

**Fig. 7.** RT-PCR of MOR variants from various brain regions. (A) Top panel: MOR-1Eii and MOR-1U were amplified in a primary PCR reaction using the primer pair of 3f1 and 19/7. MOR-1Eii and MOR-1U are found in all brain regions from C57BL/6 (B6) and DBA/2 (D2) mice. Middle panel: MOR-1Eiii and MOR-1Eiv were amplified in a primary PCR reaction using primer pair 7/19f and 8r, then in a half-nested secondary PCR reaction using primer pair 7/19f and 8+ Cr. See Table 1 for a summation of five separate experiments done. Shown is experiment number five indicating MOR-1Eiii in all brain regions except hypothalamus in both B6 and D2 strains and MOR-1Eiv in all D2 brain regions except thalamus. Bottom panel: \textit{G6PDX} was amplified in a primary PCR reaction using primer pair g6.888 and g6.1908. (B) MOR-1T was amplified in a primary PCR reaction using primer pair 3f1 and 6/4r. MOR-1T is found in all B6 and D2 brain regions analyzed, but is expressed more highly in thalamus and striatum.
exception of D2 cortex, a band of approximately 330 bp, representing MOR-1Eii, was observed in all brain regions examined (cortex, cerebellum, hypothalamus, thalamus and striatum) (Fig. 7A, top panel). The possibility that MOR-1Eii and MOR-1Eiv might have co-amplified with MOR-1Ei is unlikely because 1) the reverse primer 19/7 would not have annealed to the MOR-1Eiii/iv junction (which differs from that of MOR-1Eii, see Fig. 7A) under the PCR conditions used and 2) two rounds of PCR are required to amplify MOR-1Eiii/iv (Fig. 7A, middle panel).

A 210 bp band, representing a potentially new variant, MOR-1U, containing exon 3 and exon 19 was observed in all brain regions (Fig. 7A, top panel). The size of the band is consistent with that of MOR-1Eii lacking exon 6. Indeed, during our attempts to clone “full-length” MOR-1Eii, we cloned a new variant, MOR-1U, containing exon 3 spliced to exon 7 followed by exon 19, 8 and 9 (Figs. 1 and 5B). Sequencing of this 210 bp band confirmed that the MOR-1U splice variant is expressed in all brain regions of B6 and D2 mice.

We also cloned “full-length” MOR-1Eiii (Figs. S1 and 4A), MOR-1Eiv (Figs. S2 and 4B) and MOR-1T (Figs. S3 and 5A) during our attempts to clone full-length MOR-1Ei. RT-PCR analysis of MOR-1Eiii and MOR-1Eiv in discrete brain regions was somewhat variable across five independent experiments (Table 1). We conclude from the summation of these five experiments that MOR-1Eiii and MOR-1Eiv are expressed in all brain regions analyzed in both strains of mice (Fig. 7A, middle panel, and data not shown). The variable nature of the MOR-1Eiii/iv results can be explained by 1) low abundance of these transcripts (as suggested by the necessity of two rounds of PCR to detect them) combined with 2) inaccurate samplings of the cDNA pools between experiments. RT-PCR analysis of MOR-1T indicates that it is expressed in all brain regions examined, but is more abundant in thalamus and striatum of B6 and D2 mouse brain (Fig. 7B).

RT-PCR of G6PDX was done in separate reactions to determine the quality of the cDNA preparations as well as to verify equal sampling of the cDNA pools for the PCR step. RT-PCR of G6PDX from the various brain regions yielded bands of equal intensity in all lanes (Fig. 7A, bottom panel) indicating that the quality of the cDNA was good for all preparations and that sampling of the cDNA pool for G6PDX was accurate for relatively abundant transcripts.

### Table 1

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MOR-1Eiii and MOR-1Eiv were targeted for amplification from cortex, cerebellum, hypothalamus, thalamus and striatum of B6 and D2 mice in five separate experiments. Shown are the results of each experiment. An “ii” or “iv” indicates that MOR-1Eii or MOR-1Eiv, respectively, was amplified in that region in any given experiment, however, band intensities, indicated by “+”, “+/-”, “+/-”, etc., for MOR-1Eiii and MOR-1Eiv were variable among assays complicating quantification of each.

### 3.5. Theoretical translations of MOR-1Eii/iii/iv, MOR-1T and MOR-1X cDNAs

The theoretical translation of MOR-1Ei, MOR-1Eiii and MOR-1Eiv, containing exon 19 in their 3′ untranslated regions, is MOR-1E (Fig. 8). The theoretical translation of MOR-1T, also containing exon 19 in its 3′ UTR, is MOR-1T (Figs. 5A and 8). The theoretical translation of MOR-1U gives a novel MOR isoform with the carboxy terminus of PTL AVS VAQ IFT GYP SPT HVE KPC KSC MDS VDC YNR KQQ TGS LRE NKK KKK RRR NKQ NIL EAG ISR GMR NLL PDD GPR QES GEG QLG R (Figs. 5B and 8). We note that the amino and carboxy portions of this terminal sequence are identical to that of mMOR-1C, with the in-frame splicing of exon 19 adding 36 amino acids, many of them charged, in the middle of the exon 7 and exon 8 coding sequences. Interestingly, NLS’s are predicted to be present in the sequence KNKKKKKKKRRKN found within the 36 amino acids encoded by exon 19 in MOR-1U. In addition to the casein–kinase II (CKII), and cyclin-dependent kinase 5 (cdk5) phosphorylation sites noted by Pan (2005) for mMOR-1C, Kinase-Phos and NetPhos 2.0 predict phosphorylation sites for cell
splice variants differ in their 3′ untranslated regions in the same brain region. One explanation may be that these different splice variants that encode the same receptor isoform might be differentially regulated.

MOR-1Eii, MOR-1T and MOR-1U are readily detectable in various brain regions after a single round of RT-PCR (Fig. 7A, top panel, and B), whereas, MOR-1Eii and MOR-1Eiv require two rounds of PCR to be detected (Fig. 7A, middle panel). Thus, in contrast to MOR-1, MOR-1Eii and MOR-1Eiv are probably very rare transcripts in the brain regions examined. MOR-1 is readily detectable in various brain regions after a single round of RT-PCR (for MOR-1 results, see Doyle et al., 2007). While absolute quantification has not been done, based on the number of PCR cycles and the band intensities observed, MOR-1Eii, MOR-1T and MOR-1U variants are estimated to be expressed in the brain at levels less than MOR-1, but greater than the exon-11-containing variant. Of course, for various reasons, mRNA expression levels are not always a good indication of protein expression levels in the brain.

The MOR-1Eii, MOR-1Eiii and MOR-1Eiv variants are of particular interest as they all encode the same MOR isoform as defined by MOR-1E transcripts, but have different portions of exon 19 contained in their 3′ untranslated region (Figs. 2, 4A and B). The MOR-1E splice variant is expressed weakly in cortex, strongly in hypothalamus, striatum and periaqueductal grey, and is not expressed in the thalamus or cerebellum (Pan et al., 1999). We find the MOR-1Eii variant expressed in hypothalamus and striatum, as well as in the thalamus, cortex and cerebellum (Fig. 7A). The MOR-1Eii and MOR-1Eiv splice variants are also found expressed in hypothalamus and striatum, as well as in cortex and cerebellum (Fig. 7A). Thus, there is both overlapping and discordant patterns of expression of these three splice variants with the MOR-1E splice variant (Pan et al., 1999).

We (Doyle et al., 2007) and others (Pan et al., 1999) have shown strong expression of MOR-1 transcripts in all brain regions examined. Here, we find that the MOR-1T splice variant, which also encodes the MOR-1 isoform, is expressed in all brain regions examined, with high levels in thalamus and striatum (Fig. 7B). These findings, as well as those stated above for the MOR-1E isoform encoding splice variants, raise the question of why there should be overlapping expression of different splice variants that encode the same receptor isoform in the same brain region. One explanation may be that these splice variants differ in their 3′ untranslated regions and can therefore be differentially regulated.

The 3′ untranslated region of an mRNA can regulate the stability, translation and/or localization of the transcript (Hughes, 2006; Hesketh, 2004; Ross, 1995). Indeed, alternative 3′ untranslated regions, created by alternative polyadenylation site usage or alternative splicing of 3′ exons (Hughes, 2006), have been shown to regulate the expression of β-catenin and T-cell receptor ε (Thiele et al., 2006; Chowdhury et al., 2005). This raises the possibility of differential expression, localization and/or regulation (stability or translation) of alternatively spliced MOR-1 and MOR-1E encoding transcripts in the brain. mRNA-binding proteins, such as chicken zipcode-binding protein 1 for β-actin, transport mRNA for localized translation in neurons and other cell types (Huttelmaier et al., 2005). With that in mind, there might be localized translation of MORs within neurons both in the cell body and along the axon at synaptic loci. Localized MOR isoforms might then be in the appropriate location to interact with different MOR-interacting proteins (Onoprishvili et al., 2003; Guang et al., 2004) leading to “rafts” of receptor subtypes along the plasma membrane of the cell.

The MOR-1U splice variant encodes a newly identified MOR isoform that differs from other MORs at its carboxy terminus (Figs. 5B and 8). The cytoplasmic tail of MOR-1U contains a putative NLS as defined by the sequence RKKKKKKRRK. This feature of MOR-1U is unusual for G-protein coupled receptors (GPCR), but is not unprecedented. The known MORs do not have nuclear localization signals, but the apelin, angiotensin type 1 (AT1), and bradykinin B2 receptors transit to the nucleus in an agonist-independent manner (Lee et al., 2004). Indeed, angiotensin II induces the translocation of the AT1, but not the AT2 receptor, to the nucleus of brain neurons (Lu et al., 1998). The AT1 receptor contains an NLS (KKFKK) in its cytoplasmic tail, its location being similar to that observed in MOR-1U. This suggests that this MOR isoform may transit to the nucleus in either an agonist-independent or –dependent manner. A role for the carboxy terminus and NLS in internalization would be consistent with reports of agonist-induced internalization of MORs (Abbadie and Pasternak, 2001; Evans, 2000; Keith et al., 1998; Kelly et al., 1996; Koch et al., 2001; Sternini et al., 1996; Zaki et al., 2000; Zimprich et al., 1999). Therefore, it is not unreasonable to suggest that the distinct carboxy terminus of the newly identified MOR-1U isoform reported here might lead to differential regulation and/or subcellular localization in response to agonist binding.

The carboxy terminus of the MOR-1U isoform contains a number of putative phosphorylation sites (Fig. 8) raising the possibility that phosphorylation of the cytoplasmic tail of MOR-1U in different brain regions might serve to regulate its internalization and/or subcellular localization. GPCR desensitization and resensitization often involves the phosphorylation of the carboxy terminus of the receptor (Ferguson, 2001). Indeed, phosphorylation of threonine 394 in the carboxy terminus plays a role in receptor desensitization, resensitization and internalization of rat MOR (Pak et al., 1997; Wolf et al., 1999). Therefore, it will be important to study the involvement of the various kinases with predicted phosphorylation sites in MOR-1U regarding their involvement in receptor desensitization, resensitization and internalization.

In conclusion, we have identified three new, brain expressed, alternatively spliced MOR variants encoding MOR-1E, and a novel MOR variant, MOR-1U. The functionality and regulation of these new variants will be important to understand as they change the way in which we envision MOR expression and localization.
subcellular localization, respectively. Finally, we have confirmed the existence of the MOR-1T variant (Kvam et al., 2004) and have identified its complete exon structure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2007.02.004.

References


