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Review Article

miRNAs Mediated Regulation of Neuronal Proliferation and Differentiation during Zebrafish Development

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Abstract

Morphine is one of the first-line therapies used in pain treatment, although its side effects and abuse as recreational drug make its use controversial. This opioid is involved in several biological processes and has remarkable effects in Central Nervous System (CNS) development. In this sense, miRNAs have been postulated as possible targets to control the molecular pathways triggered by exposure to morphine. Previous research has shown that in zebrafish embryos morphine alters the dopaminergic pathway-related genes *th, dat* and *pitx3*, as occurs with other drugs of abuse such as cocaine. These changes observed lead to a failure in the correct differentiation of the dopaminergic neurons. The modification of those genes is similar and it is related to the changes induced in the expression of other genes such as *wnt1*. Besides, morphine is also able to alter proliferation by changing the pattern of the proliferative cells around the periventricular area. *mir133b, mir212* and *mir132* are involved in these changes induced by morphine during zebrafish CNS development. On the one hand, *mir133b* is strongly related to the dopaminergic system changing the expression of *th* and *dat* through *pitx*3 regulation after morphine exposure. On the other hand, *mir212* and *mir132* are altered by morphine administration but they are also involved in *oprm1* and *mecp2* expression by targeting their mRNA sequences. This negative regulation of *Mecp2* induces the over expression of Bdnf. This review highlights the importance of assessing morphine effects on development and the key role of miRNAs in this process.

ABBREVIATIONS

CNS: Central Nervous System; hpf: hours post fertilization; miRNA: microRNA; Mo: morphants; NMDA: N-methyl-D-aspartate; SCI: Spinal Cord Injury; VTA: Ventral Tegmental Area

INTRODUCTION

The opioid morphine, an alkaloid and the main active compound of *opium*, can be obtained from the seed of the poppy plant, *Papaver Somniferum.* This drug has been used for centuries as a medical and recreational agent [1] although its use is known to cause undesirable side effects on peripheral tissues and on the central nervous system (CNS). Moreover, morphine has been related to several alterations on the normal development of the CNS. These findings have special relevance in relation to pregnant women, in which the administration of this drug could induce the modification in the pattern of expression of several genes, which at the same point could cause alterations in the brain structure of the fetus or possibly later neurobehavioral problems [2]. In addition, morphine administration has been related to alterations in cell proliferation, which is of great relevance in oncology patients, in which morphine could induce division of tumor cells [3,4].

Morphine, as other opioid drugs, mainly exert it saction by the activation of the μ opioid receptor (*Oprm1*) [5-8], inducing a molecular cascade that involve several processes, such as cell proliferation, apoptosis, alteration in gene expression, epigenetic regulation and neuronal differentiation [9,10].

The zebrafish (*Danio rerio*) is used as an experimental model, to study genetics and development and also to study disease-related pathways, given its easy *in vivo* manipulation [11,12]. In contrast to mammalian embryos, which develop in the uterus

and are influenced by the maternal biochemical processes, zebrafish embryos develop externally and are protected by a transparent chorion, avoiding the maternal effect on these embryos and allowing observation of possible morphological alterations. This is essential when dealing with drug exposure, as the effects observed in mammalian embryos might be due to the susceptibility of the mother and not the embryo *per se*. In this sense, the study of the effects of the drugs using zebrafish is rapidly growing [13,14]. In relation to opioids, the endogenous opioid system in the zebrafish has been characterized including a mu opioid receptor (*Oprm1*), two delta duplicates *(Oprd1* and *Oprd2*), a kappa opioid receptor (Oprk) and an opioid receptor like (*Oprdl*) gene [15-18]. Hence, the extensive characterization of opioid receptors in zebrafish and the characteristics of this model allow us to extrapolate key components of the opioid system in the zebrafish to other biological organisms. Besides, zebrafish is an excellent model to study early differentiation, since the transparent embryos and the fast development allow the observation of proliferative and apoptotic cells. Moreover, this model is extremely efficient for the study of the response to chemicals during the early developmental stages [19,20].

Morphine effects on these processes are mainly exerted by the action of CREB, a transcription factor closely related to mitotic regulation and the expression of genes involved in differentiation, such as *nurr1*. Morphine administration and withdrawal have been related to the alteration of other pathways, such as modifications in serotonin levels after the exposure to this drug [21].

miRNAs are a group of 19-25 nucleotides non coding RNAs, which post-transcriptionally regulate gene expression [22,23]. miRNAs are evolutionary well conserved and affect 60% of mammalian genes, becoming a central topic of research [24-27]. In mammals, the binding between miRNAs and their targets is inducing the blockage of mRNA translation [28]. In addition, the perfect complementarity between both sequences produces mRNA degradation [29]. Besides this type of binding, a seed region, which represents 2-8 nucleotides from the 5' end of the miRNA, needs perfect complementarity with the target to, at least, block translation. Several miRNAs have seed regions which bind to several mRNAs [30]. This fact means that a single miRNA can control the expression of hundreds of genes.

Current studies concerning miRNAs involve several biological functions such as cellular differentiation, development, metabolism pathways and disease biogenesis [31-33]. It has been described in zebrafish that miRNAs exert a key regulation through several developmental stages [34-36].

Here, we summarize some of the alterations that morphine, as many other addictive drugs, causes in the development and differentiation of the zebrafish CNS. These changes involve several pathways, but many of these effects are mediated by the alteration of the levels of several miRNAs, which have important transcription factors as targets.

Morphine effects in zebrafish development and differentiation

Morphine alters dopaminergic differentiation by modifying the levels of expression of several transcription factors: Drugs of abuse have been related to alterations in the levels of biogenic amines [37]. In particular, morphine increases dopaminergic neurotransmission in several brain regions, such as the ventral tegmental area (VTA) and in the *nucleus accumbens*. These alterations in the dopaminergic pathway are directly linked to the addictive properties of morphine [38], and similar systems have been described in zebrafish [39]. A specific subset of dopaminergic neurons (A11), the far projecting neurons in this teleost, is located in the ventral diencephalic and posterior tuberculum and expresses specifically the transcription factor Otp [40]. The alterations observed in this area after morphine administrations are similar to those observed with other social used drugs as cocaine. Cocaine has been described to alter the expression of several genes involved in dopaminergic differentiation, thus modifying the normal development of this system [41]. This process is mediated by the alteration of the expression of several transcription factors involved in early development, such as Ndr2, Otpa/Otpb, and lmx1b1/lmx1b2. Ndr2 is known to be a positive regulator of Otp duplicates, and its absence induces a complete lack of dopaminergic neurons from the pretectum to the posterior tuberculum [42]. Moreover, Opta and Optb are required for the correct development of dopaminergic neurons [43].

The exposure of zebrafish embryos to cocaine [41] from 5 to 24 hours post fertilization (hpf) increased the expression of both *lmx1b1* and *nurr1*, upregulating the expression of tyrosine hydroxylase (*th*) which results in an increased dopaminergic differentiation at this stage. However, if the embryos were exposed to morphine until 48 hpf, a downregulation of *th* was observed, probably due to a decrease of *ndr2* and *optb*. Cocaine exposure also modified the expression of the dopamine receptors of the zebrafish [44]. This alteration of dopaminergic differentiation was described to be mediated by the transcription factor *pitx3*. This factor is a positive regulator of *th*, the dopamine transporter (*dat*) and the dopamine receptors (mainly *drd2*) [45].

This alteration in dopaminergic differentiation observed after cocaine administration was also present after morphine exposure [46]. Morphine, via activation of mu opioid receptor (*Oprm1*) regulates several signaling pathways. In particular, the mitogen-activated protein kinases (MAPK) are directly involved in dopaminergic differentiation, as observed in 24 hpf zebrafish embryos. These results showed that after morphine administration, the expression levels of *th* were downregulated, and the levels of *dat* and *pitx3* upregulated. These results were reversed after the inhibition of the pathway, showing that the activation of MAPK was necessary to the correct differentiation of these cells. These observations correlate with previous findings that relate MAPK and *Oprm1* activity with TH activation [47].

Morphine alters other differentiation pathways through several genes: In addition to altering dopaminergic differentiation in the early stages of the development, morphine is known to modify the levels of expression of other transcription factors, such as *wnt1* [48]. This factor plays an important role in the development of the central nervous system, regulating neurogenesis and neuronal differentiation. Besides, it is important in adult neuronal plasticity [49]. Moreover, *Wnt1* has been proven to be involved in the differentiation of the dopaminergic neurons

[50], thus it may be related with the other alterations previously described [48]. Morphine administration downregulates *wnt1* expression in the early stages of development (8 hpf) but induces an upregulation at the later stages (16, 24, 48 and 72 hpf). However, those changes are not induced by other important mitogenic factors such as Shh [48].

In addition to these changes, a microarray study [51] showed a differential regulation of several genes in other pathways in zebrafish embryos treated with morphine up to 24 hpf. *Copb2*, a gene involved in dopaminergic receptor D1 transport [52], was shown to be dysregulated. Moreover, several other genes as *dao.1*, involved in glutamatergic activity and addiction [53] was also altered. Another gene down-regulated by exposure to morphine and identified as a gene related to *oprm1* expression is *wls*, a putative orphan G-protein coupled receptor conserved from worms to human [54]. Wls is closely related to development, as it can inhibit the secretion of Wnt, indicating that this factor could be critical in neuronal development and morphogenesis [55].

Morphine alters cell proliferation and apoptosis: BNIP3, a protein related to cell death/survival [56] has been described to be altered in zebrafish embryos exposed to morphine [51]. The alteration of the normal expression of this gene indicates that morphine in zebrafish embryos may produce an unfavorable pro-apoptotic state of the neuronal cells. In zebrafish, morphine upregulates Bdnf and TrkB at 48 hpf [10]. Also, the proliferation pattern of the cells around the periventricular area is altered in morphine exposed embryos (Figure 1). This mechanism shows that morphine is closely related to proliferation, as described by many other authors [57,58].

In addition to the changes observed after morphine administration, *Oprm1* has an important activity in cell proliferation [59]. The knock-down of the receptor altered the normal proliferation pattern around the periventricular area, in a similar fashion than that observed after morphine administration [10]. These results indicate that the endogenous opioid system is related to normal proliferation. In addition, morphine administration enhances cell proliferation at several cell populations at 24 and 48 hpf, and act as a neuro-protector against glutamatergic excitotoxicity [60]. However, other authors have described a differential regulation of cell proliferation depending on the dose used [61]. Low doses of morphine promote cell proliferation in undifferentiated SH-SY5Y cells, while higher doses inhibited proliferation [62].

miRNAS roles in zebrafish development after morphine treatment

***mir133b* role during development:** The characterization of *mir133* was first done in mice [63], after which its homolog's were discovered in several other species. Three different miR-133 sequences are known: *mir133a-1*, *mir133a-2*, and *mir133b*.

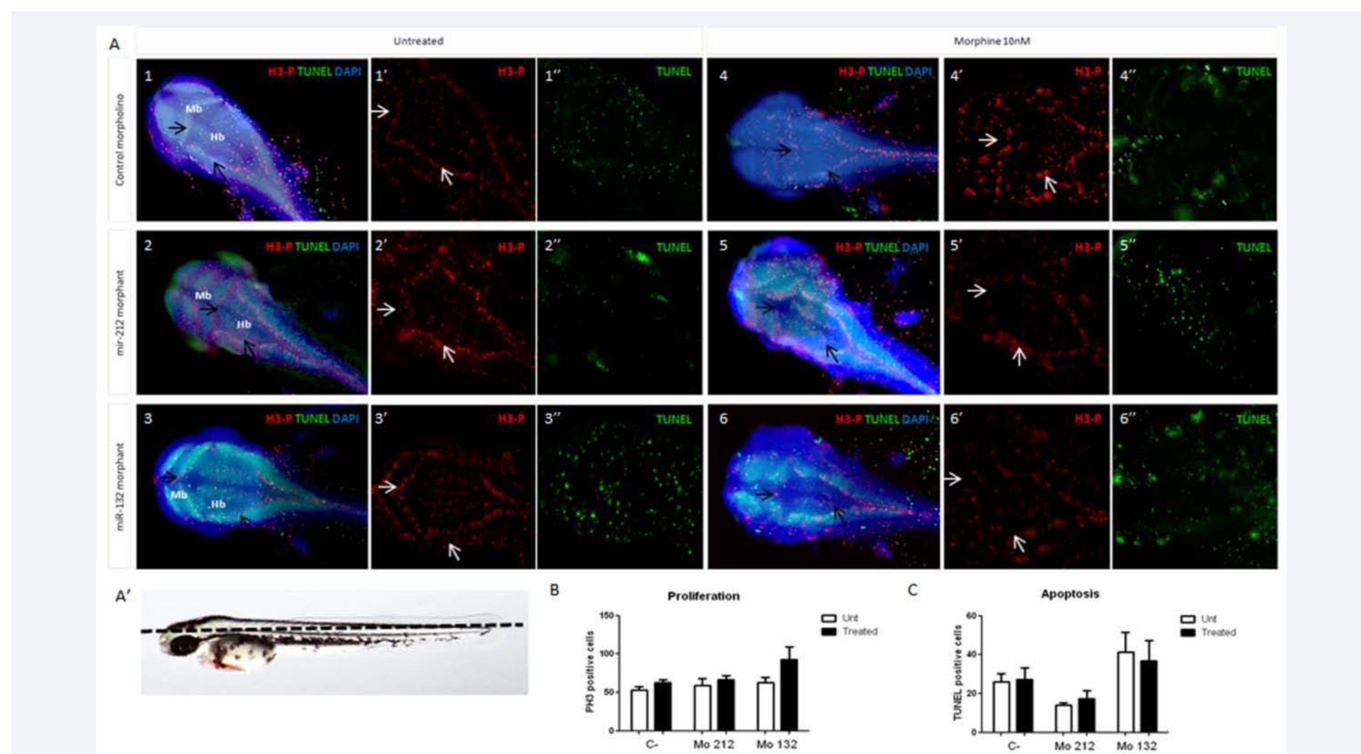

Figure 1 Morphine effects on cell division and apoptosis after morphine exposure and knocking down *mir212* and *mir132*. The mitotic marker phospho-histone 3 (H3-P) (red) and TUNEL technique (green) were used to determine whether morphine treatment (10 nM) alters proliferation and apoptosis in the CNS. Changes in the pattern of proliferation (Arrows) were observed between control (A: 1-3) and treated embryos (A: 4-6) at 48 hpf and *mir212* and *mir132* morphants (Enlarged in 1'-6'). Mitotic marker and TUNEL positive cells (Enlarged in 1''-6'') were quantified around the peri ventricular area and the midbrain-hindbrain boundary (B-C) (n=3). Cells nuclei were stained with DAPI (blue). Embryos are oriented anterior toward the left and posterior toward to the right (A'). Mb: Midbrain. Hb: Hindbrain. Mo: Morphant. Unt: Untreated embryos. Treated: 10 nM morphine treated embryos. Taken from Jimenez-Gonzalez et al., (BBA General Subjects, 2016).

Table 1: *mir133b* involvement in several diseases and biological processes.

Disease	Mechanism	Role	Model	Reference
Parkinson disease	Axonal degeneration	Regulating RhoA/Decreasing α-synuclein mRNA levels	Rat dopaminergic neuron primary culture/ PC12 cells	[62]
Renal carcinoma	Apoptosis/Cell viability	Targeting JAK2/STAT3	Human renal carcinoma cell lines	[63]
Gastric cancer	Warburg effect	Targeting PTBP1	Human cell lines	[64]
Gliobastoma	Migration	Targeting matrix metalloproteinase 14	Human glioma cell lines	[65]
Diabetic nephropathy	---	Biomarker	Human	[66]
Glioma	Proliferation	Targeting Sirt1	Human Glioma cells	[67]
Acute Myocardial Infarction	---	Biomarker	Human	[68]
HIV-associated dementia	Apoptosis	Targeting Hsp70	Rats	[69]
Tumor proliferation	Mitosis	Targeting NUP124	Cell lines	[70]

In particular, *mir133b* plays an important role in several diseases and biological processes, summarized in table (1). This miRNA has an effect on zebrafish spinal cord regeneration [73] since one of its multiple targets is RhoA [74]. This protein increases after spinal cord injury (SCI) [64] therefore the inhibition of RhoA exerted by *mir133b* enhances the re-growth of the corticospinal tract after SCI. In cancer, *mir133b* can participate in promoting or suppressing tumors. When overexpressed, *mir133b* can behave as an oncogene, inducing tumor cell proliferation [69], or when under-expressed, it functions as a tumor suppressor, negatively regulating oncogenes [65].

Additionally, *mir133b* regulates the differentiation, maturation, and function of dopaminergic neurons by downregulating the homeobox gene *pitx3* [75] which is related to CNS development. In particular, at 24 h post fertilization, the dopaminergic system begins its differentiation and the first TH-positive neurons are detected [76].

Morphine modulates the expression of *mir133b* and dopaminergic markers through *Oprm1* during zebrafish CNS development: The analysis of a microarray carried out in zebrafish embryos after morphine administration revealed a decrease in the expression of several miRNAs at three developmental stages: 16,24,and 48hpf. *mir133b*was chosen due to its reported effect on dopaminergic neurons, an essential component in drug addiction processes and CNS development [75]. The qPCR validation of *mir133b* showed its levels were decreased in 24hpf embryos exposed to10nMand1 nM morphine. The opioid antagonist naloxone did not significantly change the expression of this miRNA, but it blocked the effect of morphine, proving that the changes in *mir133b* were mediated by morphine binding to the opioid receptors [46]. The transcription factor Pitx3 Is a well-known *mir133b* target. Pitx3 regulates the transcription of the dopaminergic markers Th and Dat. Since miRNAs normally regulate the stability or the translation of their targets, morphine exposure should increase the expression levels of these transcripts by reducing *mir133b*.

qPCR studies proved that the RNA levels of *pitx3, th* and *dat* increased after morphine exposure in 24hpf zebrafish embryos while the treatment with naloxone effectively abolished the morphine induced changes in the expression levels of *pitx3*, *th*, and *dat*, suggesting that morphine regulates the level of the dopaminergic genes via the control of *mir133b*.

In order to establish the role of *Oprm1* in regulating *mir133b*, *oprm1* was knocked-down by morpho lino oligonucleotide injection. The amount of *mir133b* increases with in embryos injected with *oprm1* morpholino (morphants). Furthermore, 1or10 nM morphine exposure did not alter the *mir133b* level in *oprm1* morphants while the same concentrations of morphine treatment resulted in a decrease of *mir133b* levels in embryos injected with control morpholino. The increased expression in *mir133b* detected in the *oprm1* knock-down embryos also led to a decrease of the subsequent *mir133b* targets, i.e., *pitx3*, *th*, and *dat*. These results clearly indicate that *Oprm1* is the mediator for the morphine-induced regulation of *mir133b* and its targets [46].

***mir212/132* cluster role in the molecular cascades triggered after morphine administration:** *mir212* and *mir132*, both codified in the same locus, located on chromosome 10 in rat and 11 of mouse [78,79]. In humans they also share a primary transcript encoded by a locus on chromosome 17 [80]. In addition, *mir212* and *mir132* share the seed region although they have specific targets and are regulated by CREB and REST transcription factors, so most of their functions are strongly related to the neural compartment. In fibroblast culture it has been proved that *mir132* increases in the presence of negative form of REST [81]. Similarly, CREB was firstly identified as regulator of this locus in cortical neurons of rats stimulated with neurotrophins [79].

Besides, *mir132* induces neurite out growth and modulates the dendritic morphology of immature neurons in the hippocampus and cortex after the inhibition of one of its targets, P250GAP. This target (a brain enriched GTPase-activating protein) is important in neuronal development as it controls N-methyl-D-aspartate (NMDA) receptor signaling [82]. It also modifies the dendritic plasticity by controlling *MeCP2* expression [83] and this protein is fundamental for the correct neural maturation [84]. These results show the relevance of this locus in neural morphogenesis.

To determine the relevance of *mir212* and *mir132* during zebrafish development, their temporal expression was studied at 5, 8, 16, 24 and 48hpf. Although both are highly expressed, *mir212*expression decreases during development while *mir132* increases from 5 to16 hpf. Besides, the number of copies for *mir132* is greatly increased at 48hpf when compared to *mir212*,

suggesting the importance of this miRNA at this particular stage [7]. The levels of expression of *mir212* in zebrafish embryos were measured by qPCR after the administration of two different doses of morphine (10nM and 10μM). These morphine doses have been also antagonized with naloxone, obtaining a clear reversal of the opioid effects [60]. At 24 hpf, an increase in the levels of the miRNA was observed for both concentrations of morphine, although it was higher at10μM. In contrast, *mir212* was down-regulated at 48 hpf. Additionally, the implication of mu opioid receptor in *mir212* expression was analyzed. After knocking-down the mu opioid receptor, the levels of *mir212* were back to normal after morphine administration at 24 hpf. However, *oprm1* morpholino did not revert the effects of morphine at 48 hpf. Also, the levels of *mir212* were not modified in the control conditions in *oprm1*morphants pointing to the relevance of *oprm1* for morphine effects on *mir212* expression but not in its physiological levels. As *mir212* expression is affected by morphine administration, it was analyzed if this miRNA was regulating mu opioid receptor. After morphine treatment, mu opioid receptor was strongly up-regulated at 24 hpf. In contrast, morphine administration induced a decrease in the levels of *oprm1* at 48 hpf. However, the knock-down of *mir212* induced an increase in the expression of the receptor, much higher than the observed when *mir212* was present at both 24 and 48 hpf [7]. 10nM morphine exposure increased the expression of *mir132* at 48 hpf. The incubation with opioid antagonist, naloxone, induced the opposite effect exerted by morphine exposure, whereas both, morphine and naloxone treatment, did not change the levels of 132 [10]. These results show that morphine is changing both *mir212* and *mir132* by the specific activation of *Oprm1*.

***miR-212/132* cluster regulates *oprm1* mRNA expression binding to its 3'UTR:** In order to determine whether *mir212/132* cluster was effectively regulating *oprm1* by targeting its mRNA, a bioinformatic analysis of putative binding sites of μ opioid receptor was performed [85]. A possible binding site form iR-212/132 was observed in the 3'UTR of *oprm1*, as well as an additional site in the second exon of the mRNA. By means of aluciferase assay it was confirmed that the binding site in *oprm1* 3'UTR was actively repressing μ opioid receptor. miRNA mimics (small, chemically modified double-stranded RNAs that mimic endogenous miRNAs by the up-regulation of miRNA activity) were co-transfected with the plasmids inducing a significant decrease in the lumine scence on the non-mutated group. Moreover, the increase in the concentration of miRNA mimics reduced the luciferase activity, but only when co-transfected with the wild type plasmid. These results prove that *mir212* and *mir132* are binding to the 3'UTR region of *oprm1* mRNA, and physiologically repressing *oprm1*expression [7].

It has been described that *mir212* levels are modified after cocaine administration in the hippocampal region of adult rat brains [86]. These results suggest that the addictive properties of cocaine and morphine, and probably other drugs, could be controlled by this miRNA. In addition, cocaine has also been related to the alteration of the levels of expression of *mir-let7d* in zebrafish embryos [87] and with the regulation of *oprm1* mRNA expression in mice [88].

***mir212* expression is regulated by MAPK, calmoduline and PKA:** To analyze in detail the signaling cascade triggered by the activation of *Oprm1*, the levels of both *mir212* and *oprm1* were studied after the co-administration of morphine and inhibitors of MEK1/2 and calmoduline or an activator of PKA pathway [7]. *mir212* quantification revealed the relevance of MEK1/2 on its expression after morphine treatment at 24 hpf. CaM/CaMKII and PKA also showed an effect on *mir212* expression at 24 hpf but not 48 hpf. *oprm1* did not change at 24 hpf while its expression decreased in all the experimental groups at 48 hpf. These results point to the great relevance of the developmental stage analyzed and the importance of MEK1/2 and the balanced effect between CaMKII and PKA on the expression of *mir212* at the earlier stages.

Bdnf and TrkB expression analysis after morphine exposure and in miRNAs morphant embryos: The modification induced by morphine in the localization of mitoticcells at 48 hpf points to a possible role of neurotrophins in morphine effects (Figure 1) [10]. It has also been observed that miR-132 and miR-212 are regulating the pattern of expression of proliferating cells around the periventricular area in 48 hpf zebrafish embryos. As miR-212/132 cluster has been previously related to Bdnf pathway [80], Bdnf and Trk Bexpressions were studied in *mir212* and *mir132* morphants. Inaddition, *oprm1* morphants were also analyzed to assess if *Oprm1* is one of the possible effectors in the changes induced by morphine in the expression of Bdnf. In all groups, a significant decrease of Bdnf expression was found. When TrkB was analyzed, the levels in control embryos were higher after knocking down *mir212*, where as morphine treatment induced a decrease in the levels of this protein in those morphants. No significant changes could be observed in the expression levels of TrkBin *mir132* or *oprm1*morphants.

Bdnf expression is inhibited by *MeCP2*, which was firstly identified as an epigenetic factor which binds to methylated DNA, avoiding its transcription [86] and bioinformatic predictions showed a target region for both miRNAs in the third exon of the *mecp2* sequence. In this sense, a luciferase assay confirmed the regulation of *mir212* and *mir132* on *mecp2* gene expression.

DISCUSSION AND CONCLUSION

The use of morphine is known to cause undesirable effects, which includes changes in cell proliferation (modification in the number of dividing and apoptotic cells) and in the differentiation of several neuronal populations. In the last years, zebrafish has been proven to be an excellent model to study these processes triggered after morphine administration.

Morphine, as well as other drugs of abuse, is known to induce alterations in the dopaminergic and serotoninergic positive cells. These changes are mediated by several transcription factors such as CREB, *nurr1* or *pitx3*, which are also involved in the responses after cocaine intake. These modifications in the normal development of those specific neuronal groups observed in zebrafish embryos are likely related to the appearance of the addictive symptoms.

Moreover, miRNA alterations observed after drug exposure also interfere with the normal physiological development, through the modification of the levels of expression of their targets. *mir133b* has been related to alterations in zebrafish

development through the transcription factors *pitx3* and *wnt1*. Besides, *mir212* and *mi132* are involved on the expression of neurotrophins such as Bdnf and TrkB through *Mecp2* expression, and can also modify the number of proliferative cells around the periventricular area of the zebrafish hindbrain. Hence *mir133b, mir212* and *mir132*are novel regulators of morphine effects during two relevant stages of zebrafish development (24 and 48 hpf). These findings have also established a relationship between these miRNAs and several systems with key roles for CNS formation such as opioid, dopaminergic, neurotrophins and several other regulatory pathways.

Here we have discussed the most relevant contributions highlighting the importance of miRNAs in cell proliferation and differentiation using zebrafish as a research model. These processes are involved in morphine response and may be related to tolerance and the appearance of addiction. Thus, the better understanding of these mechanisms could lead to the design of new drugs lacking the drawbacks that the chronic use of morphine induces.

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