Morphine signaling in *Bos taurus* and *Equus caballus*

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Abstract

The presence of morphine and the mu opioid receptor has been demonstrated in cells from the nervous, endocrine, immune, vascular, reproductive, respiratory, and digestive systems of mammals. The understanding of morphinergic signaling in domestic animals is vital for properly managing this commercially valuable resource. This review discusses the known functions of mu opioid receptors in *Bos taurus* and *Equus caballus*. Morphine has been shown to regulate the release of hormones and neurotransmitters in these animals in diverse tissues. The mu opioid receptor is also present on equine gametes and controls key functions of these reproductive cells. Evidence for the presence of the mu opioid receptors in bovine blood, heart, spleen and bone marrow is discussed. Given the widespread occurrence of mu opiate processes it is surmised that it transcend functions only focused on pain.

Key words: morphine receptor, endocrine, domestic animals, equine, cattle.

Introduction

Opiate receptors and endogenous opiates along with their synthesizing enzymes are known to exist in many animal phyla [1-11]. Invertebrates and vertebrates have functional endogenous morphine signaling in numerous organ systems [4, 12-15]. Recently, it has been shown that substances of abuse appear to release endogenous morphine from immune and neural tissues [16-23].

Opiate receptors in mammals contain conserved gene sequences [24] and consequently the functions are also conserved. Specifically, the region of the μ-opioid receptor between the first intracellular loop and the third transmembrane domain is the most highly conserved in vertebrates [24]. An examination of the literature relating to domesticated animals reveals that both the bovine and the equine families possess opioid receptor and morphine reactivity. Interestingly, some opiate receptor subtypes are coupled to nitric oxide release, thus, associating some opiate actions to those previously linked to nitric oxide [9, 22, 25-30]. Even more interesting are the findings that associate estrogen signaling via nitric oxide, making the phenomena even more complex [31-36]. This review will discuss the functions of morphine and mu opioid receptors in *Bos* and *Equus*. We also demonstrate the presence of mu opioid receptors in vital cow and horse tissues.

Bos taurus morphine signaling

The *Bos taurus* mu opioid receptor was first cloned from bovine brain tissue in 1999 by Onoprishvili *et al*. [37]. The polypeptide has a 94%

sequence identity with human mu opioid receptor. Like its human counterpart, the receptor was shown to be down regulated by long term exposure to opioid agonists [37]. These researchers found no evidence for multiple types of mu opioid receptors in the brain tissue tested. Prior and subsequent studies have revealed the presence of mu opioid receptors in bovine pinealocytes [38-40]. The stimulation of these receptors has been linked to the production of melatonin [38, 41]. The opioid receptor antagonist, naloxone, was able to prevent this melatonin release [39]. Morphine's effects on other parts of the brain have been demonstrated by the inhibition of oxytocin release in dairy cows [42]. This effect was also found to be reversible by naloxone [42]. Morphine receptors in the brain play a vital role in hormonally controlled actions in the cow.

The opioid receptor has functionality in other cell types in the cow. The mu opioid receptor has also been found in bovine oocytes and has been shown to assist in maturation of these vital cells [43]. Bovine adrenal medulla tissue contains mu opioid receptors but not delta or kappa subtypes [44]. Cells lining the bovine airway also possess opioid receptors [45] and exist in differing amounts depending on the type of tissue they are found on [46]. The receptors present in bovine trachealis muscle can reduce airway constriction when stimulated [45, 46]. This inhibitory effect was shown to be controlled by the amount and type of opioid receptor on the specific tissue type. The trachealis and the bronchial muscles both possess mu opioid receptors and constriction of the airway can be controlled by mu opioid specific receptor agonists [46]. The opioid agonists were shown to be acting via the inhibition of cholinergic neurotransmission [45]. Much like the morphine signaling in the brain, the reproductive, respiratory and endocrine systems in the cow are regulated at some level by mu opioid receptors.

Detecting mu opioid receptor expression in cattle tissue

We have validated the presence of mu opioid receptors in the tissues present above and extended the observations into other tissues. Figure 1 demonstrates that the cow splicing from the known database sequence is analogous to the human splicing of the mu1. Bovine lung, bone marrow, heart, brain, and spleen tissue samples (100 mg) were homogenized in 1 ml of Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) using a polytron homogenizer. The homogenates were stored at room temperature for 5 min to allow complete dissociation of nucleoprotein. 0.1 ml of 1-bromo-3-chloropropane was added to the homogenates. The samples were vortexed vigorously for 15 s and then stored at room temperature for 7 min. After centrifugation of the samples for 15 min at 12,000 g, the aqueous phase was transferred to a fresh tube. RNA was precipitated by mixing with 0.5 ml of isopropanol.

gcctgacgctcctctctggctccgccggggttggtcgctgtaagaaataacaggagctgtggcagcggcgaagacgaagcggctcgcgcgtggaacccgaaaagtcagg
gtgctcgcggttactcccaacgtggtcccagccggcggtcagcaccatggacagcggcgccgtccccacgaacgccagcaactgcactgatcccttcacacacccttcaagt
tgctccccagcacctagtcccagctcctgggtcaacttctcccacttagaaggcaacctgtccgacccatgcggtccgaaccgcaccgagctgggagggagcgacagac
tgtgcccttcggccggcagcccttccatgatcacggccatcatcatcatggccctctactccatcgtgtgcgtggtggggctcttcggaaacttcctggtcatgtatgtgatt
gtcaggtacaccaaaatgaagactgccaccaacatctatattttcaacctcgccctggcagatgccctggcaaccagtaccctgcctttccagagtgtcaattacctgatgg
gaacatggccgtttggaaccatcctgtgcaagattgtgatctccatagattactacaatatgttcaccagcatattcaccctctgcaccatgagtgtggatcgctacattgca
gtctgccatcctgtcaaggccctggatttacgcactccccgtaatgccaagatcatcaacatctgcaactggatcctctcttcagccattggtctgcctgtgatgttcatggc
aacgacaaagtaccggcaaggttccatagattgtacactaacattctctcacccaacgtggtactgggaaaacctgctgaaaatctgtgttttcatctttgccttcatcatgc
ctatcctcatcattacagtgtgttatgggctgatgatcttacgcctcaagagtgtccgcatgctctctggctccaaagaaaaggacaggaacctgcgaagaatcaccagga
tggtgctggtggttgtggctgtgttcattgtctgctggacgcccattcacatctacgtcatcattaaagccttgatcacaatcccggaaactactttccagaccgtttcctggc
acttctgcattgctctaggttataccaacagttgcctcaaccccgtcctttatgcatttctggatgaaaacttcaaacgatgcttcagagagttctgtatcccaacttcctccac
cattgagcagcaaaactccactcgaattcgtcagaacaccagagaccacccctccacagccaatacggtggataggactaaccatcagctagaaaatctggaagcaga
aaccactccgttaccctaactgggtctcataccattcagaccctcactgagcttagacgccacatctatatga

Full-length *Bos taurus* mu1 opioid receptor amino acid sequence

MDSGAVPTNASNCTDPFTHPSSCSPAPSPSSWVNFSHLEGNLSDPCGPNRTELGGSDRLCPSAGSPSMITAIIIMALYSIVCVVGLFGNF
LVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDLRTP
RNAKIINICNWILSSAIGLPVMFMATTKYRQGSIDCTLTFSHPTWYWENLLKICVFIFAFIMPILIITVCYGLMILRLKSVRMLSGSKEKDRNL
RRITRMVLVVVAVFIVCWTPIHIYVIIKALITIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREFCIPTSSTIEQQNSTRIRQNTR
DHPSTANTVDRTNHQLENLEAETTPLP

Bos taurus genomic DNA chromosome 9
Length = 108145351

Exon positions (*Bos taurus*)

94456715-94457165 94493812-94494169 94495031-94495553 94511994-94512090

Figure 1. Full-length *Bos taurus* mu1 opioid receptor nucleic acid sequence

Samples were stored at room temperature for 6 min and then centrifuged at 12,000 g for 8 min at 4°C. After removing the supernatant, the RNA pellet was washed with 1 ml of 75% ethanol, and subsequently centrifuged at 7,500 g for 5 min at 4°C. The ethanol was discarded, and the RNA pellet air-dried for 5 min. The RNA pellet was dissolved in 60 µl water and denatured at 55°C for 10 min.

An aliquot of each RNA sample was separated in 1% agarose gel stained with ethidium bromide. Two predominant bands of 18s and 28S ribosomal RNA were observed. In addition, spectrophotometric measurements of the RNA samples were made at 260 and 280 nm. The 260/280 ratios from all of the samples were above 1.6.

RT-PCR analysis was used to study the expression of mRNA encoding a bovine mu opioid receptor. RNA (1 µg) was reverse transcribed using Superscript III Rnase H-Reverse Transcriptase with random hexamers (Invitrogen, Carlsbad, CA). PCR analysis was performed using the following primers: forward primer 5'-GGTACTGGGAAAACCTGCTGAAGATCTGTG-3' and reverse primer 5'-GGTCTCTAGTGTTCTGAC-GAATTCGAGTGG-3'. Separation of the PCR products by gel electrophoresis revealed the expected 441 bp band in all tissue samples.

In another assay using the same primers listed above, PCR analysis was performed on bovine blood samples the using procedure described below for horse blood. Agarose gel electrophoresis indicated the presence of a mu opioid receptor in cow blood.

Equus caballus morphine signaling

Similarities between cow and horse mu opioid receptor functions can be seen when the presence of morphinergic signaling is noted in the endocrine systems of both species. An example of this is given by the findings of Hon and Ng [47] who show that opiate like material was present in the equine pancreas. In addition, they show that this endocrine organ possesses opiate receptor binding activity [47]. Another striking parallel between cows and horses can be seen in the reproductive cells. Similar to cows, the horse's oocytes contain mu opioid receptors that regulate meiosis [48]. The mu opioid receptor's density on these cells varies seasonally and influences the maturation of the oocyte [48]. Mu opioid receptors are not only present on female gametes, they have also been detected in equine spermatozoa [49]. The function of the receptors on these cells is thought to be regulation of motility. Naloxone has a biphasic effect on sperm motility. High concentrations inhibit movement while lower concentrations increased it [49]. The functional presence of mu opioid receptors in reproduction and hormone regulation underscores the vital importance of morphine signaling in domesticated animals.

Cow and horse intestinal motility are known to be influenced by opioid mediated systems [50]. Morphine signaling is indeed present in the gut of mammals [14]. Stimulation of opioid receptors can decrease gut motility in the equine ileum and this stimulation can be prevented by naloxone treatment [51]. Studies of the intestinal transit in horses and ponies reveal that transit times are slowed by morphine but can be improved with naloxone and other opioid receptor antagonists [52-54]. Equine medicine has benefited by the discovery of opioid receptor mediated phenomenon in the mammalian digestive tract.

Equus caballus sequence identifier XM_001501436

agatgctcagctatccccttctgctcctctctggctccgccagggatggtctctgtaagaaacagcaggagctgtggcagcagctaaaggaagcggctgaagcgcgtgg
aaccagaacagtccgggcgctcgcggttacctcacatcgtggtcacagccggccgtcagcaccatggacagcagcaccgtccccgcaaacgccagcaattgcaatgatc
cctttacgcactcttcaagttgctccccagcacccagccccggttcctgggtcaacttctcccacgcagatggcaacctctccgacccatgcggtccgaaccgtaccgaact
gggcgggagcgacagcctgtgccctccgaccggcagtccttctatgatcacagccatcacaatcatggccatctactccatcgtatgcgtggtgggtctctttggaaacttc
ctggtcatgtatgtgattgtcagatacaccaaaatgaagactgccaccaacatctatattttcaatcttgctctggcagatgccttagcaaccagcaccctgccattccaga
gtgttaattacctaatgggaacatggccatttggaaccatcctctgcaagatcgtgatctccatagattactataatatgttcaccagcatattcaccctctgtactatgagt
gttgatcgctacattgcagtctgccatcccgtcaaggccctggatttccgtactccccgcaatgccaaaatcgtcaacgtctgcaactggatcctctcttcagccattggtct
gcctgtaatgttcatggcaacaacaaaatacaggcatggttccatagactgtacactaacattctctcacccaacatggtactgggaaaacctgctgaaaatctgtgttttc
atctttgccttcatcatgccggtcctcatcattacggtgtgttatggactgatgatcttacgcctcaagagtgtccgtatgctctctggctccaaagaaaaagacagaaacct
gagaagaatcaccaggatggtgcttgtggttgtggctgtgttcattgtctgctggactcccattcacatttatgtcatcattaaagccttgattacgatcccagaaactactttt
ccagaccgtctcttggcacttctgcattgctctaggttacacaaatagctgcctgaacccagtcctttatgcatttctggatgaaaacttcaaacgatgcttcagagagttctg
tatcccaacttcctccaccattgagcagcaaaactctactcgagttcgtcagaacactagagaccacccctccacggccaatacagtggataggactaaccatcagctaga
aaatctggaagcagaaactgctccgttgccctaagtgggtctcatgccattcagagcctcactgagcttaaaagccacc

Predicted horse mu opioid receptor amino acid sequence

MDSSTVPANASNCNDPFTHSSSCSPAPSPGSWVNFSHADGNLSDPCGPNRTELGGSDSLCPPTGSPSMITAITIMAIYSIVCVVGLFGN
FLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCTMSVDRYIAVCHPVKALDFRT
PRNAKIVNVCNWILSSAIGLPVMFMATTKYRHGSIDCTLTFSHPTWYWENLLKICVFIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKEKD
RNLRRITRMVLVVVAVFIVCWTPIHIYVIIKALITIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREFCIPTSSTIEQQNSTRVR
QNTRDHPSTANTVDRTNHQLENLEAETAPLP

Figure 2. Predicted horse mu opioid receptor mRNA sequence

The presence of mu opioid receptors has also been discovered in the equine brain [55] and synovial tissue [56]. These findings support the use of opiate analgesics in treating horses. Further research is required to evaluate the unwanted treatment effects of morphine on horses [57].

Mu opioid receptor expression in horse blood

Peripheral blood was collected from horses (Equus caballus) by veinipuncture and processed using the PAXgene blood RNA system (PreAnalytix, Qiagen, Valencia, CA). RNA was isolated from 2.5 ml of whole blood according to the manufacturer's detailed instructions. A 1 µl aliquot of total RNA was then analyzed using an Agilent 2100 Bioanalyzer with RNA nano chips (Agilent, Santa Clara, CA). RNA (130 ng) was then reverse transcribed using Superscript III Rnase H-Reverse Transcriptase with random hexamers (Invitrogen, Carlsbad, CA). The predicted sequence from the database supports the presence of a mu1 type receptor in horse (Figure 2).

The u_1 opioid receptor gene was screened for using real time PCR with the commercially available kit from Applied Biosystems (part number Hs 00168570_m1). This primer and probe set (detector set) is located in the second exon of the mu1 opioid receptor gene. The 2X universal master mix (Applied Biosystems) containing the PCR buffer, $MgCl_2$, dNTP's, and the thermal stable AmpliTaq Gold DNA polymerase was used in the PCR reactions. The PCR reaction mixture was transferred to a MicroAmp optical 96-well reaction plate and incubated at 95°C for 10 min to activate the Amplitaq Gold DNA polymerase and then run for 40 cycles at 95°C for 30 s and 60°C for 1 min on the Applied Biosystems GeneAmp 7500 sequence Detection System. The sequence was not detected using this assay and therefore it can be concluded that these cells probably do not express the mu1 opioid receptor.

In conclusion, opiate systems are present as expected in both horse and cattle and there is a resemblance of the two. The system appears to be present and thus mediate functions that merely focus on pain. As such, the opiate system exhibits a general level of functionality in these important commercial animals.

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