Comparative Aspects of Endogenous Morphine Synthesis and Signaling in Animals

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For many years it has been believed that animals cannot make morphine. However, within the last 30 years scientific documents have emerged reporting on endogenous animal opiate synthesis, including morphine biosynthesis in animals and specific tissues. These data are complemented by other reports demonstrating the presence of opiate receptors specifically used for morphinergic signaling in animal tissues, bringing together the lock-and-key concept for an animal morphine chemical messenger hypothesis.

Key words: **endogenous morphine; μ opiate receptor; nitric oxide**

Presence of Opiate Alkaloid in Vertebrate and Invertebrate Animals

An endogenous morphine presence[1–7] in mammals occurs in nerve tissue (bovine, rat brain),[8] adrenal gland,[9] and human plasma.[10] Furthermore, concentrations of endogenous morphine in the sub-picomol/g range have been found in rat brain,[11] as determined by a new extraction technique, HPLC and gas chromatography–mass spectrometry (GC–MS)/quadrupole time of flight (Q-Tof) MS. Recently, morphine has been identified in human brain.[12] Importantly, morphine precursor molecules, tetrahydropapaveroline (THP), reticuline, salutaridine, thebaine, and codeine were also detected in various vertebrate tissues.[5,13–15] These findings have provided strong presumptive evidence for the physiological relevance of endogenous morphine.

Morphine has also been found in human plasma,[16,17] suggesting a hormonal action with immune, vascular, and gut tissues as targets.[18,19] Our laboratory has demonstrated recently that normal, human white blood cells (WBC), specifically polymorphonuclear cells, contain and have the ability to synthesize morphine[20] as well as release morphine into their environment.

Interestingly, in invertebrates, THP, reticuline, and codeine also were identified along with morphine, suggesting that opiate signaling exists in invertebrate tissues.[11] The discovery of morphine in the nervous system, using techniques, such as MS, has generated interest in determining its distribution, sites of action, and functional significance. In invertebrates, specifically with *Mytilus edulis*, the presence of morphine, morphine 6-glucuronide, morphine 3-glucuronide, codeine, THP, and reticuline have been reported.[11,21–24] Endogenous opiate levels can be induced to change following starvation, surgical stress, and bacteria infection.[11,25–29] Morphine and morphine-6-glucuronide, a morphine metabolite, have been identified and quantified in *Modiolus deminissus* pedal ganglia as well.[22]

In the American lobster, *Homarus americanus*, morphine was found in nerve cord, eye stock, and hemolymph via the same rigorous techniques. It was quantified via radioimmunoassay (RIA) and was identified via

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Q-Tof–MS.[30] In stressed (pereiopod-ablated or lipopolysaccharide-injected) animals, the endogenous morphine levels initially increased significantly by 24% for hemolymph and 48% for nerve cord. By day 5, the stressed and control values for endogenous morphine, in both tissues, was lower and nondistinguishable. In both hemocytes and neural cells, morphine, not met-enkephalin, stimulated constitutive nitric oxide (NO) release in a naloxone antagonizable manner, demonstrating a μ opiate receptor-mediated phenomenon, suggesting the presence of the mu opiate receptor subtype μ3 since it is opiate alkaloid selective and opioid peptide insensitive.[21,31,32] Reverse transcriptase (RT)-PCR revealed the presence of a μ opiate receptor transcript in *Homarus* neural and immune tissues, which exhibits a 100% sequence identity with its human counterpart.[30,32] Thus, morphine is present in lobster tissues, potentially demonstrating hormonal and neurotransmitter functions that are involved in the animal's stress response.

Moreover, the parasitic worm, *Ascaris suum*, contains the opiate alkaloid morphine, as determined by HPLC coupled to electrochemical detection and by GC–MS.[33,34] *Ascaris* maintained for 5days contained a significant amount of morphine as did the medium used, demonstrating their ability to synthesize the opiate alkaloid since nonspecific host accumulation would only last for 2 days. The anatomic distribution of morphine immunoreactivity in the parasite reveals that morphine is in the subcuticle layers and in the animal's nerve cords. Furthermore, as determined by RT-PCR, *Ascaris* does not express the transcript of the neuronal μ receptor. Failure to demonstrate the expression of this opioid receptor as well as the morphine-like tissue localization in *Ascaris* suggests that the endogenous morphine is intended for secretion into the microenvironment, influencing host gastrointestinal functions.

By using the same analytical methods, adult *Dracunculus medinesis* and *Schistosoma mansoni* both contain the opiate alkaloid morphine, and *D. medinesis* also contains the active metabolite of morphine, morphine 6-glucuronide.[35] From these and previous observations it would appear that many helminthes are probably using opiate alkaloids as potent immunosuppressive and antinociceptive signal molecules to downregulate immunosurveillance responsiveness and pain signaling in their hosts.

TABLE 1. Presence of Opiate Alkaloids in Animals

Invertebrate	Tissue distribution
Mollusk	
Mytilus edulis	Ganglia, hemocyte
Modiolus deminissus	Ganglia
Parasites	
Ascaris suum	Nerve cord, subcuticle layers, uterus, eggs
Dracunculus medinesis	Whole body extraction
Schistosoma mansoni	Whole body extraction
Lobster	
Homarus americanus	Nerve cord, eye stock hemolymph
Vertebrate	
bovine	Brain, adrenal
toad	Skin
rat	Brain, amygdale, adrenal
pig	Arterial blood
human	Brain, heart tissue, white blood cells, plasma
Vertebrate cell lines	Rat adrenal chromaffin cells Human neuroblastoma cells Human pancreas carcinoma cells

Mammalian Cell Lines and Tumor

Morphine has also been identified in many vertebrate cell lines. Morphine is present in rat adrenal medulla chromaffin PC-12 cells,[36,37] human neuroblastoma cells, and human pancreas carcinoma cells.[38,39] Morphine-6-glucuronide was found in secretory granules and is a secretion product of SHY cells.[38] Since morphine has been found in these cancer cell lines, originating from human and animal cells, it became important to demonstrate that actual tumors also contain this opiate alkaloid. Indeed, it has been found in human gliomas,[40]

suggesting that it may exert an action that affects tumor physiology/pathology.[41,42]

Morphine distribution in various animals and their tissues is shown in Table 1.

Morphine Biosynthesis Pathway: Precursors and Key Enzymes

Recent studies from our group employing well-established, *ex vivo,* invertebrate nervous tissue preparations and primary cultures of human WBC[20,43,44] and those by Zenk and co-workers using human tumor-derived cell lines[45,46] have markedly facilitated the formulation of an evidence-based model of *de novo* formation of endogenous morphine in animal cells (Fig. 1) with remarkable similarities to the well-characterized enzymatic pathway described in poppy plant.[47] We have demonstrated that WBC, specifically polymorphonuclear cells, contain and have the ability to synthesize morphine.[20] We also show that WBC expresses CYP2D6,[20] an enzyme capable of synthesizing morphine from tyramine, norlaudanosoline, and codeine. Significantly, we also show that morphine can be synthesized by another pathway via L-3,4-dihydroxyphenylalanine (L-DOPA).[20]

The same synthesis structure occurs in *M. edulis*. In *Mytilus*, pedal ganglia incubated with either tyramine or tyrosine or whole animals receiving injections exhibited a statistically significant concentration- and time-dependent increase in their endogenous morphine and dopamine levels.[43] Incubation with quinidine and/or alpha-methyl-p-tyrosine (AMPT) diminished ganglionic morphine and dopamine synthesis at various steps in the synthesis process. We also demonstrated that CYP2D6 mediates the tyramine to dopamine step in this process, as did tyrosine hydroxylase in the step from tyrosine to L-DOPA. Furthermore via RT-PCR, we identified a cDNA fragment of the CYP2D6 enzyme in the ganglia that exhibits 94% sequence identity with its human counterpart.[20] Evidence that tyrosine and tyramine was, in part, being converted to dopamine then morphine and that this process can be inhibited by altering either or both CYP2D6 or tyrosine hydroxylase is also provided.[43]

These studies demonstrate that L-tyrosine, its monoamine homolog tyramine, and their respective catechol derivatives L-DOPA and dopamine serve as substrates for *de novo* morphine production and that pharmacological characterization of tyramine utilization as a morphine precursor indicates one or more catalytic steps mediated by microsomal CYP2D6 (Fig. 1).[20,43] The significance of tyramine as a biosynthetic intermediate is validated by *in vitro* enzyme kinetic studies demonstrating dopamine formation via CYP2D6-catalyzed ring hydroxylation of tyramine,[48–51] which in turn lends support to the existence of a potentially important tyrosine hydroxylase-independent pool of cytosolic dopamine that is available for endogenous morphine expression.[20,43,44] These data are complemented by metabolic labeling/isotope enrichment studies employing SH-SY5Y neuroblastoma cells,[45,46] indicating asymmetric isotopic labeling of the benzyl and isoquinoline chemical domains of newly formed morphine that is operationally determined by the type of L-tyrosine-derived precursor molecule that is employed: L-tyrosine and L-DOPA are incorporated in both the benzyl and isoquinoline chemical domains of morphine, whereas dopamine and tyramine are only incorporated into the isoquinoline domain. Over all, based on the findings in vertebrates and invertebrates, we surmise that morphine biosynthesis in animals has been preserved during evolution.[52]

Characterization and Function of Novel μ_3 Opiate Receptors in Animals

Kosterlitz and co-workers demonstrated that exogenous morphine can bind to receptors in the mammalian brain.[53] This report indicated that morphine binds to the same sites as those

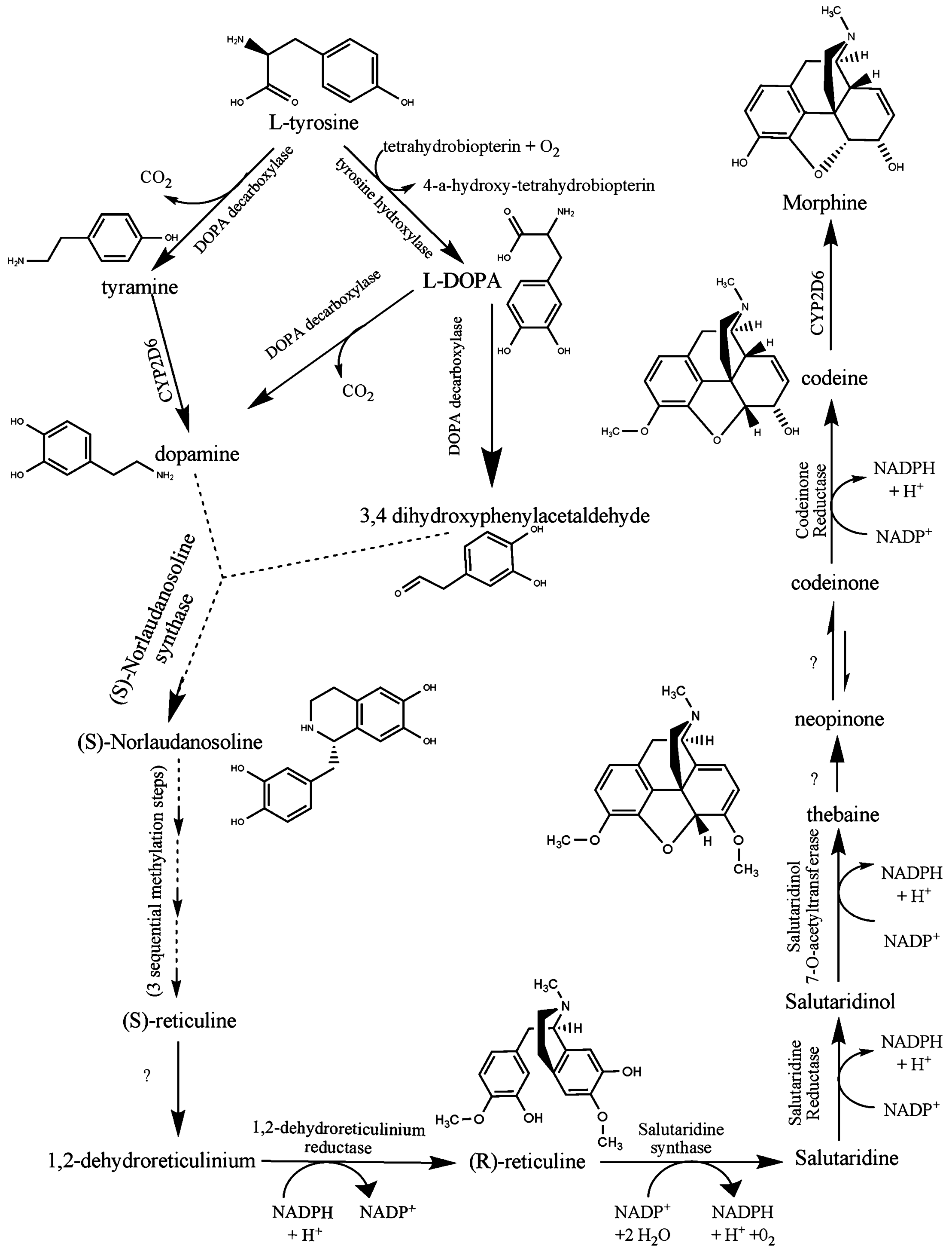

Figure 1. Evidence-based model of *de novo* morphine biosynthetic pathway in animals. Question marks represent unidentified enzymes in the pathway. (Abbreviations: L-DOPA, L-3,4-dihydroxyphenylalanine.)

used by the endogenous opioid peptides (e.g., enkephalins). Since then, demonstration of the multiplicity of receptor types has suggested that both opioid peptides and opiate alkaloids may bind to more than one opiate receptor subtype.[54–57]

By comparing affinity constants and relative strength in competitive binding assays, different degrees of selectivity have been recognized for various ligands. Both high- and low-affinity binding sites for [^{3}H]-dihydromorphine (^{3}DHM) and [^{3}H]-naloxone in the rat brain have been reported.[58] The higher affinity type was designated μ_1 and the lower affinity morphine-selective type was designated μ_2.[59,60]

Stefano, and later Cadet, *et al.*, were crucial in finding a novel μ opiate receptor subtype, designated μ_3, located on immunocytes and neural tissues of invertebrates, such as *M. edulis*, and on human monocytes, granulocytes, vascular endothelial cells, and other neuronal and non-neuronal cell types.[21,32] More recently, they have extended this finding to the human multilineage progenitor cells.[61] This receptor differed from previously described neuronal opioid receptor subtypes. It exhibited nondetectable or exceedingly low affinities for naturally occurring endogenous opioid peptides, peptide analogues, μ_1 opioid-selective endomorphins 1 and 2, and synthetic opioid alkaloids of the benzomorphan and phenylpiperidine classes.[21,31,62–65] In contrast, the μ_3 opiate receptor displayed high-affinity binding for morphine-related morphinan alkaloids, such as dihydromorphine and the clinically established antagonists naloxone and naltrexone.[21,32] It was also established that the μ_3 receptor is coupled to G protein isoforms, based on guanine nucleotide effects on agonist binding.[63]

Molecular cloning of a μ_3 receptor encoding cDNA employed a screening probe derived from a conserved region of the human μ_1 receptor and a human testis cDNA library.[32] A full-length 1338 base pair cDNA was cloned and subsequently sequenced.[32] The National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) analysis indicated that the clone exhibited 100% identity to the μ_1 opioid receptor subtype in its central conserved region, with most of exon 1 spliced out at its 5′-end (Fig. 1). The 3′-end of the μ_3 receptor cDNA contained the 3′-end of the μ_1 receptor protein coding sequence, with a spliced insert of 263 bases containing a stop codon and terminated by a μ_1-specific 202 base untranslated region.[32]

RT-PCR and subsequent sequence analysis revealed the presence of this opiate receptor subtype in human vascular endothelial cells, mononuclear cells, and polymorphic nuclear cells.[32] To determine if the cDNA clone we isolated was functional and had the biochemical properties expected of the μ_3 receptor, the cDNA was expressed in a heterologous system (Cos-1 cells). Following exposure to morphine, the transfected Cos-1 cells released NO in a naloxone-reversible manner.[32] Untransfected Cos-1 cells failed to produce any detectable NO upon addition of morphine.[32] The addition of Met-enkephalin, DPDPE, or Leu-enkephalin did not stimulate NO release in the controls or transfected cells.[32] In sum, heterologous cellular expression of the cloned μ_3 opiate receptor cDNA by Cos-1 cells resulted in morphine-selective stimulation of NO release, consistent with previous biochemical data presented above.[11,32,66]

Functional Studies

Documentation of the functional association of morphine action on this μ receptor is that NO production and release were stimulated. Interestingly, earlier reports have demonstrated that peripheral morphine analgesia involves NO-stimulated increases in intracellular cyclic guanosine monophosphate (cGMP).[67] NO has been associated with antinociception[68] as well as tolerance and dependence.[69] In addition, the morphine-induced suppression of splenic lymphocyte proliferation has been shown to involve NO.[70] Morphine and NO have been

TABLE 2. Animal μ3 opiate receptor expression

Invertebrate	*Mytilus* ganglia, hemocytes
	Homarus americanus ganglia and hemocytes
Vertebrate	Human white blood cells (polymorphonuclear, morphonuclear, Raji, U937), vascular tissues (endothelium cells), human multilineage progenitor cells
	Human brain tissue, human heart tissue
	Rat brain, gastrointestinal tract
	Human neuroblastoma cells (SHY5Y, HTB11)

linked in gastrointestinal regulation.[19,71] Furthermore, morphine, not opioid peptides, stimulate constitutive NO release in macrophages, granulocytes, various types of human and rat endothelial cells, invertebrate neurons and immunocytes, and in rat median eminence fragments.[8,31,72] All these observations were documented to be naloxone antagonizable and blocked by L-NAME, an NO synthase inhibitor.[65,73–81] These data suggest that the μ_3 receptor is coupled to constitutive NO release in these cells found in evolutionarily diverse animals.[76,82,83]

Morphine's actions in these diverse tissues (Table 2) complement what is known about NO mediating immune and vascular functions, namely that it can downregulate them from an excitatory state or prevent the excitatory state from occurring.[7,73,79,81] Additional information on opiate alkaloid signaling substances can be summarized as follows: Injection of vertebrate animals with morphine results in deficient macrophage function[84] and an alteration of T cell activity.[85] Morphine also antagonizes interleukin-1-α or tumor necrosis factor-α-induced chemotaxis in human granulocytes and monocytes.[86,87]

Conclusion

It appears that after more than 30 years of research into an animal's ability to make morphine the empirical evidence is now overwhelming. Recent reports have also demonstrated that major substances of abuse, i.e., nicotine, alcohol, and cocaine, appear to work via altering endogenous morphinergic signaling in a common manner, i.e., inducing morphine release.[25,88–93] Furthermore, endogenous morphine participates in physiological processes transcending pain, making it an important chemical messenger. It would appear that at the present time we are only skirting around the significance of this chemical messenger humans have been using exogenously for over 6000 years. It is interesting to surmise morphine gave rise to the catecholamine signaling family since it functions as an information transmitter in plants, invertebrates, and mammals, whereas the catecholamine pathway is only complete in mammals.[52]

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Conflicts of Interest

The authors declare no conflicts of interest.

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