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The Epigenetic Regulation of the Opioid System: New Individualized Prompt Prevention and Treatment Strategies

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ABSTRACT

The most well-known physiological effect associated with opiod system is their efficacy in pain reduction or analgesia, although their effect on a variety of other physiological and physiophological functions has become apparent in recent years. This review is an attempt to clarify in more detail the epigenetic regulation of opioid system to understand with more precision their transcriptional and posttranscriptional regulation in multiple pyisiological and pharmacological contexts. The opioid receptors show an epigenetic regulation and opioid peptide precursors by methylation, chromatin remodeling and microRNA. Although the opioid receptor promoters have similarity between them, they use different epigenetic regulation forms and they exhibit different pattern of expression during the cell differentiation. DNA methylation is also confirmed in opioid peptide precursors, being important for gene expression and tissue specificity. Understanding the epigenetic basis of those physiological and physiopathological procesess is essential for the development of individualized prompt prevention and treatment strategies. J. Cell. Biochem. 116: 2419–2426, 2015.

KEY WORDS: EPIGENETIC; METHYLATION; CHROMATING REMODELING; MIRNA; OPIOIDS; DRUG ADDICTION

The opioid system is a biological communication system for which activity is mediated by the so-called endogenous opioid peptides (EOPs). Pharmacologically, it has been described three opioid receptors (ORs): mu opioid receptor (MOR), delta opioid receptor (DOR) and kappa opioid receptor (KOR), which belong to the 7-transmembrane, G protein-coupled receptors super-family [Law et al., 2004]. Later it was discovered the fourth OR, the nociceptin receptor (NOP). The comparison of the amino acid sequence and nucleotides has established that four genes are highly conserved in their homolog codificant exons (73–100%), which are located in the middle of each gene. That exon codifies the 7-transmembrane domain, suggesting that four ORs come from the same ancestor gene. Also it has a single intron in the coding region. However, the four ORs diverge in their amino-end which is produced out from the

Abbreviations: Ors, Opioid receptors; MOR, mu opioid receptor protein; DOR, delta opioid receptor protein; KOR, kappa opioid receptor protein; NOP, nociceptin receptor protein; Oprm1, mu opioid receptor gen; Oprd1, delta opioid receptor gen; Oprk1, kappa opioid receptor gen; Oprl1, nociceptin receptor protein; POMC, proopiomelanocortin protein; PENK, proenkephalin protein; PDYN, prodynorphin protein; PNOC, nociceptin/orphanin FQ protein; Pomc, proopiomelanocortin gen; Penk, proenkephalin gen; Pdyn, prodynorphin gen; Pnoc, nociceptin/orphanin FQ gen; Sox2, (Sry-like high-mobility-group box gene; Sp1, specificity protein 1; iGA, Sp1 on an inverted GA motif; PCBP, poly C binding protein; CREB, cyclic adenosine monophosphate response element binding protein; Oct-1, octamer-1; PU.1, PU box binding on a 34-bp silencer region; PARP1, polyADP-ribose polymerase 1 on a double-stranded poly-C sequence; Sp3, transcription factor binding to the 5′ UTR specificity protein 3; REST, repressor element-1 silencing transcription factor; MeCP2, methyl CpG binding protein 2; RA, retinoic acid; Ets-1, E-twenty six 1; USF, upstream stimulatori factor; MBD2, methyl-CpG binding domain protein 2; PI3K, fosfatidilinositol 3-kinase; NGF, nerve growth factor; AP2, activating protein; IK, Ikaros; nGRE, one inhibiting transcription factor binding site; Hdac2, histone deacetylase 2; Dnmt1, (cytosine-5)-methyltransferase 1; MAP2K8, mitogen activated protein kinase 2; PC1, proprotein convertase 1; PC2, proprotein convertase 2; NAc, nucleus accumbens; DREAM, downstream regulatory element antagonist modulator; Orl1, opioid receptor-like receptor; BDNF, brain-derived neurotrophic factor.

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cellular surface, and also in the carboxyl-end which is extended in the intracellular space (9–20% of conservation) [Neer, 1995]. Those slight differences explain specificity between the ligand union pattern, pharmacological effects and transduction signaling pathway of each OR [LaForge et al., 2000]. But, despite their conserved structure, it has been proved that each gene has a single regulatory pathway and shows a different expression pattern [Wei and Loh, 2002]. These genes are *Oprm1*, *Oprd1*, *Oprk1*, and *Oprl1*. cDNA alignment studies with the genomic DNA and mRNA processing have established the existence of several isoforms or variants produced by each OR, by means of the use of alternative splicing, alternative promoters (*Oprm1*, *Oprk1*), alternative polyadenylation sites (*Oprk1*) or inclusions in non-coding exons [Wei and Loh, 2002].

The endogenous opioid peptides, endorphins, enkephalins, dynorphins and orphanin/nociceptins, are derived from precursors encoded by proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN) and nociceptin/orphanin FQ (PNOC), respectively [LaForge et al., 2000; Levran et al., 2012]. However, biological approaches have demonstrated that in mammals, these peptides are grouped in three major types of opioid peptides: endorphins, enkephalins and dynorphins [Koneru A, Satyanarayana S and Rizwan S., 2009]. Recent studies have shown that there are more endogenous opioid peptides which do not belong to these three major types and which precursors are not yet known. These two endogenous opioid peptides are tetrapeptides, endomorphin-1 (Tyr-Pro-Tr p-Phe-Nh2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2) [Fichna et al., 2007]. Although their better known function of the endogenous opioid peptides is to suppress pain (analgesia), currently other different physiological functions have been reported. The system is involved in brain development, regeneration and plasticity phenomena, taking part even in higher functions as memory and learning; sensory functions regulation; production of changes in eating behavior; modulation of mental illnesses such as anxiety or depression and mood; in gastrointestinal, renal and hepatic functions; modulation of cardiovascular response and blood pressure; modulation of breathing, causing deficiencies in respiratory and thermoregulatory responses; modulation of immunologial response, particularly immune-suppression; regulating the movement and general activity; and finally in the regulation of reproductive function [Bodnar, 2007; Subiran et al., 2011]. This review tries to on clarify the epigenetic mechanisms of opioid system, focusing more detail on methylation patterns, chromatin remodeling, and microRNA regulation to understand with more precision their transcriptional and posttranscriptional regulation in multiple physiological and pharmacological contexts.

THE EPIGENETIC REGULATION OF OPIOID RECEPTORS

Four ORs are subjected to epigenetic regulation since they maintain several features, making them susceptible to be controlled by this machinery: all of the genes are rich in CpG islands and hence, they can be highly methylated, their promoters show several types of modifications in different cellular stages or culture conditions, and chromatin remodeling occurs in their promoters producing changes in their expression patterns in the differentiation process [LaForge et al., 2000].

MU OPIOID RECEPTOR (*OPRM1*)

MOR is codified by the gene *Oprm1*, which has an important role in clinical effects of opiates, such as analgesia, tolerance development, and physical dependency under long drug treatment. MOR is one of the four ORs receiving further action by endogenous opioids, opiates and opioid analgesic drugs and also by exogenous opioid drugs as follows methadone, heroin and morphine [Kreek et al., 2002]. Certain numbers of splicing variants have been named, they are variants of the MOR mRNA which are modified in their 5′UTRs and are differentially regulated at transduction level [Song et al., 2009]. This receptor uses two closely positioned promoters, the distal promoter and the proximal promoter, which is responsible for most of the activity of the gene. Both of them belong to TATA-less type promoter, which are rich in CGs and they have several regulatory elements. Lots of transcription factors have been examined as transcription regulators of the activity of *Oprm1*: Sox2 activators (Sry-like high-mobility-group box gene), Sp1 (specificity protein 1), iGA (Sp1 on an inverted GA motif), PCBP (poly C binding protein), and CREB (cyclic adenosine monophosphate response element binding protein). In contrast, the repressors are, Oct-1 (octamer-1), PU.1 (PU box binding on a 34-bp silencer region), PARP1 (polyADP-ribose polymerase 1 on a double-stranded poly-C sequence), Sp3 (two TFs binding to the 5′UTR specificity protein 3) and REST (repressor element-1 silencing transcription factor) [Hwang et al., 2009; Wei and Loh, 2011].

The *Oprm1* promoter is heavily methylated in the CpG islands, in the undifferentiated embryonic carcinoma cells, thus the *Oprm1* is silenced. The silenced *Oprm1* can be activated in two ways, the first one by decreasing the expression of the methyl CpG binding protein 2 (MeCP2) and the second one by adding a histone acetylation inducer, that is the trichostatin A (TSA) [Lin et al., 2008]. In addition, DNA methylation on the *Oprm1* can be reduced adding an artificial demethyilation agent such as the 5′-Aza-2′-deoxycytidine (5-Aza-C), but it is not clear which type of endogenous signal triggers the DNA demethylation on the promoter of undifferentiated cells.

Other studies further provided that a higher-order chromatin conformational remodeling of the *Oprm1* promoter occurs during neuronal differentiation in embryonic carcinoma cells [Hwang et al., 2010]. Together with the silencing of *Oprm1* in pre-differentiated cultures, the promoter region is also organized into an ordered nucleosome. Whereas neuronal differentiation occurs by the retinoic acid (RA) induction, nucleosomes of the promoter region change their positions, and they start to recruit specific chromatin remodelers, which remodel the promoter and activate the gene. In that way, the activation or silencing of *Oprm1* is correlated with histone modification, as in brain studies is observed, that to achieve *Oprm1* activation, it is necessary the hyperacethylation of H3 and H4 [Song et al., 2009].

Recent studies have demonstrated that in term of non coding RNA, there are some miRNA linked with epigenetic regulation: miR-23b, miR-339, and Let-7 miR. On the one hand, miR-23b interacts with the 3′UTR of *Oprm1* through the K box motif (5′-UGUGAU-3′), which is a conserved sequence. That interaction blocks the attachment between *Oprm1* mRNA and the polysomes, hence, the transduction is arrested and MOR expression is canceled at protein level [Wu et al., 2013]. On the other hand, the binding of

TABLE I. The Epigenetic Mechanisems Involved in the Opioid Receptors Gene Expression

	OPRM1	OPRD1	OPRK1	OPRL1
Silencing				
Regulatory elements	Oct1, PU.1, PARP1, Sp3, REST	Sp3, IK	IK	
Metilation mechanisems	Metilation ↑MeCp2	Metilation ↑MeCP2		
Histon modifications		Histone deacetilases H3, H4	H3K9 me2	
MiroRNA	miR-23b, miR-339, Let-7			
Activation				
Regulatory elements	Sox2, Sp1, iGA, PCBP, CREB,	STAT6, Sp1, Ets-1, USF, NF-kB, AP2	c.Myc, Sp1, AP2	
Metilation mechanisems	Hipometilation ↓MeCp2	Hipometilation ↓MeCp2		Hipometilation
Histon modifications	Histone acetilases H3, H4	Demetilation of H3K9me3 by NGF/PI3K signalling	H3K4 me2	
MiroRNA				

miR-339 with *Oprm1* in the 3′UTR, suppress the receptor expression which is recovered after the addition of miR-339 inhibitor or mutating the binding target [Hwang et al., 2012]. Finally, the last miRNA discovered in *Oprm1* regulation is Let-7 miR, which also regulates opioid tolerance. Let-7 miR binds to its target in the *Oprm1* 3′UTR and represses its expression, sequestering *Oprm1* mRNA to P-bodies and finally leading to translational repression. Thus, binding between *Oprm1* transcript and polysomes is decreased in a Let-7 dependent way [He et al., 2010]. (Table I)

DELTA OPIOID RECEPTOR (*OPRD1*)

DOR, which is codified by the gene *Oprd1*, is involved in the modulation of addition, affective state, pain perception, and analgesia. It has a single promoter, TATA-less and is rich in CGs and although some studies have shown that *Oprd1* initiates its transcription in two adjacent sites since there is no study which confirms that alternative splicing [Wang et al., 2003]. Studies of transcriptional regulation of DOR have focused on activating factors, such as, STAT6, Ik (Ikaros), Sp1/Sp3, Ets-1 (E-twenty six 1), USF (upstream stimulatori factor), NF-kB, and AP2 [Wang et al., 2003; Wei and Loh, 2011].

In studies using embryonic carcinoma cells, the *Oprd1* is constitutively active in undifferentiated cells, but it becomes inactive in the neuronal differentiation [Wang et al., 2003]. It has been shown a heavy DNA methylation on *Oprd1* promoter in neural crest-derived cells, where it is not expressed and a demethylation in neuroblastoma cells, where *Oprd1* gene is highly expressed, displaying an inverse correlation between both cell types. In addition, 5-Aza-C treatment on the culture generates an increased *Oprd1* expression in neural crest-derived cells [Wang et al., 2005].

Through different studies, scientists have been trying to establish a connection between promoter's methylation state and chromatin modifications, concluding that, this connection is important to regulate *Oprd1* expression. In lack of methylation, *Oprd1* promoter in neuroblastoma cells maintains its accessibility if comparing with partially methylated *Oprd1* promoter in neural crest-derived cells [Wang et al., 2005]. It suggests that the silencing induced by the methylation generates a modification in the chromatin structure limiting the accessibility to the promoter during the transcription. For example, *Oprd1* promoter methylation contributes to MeCP2. In consequence, the histone deacetylase can access to the chromatin, generate decreased levels of H3 and H4 acetylation in *Oprd1* promoter region and change the chromatin structure. In contrast, when *Oprd1* promoter is completely methylated, it is correlated with low acetylation level in H3 [Wang et al., 2005]. Furthermore, other studies show the signaling role of fosfatidilinositol 3-kinase (PI3K) in the regulation of H3K9 state during neuronal differentiation induced by the nerve growth factor (NGF). That is to say, NGF/PI3K signaling is involved in demethylase activity of H3K9me3 in rat adrenal pheochromocytoa cells changing from a chromatin repressive mark to a activating mark [Chen et al., 2008]. (Table I)

KAPPA OPIOID RECEPTOR (*OPRK1*)

KOR is codified by *Oprk1*, and has an important role in a wide range of physiological systems, such as, pain regulation, drug abuse addiction, neuroendocrine regulation, cardiovascular functions, breathing, temperature regulation, nourishment behavior, and ability in stress response [Bruchas et al., 2010; Knoll and Carlezon, 2010]. This OR also modulates the effect of opiates, cocaine and other drugs, through the modulation of the basal level and dopaminergic tone induced by drugs [Knoll and Carlezon, 2010]. Several alternative splicing variants have been named, some of them are disturbed in the 5′UTR of *Oprk1* mRNA and they are differentially regulated at transduction level; others are disrupted in the 3′UTR and they are differentially regulated at stability level or RNA transport [Hu et al., 2002]. In different studies, at least six mRNA variants have been validated and they are generated from the same gene but through the use of two alternative promoters (P1 and P2, TATA-less and rich in GCs) and two alternative polyadenylation sites, besides the inclusion of one non codifying exon (upstream), where the alternative splicing occurs to produce different 5′UTRs [Lu et al., 1997]. The transcription factors which regulate this gene include three positives, the proto-oncogene c-Myc, Sp1 and activating protein (AP2) and one negative, Ikaros (IK) [Wei and Loh, 2011].

Oprk1 is constitutively active and highly expressed in embryonic carcinoma cells in proliferative state, and once the cells complete that differentiation induced by RA, *Oprk1* expression decreases. Because there is no evidence on charges in methylation pattern, it has been suggested that the acquisition of chromatin repressive marks is the main epigenetic regulator of *Oprk1* expression [Chen et al., 1999; Bi et al., 2001]. In proliferative embryonic carcinoma cells that are in differentiation, the P1 promoter of *Oprk1* has a totally accessible chromatin conformation, while, the promoter P2 shows a totally inaccessible chromatin [Lu et al., 1997]. The transcription factors, such as, Sp1 can bind promoter P1 and activate transcription. However, after embryonic carcinoma cell differentiation, the chromatin structure gets ordered and organized, and in

that way Sp1 loses the possibility to bind [Park et al., 2005]. Studies using the embryonic carcinoma cells have revealed a biphasic pattern, which shows that *Oprk1* expression is stated again later in differentiated cell populations. This reactivation is explained through the action of transcription factor NGF, because differentiated cells start to express NGF-binding receptors. The NGF binding transmits signals to activate AP2 transcription factor to bind this latter to P2 promoter, inducing changes in the *Oprk1* promoter epigenetic marks from silenced H3-K9-me2 to activated H3-K4-me2 [Park et al., 2008]. (Table I)

NOCICEPTIN RECEPTOR (*OPRL1*)

NOP, which is encoded by opioid receptor like 1 gene (*Oprl1*), is a G-protein coupled receptor with high homology to opioid receptors *Oprm1*, *Oprd1*, and *Oprk1* [Mollereau et al., 1994; Wick et al., 1995]. It shares many structural traits with other OR genes, especially in terms of primary structure (60% homology), yet its pharmacological profile in not opioid. Anatomic data reveal that the *Oprl1* receptor is widely expressed in the brain, spinal cord, and peripheral nervous system, and is found in areas involved in various processes: pain and sensory perception, memory, stress, motricity and hormonal regulation [Mollereau et al., 1994]. *Oprl1* mRNA is expressed as two alternatively spliced forms, which differ only in their 5′UTRs [Wick et al., 1995]. It consists of five exons with a TATA-box in its 5′ flanking region, and the protein coding region starts in exon 2 and ends in exon 4.

Regarding epigenetic regulation, it is the less studied receptor. A recent study on environmentally regulated gene expression shows, that *Oprl1* gene together with other candidate genes is epigenetically regulated by the methylation of CpG islands [Zhang et al., 2013a]. It reports a hypermethyation in the CpGs of the *Oprl1* gene, and an elevated overall methylation levels in the promoter region too, in a changing environment exposure, leading to changes in gene transcription and an increased risk for other disorders [Zhang et al., 2013a].

Others have worked with different epigenetic modifications, as histone methylation, highlighting that the reduction of mRNA levels of the *Oprl1* gene are supported by the decrease of H3K4me3 and the increase of H3K27me3, it is said, a decreased level of the activating marker and the increased level of the repressive mark [Caputi et al., 2014]. To the date, there is no evidence of research in miRNA regulation of this gene. (Table I)

THE EPIGENETIC REGULATION OF OPIOID PROTEIN PRECURSORS

The endogenous opioid peptides are derived from precursors encoded by POMC, PENK, PDYN, and PNOC [Tseung, 1995]. The importance of the peptide processing enzymes is evident from studies examining the forms of the opioid peptides in different tissues, because each one has different bioactive properties [Tseung, 1995].

PROOPIOMELANOCORTIN GENE (*POMC*)

The pro-opiomelanocortin (*Pomc)* gen, encodes a cDNA spanning 3 exons and 2 introns. The sequence covers four activating transcription factor binding sites (FoxO1, STAT3, Sp1, NF-κB) and one inhibiting transcription factor binding site (nGRE) [Plagemann et al., 2009]. *Pomc* is a precursor polypeptide, which is cleaved in a tissue specific fashion by prohormone convertases to yield a variety of bioactive peptides, including α-melanocortin stimulating hormone (α-MSH), β-lipotropin (β-LPH), β-endorphin and adrenocorticotropin (ACTH), these two latter, are the principal components of the hypothalamic-pituitary-adrenal axis. β-endorphin and met-enkephalin are created from β-LPH and they both are the most powerful opioids. These peptides play diverse roles in pathophysiology, including obesity, depression, skin pigmentation, adrenal development and regulation of the HPA axis. In other tissues, including the hypothalamus, placenta and epithelium, all potential cleavage sites may be used to produce peptides responsible for energy homeostasis, pain, perception, melanocyte stimulation and immune responses. *Pomc*-derived peptides actively regulate drug-related behaviors [O′Malley et al., 2002; Levran et al., 2012].

It has been suggested that there is a variable tissue specific CpG island mehtylation, and that it has important implications for gene expression. Some studies revealed 2 CpG islands within the mouse *Pomc* gene locus [Gardiner-Garden and Frommer, 1994]. The called CpG island 1, flanking the *Pomc* transcription start site, which is highly tissue-restricted, and the CpG island 2, approximately 5 kb downstream, comprising the third exon of the *Pomc* gene, which is weakly active in many tissues [Lavender et al., 1991]. In ACTH-secreting tumors and *Pomc* expressing cell lines, *Pomc* is unmethylated at the pituitary-specific promoter region. In contrast, in non-ACTH-secretion tumors, this region is heavily methylated. In addition, *Pomc* is heavily methylated at the same region in a number of normal ACTH-non-expressing tissues including: pancreas, spleen, lung, testes, and peripheral blood leukocytes [Lavender et al., 1991]. Moreover, studies in rodents have shown that *Pomc* DNA methylation can be altered by environmental conditions. For example, in a neonatal model of obesity, the hypothalamus revealed hypermethylation of CpG dinucleotides in the *Pomc* promoter, and stressor factors elevate *Pomc* mRNA levels in the pituitary [Wu et al., 2014]. The DNA methylation appears to be responsible for controlling *Pomc* gene expression by recruiting MeCP2 to silence the gene. MeCP2 is phosphorylated at serine 438 and generates dissociation from the *Pomc* promoter. As a result of that, the lack of MeCP2 prevent the binding of co-repressor complex such as histone deacetylase 2 (Hdac2) and DNA (cytosine-5)-methyltransferase 1(Dnmt1) at the promoter losing the capacity to maintain of DNA methylation pattern during cell replication [Wu et al., 2014].

On the other hand, other epigenetic mechanism seems to increase H3K9 acetylation levels in late-gestation ewe fetal hypothalami, although it shows no change in the expression of *Pomc* mRNA [Stevens et al., 2010]. Some studies have been carried out on beta-endorphin that reduces its production in POMC neurons, by decreasing levels of activation histone marks H3K4, aceH3K9, and pH3S10 and increasing the levels of the repressive histone mark H3K9 [Bekdash et al., 2013].

In the case of non coding RNA regulation, different studies have demonstrated that miR-375 dramatically inhibits *Pomc* expression both at the gene and protein levels by targeting mitogen activated protein kinase 2 (MAP2K8) and mediating the Corticotropin releasing factor signaling pathway in AtT-20 cells [Zhang et al.,

2013b]. Finally, a recent study has highlighted, that Dicer-derived miRNAs are essential for survival and maintenance of *Pomc* expressing neurons during post-natal and early adulthood, suggesting that the deletion of Dicer controls *Pomc* gene expression [Schneeberger et al., 2012]. (Table II)

PROENKEPHALIN GENE (*PENK*)

PENK is a large precursor, which is processed through the action of proprotein convertase 1 (PC1) and proprotein convertase 2 (PC2) to produce several peptide sequences: 4 met-enkephalin copies and 1 leu-enkephalin, met-enkephalin-Arg^{6}-Phe^{7}, met-enkephalin-Arg^{6}-Gly^{2}-Leu^{8}, sin enkephalin copy, and C- or N- terminally extended variants of these peptides [Goumon et al., 2000]. The gene consists of four exons separated by three introns, and there are also identificated several repetitive DNA sequences within and flanking the gene. PENK-derived peptides act on MOR and DOR to produce rewarding actions of substances of abuse in different brain regions, including the ventral tegmental area and nucleus accumbens (NAc) [Levran et al., 2012]. They are also involved in analgesia, responses to stress and pain and regulation of appetite and sleep [Kieffer and Gaveriaux-Ruff, 2002]. The structural organization of the *Penk* gene exhibits similarities to the organization of the *Pomc* gene suggesting that two opioid peptide precursors may have arisen by duplication from a common ancestral gene.

Data shows that *Penk* expression is specific for cell-type and tissue-compartment and that this expression can be regulated by environmental factors, such as, natural sunlight, salt water bathing, ultraviolet A irradiation and certain pathologies (psoriatic) [Nissen et al., 1999; Slominski et al., 2011]. Previous studies reveled that approximately 80–90% of the CpG dinucleotides occurring in the *Penk* gene are concentrated at the 5′ and 3′ ends, with a nonrandom distribution [McClelland and Ivarie, 1982], and some CpG sites have been shown, by analysis of genomic DNA, to be methylated in a tissue specific fashion suggesting a control of the gene expression [Comb and Goodman, 1990]. DNA methylation may also control the level of *Penk* expression since the methylation of CpG dinucleotides within the promoter inhibits its expression. In addition, that methylation affects a site located within a binding site for the AP-2 transcription factor. Thus, the methylation inhibits *Penk* expression by inhibition of AP-2 binding site [Comb and Goodman, 1990].

The studies of histone methylation have demonstrated the regulation of *Penk* expression by repressive marks, such as the methylation of H3K9me2 in upstream regions of the gene, typically enriched at peri-centromeric heterochromatin and sites of repressed chromatin; and by activating marks such as the methylation of H3K4me3 across the *Penk* promoter [Tomasiewicz et al., 2012]. This suggests that the distinct epigenetic profiles during the different cellular periods may allow the *Penk* to respond differentially to similar environmental cues.

At the miRNA level, it has been suggested an interaction between *Penk* and miR-29c [Slominski et al., 2011], but there is too little information on this topic in current studies. (Table II)

PRODYNORFHIN GENE (*PDYN*)

PDYN is the precursor for the next opioid peptides 3 leu-enkephalin sequences, and other bigger peptides such as alpha- and beta-neoendorphins, dynorphin A and dynorphin B, which act as endogenous ligands for the Oprk1 [Levran et al., 2012]. Dynorphin peptides reduce basal and drug-induced dopamine levels in different areas of the dopaminergic, nigrostriatal, and mesolimbic, meso-cortical systems. Expression of *Pdyn* is increased by cocaine behavior in anxiety tests demonstrating the anxiogenic role of prodynorphin-derived peptides [Wittmann et al., 2009] Pdyn contains four exons: exon 1 and 2 encode the 5′UTR, exon 3 encodes a signal peptide, and exon 4 encodes the dynorphin peptides and has multiple transcription start sites located in exons 1 and 4 and introns 1 and 2 [Nikoshkov et al., 2005; Tejeda et al., 2012]. It has been identified some alternatively spliced Pdyn transcripts, which contribute to dynorphin/ Oprk1 system diversity [Kimura et al., 2006]. Several potential transcription factor binding sites within the *Pdyn* promoter have been reported to play a role in regulating *Pdyn* expression. A polymorphic 68-bp tandem repeat polymorphism (rs35286281) that contains a putative AP-1, a site at −156 in the proximal promoter; and −2745 microsatellite [Babbitt et al., 2010], and a calcium sensitive transcription repressor DREAM (downstream regulatory element antagonist modulator) that binds to the regulatory element (DRE) locate in the 5′ UTR within exon 1 [Yuferov et al., 2011].

Epigenetic factors, mainly DNA methylation, play an important but still unknown role in modulation of *Pdyn* expression. It has been analyzed the DNA methylation patterns of three CpG-rich regions of the *Pdyn*, a CpG island, cluster A in the proximal promoter and cluster B in coding exon 4 [Yuferov et al., 2011]. Those results have suggested that the CpG island is implicated in tissue- or cell-specific regulation of gene expression, while the CpG cluster A may be associated to regulation of basal activity of the *Pdyn* [Yuferov et al., 2011]. In addition, the decrease in association of the methyl DNA

TABLE II. The Epigenetic Mechanisems Involved in the Opioid Protein Precursors Gene Expression

	POMC	PENK	PDYN	PNOC
Silencing				
Regulatory elements	nGRE		DREAM	
Metilation mechanisems	Metilation ↑MeCp2	Metilation ↑MeCp2	Metilation ↑MeCp2	Metilation
Histon modifications		H3K9 me2	H3K27me3	H3K27me3
MiroRNA	miR-375	miR-29c		
Activation				
Regulatory elements	FoxO1, STAT3 Sp1, NF-kB	AP2, Sp1, CREB	AP1	CREB, NF-kB
Metilation mechanisems	Hipometilation		H3K4me3, H3K9ac	H3K9ac
Histon modifications	H3K9ac	H3K4 me3		
MiroRNA				

binding protein MeCP2; to the promoter of *Pdyn* suggests the possibility that alterations in DNA methylation may be concomitant with altered *Pdyn* transcription [Reed et al., 2012].

To study the chromatin modifications of the *Pdyn*, human neuroblastoma SH-SY5Y cells model is used by several groups, which is endogenously expressing the opioid system genes. It is shown that there is a relationship between chromatin modifications and Pdyn expression, thus epigenetic changes may precede gene transcription [D′Addario et al., 2011]. It has been demonstrated that there is an increase in H3K27me3 and a decrease in H3K4me3 and H3K9Ac in promoter when the gene expression is repressed [D′Addario et al., 2011]. In contrast, there is an increase in H3K4me3 and H3K9Ac together with a return on unmethylated H3K27me3 when the *Pdyn* expression back to normal levels after the original repression [D′Addario et al., 2013]. This hypothesis is also supported in the same study by temporal changes in RNA polymerase II (RNAPII) recruitment and activation consistent with epigenetic changes [D′Addario et al., 2013].

On the other hand, a gene encoding a long non-coding RNA, AK090681, is transcribed from the opposite strand of *Pdyn*, and both are separate but overlapping transcription units. Some studies show that this gene appears to be actively transcribed in human embryonic stem cells while *Pdyn* does not. Interestingly, the promoter of AK090681 contains a CpG island which methylation status may correlate with that of H3K27, suggesting also an epigenetic regulation[Tejeda et al., 2012]. (Table II)

PRONOCICEPTIN GENE (*PNOC*)

PNOC is a precursor for 3 peptides, 1 nociceptin/orphanin FQ copy, 1 nocistatin copy and one prepronociceptin copy and it is the endogenous agonist of the NOP, another opioid receptor type in terms of primary structure, but without an in vitro opioid pharmacological profile [Mollereau et al., 1996]. The overall structure and organization of the *Pnoc* codifying gen is highly homolog of those codifying *Pomc*, *Pdyn*, and *Penk*, it suggest a common evolutionary ancestor. Consists in four exons, exon 1 constitutes the majority of the 5′UTR, exon two contains the translational start site and the signal peptide, exon 3 contains the coding region for the multiple bioactive peptides and exon 4 encodes the 3′UTR and poliadenylation signal. The promoter region upstream of exon 1 contains several regulatory sites including cAMP response elements and glucocorticoid receptor binding sites [Xie et al., 1999; Arjomand and Evans, 2001], while other study shows the presence of NFκB-binding site too [Wiggins et al., 2010]. Some reports show a pattern of splicing variants: in the 3′-end of exon 3, which result in elongated and conserved C-terminal of the precursor protein, and other 2 spliced variants in the exon 2 [Arjomand and Evans, 2001]. This gene is a modulator of pain sensation and it is essentially expressed in nerve tissues, brain and spinal cord, where its distribution correlates with the localization of *Orl1* transcripts [Nothacker et al., 1996].

There are few results about epigenetic regulation in Pnoc gene. A very recent study has demonstrated a consistent increase in DNA methylation in the Pnoc promoter, suggesting that epigenetic regulation might affect its expression level upon environmental changes such as diet manipulation [Di Bonaventura et al., 2013].

Selective epigenetic changes in histone modifications, similar to *Pdyn*, are reported for the *Pnoc* gene. It seems to be an inverse relationship between the repressive mark H3K27me3 and the activating mark H3K9Ac, in the *Pnoc* promoter [D′Addario et al., 2013]. This fact has suggested that there is a long-term maintenance of epigenetic chromatin state, which determines accessibility for transcription factors, and that might be still present even when the histone modifications are decreasing [D′Addario et al., 2013]. It confirms that, at least for the *Pnoc* gene, there is a transient genomic memory triggered by histone modifications occurring immediately at exposure.

There is no evidence of epigenetic control at miRNA level in this gene. Therefore this is a field to focus future studies. (Table II)

CONCLUSION

Different physiological functions have been reported for the opioid system. Discovering and validating the complete biological roles of these three genes will require extensive studies of each in multiple physiological and pharmacological contexts and, in particular, studies that define with more precision their transcriptional and posttranscriptional regulation. In this context the opioid receptors show an epigenetic regulation. DNA methylation is confirmed in the case of *Oprm1*, *Oprd1*, *and Oprl1*, while the chromatin remodeling is reported in four of the receptors. Although four promoters have similarity between them, they use different epigenetic regulation forms and they exhibit different pattern of expression during the cell differentiation. Moreover, the opioid peptide precursors are also epigenetically regulated. DNA methylation is confirmed in four of them, being important for gene expression and tissue specificity. Histone methylation is also present in four precursors, which suggest the possible genomic memory adquisition at least in Pnoc.

In spite of thet, these genes interact with environment factors that are increasingly recognized to be complicated by intertwined biological processes, including temporal and spatial controls that must underlie the activity of any drug receptor in the whole organism. Consequently, future studies of opioid system genes must go beyond the action of transcription factors to include factors affecting their epigenetic states, physiological contexts, and posttranscriptional control to develop individualized prompt prevention and treatment strategies

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