REVIEW

Morphine's chemical messenger status in animals

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Abstract

Conventional wisdom recognizes morphine only as a plant product with profound pharmacological actions on mammalian tissues. This widely held belief ignores 30 years of empirical evidence from different laboratories, demonstrating its presence and synthesis in animal tissues, including human. Using state-of-the-art technologies, we recently demonstrated that normal healthy animal tissues, including human, have the ability to synthesize morphine in a process that both resembles that occurring in plants and one which is subject to pharmacological manipulation via existing mammalian enzymes. Importantly, this ability also occurs in invertebrate neural tissues in animals 500 million years divergent in evolution. Morphine is present in human immune, vascular and neural tissues, along with its own receptor, μ3, which we have cloned and found to be opioid peptide insensitive and opiate alkaloid selective, establishing its endogenous signaling capabilities. The functional implications of endogenous morphine expression as a parallel, but independently regulated signaling system, confers a major adaptive advantage to an expanding cadre of L-tyrosine-derived molecular species as autocrine, paracrine, and hormonal regulators of cellular systems involved in immune function, neural-immune coupling in the mediation of nociception and antinociception, and cardiovascular integrity linked to functional recruitment of constitutive nitric oxide (NO). These linkages are the driving knowledge that now supports a role for intracellular morphine expression and its biosynthetic intermediates as developmental chaperones in the evolutionary adaptation of dopamine and its catecholamine derivatives norepinephrine and epinephrine as signaling molecules.

Dopamine Linkage

The historical weight of morphine's dualistic potential to provide highly efficacious pain control, inextricably linked to debilitating side-effects and addiction, has maintained morphine's ominous pride of place as a DEA Schedule II drug (Esch *et al* 2004b; Fricchione & Stefano 2005; Stefano & Scharrer 1994; Stefano *et al* 1996b; Stefano *et al* 2005b). Curiously, initial speculations as to the existence and potential physiological role of endogenous morphine were made over 30 years ago by prominent researchers in the field of alcohol abuse, not opiate abuse, who advanced the hypothesis that the reinforcing or addictive effects of ethanol were functionally linked to the cellular effects of dopamine (DA)-derived isoquinoline alkaloids, notably the tetrahydroisoquinoline (TIQ) salsolinol (Davis & Walsh 1970; Davis *et al* 1970; Yamanaka *et al* 1970) and the benzylisoquinoline (BIQ) morphine precursor (tetrahydropapaveroline) THP (Halushka *et al* 1970; Walsh *et al* 1970; Weiner 1978). Recognition of TIQs, THP, and endogenous morphine as active principles of alcohol abuse was inherently linked to their normal presence in dopaminergic neurons, enhanced cellular

expression following chronic ethanol intake (Collins *et al* 1979; Turner *et al* 1974; Weiner 1978; Weiner 1981; Zhu *et al* 2006a; 2006b), and concentration-dependent disregulation of DA metabolism and/or DA-ergic signaling in mesocortical/mesolimibic areas such as the nucleus accumbens and ventral tegmental area traditionally associated with reinforcement of alcohol-related behaviors (Clow *et al* 1983; Duncan & Fernando 1991; Myers 1990; Myers & Robinson 1999; Sallstrom *et al* 1999). The causal relationship and functional association of central nervous system (CNS) expression of TIQ and BIQ alkaloids to alcohol abuse remains controversial despite anatomical, physiological, pharmacological, and behavioral evidence linking DA-ergic and opioidergic systems in limbic areas associated with reinforcement of ethanol intake behaviors (Haber *et al* 1997; McCoy *et al* 2003; Naoi *et al* 2004; Shearman & Herz 1983).

The functional association between aberrant DA metabolism, cellular expression of isoquinoline alkaloids, and the etiology of Parkinson's Disease has also been extensively studied and debated for three decades (Britton 1982; Collins 2004; Greenberg & Cohen 1973; Heikkila *et al* 1971; Katz & Cohen 1976; Naoi *et al* 1998; Niwa *et al* 1992; Sandler *et al* 1982; Suzuki *et al* 1990). In contrast to the hypothesized role of isoquinoline alkaloids to activate neural circuits involved in the reinforcement of alcohol dependence, these same conjugate molecules were proposed as pathophysiological agents responsible for Parkinson's Disease-associated symptomatology. Interestingly, by the early 1970s a functional association between L-3,4-dihydroxyphenylalanine (L-DOPA) therapy and *in vivo* formation of BIQs had been proposed (Coscia *et al* 1977; Davis *et al* 1975; Johnston 1971; Sandler *et al* 1973). It was subsequently demonstrated that urinary levels of morphine, codeine, and THP in L-DOPA-treated Parkinsonian patients are dramatically elevated as compared to matched controls and abstinent alcoholics (Matsubara *et al* 1992). Not surprisingly, enhanced production of THP in Parkinsonian patients was peremptorily linked to the mediation of adverse side effects and cellular toxicity evolving from chronic L–DOPA therapy (Galloway *et al* 1982; Kim *et al* 2005; Nimit *et al* 1983; Okada *et al* 1998; Shin *et al* 2004; Soh *et al* 2003), despite clinical evidence supporting positive effects of morphine on L-DOPA-associated dyskinesias (Berg *et al* 1999; Berg *et al* 2001; Cadet *et al* 2003b; Fricchione & Stefano 2005).

By the mid 1970s, evidence of endogenous morphine expression in animal systems was provided by Spector and coworkers who characterized a nonpeptide morphine-like compound (MLC) extracted from mammalian brain which bound with high affinity and selectivity to an anti-morphine serum originally intended for radioimmunoassay of morphine in blood and urine phenotype and exhibited opiate-like inhibitory effects in established bioassays (Gintzler *et al* 1976; Gintzler *et al* 1978). The same anti-morphine serum was employed to provide immunohistochemical detection of MLC in CNS areas including vestibular, cerebellar, and raphe systems (Gintzler *et al* 1978). Subsequently, Bianchi and coworkers replicated and extended the original anatomical observations of Spector and coworkers by demonstrating uptake and accumulation of 3H-labeled morphine within defined rat brain areas (Bianchi *et al* 1993a; 1994b) and providing immunohistochemical localization of morphine-like material in perikarya, fibers, and terminals of neurons in discrete areas of both rat and human brain (Bianchi *et al* 1993a; 1994b; Stefano *et al* 2000b). In the same study, morphine-like immunoreactive material associated with striatal neurons was markedly reduced following exposure of brain slices to high K+ concentrations (Bianchi *et al* 1993a; 1994b), a physiologically important observation that was subsequently addressed in great depth in later studies from this same group (Guarna *et al* 1998; 2002).

Historically, anatomical observations of intrinsically low basal levels of immunoreactive morphine-like material widely distributed across diverse CNS areas may have led members of the scientific community to cursorily disregard any compelling argument in support of a biological role for endogenous morphine expression. Because CNS distributions of immunoreactive morphine-like material did not appear to be strictly co-localized with DA-ergic systems, there was also an apparent conflict with accumulated data linking increased or aberrant production of DA metabolites to randomly formed DA-derived BIQ alkaloids. Extensive data sets evolving from alcohol and Parkinson's Disease research introduced inconclusive, often contradictory, evidence indicating that non-physiological concentrations of isoquinoline alkaloids, often in the millimolar range, were required to mediate cellular toxicity via down-regulation of necessary DA metabolism and turnover linked to free radical production. Because biologically meaningful concentrations of BIQ alkaloids were often observed to have little or no effect on DA metabolism and cellular integrity, a null hypothesis was apparent indicating different, potentially important, regulatory activities for this class of biomolecules outside the realm of DA signaling.

CYP2D6 Importance

In light of the above, the lack of a well-characterized cell- or organ-based expression system made the difficulties of monitoring *de novo* incorporation of isotopically-labeled L-tyrosine (TYR), L-DOPA or DA into endogenous morphine appears insurmountable. Spector's group, however, made considerable advances in characterizing biosynthetic events involving *in vivo* enzymatic conversion of morphinan precursors into endogenous morphine, i.e., the later stages of the biosynthetic pathway. Key studies demonstrated stereoselective conversion of the morphinan alkaloids (+)-salutaridine, (-)-thebaine, and (-)-codeine into chemically authentic

morphine in rat tissues (Donnerer *et al* 1986) and transformation of thebaine to oripavine, codeine, and morphine by rat liver, kidney, and brain microsomes in the presence of NADPH- and NADH-generating systems (Kodaira & Spector 1988). Importantly, use of chemical inhibitors indicated a critical role of cytochrome P450s (CYP) in these synthetic processes (Kodaira & Spector 1988).

Contemporaneously, Goldstein and coworkers reported the presence of morphine-like and codeine-like immunoreactivities in bovine hypothalamus and adrenal, and in rat brain, that were chemically characterized as authentic morphine and codeine (Goldstein *et al* 1985; Weitz et al, 1986; 1987). They proceeded to demonstrate *in vivo* and *in vitro* intramolecular conversion of reticuline to form salutaridine in rat liver, a critical step in generating the morphine/morphinan skeleton and the stereochemistry of the morphinan series (Weitz *et al* 1987). Subsequent studies from Zenk and coworkers provided further characterization of hepatic conversion of reticuline to salutaridine (Amann & Zenk 1991; Amann *et al* 1995), thereby reinforcing the critical involvement of CYPs in endogenous morphine expression.

In recent studies, we demonstrated that normal and healthy animals, invertebrate ganglia and human white blood cells (WBC) do make morphine from tyrosine, tyramine to DA and THP to codeine (Zhu *et al* 2005a; 2005b) (***Tab. I, Fig. 1***). These experiments demonstrated that CYP2D6 is involved with morphine synthesis, which was supported by RT-PCR analysis amplifying a 282 bp fragment, demonstrating the presence of CYP2D6 mRNA (Pai et al, 2004). Sequence analysis of this transcript fragment demonstrated a 94% similarity to human GeneBank accession number M20403 in the invertebrate tissue (Zhu *et al* 2005b). These studies provide evidence that:

(1) the synthesis of morphine by various animal tissues is more widespread than previously thought and now includes human immune cells (***Tab. I***);
(2) Moreover, another pathway for morphine synthesis exists, via L-DOPA, demonstrating an intersection between dopamine and morphine pathways (***Fig. 1***);
(3) White blood cells can release morphine into the environment to regulate themselves and other cells.

Therefore, white blood cells employ endogenously expressed morphine as a key autocrine/paracrine signaling factor. Interestingly the coupling of endogenous morphine to constitutive nitric oxide release demonstrated end product inhibition on select morphine synthesizing enzymes, further substantiating morphinergic signaling in animals (Mantione *et al* 2008).

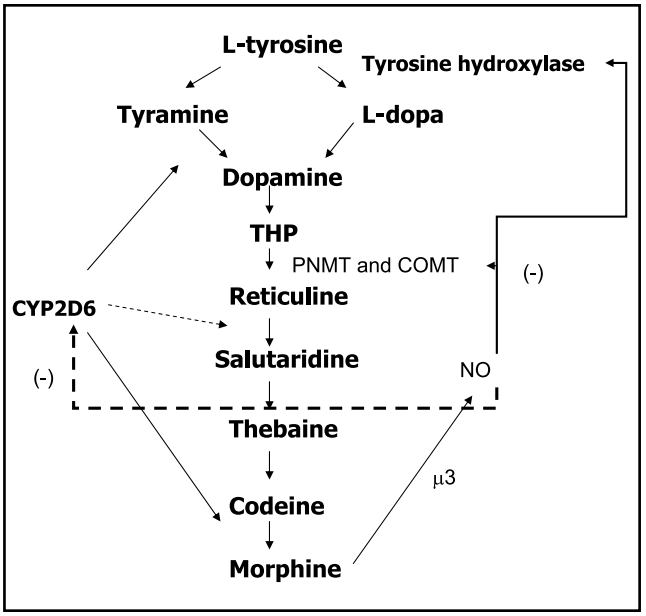

Figure 1. Schematic of morphine biosynthesis in animals (Kream & Stefano 2006). Enzymes shown are sensitive to nitric oxide exhibiting lower expression in its presence, representing inhibition via end product signaling via morphinergic stimulation of constitutive nitric oxide release via the μ3 opiate receptor (Mantione *et al* 2008). Tyrosine hydroxylase effect is unpublished.

Novel Opiate Receptor

Previous studies from our laboratory have revealed a novel μ opiate receptor, μ_3, which is expressed in different animal tissues such as human vascular endothelial cells, leukocytes and neural tissues (Cadet *et al* 2003a). This novel mu receptor is selective for the opiate alkaloid morphine, since opioid peptides do not bind to this mu-type splice variant receptor. In reporting on the acute effects of morphine exposure to human leukocytes by analyzing the expression of different genes, it was revealed that exogenously applied morphine down regulated TH expression, suggesting an end-product inhibition mechanism modulating the pathway of morphine biosynthesis (Mantione *et al* 2008; Stefano *et al* 2005a). In the same study it was demonstrated that constitutive nitric oxide synthase (cNOS) was up-regulated and inducible NOS was down regulated, confirming our previous observations (Cadet *et al* 2003a; 2007; Stefano *et al* 2000b). We have demonstrated that μ3, via morphine activation, releases cNOS in immune, vascular, gut, human stem cells and neural tissues (Cadet *et al* 2007; Pryor *et al* 2005; Stefano *et al* 2005b). In this regard, NO also inhibits dopamine β hydroxylase (Stefano *et al* 2001), suggesting that via NO the morphine biosynthetic pathway is favored by cutting off further catecholamine synthesis and diverting the extra DA to morphine synthesis. Furthermore, the μ3 opiate receptor represents a 6 transmembrane receptor with specific nitric oxide synthase links, substantiating the many

Table I. (amended from (Stefano *et al* 2008)

μ3 Opiate Receptor Binding and Expression	
Tissues or Cell lines:	
Mytilus edulis:	immunocytes (Stefano *et al* 1993), ganglia (Liu *et al* 1996b; Sonetti *et al* 1994; 1997; Stefano & Scharrer 1994; Stefano & Scharrer 1996a)
Cat:	Astrocytes and microglia (Dobrenis et al, 1995)
Rat:	Kupffler cells (Dobrenis *et al* 1995)
Murine:	Cell Lines (J774.2, RAW 264.7, BAC1.2F5) (Makman *et al* 1995b), Mouse Neuroblastoma (Cruciani *et al* 1994)
Human Tissues:	Vascular (Stefano *et al* 1995b), White Blood Cells (Makman *et al* 1995a; Stefano *et al* 1993), Neural (Fricchione *et al* 2008)
μ3 and μ4 Opiate Receptor Expression	
Mytilus edulis:	Ganglia (Cadet & Stefano 1999; Cadet *et al* 2002)
Human Tissues:	Cell lines (HTB-11, Sh-Sy5y, HUVEC, U937, Raji, HP75) (Cadet *et al* 2003a), Heart (Cadet *et al* 2000; 2001b), Vascular (Cadet *et al* 2000; 2003a; 2004), White Blood Cells (Cadet *et al* 2001a; 2003a), Neural (Fricchione *et al* 2008), Stem Cells (Cadet *et al* 2007)
Human prostate:	mu4 (not published); HP75 is a human hypothalamus cell line from ATCC (not published); HTB-11 and Sh-Sy5y are human neuroblastoma cell lines (ATCC). U937 cells is a human monocyte cell line (ATCC). (not published); Raji Cells is a human B lymphocyte cell line (ATCC). (not published)
Morphine Presence	
Invertebrates:	
Mytilus edulis:	immunocytes (Stefano *et al* 1993), ganglia (Zhu *et al* 2001a; 2002b; 2003; 2005b; Zhu & Stefano 2004c), hemolymph (Stefano *et al* 1993)
Modiolus deminensus:	ganglia (Goumon *et al* 2001)
Ascaris suum:	ganglia (Goumon *et al* 2000b),muscle (Zhu *et al* 2004b), uterus (Zhu *et al* 2004b)
Dracunculus medinensis:	Whole body extraction (Zhu *et al* 2002a)
Schistosoma mansoni:	Whole body extraction (Zhu *et al* 2002a)
Homarus americanus:	ganglia (Casares *et al* 2005)
Vertebrates:	
Human:	Cell line SH-SY-5Y (Poeaknapo *et al* 2004), Heart (Zhu *et al* 2001c), White Blood Cells (Zhu *et al* 2005a), Plasma (Brix-Christensen *et al* 1997; Liu *et al* 1996a), Cerebrospinal fluid (Cardinale *et al* 1987)
Other Mammals (rat, mice, cattle):	Cell line PC-12 (Goumon *et al* 2000d; Zhu *et al* 2001b), Neural (Goumon *et al* 2000a; Zhu *et al* 2004a), Adrenal (Goldstein *et al* 1985; Goumon & Stefano 2000; 2005; 2006), Brain/Neural (Bianchi *et al* 1993b; 1994a; Donnerer *et al* 1986; 1987; Gintzler *et al* 1976; 1978; Goldstein *et al* 1985; Guarna *et al* 1998; Horak *et al* 1993; Kodaira & Spector 1988; Lee & Spector 1991; Neri *et al* 2004; Oka *et al* 1985; Weitz *et al* 1986)

reports associating the two signaling systems (***Figure 2***, Kream *et al* 2007).

Taken together, we have surmised that the low concentrations of morphine found in various tissues serve to limit their excitability (e.g., micro environmental noise, Stefano *et al* 2000a) which upon trauma, after a latency period, increase in an attempt to again limit their excitability (Stefano & Scharrer 1994; Stefano *et al* 1996b). This suggests that the respective tissues are always in the "on" state, allowing them to emerge immediately from this diminished excitability state and spring into action, which is critical for survival, and after a period of time they are brought back into the down state via cNOS (Stefano *et al* 1994; 2000a; 2000b). This hypothesis is also supported by a microarray study of human genes, whereby morphine exposure to polymorphonuclear cells down regulated proinflammatory mediator expression (Stefano *et al* 2005a). Recently, we have also demonstrated that exogenous morphine exposure can have positive implications for Alzheimer's and Parkinson's disorders (Pak *et al* 2005; Rambhia *et al* 2005). We have even demonstrated that morphine may be a key component of relaxation associated with various human activities (Esch *et al* 2004; Esch & Stefano 2004; Stefano *et al* 2004b; Zhu *et al* 2004a). It appears to also represent a vital signaling component in pleasure, love and eating (Esch & Stefano 2004; Esch & Stefano 2005; Esch *et al* 2006). The fact that this signaling system is present in organisms 500 million years divergent in evolution, performing identical functions, also supports a protective role for morphine.

The *de novo* biosynthesis of morphine in animal cells provides us with an expanded set of cellular processes that require BIQs as defined biosynthetic intermediates/enzyme substrates, alternative explanations of the biological relevance of isoquinoline alkaloids over and beyond those linked to DA-induced cellular toxicity, and establishes an underlying chemical basis for phylogenic conservation and adaptation of reciprocally interactive catecholamine and opioid signaling pathways (Stefano & Kream 2007b).

Evolution

Adaptive nociceptive, nocifensive, and anti-nociceptive behaviors have evolved from paracrine cellular processes. As a prime example, in *Papaver somniferum*, pyridoxal phosphate-dependent progenitor isoenzymes with dual L-TYR decarboxylase and L-DOPA decarboxylase activities, as well as berberine bridge enzyme, i.e., key players in the biosynthesis of BIQ alkaloids (Boettcher *et al* 2005; Facchini & De Luca 1994; Facchini & Park 2003), are induced following traumatic insult to opium poppy cells via activation of wound-responsive regulatory elements on their respective genes (Park *et al* 1999). The differential expression of these essential gene products and the organ-dependent accumulation of different alkaloids suggest a coordinated regulation

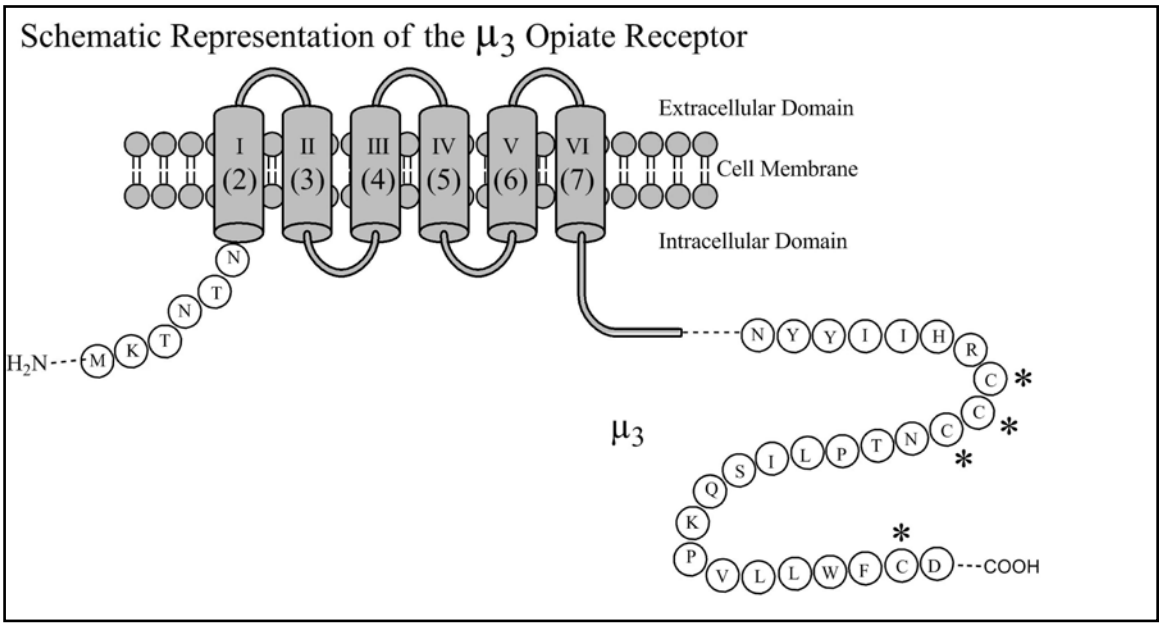

Figure 2. 6-Transmembrane μ_3 Opiate Receptor (From (Kream et al, 2007)
Graphic includes a schematic representation of the μ_3 opiate receptor. Trans-Membrane Helical (TMH) domains of the μ_3 opiate receptor are numbered I–VI and correspond to conserved TMH domains 2–7of the μ_1 receptor. Conserved Extracellular Loops (ELs), Intracellular Loops (ILs), and C-terminal intracellular sequences common to traditional μ_1 and μ_3 receptors are represented by thick grey lines. The unique 26 amino acid C-terminal intracellular domain of the μ_3 receptor is represented by the single letter amino acid code with starred Cysteine residues, indicative of nitric oxide synthase docking. Similarly, the conserved intracellular N-terminus of the μ_3 receptor expressed from Exon 1 of the μ receptor gene is represented by the single letter amino acid code.

of specific alkaloid biosynthetic genes. Accordingly, we propose that positive evolutionary pressure that has preserved a primordial, phylogenic broad, biochemical mechanism by which the prototype opiate alkaloid morphine is expressed and utilized as a physiological regulator of relatively simple, as well as significantly more complex, cellular functions (Stefano & Scharrer 1994; Stefano & Kream 2007b).

Recent findings by Goumon et al., also demonstrate that endogenous morphine-6-glucuronide is synthesized in chromaffin cells and secreted into the incubation medium upon stimulation (Goumon *et al* 2006; Muller *et al* 2008). This finding strongly suggests that this material may be released from adrenal tissues in response to stressors. In the peripheral circulation, morphine-6-glucuronide may mediate several systemic actions (e.g., on immune cells) based on its affinity for the μ3 opiate receptor. In sum, these data represent an important observation on the role of morphine-6-glucuronide as a new endocrine factor, which may be released from adrenal tissues in response to stressors, thereby mediating neural-immune coupling events (Goumon *et al* 2005; Mantione *et al* 2002; 2005).

Throughout the 1970s, considerable cross-fertilization of ideas and hypotheses between alcohol abuse and Parkinson's Disease researchers into the origin and biological significance of isoquinoline alkaloids resulted in a historical convergence of numerous studies attempting to define DA-derived TIQ and BIQ alkaloids as addictive agents responsible for alcohol dependence and as major neurotoxic agents responsible for the etiology and persistence of Parkinson's Disease. Importantly, the demonstration of *in vivo* conversion of THP to morphine provides invaluable mechanistic insight into previous pharmacological studies involved with focal administration of THP into DA-ergic limbic areas associated with ethanol abuse (Myers 1990; Myers & Robinson 1999; Zhu *et al* 2006b), supports the critical involvement of morphine-preferring mu opioid receptors (MORs) in limbic areas associated with ethanol intake behaviors (Burattini *et al* 2006; Ghozland *et al* 2005; Lee *et al* 2005; Zhu *et al* 2004a), and adds an additional dimension to alcohol abuse research using both pharmacological inhibitors of morphine biosynthesis as well as type selective opioid antagonists (Ciccocioppo *et al* 2002; Gonzales & Weiss 1998; McBride *et al* 2002).

Highlighting the importance of endogenous morphine are recent studies demonstrating that addictive properties of nicotine, alcohol and cocaine may arise from their ability to enhance endogenous morphine levels and its neuronal release, opening up a new level of understanding in substance abuse induced addiction and behavioral effects, as well as morphine regulation (Stefano *et al* 2007a; 2008; Zhu *et al* 2006a; 2006b). In the past, these substances of abuse have again been linked into a common pathway because of the common DA connection (Bainton *et al* 2000; Nestler 2005). Now, they are additionally linked because of their common effect on morphinergic processes. It is highly significant that both nicotine and ethanol increase ganglionic

morphine levels rapidly, providing a mechanism to initiate their pleasure and addicting actions with continued frequent use.

At the present time we have demonstrated that there is a morphine presence in immune tissues (see above), in vascular endothelial cells (Bilfinger *et al* 1998; Stefano *et al* 1995b; 1998a; 1998b; 1998c; Stefano 1998), including human heart tissues (Cadet *et al* 2000; Zhu *et al* 2001c), various neural tissues (see above), and in human limbic tissues (Stefano, in preparation), as well as in animal gut tissues (Stefano *et al* 2004a). These reports also demonstrate the presence of the mu3 opiate receptor subtype, which appears always to be associated with nitric oxide release. Clearly, just based on these studies, the endogenous morphine "story" transcends analgesia and addiction related phenomena and appears to exert physiological actions in most of the physiological systems. We've even demonstrated that parasites employ this signaling system (Goumon *et al* 2000b; Leung *et al* 1995; Pryor *et al* 2004; Zhu *et al* 2002a). Additionally, the impact to mental health also promises to be highly significant (Fricchione *et al* 1994; Fricchione & Stefano 2005; Stefano & Fricchione 1995a; Stefano *et al* 1996b; 1996c; 2001; 2005b; 2006).

In conclusion, scientific orthodoxy has attempted to establish rational guidelines by which it may construct an empirically-driven, consistently tame, superstructure to encompass cellular regulation of complex biological processes in higher organisms. As a corollary, the collective effort to codify general principles of cellular organization has effectively resulted in the marginalization of many important lines of empirical investigation that are perceived to inject varying degrees of disorder and/or controversy into well-ordered regulatory schemes. A prime example, the biochemical and physiological investigation into the expression and functional roles of endogenous morphine by animal cells has presented an ongoing challenge to several research groups for over thirty years. A compelling body of evidence now supports the existence of a de novo biosynthetic pathway for endogenous morphine in mammalian and invertebrate cells, with remarkable similarities to the well-characterized enzymatic pathway described in *Papaver somniferum*. Elucidation of the potential biological significance/impact of evolutionarily conserved opiate alkaloid plant products in animal cells awaits further investigation (Stefano & Miller 2002).

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