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TITLE: Morphine regulates dopaminergic neuron differentiation via miR-133b

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RUNNING TITLE: Morphine regulation of dopaminergic differentiation

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ABBREVIATIONS: ERK, extracellular signal-regulated kinase; zfMOR: zebrafish mu opioid receptor; miR, microRNA; PCR, polymerase chain reaction; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; UTR, untranslated region; Pitx3, paired-like homeodomain transcription factor 3; CTOP, Cys^2-Tyr^3-Orn^5-Pen^7-amide; SB203580, $C_{21}H_{16}FN_3OS$; JNKII, Jun N-terminal Kinase II; PD98059, $C_{16}H_{13}NO_3$;

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ABSTRACT

Morphine is one of the most used analgesics to treat chronic pain, although its long-term administration produces tolerance and dependence through neuronal plasticity. The ability of morphine to regulate neuron differentiation *in vivo* has been reported. However, the detailed mechanisms have not yet been elucidated due to the inability to separate maternal influences from the embryonic events. Using zebrafish embryos as the model, we demonstrate that morphine decreases miR-133b expression, hence increasing the expression of its target, Pitx3, a transcription factor that activates tyrosine hydroxylase (TH) and dopamine transporter (DAT). Using a specific morpholino to knock down the zfMOR in the embryos and selective MAPKinase inhibitors, we demonstrate that the morphine-induced miR-133b decrease in zebrafish embryos is mediated by zfMOR activation of ERK1/2. A parallel morphine-induced downregulation of miR-133b was observed in the immature but not in mature rat hipoccampal neurons. Our results point for the first time that zebrafish embryos express a functional mu opioid receptor, and that zebrafish serves as an excellent model to investigate the roles of miR in neuronal development affected by chronic morphine exposure.

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INTRODUCTION

Opioids are the most potent compounds known today to control pain, and are also amongst the most used drugs of abuse (Corbett et al., 2006). They bind to the three classical opioid receptors, mu (MOR), delta (DOR) and kappa (KOR). Although great efforts have been made on the study of the different mechanisms that are activated by the opioid system, using mammalian models, many issues regarding opioid regulation remain unknown. The zebrafish (*Danio rerio*) has been used as an experimental model, not only to study genetics and development, but also to study disease-related pathways, given its easy *in vivo* manipulation. In this sense, the zebrafish can be an important tool to analyze *in vivo* the molecular mechanisms related to the activity and function of the opioid system that cannot be fully established in other models. For instance, in contrast to mammalian embryos, which develop in the uterus and are influenced by the maternal biochemical processes, zebrafish embryos develop externally, avoiding the maternal effect on these embryos. 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*. The study of the morphine direct effects in the embryos will provide a better understanding on the molecular mechanisms that underlie the physical and neurobehavioural defects shown in fetuses and offsprings after maternal morphine consumption (Nasiraei-Moghadam et al., 2009). Also, the endogenous opioid system has been characterized in the zebrafish, which has a mu opioid receptor (zfMOR), two delta opioid receptor duplicates (zfDOR1 and zfDOR2), a kappa opioid receptor (zfKOR) and an opioid receptor like (zfORL) (Alvarez et al., 2006; Barrallo et al., 2000; Pinal-Seoane et al., 2006; Rodriguez et al., 2000). The

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opioid-induced drug addiction pathway has been suggested to involve the midbrain dopaminergic neurons located within ventral tegmental area and the nucleus accumbens (NAc). The alteration of dopamine levels in this region produces neuronal sensitization or desensitization, depending on the drug used. It has also been established that morphine increases dopamine level through the MOR in the NAc, which may mediate the reinforcing effects of morphine (Gianoulakis, 2009). In relation to these observations, the endogenous opioid peptides, such as enkephalins or dynorphin, are upregulated in the NAc after exposure to morphine and modulate dopamine release in the midbrain (Gieryk et al.). Therefore, the studies on the probable opioid regulation of dopaminergic activities in zebrafish could provide insights on the mammalian embryonic development during chronic exposure to the drug.

Micro RNAs (miRNAs), a class of ~22 nucleotide-long RNA molecules, are known to bind to their mRNA targets to inhibit the transcripts translation and/or destabilize them (Valencia-Sanchez et al., 2006). They have been shown to regulate the expression of many genes, including those in the central nervous system (CNS). For example, miR-134 regulates dendritic spine morphology by controlling actin filament dynamics (Schratt et al., 2006) while miR-190 regulates NeuroD level, a transcription factor that is known to regulate the differentiation and maturation of neurons (Zheng et al., 2010). Another class of miRNA, miR-133b, regulates the differentiation, maturation and function of dopaminergic neurons by down-regulating the transcription of its target, the homeobox gene *pitx3* (Hebert and De Strooper, 2009). *Pitx3* activates the transcription of genes

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directly involved in the differentiation of dopaminergic neurons, genes such as the tyrosine hydroxylase (*th)* and the dopamine transporter (*dat*) (Kim et al., 2007).

In the current study, we analyze the effect of morphine on the miR-133b regulatory pathway using zebrafish embryos as a model, which have been widely used to study the role of miR on development (Schier and Giraldez, 2006). At 24 hr post fertilization (hpf), the dopaminergic system begins its differentiation and the first TH positive neurons begin to be detected at this particular developmental stage (Filippi et al., 2007). Our previous studies also indicated at 24 hfp, there is a robust expression of zfMOR, the putative target of morphine (de Velasco et al., 2009). Therefore, the use of 24 hpf zebrafish embryos not only will provide information on the implication of the opioid system in the maturation and differentiation of dopaminergic neurons compared to any other stages of development, but also, will demonstrate that the mu opioid receptor is functional in the zebrafish and has a specific role in the development of the CNS and a possible pathway that leads to addiction.

MATERIAL AND METHODS

Zebrafish

Zebrafish from the AB strain were bred and raised in the Fish Facilities of the University of Salamanca and the University of Minnesota following standard protocols (Westerfield, 1995). Embryos were separated into two experimental groups for the miRNA microarray: control (untreated) group and embryos exposed to 10 nM morphine. For the analysis of the effects of morphine on the expression of miRNA-133b, *Pitx3*, *TH* and *DAT*,

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zebrafish embryos were divided into four experimental groups: control, exposed to 10 nM morphine, exposed to 1 nM morphine and exposed to 1 µM naloxone.

miRNA microarray

Custom miRNA microarray experiments and data analyses were performed as previously described (Kalscheuer et al., 2008). Microarray data have been deposited into the National Center for Biotechnology Information's Gene Expression Omnibus database under the series GSE18847. Zebrafish embryo-samples were prepared in triplicate. The data were normalized against internal control in each chip. The expression of miRNAs in morphine-exposed embryos was compared with those in control embryos. The miRNAs with significant expressional change (> 125 or < 80%; $p < 0.225$ by Student *t* test) were identified and the miRNA-133b was chosen for this study, given its implication in addiction.

RNA extraction and qRT-PCR

Total RNA, including miRNA was extracted using Tri-Reagent (Molecular Research Center), following the manufacturer's protocol. NCode™ miRNA First-Strand cDNA Synthesis (Invitrogen) was used to synthesize cDNA from miRNA and mRNA. cDNA concentration was determined by measuring the absorbance at 260 nm with a spectrophotometer (SmartSpec™ Plus from BioRad). The absolute quantification of the PCR products was accomplished with a standard curve using the SYBR-Green method. The SYBR-Green was included in a 2X Master Mix from QIAGEN (QuantiTect SYBR Green PCR Kit). The oligonucleotides used to amplify the different genes studied in this work are:

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EF1α F: GTACTTCTCAGGCTGACTGTG; *EF1α* R: ACGATCAGCTGTTTCACTCC; dre-miRNA-133b: TTTGGTCCCCTTCAACC AGCTA; *zfPitx3* F: GACAACAGTGACAC AGAGAAGT; *zfPitx3* R: TGTCGGGATAA CGGTTTCTC; *zfTH* F: TTTGAAGAGAAGT GCAGAGGAT; *zfTH* R: TCAGTAAATCCT GGGTGATCC; *zfDAT* F: AGACATCTGGGAAGGTGGTG; *zfDAT* R: ACCTGAGCAT CATACAGGCG. The final volume of each reaction was 20 µl: 10 µl of Master Mix, 0.8 µl of each oligonucleotide, 7.4 µl of distilled water and 1 µl of cDNA in a concentration of 25 ng/µl. A standard curve was constructed for each experiment by serial dilutions of cDNA: 0.1 ng/µl, 0.01 ng/µl, 0.001 ng/µl and 0.0001 ng/µl. The amplification reaction took place in an iCycler System (BioRad), with the following conditions: 15 min at 95 ºC followed by 35 cycles of 15 s at 95 ºC, 30 s at 57 ºC and 30 s at 70 ºC. Three PCR reactions were performed for each sample per plate, each experiment was repeated with two different samples. We have used *EF1α* as an endogenous control.

Pitx3 3'UTR cloning and microinjection

QuantiTect SYBR Green PCR Kit from QIAGEN was used to amplify the 3'UTR from *Pitx3* using primers based on the sequence of the full-length cDNA from Ensembl (Acc. No: ENSDARG00000070069). The following primers were used: *zfPitx3* 3'UTR F: CGGTATGAAAGCGATGCGTCTA; *zfPitx3* 3'UTR R: AGACAAAGCAGGCTACACCAG GA. The program used for the amplification was: 15 min at 95 ºC followed by 35 cycles of 15 s at 95 ºC, 30 s at 57 ºC and 1 min at 70 ºC. At the end of the cycles a final extension temperature of 70 ºC was added during 10 min. The PCR product was purified and cloned into a TOPO-TA 2.1 vector (Invitrogen). TOP 10'F cells

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(Invitrogen) were transformed with the construct and a maxi-prep was performed to obtain high quantities of the construct. This construct was digested with EcoRI 1 h at 37 ºC and sent for sequencing. The digested product was injected at a concentration of 0.1 ng/µl into the cell of one-cell zebrafish embryos with a micromanipulator-microinjector system from Eppendorf.

Morpholino microinjection

The morpholino antisense (MO) oligomer used to knock down *zfMOR* was purchased from Gene Tools, LLC and its sequence is: AATGTTGCCAGTG TTTTCCATCATG. The MO was diluted in sterilized water to a stock concentration of 0.3 mM. In addition to the three MO experimental groups (untreated, 10 nM morphine, 10 nM morphine plus 1 µM naloxone), each experiment included a control MO group injected with morpholino that exhibits no binding target or biological activity, as well as a control group (uninjected) for each experimental group (untreated, 10 nM morphine, 10 nM morphine plus 1 µM naloxone). Zebrafish embryos were injected into the yolk at one-to-four cell stage with the morpholino oligonucleotide according to the published protocols (Nasevicius and Ekker, 2000). Several MO concentrations were used so as to establish the concentration that produced the greatest effect on the expression level of the studied genes and the lowest embryonic death. To calibrate the amount of solution injected, 10 ms pulses are injected into a 1-l microcapillary (Drummond Scientific, Broomall, PA). The amount of solution in the capillary is measured using a mm ruler; these capillaries have 1 µl of total capacity and are 33 mm long; thus, 1 mm represents 30 nl of solution.The concentrations of *zfMOR* MO and control MO used were 0.2 µM and 1 µM respectively (3 nl were injected into each

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embryo). Embryos were maintained in E3 medium at 28.5 ºC until sacrificed at 24 hpf.

Embryonic treatment with MAPK inhibitors

Zebrafish embryos were first divided into five experimental groups: control (untreated), exposed to 0.6 µM JNKII inhibitor, exposed to 60 µM SB203580 (p38 inhibitor), exposed to 50 µM PD98059 (ERK 1/2 inhibitor) and exposed to 50 µM U0126 (ERK 1/2 inhibitor). Exposure to the MAPK inhibitors was done after 5 hpf. As only the ERK 1/2 inhibitors produced the desired effect, different zebrafish embryos were then divided into other experimental groups: control, exposed to PD98059 from 5 hpf to 24 hpf, exposed to PD98059 from 16 hpf to 24 hpf (the exact period in which the CNS is developing and differentiating), exposed to U016 from 5 hpf to 24 hpf and exposed to U0126 from 16 hpf to 24 hpf. All embryos were sacrificed at 24 hpf.

Primary neuronal culture and RNA extraction

Hipoccampal neurons from P1 rats were isolated and cultured in 6-well plates according to previously described protocols (Liao et al., 2007). The one-week cultured neurons were divided into four experimental groups: untreated, treated with 100 nM morphine, treated with 1 µM naloxone and treated simultaneously with morphine and naloxone. Total RNA (including miRNAs) was isolated using the QIAGEN RNA-easy purification system based on columns. qRT-PCR was performed as described in the *RNA extraction and qRT-PCR* section using the same miRNA-133b oligonucleotide that was used with the zebrafish samples, as both species share the same sequence.

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RESULTS

Morphine modulates the expression of miR-133b

Using a miRNA array, we observed a decrease in the expression of several miRNAs after embryonic exposure to morphine at three developmental stages: 16, 24 and 48 hpf (array data were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus database (USA) under the series GSE18847). Considering the pathways in which each miRNA could be involved, we focus on miR-133b in our studies, due to its reported effect on dopaminergic neurons, an essential component in drug addiction process. Our studies were carried out with the 24 hpf embryos, when the differentiation of the zebrafish CNS begins.

In order to validate our microarray results, quantitative real time PCR (qRCR) assays were carried out. As shown in Fig. 1A, miR-133b level was decreased in 24 hpf embryos exposed to morphine, at two different morphine concentrations, 10 nM and 1 nM. The antagonist naloxone did not significantly change the expression of this miR, but it could block the morphine effect. Although more selective agonists such as DAMGO or antagonists such as CTOP were not used to define the receptor involved due to the lack of affinity of such ligands for zfMOR (de Velasco et al., 2009), such morphine effect of miR-133b level was mediated probably by the activation of zfMOR.

Morphine modulates the expression of miR-133b targets

One of the miR-133b targets is the transcription factor Pitx3. Pitx3 in turn has been shown to regulate the transcription of *th* and *dat*. Since microRNAs normally regulate the

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stability or the translation of the transcripts, by decreasing the miR-133b, morphine should either increase the levels or the activities of these transcripts. As shown in Fig. 1 B-D, treatment of zebrafish embryos with 1 and 10 nM morphine increases the mRNA levels of *pitx3* and *dat* while morphine treatment decreases miR-133b level. However, the level of *th* increases only after treatment with 1 nM morphine, whereas higher concentrations induce a decrease, producing a biphasic effect.

Addition of naloxone effectively abolished the morphine-induced changes in the expression levels of miRNA-133b, *pitx3*, *th* and *dat* (Fig 1). These data suggest that morphine regulates the level of the dopaminergic genes via the control of miR-133b by activating zfMOR.

Although treatment of embryos with morphine clearly decreases the miR-133b level and increases the Pitx3 and its subsequent targets: TH and DAT levels, whether miR-133b indeed interacts with Pitx3 thereby destabilizing the transcript has not been demonstrated in zebrafish. Hence, in order to demonstrate that miR-133b does indeed interact with Pitx3 and regulates Pitx3's targets levels in zebrafish, the 3'UTR sequence of Pitx3 was amplified and cloned into TOPO-TA pCR 2.1 vector for the injection into one-cell embryos. As shown in Fig. 2A, the embryos injected with the Pitx3 3'UTR sequence displayed a decrease in the miR-133b level as compared to control embryos (non-injected embryos). At the same time, an increase in the Pitx3, TH and DAT transcript levels were observed in embryos injected with the 3'UTR sequence (Fig. 2B-D). Thus, the presence of the 3'UTR sequence of Pitx3, the putative target of miR-133b, decreased the free miR-133b

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level in zebrafish embryos resulting in an increase in the expression of Pitx3 and its subsequent targets.

The role of zfMOR in morphine-induced regulation of miR-133b pathway

The effects of morphine in the embryos are probably mediated by zfMOR, the opioid receptor that exhibits highest affinity towards morphine (de Velasco et al., 2009). In order to establish the role of zfMOR in regulating miR-133b without the availability of a zfMOR selective antagonist, we decided to silence zfMOR by morpholino oligonucleotide injection. The efficiency of the morpholino oligonucleotide to decrease the zfMOR level was determined with qRT-PCR. Injection of 0.2 μM of the morpholino oligonucleotide per embryo reduced the zfMOR transcription level by 95% (the injection of ZfMOR decreased the expression of both ZfDOR1 and ZfDOR2 by approximately 2,5%, which is not statistically significant, showing the specificity of the ZfMOR MO; *data not shown*).

The absence of zfMOR increases the number of miR-133b molecules within the embryos (Fig. 3A). Such an increase was not observed after the injection of a control morpholino. Furthermore, 1 nM or 10 nM morphine exposure did not alter the miR-133b level in embryos injected with zfMOR morpholino, while the same concentrations of morphine treatment resulted in a decrease of miR-133b level in embryos injected with control morpholino (Fig. 3A). The increased expression in miR-133b detected in the ZfMOR knock down embryos also led to a decrease of the subsequent miR-133b targets, i.e., Pitx3, TH and DAT (Fig. 3B-D). Clearly, the morpholino and the opioid antagonist naloxone studies indicate zfMOR is the mediator for the morphine-induced regulation of miR-133b and its targets.

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Morphine-induced regulation of the miR-133b pathway depends on ERK 1/2 activity.

Morphine via the mammalian MOR regulates multiple signalling pathways. In the rat hippocampus, morphine activates ERK1/2 and decreases the expression level of miR-190 (Zheng et al.). Whether similar signaling mechanism is involved in morphine-induced regulation of the miR-133b pathway in zebrafish is unknown. Hence, several MAPK inhibitors, such as JNK inhibitor II for JNK, SB203580 for p38 and PD98059 and U0126 for ERK1/2 pathways were used to identify the signals involved in morphine-induced miR-133a regulation. The inhibition of JNK and p38 did produce a significant decrease in the miR-133b level, and hence, an increase in the level of Pitx3, TH and DAT (Table 1). In contrast, the inhibition of MEK1/2 by either U0126 or PD98059 enhanced miR-133b expression, and as a consequence, it decreased the level of Pitx3, TH and DAT transcripts (Fig. 4A-D). Simultaneous exposure to morphine and one of the MEK1/2 inhibitors did not alter the miR-133b level or its related genes transcript levels as compared to embryos that were only exposed to the MEK1/2 inhibitors. Parallel treatment of embryos with morphine in the presence of either JNK or p38 inhibitor did not eliminate the morphine-induced decrease in the miR-133b level (Table 1). Probably, by activating zfMOR, morphine, via the ERK1/2 pathway, regulates the miR-133b level in the zebrafish embryos.

Morphine regulates miR-133b expression in hippocampal neurons

In order to determine whether the observed regulation of miR-133b by zfMOR in the zebrafish embryos has any mammalian counterparts, hipoccampal neurons obtained from P1 rats were treated with 100 nM morphine. Similar to previously reported studies using mature hippocampal neuron cultures from mice chronically treated with morphine, in which

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microRNA array and qRT-PCR studies did not reveal any effect on the miR-133b level (10), our current studies with neurons that were mature and differentiated (3-week culture) revealed the absence of morphine or morphine and naloxone effect on the expression of miR-133b (Fig. 5A). In contrast, the level of miR-133b was decreased in 1-week old neurons treated with morphine but not with naloxone, an effect that was abolished by the co-administration of morphine and naloxone (Fig. 5B). Thus, similar to what we have observed in the zebrafish embryos, only the miR-133b level within the immature neurons could be affected by the addition of morphine.

DISCUSSION

Opioid receptors are involved not only in endogenous analgesia and development of tolerance, dependence and addiction, but also in certain aspects of the maturation of the CNS, such as neurogenesis or differentiation of neuronal stem cells (Kim et al., 2006). The molecular signals for such opioid activity is being described, but many issues concerning how opioids regulate the maturation of the CNS remain widely unknown, including the interaction between opioids and miRNAs. miRNAs are one of the most important regulatory systems in the organism (Bonauer et al., 2009; Giraldez et al., 2006) (Zeng et al., 2009). Their main function is to activate cell proliferation by targeting and, therefore, promoting the down-regulation of genes involved in differentiation, or genes that inhibit proliferation-inducer genes. One of these miRNAs, miR-133b, targets the transcription factor Pitx3, which activates dopaminergic differentiation by up-regulating the expression of genes that are dopaminergic-neuron specific, such as *th* and *dat* amongst others (Kim et

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al., 2007). Similar to the observations in mammals, miR-133b controls *pitx3* transcription by targeting its 3'UTR (Fig. 2) in zebrafish. Taking into consideration the implication of the dopaminergic system in addictive disorders, including addiction to opioid drugs (Flores et al., 2004) (Leggio et al., 2009), we have established in this work a pathway that could account for the observed morphine-induced increase in dopamine production (Gianoulakis, 2009). By the modulation of miR-133b regulatory pathways, and hence, dopaminergic differentiation, zfMOR has a specific role in the CNS and is capable of regulating transcription through miRNAs.

In our miRNA arrays screen, morphine regulates multiple miRs (the identities of the miRs regulated have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus database). The dose-dependent regulation of these miRs is unknown. Furthermore, the agonist concentrations to regulate various signalling pathways are not identical. An excellent example for apparent opposite effect of the drug is reported with neurite growth or regeneration. At lower concentrations, ~10^{-14} M, morphine promotes neurite outgrowth and has a neurotrophic role in rat spinal and cortical neurons, but at higher doses ~10^{-6} M, it inhibits axon regeneration (Sinatra and Ford, 1979) (Brailoiu et al., 2004). Both actions are as antagonized by naloxone, indicative of the involvement of opioid receptor. Although the difference in the morphine doses used in our study is not as obvious, the changes in the transcription of TH induced by 1 and 10 nM morphine could be antagonized by naloxone, and so could be blocked by reducing the receptor concentration with specific morpholinos. Probably, the TH biphasic responses to these morphine concentrations reflect the interactions between myriad of signals and miRs

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regulated by the opioid receptor expressed in the embryos.

The mammalian mu opioid receptor activates ERK 1/2 (Zheng et al., 2008), and subsequently activates different sets of transcriptional factors leading to the many functions displayed by the receptor, such as proliferation (Persson et al., 2003). Also, chronic exposure to morphine disrupts ERK 1/2 signalling, which is thought to enhance the development of tolerance. (Macey et al., 2009). Although in zebrafish there is no evidence yet that zfMOR induces phosphorylation of ERK 1/2, given the close molecular, pharmacological and functional homology between mammalian and the zebrafish MOR, we use different MAPK inhibitors to demonstrate that only ERK 1/2 activity was involved in the zfMOR regulation of miR-133b and its targets expression (Fig. 4). As observed in Table 1, the inhibition of other MAPK such as p38 or JNK decreased the level of miR-133b, an opposite to the inhibition of ERK 1/2, which induced an increment of miR-133b copies. Considering that the absence of the mu opioid receptor produced a similar effect to that observed after the inhibition of ERK 1/2, these results point out that it is through ERK 1/2 and not the other MAPK that zfMOR regulates the differentiation of dopaminergic neurons. Apart from its implication in MOR function (Zhang et al., 2009), phosphorylation by ERK 1/2 is involved in TH activation in order to synthesize dopamine (Haycock et al., 1992), which is the main step in the differentiation of neuronal precursors to the dopaminergic phenotype. Hence, by inhibiting ERK1/2, dopaminergic differentiation is reduced in two different ways: (1) by attenuating MOR regulation of miR-133b, which leads to a decrease in *pitx3* expression; and (2) by preventing TH phosphorylation. ERK1/2 is also known to be involved in embryogenesis (Krens et al., 2008a; Krens et al., 2008b).

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Hence, the consequences of maternal morphine intake on the fetus could take place through the intracellular pathways of miR133b and Pitx3. As our results suggest, the neonate abstinence syndrome might be caused by the alteration in dopaminergic differentiation induced by morphine. In addition, the inhibition of ERK 1/2 at two different developmental stages (5 ad 16 hpf) have shown that the closest the treatment is to the beginning of the differentiation of the CNS, the greatest is the effect of this inhibition in the expression levels of the genes involved in the maturation and differentiation of dopaminergic neurons. These data point out the importance of the developmental stage at which embryos are exposed to drugs, as exposure at different stages vary the impact of such drugs on the embryo's development. Thus the control of miR-133b level could be a possible mechanism responsible for the development of morphine addiction, or other drugs of abuse that also increase dopamine levels in the extracellular space. These results show for the first time that the miRNA-133b is a possible new target for the design of new treatments against addictive disorders.

The differences observed on the effect of morphine on the expression of miR-133b in 1-week and 3-week neurons (Fig. 5) demonstrate that morphine induces differentiation by decreasing the expression of this particular miRNA only in the immature neurons. These results point out that the effects of morphine consumption during pregnancy could reflect the alteration in neuronal differentiation by changes in miR-133b expression. These data also points out that in mammals, morphine has the same effect as in the zebrafish in neuronal differentiation through miR-133b, therefore proving the validity of the zebrafish model to study neurological development during drug treatment.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effect of morphine on the expression of miRNA-133b, Pitx3, TH and DAT. A) qRT-PCR analyses of miRNA-133b levels in 24 hpf zebrafish embryos at two different morphine concentrations, 10 nM and 1 nM. B) The Pitx3 levels measured at both concentrations of morphine, with qRT-PCR. C) The level of TH transcript measured with qRT-PCR in morphine-exposed embryos. D) The level of DAT transcript measured with qRT-PCR in embryos exposed to morphine. In all cases, the levels of miR-133b and all 3 transcripts were measured by qRT-PCR in control embryos (no drug exposure), embryos exposed to naloxone only, or embryos exposed to morphine and naloxone. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$ (unpaired Student *t*-test with Welch correction). N=3

Fig. 2. Effect of the injection of Pitx3 3'UTR on the expression of miRNA-133b, Pitx3, TH and DAT. Analyses of the level of (A) miR-133b; B) Pitx3; (C) TH; and (D) DAT by qRT-PCR in 24 hpf zebrafish embryos injected with 100 pg of Pitx3 3'UTR. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$ (unpaired Student *t*-test with Welch correction). N=3

Fig. 3. Effect of MOR knock-down on the expression of miRNA-133b, Pitx3, TH and DAT. Using the morpholino oligonucleotide technique the mu opioid receptor from zebrafish was knocked down as described in text. The consequence of MOR knockdown on the levels of (A) miR-133b; (B) Pitx3; (C) TH; and (D) DAT in control and 1 nM or 10 nM morphine-treated embryos were determined by qRT-PCR as described. Data were analyzed by two-way ANOVA with post-hoc Bonferroni test for comparisons. Error bars, SEM. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$. N=3

Fig. 4. Effect of the inhibition of ERK 1/2 on the expression of miRNA-133b, Pitx3, TH and DAT. A) qRT-PCR analyses of miRNA-133b expression levels in 24 hpf zebrafish embryos exposed to the ERK 1/2 inhibitors PD98059 (PD) and U0126 (U) added at 5 hpf or at 16 hpf. B) The *Pitx3* gene expression levels are also measured in the same embryos, as well as (C) *TH* and (D) *DAT*. qRT-PCR analyses were carried out also in 24 hpf zebrafish embryos treated simultaneously with 10 nM morphine and ERK 1/2 inhibitors. Data were analyzed by two-way ANOVA with post-hoc Bonferroni test for comparisons. Error bars, SEM. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$. N=3

Fig. 5. Effect of morphine on the expression of miRNA-133b in mammalian neurons. (A) The level of miRNA-133b in 1-week hipoccampal neuron culture treated with 100 nM morphine, with 1 μM naloxone or with morphine and naloxone simultaneously were determined by qRT-PCR. (B) of the level of miRNA-133b in 3-week hipoccampal neuron culture treated with 100 nM morphine, with 1 μM naloxone or with morphine and naloxone simultaneously were determined by qRT-PCR. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$ (unpaired Student *t*-test with Welch correction). N=4

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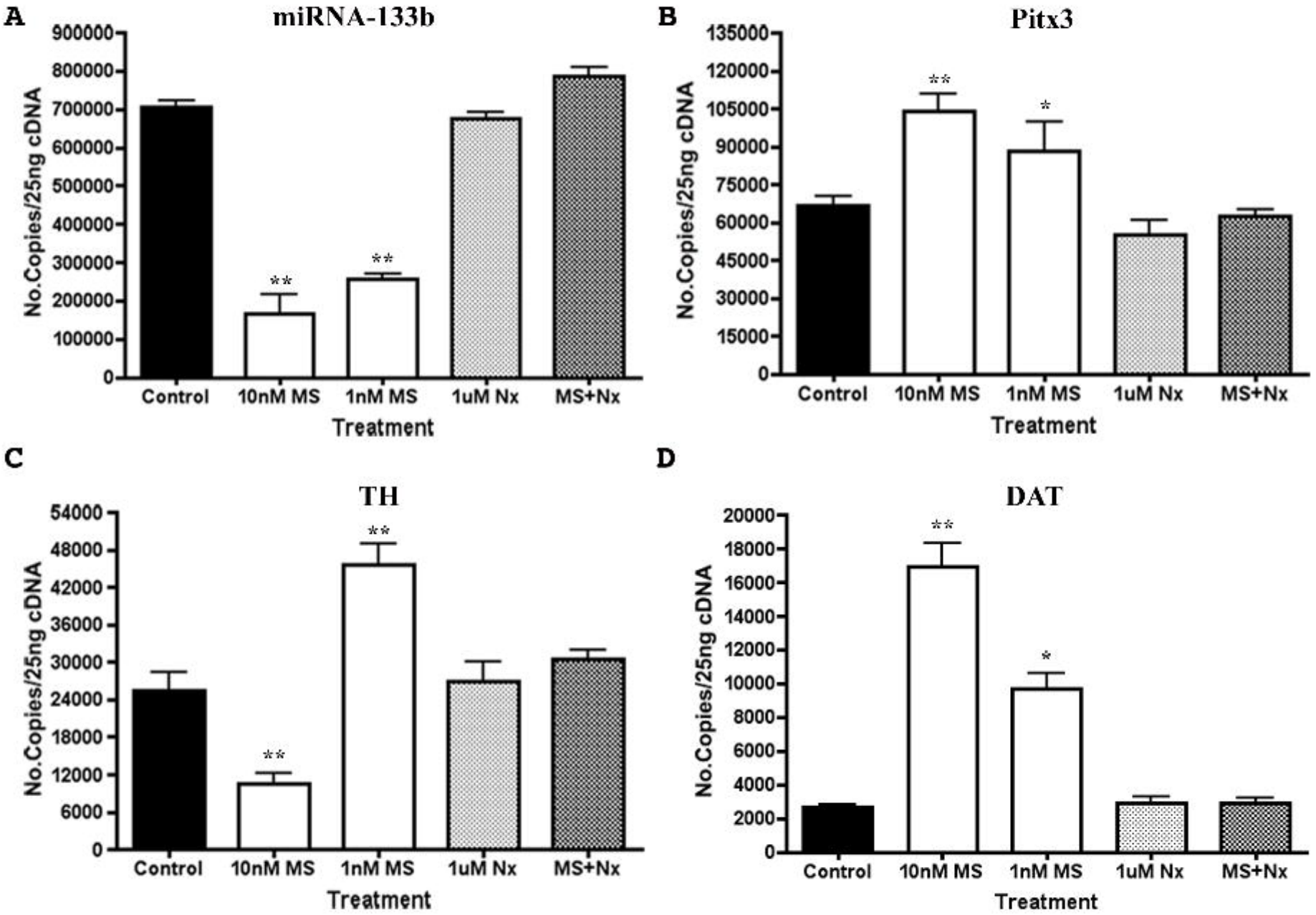
TABLE 1. *Effect of the inhibition of different MAPK on the level of miRNA-133b, Pitx3, TH and DAT in zebrafish embryos.*

	miR-133b	**Pitx3**	**TH**	**DAT**
control	$7.10x10^5$±10260	66684± 3537	25465± 3006	2730±508
0.6 μM JNKII-inhibitor	$1.73x10^5$±9229**	$1.33x10^5$±5786**	50799±3031**	11777±1120**
60 μM SB203580	$2.97x10^5$±15142**	$1.51x10^5$±7494**	52061±2575**	18492±2311**
50 μM PD98059	$1,04x10^6$±19805*	40060±2797*	17546±771*	1231±250*

Summary of the results obtained by qRT-PCR analysis of miR-133b, Pitx3, TH and DAT levels in 24 hpf zebrafish embryos exposed to different MAPK inhibitors. The values represent average ± SEM of the miR and mRNAs copies numbers determined as described in Method. * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$ (unpaired Student *t*-test with Welch correction)

Figure 1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800000
700000
600000
500000
400000
300000
200000
100000
0
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**
Control
10nM MS
1nM MS
1uM Nx
MS+Nx
Treatment
B
Pitx3
No.Copies/25ng cDNA
135000
120000
105000
90000
75000
60000
45000
30000
15000
0
**
*
Control
10nM MS
1nM MS
1uM Nx
MS+Nx
Treatment
C
TH
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48000
42000
36000
30000
24000
18000
12000
6000
0
**
**
Control
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1nM MS
1uM Nx
MS+Nx
Treatment
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DAT
No.Copies/25ng cDNA
20000
18000
16000
14000
12000
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4000
2000
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*
Control
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1nM MS
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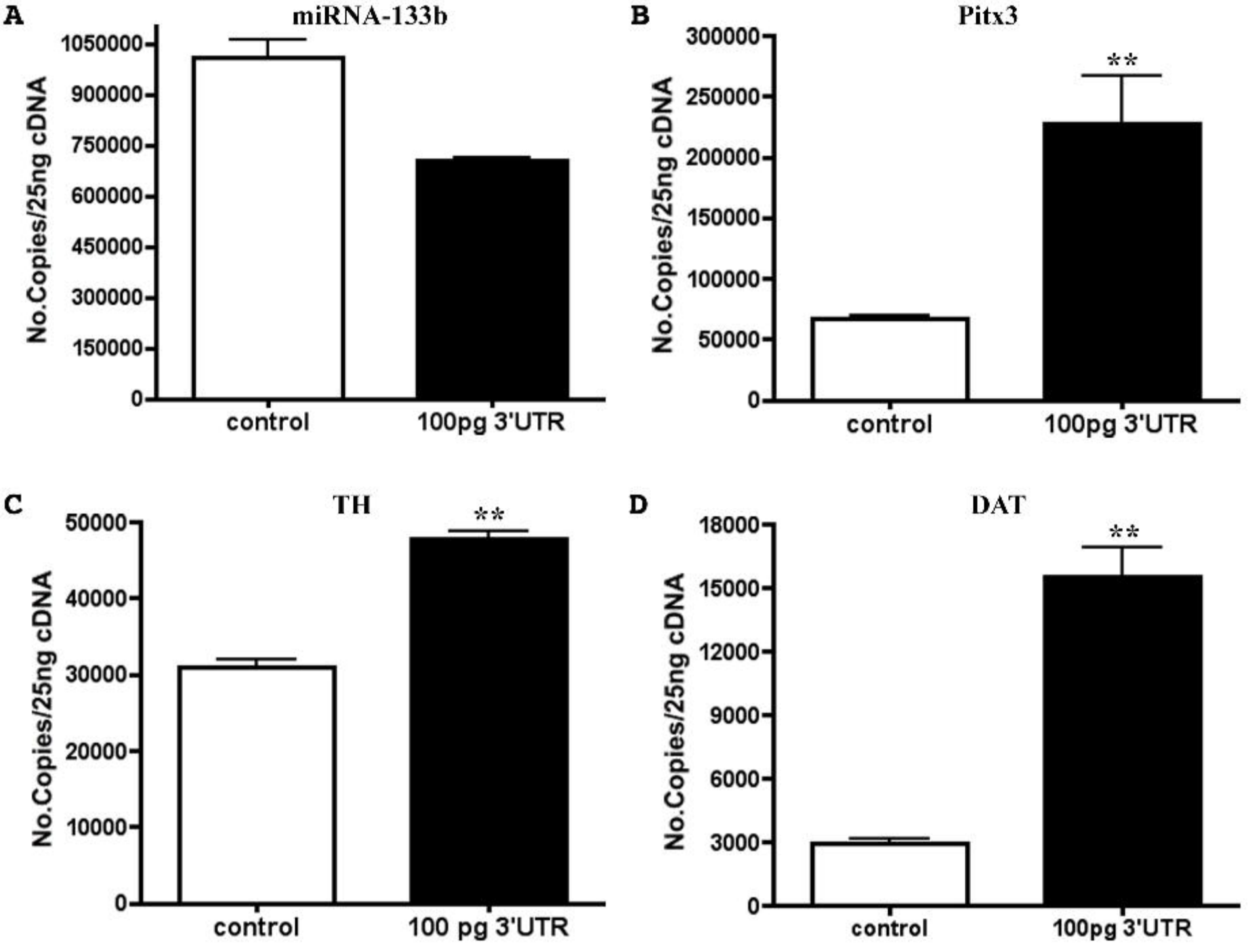
MS+Nx
Treatment

Figure 2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900000
750000
600000
450000
300000
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0
control
100pg 3'UTR
B
Pitx3
No.Copies/25ng cDNA
300000
250000
200000
150000
100000
50000
0
**
control
100pg 3'UTR
C
TH
No.Copies/25ng cDNA
50000
40000
30000
20000
10000
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15000
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6000
3000
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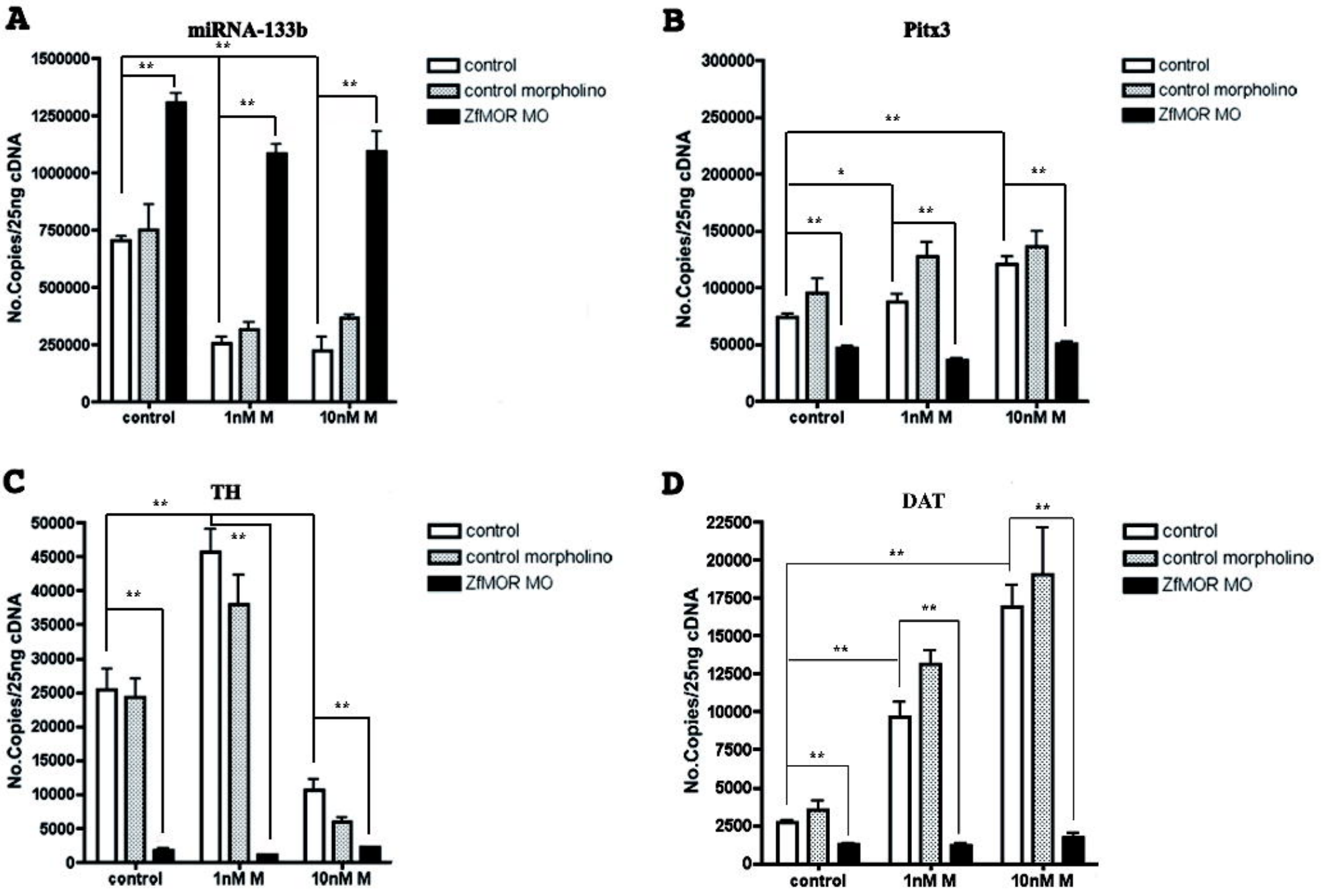
control
100pg 3'UTR

Figure 3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1nM M
10nM M
control
control morpholino
ZfMOR MO
B
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No.Copies/25ng cDNA
300000
250000
200000
150000
100000
50000
0
control
1nM M
10nM M
control
control morpholino
ZfMOR MO
C
TH
No.Copies/25ng cDNA
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
control
1nM M
10nM M
control
control morpholino
ZfMOR MO
D
DAT
No.Copies/25ng cDNA
22500
20000
17500
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7500
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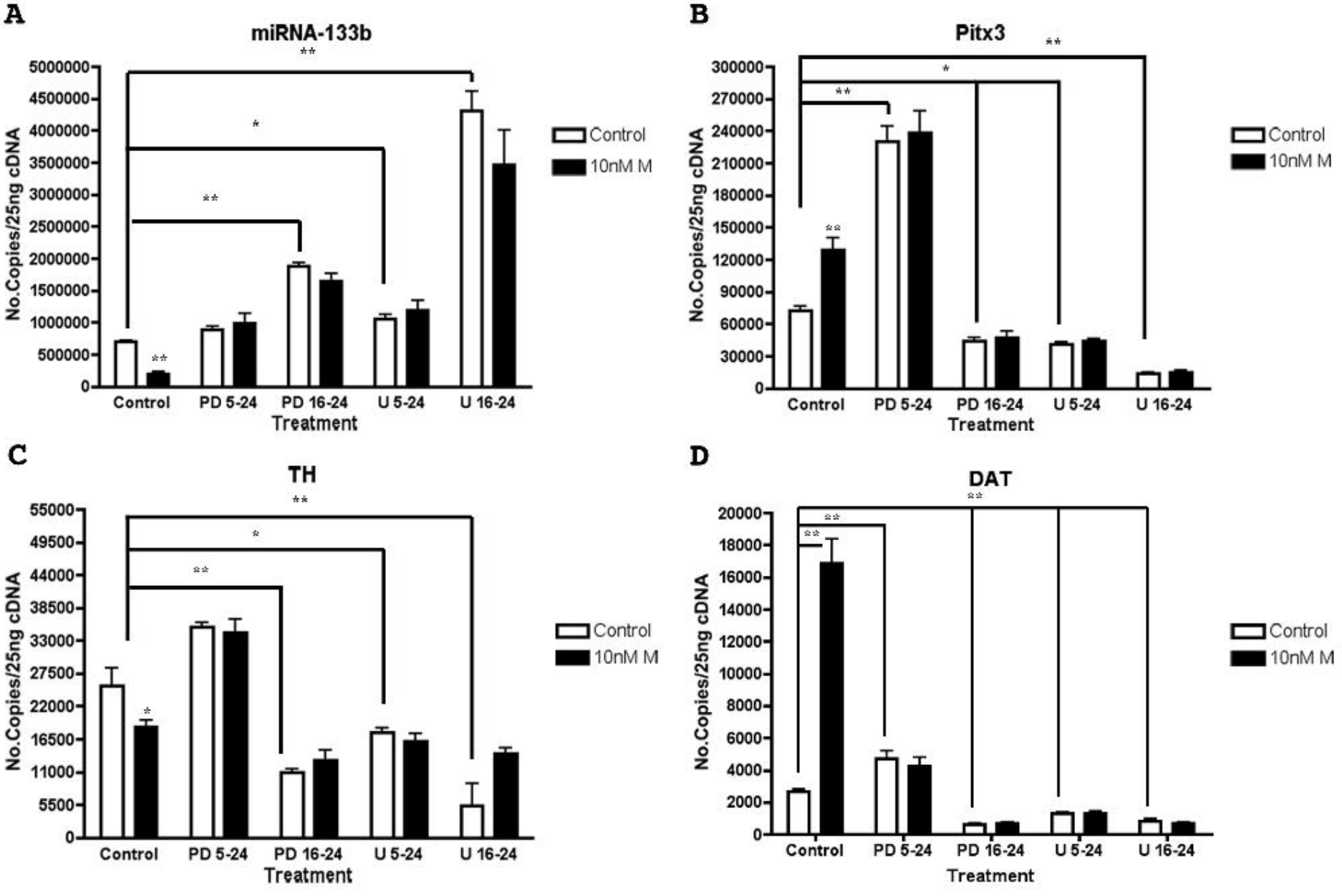
10nM M
control
control morpholino
ZfMOR MO

Figure 4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No.Copies/25ng cDNA
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PD 5-24
PD 16-24
U 5-24
U 16-24
Treatment
Control
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B
Pitx3
No.Copies/25ng cDNA
Control
PD 5-24
PD 16-24
U 5-24
U 16-24
Treatment
Control
10nM M
C
TH
No.Copies/25ng cDNA
Control
PD 5-24
PD 16-24
U 5-24
U 16-24
Treatment
Control
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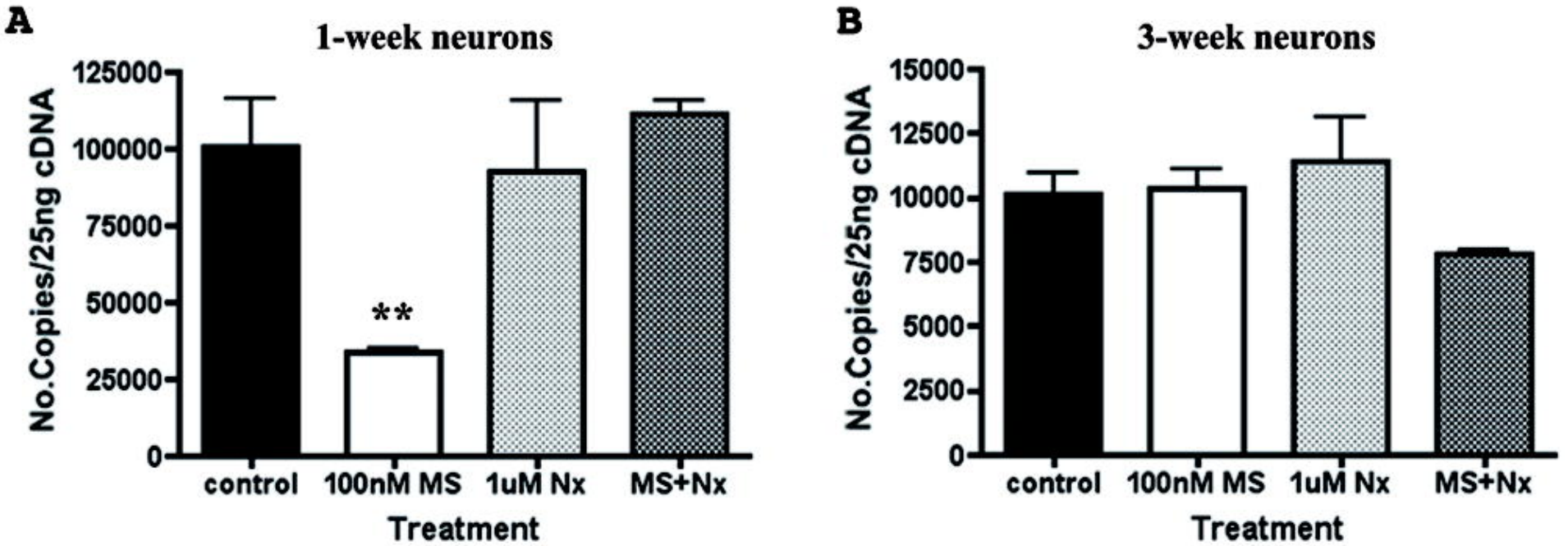
D
DAT
No.Copies/25ng cDNA
Control
PD 5-24
PD 16-24
U 5-24
U 16-24
Treatment
Control
10nM M

Figure 5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1-week neurons
No.Copies/25ng cDNA
125000
100000
75000
50000
25000
0
**
control
100nM MS
1uM Nx
MS+Nx
Treatment
B
3-week neurons
No.Copies/25ng cDNA
15000
12500
10000
7500
5000
2500
0
control
100nM MS
1uM Nx
MS+Nx
Treatment