Prediction of a Novel "Short" μ Opiate Receptor in Domestic Chicken

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Abstract

Previous work from our laboratory has established that cellular signaling processes of endogenous morphine are mediated by cognate G protein coupled receptor (GPCR) proteins, designated μ_3 and μ_4 opiate receptors. μ_3 and μ_4 opiate receptors are structurally unique "short" 6 transmembrane helical (TMH) domain GPCRs that are selectively responsive to endogenous morphine and not to families of endogenous opioid peptides. The goal of the present study was to provide protein sequence and conformational analysis of a predicted novel "short" μ opioid receptor mRNA in domestic chicken utilizing well established computer-assisted tools.

The nucleic acid sequence of the predicted mRNA was derived by Gnomon, an automated computational analysis algorithm provided by the NCBI and was translated into corresponding amino acid sequences using the widely accepted proteomics server, ExPASy. Conformational analysis of predicted amino acid sequences, as derived by ExPASy, was performed using the TMHMM computer program. BLAST software was used to perform comparative amino acid sequence analyses of the predicted μ opiate receptor protein in chicken to previously deposited μ opiate receptor sequences from other species.

Based on high resolution predictive measures, it appears likely that poultry express a μ opiate receptor mRNA encoding one or two novel "short" GPCRs with similar biochemical characteristics as described for μ_3 and μ_4 opiate receptors as well as traditional μ1 opioid receptors.

The expression of biologically unique, functional "short" μ opiate receptor proteins in chicken not only reinforces the primacy of said receptor, but in doing so provides us with a window for understanding evolutionary processes underlying GPCR diversity.

INTRODUCTION

Previous work from our laboratory has focused on the elucidation of biochemical, cellular, and molecular mechanisms underlying the regulatory roles of endogenously expressed, chemically authentic, morphine in animal cells and organ systems. As a critical corollary, we have established that cellular signaling processes of endogenous morphine are mediated by cognate G protein coupled receptor (GPCR) proteins, designated μ_3 and μ_4 opiate receptors. μ_3 and μ_4 opiate receptors are structurally tailored to be selectively activated by morphine and morphine-related opiate alkaloids and not by related families of endogenous opioid peptides (Cadet *et al* 2003; Cadet *et al* 2007) and are functionally coupled to activation of constitutive nitric oxide (NO) production and release (Atmanene *et al* 2009; De Gennaro Colonna *et al* 2009; Fricchione *et al* 2008; Pflueger *et al* 2009; Stefano *et al* 2009a,b; Tanii *et al* 2008).

The unique structural features of μ_3 and μ_4 opiate receptors are determined post-transcriptionally via selective splicing of the primary transcript of the μ_1 opioid receptor (MOR) gene. Mature μ_3 and μ_4 opiate receptor-encoding mRNAs translate into receptor proteins lacking an amino acid sequence of approximately 90 amino acids that constitute the extracellular N-terminal and transmembrane helical (TMH)1 domains and part of the first intracellular loop of the μ_1 receptor, but retain the empirically defined ligand binding pocket distributed across conserved TMH2, TMH3, and TMH7 domains of the μ_1 sequence. In effect, μ_3 and μ_4 opiate receptors are "short" 6TMH domain GPCRs that are selectively responsive to endogenous morphine. The evolutionary and biological significance of the μ_3 opiate receptor is further enhanced by our recent finding that it is present on human stem cells as is its variant, the μ4 opiate receptor, in the absence of the traditional 7 TMH domain μ opioid receptor (Cadet *et al* 2007). A schematic representation of 6 TMH μ opiate receptors is depicted in Fig. 1, below.

Our compelling demonstration of novel "short" 6 TMH domain μ_3 and μ_4 opiate receptors stimulated an exhaustive search of existing databases to determine whether additional 6TMH domain μ opiate receptors were expressed in various animal species. Interestingly, access of the National Center for Biotechnology Information (NCBI) database yielded a predicted chicken μ opioid receptor mRNA sequence (Fig. 2) that provided putative evidence supporting the existence of one or two novel "short" μ opiate receptors in the domestic chicken. Upon further analysis utilizing well established computer-assisted prediction tools, it appears likely that poultry express novel "short" GPCRs with similar biochemical characteristics as described for μ_3 and μ_4 opiate receptors as well as traditional MOR's.

MATERIALS AND METHODS

The NCBI database provided access to a predicted mRNA sequence with a shortened open reading frame, accession number XR_026927, corresponding to a potentially novel μ opiate receptor in chicken. The nucleic acid sequence (Fig. 2) was derived by Gnomon, an automated computational analysis algorithm provided by the NCBI. The algorithm accesses the Reference Sequence Collection (McEntyre & Ostell 2002; Pruitt *et al* 2007), a computer database that aims to provide a comprehensive and integrated well annotated set of sequences for taxonomically diverse eukaryotes. Because the 5' end of predicted mRNA sequence was observed to contain three potential ATG start codons, consensus sequence analysis of Translation Initiation Sites (TIS) was performed as previously described (Cavener & Ray 1991; Kochetov 2005; Kochetov 2008; Kozak 2002; Kozak 2005a,b).

Nucleic acid sequence information contained within the predicted mRNA was translated into a corresponding amino acid sequence using the widely accepted proteomics server, ExPASy (Expert Protein Analysis System) provided by the Swiss Institute of Bioinformatics. Conformational analysis of predicted amino acid sequences, as derived by ExPASy, was performed with the aid of the computer program TMHMM Server v. 2.0 provided by the Center for Biological Sequence Analysis at the Technical University of Denmark. TMHMM is a membrane protein topology prediction method based on a hidden Markov model (Churchill 1992; Durbin *et al* 1998; Krogh *et al* 1993; Krogh *et al* 2001) and is designed to predict the placement of TMH domain regions within 97–98% accuracy (Krogh *et al* 2001). The model consists of a set of states, each one corresponding to a different region of the total protein sequence to be modeled. Each state has an associated probability distribution over the 20 amino acids (emission frequency) characterizing the compositional bias (e.g., hydrophobicity and charge) of amino acids in the corresponding region. The architecture of the model specifies how these states are connected to one another, so the transitions from state to state reflect how biologically the different regions are assembled to form the entire protein (Kahsay *et al* 2004).

Finally, the bioinformatics software BLAST (Basic Local Alignment Tool) (Altschul *et al* 1997) was used to perform comparative amino acid sequence analyses of the predicted μ opiate receptor protein in chicken to previously deposited μ opiate receptor sequences from other species contained within the NCBI's database (Altschul *et al* 1997). A "global alignment" was utilized in the comparisons below. This type of alignment attempts to align two sequences over their entire length.

RESULTS

1. A predicted mRNA sequence corresponding to a novel "short" μ opiate receptor in domestic chicken

An in depth search of the NCBI's database yielded a predicted mRNA sequence corresponding to a novel "short" μ opiate receptor in domestic chicken, accession number XR_026927 (Fig. 2). The predicted mRNA sequence was derived by Gnomon from the annotated genomic sequence (NW_001471669.1) found on Gallus gallus chromosome 3 and is schematically depicted in Fig. 3 along with a comparison depiction of the maturation of human $_1$ opioid receptor mRNA from the MOR gene. The TIS for the human MOR is contained within the first Exon. The chicken receptor has multiple potential in frame TISs. The first and second are contained within Exon 1 and the third within Exon 2.

These bioinformatics data indicate that in the domestic chicken, mature μ opiate receptor mRNA contains 4 contiguous spliced exons of approximately 1.1 kb derived via post-transcriptional processing of a 21.5 kb primary transcript, as enumerated in Tab. 1 and illustrated in Fig. 3. Interestingly, mature μ_1 opioid receptor mRNA also contains 4 contiguous spliced exons of approximately 2.2 kb, but is derived via post-transcriptional processing of an approximate 80 kb primary transcript. From a comparative perspective, it is apparent that the relative proportion of intronic DNA found in the human MOR genomic sequence far exceeds that found in the corresponding chicken MOR genomic sequence. Comparative maps of Exon and Intron domains found in human and chicken MOR genomic sequences are schematically depicted in Fig. 3.

2. ExPASy translation of predicted mRNA open reading frames into corresponding amino acid sequences

Consensus sequence analysis of probable TIS (Fig. 2, bolded initiation codons) was performed as according to guidelines, as previously described (Cavener & Ray 1991; Kochetov 2005; Kochetov 2008; Kozak 2002; Kozak 2005a,b). Accordingly, predicted nucleotide sequences utilizing the first and the third initiation codons (Fig. 4) as likely TIS candidates were translated into two probable protein sequences of 327 and 300 amino acids (Fig. 5), respectively, using the Translation Web Tool provided by EXPASY (Expert Protein Analysis System). The two predicted protein sequences are schematically represented in Fig. 5.

3. TMHMM-mediated conformational analysis of probable μ opiate receptor protein sequences

TMHMM software was employed to provide theoretical topological mapping of TMH domains within the predicted 327 and 300 amino acid opiate receptor protein species. Fig. 6 presents a theoretical map of the TMH domains in the 300 amino acid protein species in its native, membrane-associated, conformation. Schematic depictions of the predicted 300 and 327 amino acid μ opiate receptor protein species are depicted in Figs. 7 and 8, respectively. Computational analysis of the smaller 300 amino acid μ opiate receptor protein species indicates a 6TMH domain GPCR with an identical membrane topology to native μ_3 and μ_4 opiate receptors. Interestingly, computational analysis of the larger 327 amino acid μ opiate receptor protein species indicates a novel 7TMH domain GPCR lacking a typical extracellular domain containing consensus N-linked glycosylation sites. In both cases, predictive measures indicate that the domestic chicken expresses one or two novel "short" GPCRs with similar biochemical characteristics as previously described for μ_3 and μ_4 opiate receptors as well as traditional MOR's.

4. BLAST (Basic Local Alignment Tool)-mediated comparative amino acid sequence analyses

BLAST analysis yielded an N-terminal sequence homology of 96% for an alignment containing amino acid residues 1–290 of the predicted 300 amino acid chicken μ opiate receptor in comparison to amino acid residues 1–290 of the 292 amino acid μ_4 opiate receptor (MOR1W [Homo sapiens]Accession Number AAR11568).

BLAST analysis yielded a C-terminal sequence homology of 96% for an alignment containing amino acid residues 1–300 of the predicted 300 amino acid chicken μ opiate receptor in comparison to amino acid residues 101 to 400 of the 400 amino acid μ_1 opiate receptor (MOR-1 [Homo sapiens] Accession Number NP_000905).

DISCUSSION

1. Principal predictions

A predicted mRNA sequence corresponding to a novel "short" μ opiate receptor in domestic chicken was derived by Gnomon from the annotated genomic sequence (NW_001471669.1) found on Gallus gallus chromosome 3 (Fig. 3). Consensus sequence analysis of probable TIS was performed as according to Kozak sequence guidelines, as previously described (Cavener & Ray 1991; Kochetov 2005; Kochetov 2008; Kozak 2002; 2005a,b). Accordingly, predicted nucleotide sequences utilizing the first and the third initiation codons (Fig. 4) as likely TIS candidates were translated into two probable protein sequences of 327 and 300 amino acids (Fig. 5). Computational/conformational analysis of the smaller 300 amino acid opiate receptor protein species indicates a 6TMH domain GPCR with an identical membrane topology to native μ_3 and μ_4 opiate receptors, whereas a similar analysis of the larger 327 amino acid μ opiate receptor protein species indicates a novel 7TMH domain GPCR lacking a typical extracellular domain containing consensus N-linked glycosylation sites (Figs. 7 and 8, respectively). In both cases, predictive measures indicate that the domestic chicken expresses one or two novel "short" GPCRs with similar biochemical characteristics as previously described for μ_3 and μ_4 opiate receptors as well as traditional MOR's.

As confirmed by BLAST analysis, the 300 amino acid 6 TMH domain chicken μ opiate receptor may be operationally defined as an N-terminally truncated "short" homolog of the μ_1 opioid receptor (Fig. 9). Furthermore, the selectively tailored chicken μ opiate receptor is predicted to retain the obligate morphine binding pocket conserved throughout the 2, 3, and 7 helical domains of the μ_1 receptor. We have previously hypothesized that conformational stabilization provided by conformational interaction of glycosylated extracellular N-terminal protein domains and the extracellular loops is required for binding of endogenous opioid peptides as well as synthetic flexible opiate alkaloids. Accordingly, the absence of the glycosylated extracellular N-terminal domains in the primary sequences of both μ_3 and μ_4 opiate receptors is hypothesized to confer high selectivity for morphine and related rigid morphinan alkaloids due to the absence of secondary stabilization. Due to the novel 6 TMH domain configuration and sequence identity to μ_3 and μ_4 opiate receptors at its N-terminus, we predict that the 300 amino acid chicken μ opiate receptor functions as a "hybrid" signaling GPCR with selective preference for morphine and related morphinan alkaloids with the exclusion of endogenous opioid peptides (Cadet *et al* 2003; Cadet *et al* 2007). Similar criteria relating to ligand selectivity will most certainly apply to the predicted novel "short" 7TMH domain 327 amino acid chicken μ opiate receptor due to the genetic deletion of the glycosylated extracellular N-terminal domain.

2. Biological ramifications of the predictions

Based on guidelines established by Kozak and coworkers (Kochetov 2005; Kozak 2005b) it appears that mature chicken μ opiate receptor-encoding mRNA contains two probable TISs with the potential for translation of two distinct 300 and 327 amino acid μ opiate receptor proteins. Prior literature indicates that many mRNAs are capable of producing functionally distinct proteins using different in frame start codons within the same mature fully spliced mRNA (Gray & Wickens 1998; Kochetov 2008). Furthermore, the effects of upstream start codons can vary with cell type during differentiation (Descombes & Schibler 1991; Imataka *et al* 1994; Lin *et al* 1993; Zimmer *et al* 1994). Accordingly, mature chicken μ opiate receptor-encoding mRNA may be similarly translated into one or two functional receptor proteins that are sorted or expressed according to tissue or cell type. Morphine and other chemically related opiate alkaloids represent classical and reliable analgesic principles for management of severe pain associated with disease (Merlin 1984; Stefano *et al* 2005). Paradoxical morphine-mediated hyperalgesia in the presence of typical morphine-mediated respiratory depression has been observed in the domestic chicken (Hughes *et al* 2009; Hughes & Sufka 1991; Sufka *et al* 1991). It is a reasonable, therefore, to speculate that these markedly different physiological responses to administered morphine may be due differential expression of the 300 vs. the 327 amino acid μ opiate receptor in spinal cord and brain stem loci.

From a comparative perspective, it is apparent that the relative proportion of intronic DNA found in the human MOR genomic sequence far exceeds that found in the corresponding chicken MOR genomic sequence (Table 1). The link between genome size and metabolic rate was first made in 1970 by Henryk Szarski (Szarski 1970; Szarski 1976; Szarski 1983). Birds have the smallest genome when compared to other vertebrates including humans (Gregory 2002). Avian species require a high metabolic rate to carry out basic physiological functions. The metabolic advantage of a smaller, relatively streamlined genome within all cells allows cells not only to be smaller but operationally expands cellular surface area to volume ratios. Smaller genomes do not necessarily mean fewer genes, but rather a more succinct use of space on the chromosomes. In effect, smaller cells are more energy efficient that larger ones, resulting in an increased metabolic advantage. These complementary data may also contribute to understanding the physiological role of nucleated red blood cells in birds. It is possible that avian species due to their relatively streamlined genome and smaller size do not require expulsion of the nucleus from the red blood cells before its entry into the blood stream. In effect, the high surface to volume ratio of nucleated red cells in avian species facilitates markedly efficient gas exchange with surrounding tissues.

CONCLUSION

The presence of this biologically unique, functional "short" membrane bound receptor protein in the chicken not only reinforces the primacy of said receptor but in doing that it also gives us a view into a window of learning and understanding the history of evolution. The chicken is evolutionarily placed to bridge the gap between mammals and non-amniote vertebrates, and is therefore the best studied representative of all avian species, thus providing a valuable resource for understanding comparative genomics (Antin 2009). It has already been demonstrated that animal cells have the ability to synthesize chemically authentic morphine via multienzyme mediated processes restricted by numerous feedback inhibitory steps. Further understanding of "morphinergic" signaling mechanisms via activation of novel "short" μ opiate receptors in poultry will provide a window of high resolution for better understanding basic physiological processes underlying human health.

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