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Exposure to Morphine and Cocaine Modify the Transcriptomic Landscape in Zebrafish Embryos

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Abstract—Morphine and other opioid analgesics are the drugs of election to treat moderate-to-severe pain, and they elicit their actions by binding to the opioid receptors. Cocaine is a potent inhibitor of dopamine, serotonin, and noradrenaline reuptake, as it blocks DAT, the dopamine transporter, causing an increase in the local concentration of these neurotransmitters in the synaptic cleft. The molecular effects of these drugs have been studied in specific brain areas or nuclei, but the systemic effects in the whole organism have not been comprehensively analyzed. This study aims to analyze the transcriptomic changes elicited by morphine (10 uM) and cocaine (15 uM) in zebrafish embryos. An RNAseq assay was performed with tissues extracts from zebrafish embryos treated from 5 hpf (hours post fertilization) to 72 hpf, and the most representative deregulated genes were experimentally validated by qPCR. We have found changes in the expression of genes related to lipid metabolism, chemokine receptor ligands, visual system, hemoglobins, and metabolic detoxification pathways. Besides, morphine and cocaine modified the global DNA methylation pattern in zebrafish embryos, which would explain the changes in gene expression elicited by these two drugs of abuse.

Key words: Transcriptomic assay, Morphine, Cocaine, DNA methylation, Zebrafish.

INTRODUCTION

Drugs of abuse such as morphine and cocaine are considered severe public health problems due to their addictive properties. Morphine, a natural alkaloid that constitutes 4–21% of the opium poppy (*Papaver somniferum*) capsules (Devereaux et al., 2018), is widely used to treat moderate and severe pain, despite its undesirable side effects (Stein et al., 2000). Morphine blocks the transmission of nociceptive signals by activating the opioid receptors (Pacifici 2016). It displays a high affinity for the mu-opioid receptor, and its activation mediates analgesia, respiratory depression, and gastrointestinal motility impairment (Sverrisdottir et al., 2015). While acute morphine exposure activates several mechanisms that produce analgesia, chronic exposure induces long-term changes at the cellular and molecular level, thus leading to the phenomenon of addiction. It is shown that chronic morphine use causes physical dependence and psychological addiction and several CNS diseases such as mood disorders, conduct disorder, Attention Deficit Hyperactivity Disorder (ADHD) and anxiety, leading to personality disorders, comorbidity, and premature death from overdose (Korsgaard et al., 2016; Fridell et al., 2019).

Cocaine, a tropane alkaloid obtained from the coca leaf (*Erythroxylum coca*), is a recreational drug with millions of users worldwide (Drake and Scott, 2018). Changes in noradrenergic and dopaminergic activity mainly characterize the pharmacological actions of cocaine. It is a potent inhibitor of dopamine, serotonin, and noradrenaline reuptake, as it blocks dopamine transporter, causing an increase in the local concentration of this neurotransmitter in the synaptic cleft and enhancing its effects on the post-synaptic neuron. The physiological effects of an acute cocaine dose primarily affect the cardiovascular and CNS system (Goldstein et al., 2009). Chronic cocaine consumption has been associated with an increased risk of stroke (Fonseca and Ferro, 2013), inflammation of the cardiac muscle, impaired cardiac con-

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Abbreviations: APID, Agile Protein Interaction DataAnalyzer; HAND, HIV-associated neurocognitive disorders; hpf, hours post-fertilization; NAc, nucleus accumbens; NGS, Next Generation Sequencing.

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traction, and aortic rupture (Maraj et al., 2010). Cocaine cardiotoxicity comprises arrhythmias, acute myocardial infarction, and stroke (Riezzo et al., 2012), leading to sudden death. The central chronic disorders are cardiomyopathy and coronary artery disease (Kim and Park, 2019). Cocaine use is also related to neurological diseases, such as motor disorders, including Parkinson's disease (Buttner, 2012; Riezzo et al., 2012), impairment of cognitive functions, and Attention-Deficit/Hyperactivity Disorder (Spronk et al., 2013). Cocaine also reduces the blood flow in the gastrointestinal tract, resulting in loss of appetite and weight (Riezzo et al., 2012; Fonseca and Ferro, 2013).

Opioid exposure causes long-term changes in those brain regions related to the reward pathway and motivation, and these neuroadaptations are partly mediated by epigenetic modifications that alter gene expression programs in several brain regions. DNA methylation seems to increase after long-term heroin use, as people with heroin use disorder displayed higher methylation levels at LINE-1 retrotransposon sites in blood leukocytes than in the control group (Doehring et al., 2013). Besides, differential methylation patterns have been observed in neurons within the frontal cortex from people with heroin addiction (Kozlenkov et al., 2017), and higher levels of methylation were also observed at CpG-rich islands within the OPRM1 gene in blood cells and brain tissue from people who use heroin (Nielsen et al., 2009; Chorbov et al., 2011; Doehring et al., 2013). The same effects were also observed in those patients who received long-term opioid treatment for chronic pain, thus reflecting the pharmacological influence of long-term opioid use (Doehring et al., 2013). In addition to this, changes in global or promoter-specific 5meC and 5hmeC levels have been observed across several brain regions after chronic morphine exposure in rats (Barrow et al., 2017). However, whether these changes can modify gene expression or even the behavior has not been determined yet.

Gene expression changes elicited by cocaine are regulated by complex interactions, including transcription factors, chromatin, and epigenetic processes (Nestler, 2004; Robison and Nestler, 2011). Although cocaine exposure modifies gene expression in several brain regions, the *nucleus accumbens* (NAc) within the limbic system is the most affected area. It has been reported that morphine and cocaine exposure can cause DNA hypomethylation, associated with neuroadaptive synaptic plasticity and memory changes. This epigenetic modification can be long-lasting and potentially transmitted to the offspring, an emerging field of study in recent years (Vaillancourt et al., 2017).

Although there are many studies regarding the differential regulation of gene expression elicited by drugs of abuse in specific brain areas or nuclei, little is known about the gene expression changes underlying the systemic effects of these drugs. This paper aims to analyze the transcriptomic changes elicited by morphine and cocaine exposure in a whole organism. The effect of different pharmacological agents in the CNS has been widely evaluated using zebrafish embryos, including those chemicals targeting dopaminergic and opioid neurotransmitter systems (Guo, 2009; Rosa et al., 2022). In fact, zebrafish models for neuropsychiatric disorders have been developed, including drugs of abuse, and their importance for high-throughput chemical discovery using zebrafish embryos is well documented (Khan et al., 2017; Vaz et al., 2019); thus, it can contribute to analyze plasticity and behaviour (Nelson and Granato, 2022), two processes that are controlled by brain circuits. Zebrafish neurotransmitter systems share similar molecular, pharmacological, and biochemical profiles with their human homologs, specifically the opioid and the dopaminergic system (Gonzalez-Nunez and Rodriguez, 2009). Thus, transcriptomic analysis has been performed on zebrafish embryos of 72 hpf, which is a valuable tool to evaluate the *in vivo* effects of pharmacological agents.

EXPERIMENTAL PROCEDURES

Chemicals

Morphine was kindly provided by Prof. Raquel E. Rodríguez Rodríguez. Dirección General de Policía supplied cocaine hydrochloride, and the Mass Spectrometry Core Facility at the University of Salamanca analyzed it to establish its purity. These compounds were dissolved in water to obtain stock solutions of 1 mM of morphine and 1.5 mM of cocaine, and these solutions were stored at −20 °C until use.

Zebrafish care and breeding

Zebrafish were maintained in racks (Aquaneering) at the zebrafish facility at INCyL on a 14 h/10 h light/dark cycle. Embryos were obtained by natural breeding, distributed in 6-well plates and kept at 28.5 °C in an incubator with E3 medium (NaCl 5 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM, $MgSO_4$ 0.33 mM dissolved in ddH_2O with methylene blue and pH = 7). All protocols and experimental procedures followed the current guidelines and regulations approved by the Spanish legislation (RD53/2013, BOE34, of 8th February 2013, pp. 11370–11421), the European Communities Council Directive 2010/63/EU, and agree with the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals, adopted and promulgated by the US National Institutes of Health. All the experiments performed were approved by the Bioethics Committee at the University of Salamanca (registration number 0099).

Experimental design

20 or 50 embryos per well of a 6-well plate were exposed to morphine 10 μM or cocaine 15 μM from 5 hpf to 72 hpf, and a control group was run in parallel. The medium was renewed daily (100%), checked embryo viability, and eliminated dead embryos. No significant increase in the mortality rate was observed in any experiment (the maximum acceptable limit was set at 5% mortality), and no variation in pH and temperature of the medium was found along with the exposures. In the case of morphine, a concentration of 10 μM was selected as the

working concentration, as it correlates with the most common administered doses in rats (ranging from 0.1 mg/kg to 10 mg/kg) (Wang et al., 2020). A concentration of 15 μM cocaine was selected because it is also an analogous dose to those administered in rats (between 15 mg/kg and 50 mg/kg) (Zhang-James et al., 2019). Embryo viability was assessed by observing the presence of a heartbeat, their movements inside the eggs, or their swimming after hatching with a stereomicroscope (Zeiss Stereomicroscope Discovery.V8). No morphological alterations, such as developmental and structural abnormalities, were observed in any case. At 72 hpf, embryos were sacrificed by cold exposure, incubating the multiwell plates on crushed ice. Embryos were transferred to Eppendorf tubes, removed the medium, and frozen in $N_{2(l)}$. The tissue was stored at −80 °C until use.

RNAseq

RNA was obtained using PureLink™ RNA Mini Kit (Ambion Fisher Scientific) following the manufacturer's instructions. In-column DNase I digestion (Invitrogen Fisher Scientific) was performed to remove gDNA traces. RNA concentration was initially determined with a NanoDrop 2000C spectrophotometer (Thermo Scientific), the purity of the sample was established according to the ratio of absorbances measured at 260/280 nm, and RNA integrity was checked with a Bioanalyzer RNA (Agilent). Ribosomal RNA was depleted using RiboMinus™ (ThermoFisher), following the manufacturer's instructions. RNAseq experiment was carried out at the *Servicio de Secuenciación de ADN* of the University of Salamanca, which consisted of several steps: RNA fragmentation, retrotranscription using random hexamers, 3′ and 5′ adapter attachment, and PCR amplification. RNAseq was performed with the Illumina HiSeq 4000 platform, using 125 bp paired-end readings, ensuring more than 30 million readings per sample. RNAseq analysis was carried out using three biological replicates.

Bioinformatic analysis

Bioinformatic analysis was carried out by the *Servicio de Bioinformática* of the University of Salamanca. This analysis involved *de novo* assembly, alignment of the readings with the reference genome, determination of the identified transcripts and their abundance, determination of new transcripts, establishment of a possible differential expression, and functional analysis using GO Terms and KEGG to identify overrepresented pathways (enrichment analysis). Firstly, FASTQC software (Andrews, 2010) was used to determine the samples' quality and select the most suitable parameters for alignment. All samples passed this quality control. The distribution of the expression values was studied by normalizing the data with EdgeR (Robinson et al., 2010), and none of the samples differed in their distribution values compared to the rest.

Sequences were aligned with TopHat v2.0.9 (Trapnell et al., 2009), and sequence reads were mapped to each gene by BedTools (Quinlan and Hall, 2010) using the zebrafish reference genome ENSEMBL GRCz10.85 (https://www.ensembl.org/Danio_rerio/Info/Index) (Yates et al., 2016). Differential expression analysis was assessed using the EdgeR package (Robinson et al., 2010).

Functional characterization of deregulated genes

Deregulated genes were searched in the zebrafish reference genome ENSEMBL GRCz10.85 (https://www.ensembl.org/Danio_rerio/Info/Index) to determine their location in the genome and biological role. Additional information was also obtained from ZFIN (The Zebrafish Model Organism Database, https://zfin.org/), GeneCards (https://www.genecards.org/), and UniProt (https://www.uniprot.org/). Functional characterization was performed using the following databases.

GO Terms (https://geneontology.org/) (Huber et al., 2015): Genes were grouped according to their GO Terms (Gene Ontologies): biological processes, cellular components, and molecular functions. Bioconductor website (https://www.bioconductor.org/) was used for data analysis and visualization.

REACTOME (https://reactome.org/) (Wu and Haw, 2017): Genes were also classified according to their annotated REACTOME pathways; as this database identifies the metabolic pathways present in humans, it also makes ontology-based inferences in other organisms such as zebrafish.

PANTHER (https://www.pantherdb.org/) (Mi and Thomas, 2009): Deregulated genes were classified according to their function based on the evolutionary families and subfamilies, their molecular function, the biological process involved, or the metabolic pathway.

Network analysis: To determine whether the proteins encoded by the deregulated genes could interact and build functional networks, *in silico* analysis was carried out using APID software (Agile Protein Interaction DataAnalyzer, https://cicblade.dep.usal.es:8080/APID/init.action) (Prieto and De Las, 2006). The visualization of the results was performed with RJSplot software (https://rjsplot.net/), developed by the Bioinformatics Core Facility of the University of Salamanca (Barrios and Prieto, 2018).

Real-time PCR (qRT-PCR)

qPCR assays were performed as previously described (Gonzalez-Nunez et al., 2013). Briefly, total RNA from zebrafish embryos was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Specific primers for the selected target genes were designed using the Universal Probe Library Assay Design Center web tool and appear listed in Suppl. Table S1. qPCR experiments were carried out in 25 μL final volume using PowerUp SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific). The real-time polymerase chain reaction was performed using the QuantStudio 7 Flex Real-Time System (Applied Biosystems, Thermo Fisher Scientific). Ct values were calculated for each gene with the QuantStudioTM Real-Time PCR Software (Applied Biosystems, Thermo Fisher

Scientific). Relative gene expression data were calculated by the ΔΔCt method, using β-actin as a housekeeping gene. Also, standard curves for each gene and negative controls (NTC: no template control; and RNA: RNA which was not reverse transcribed) were included in each PCR reaction. Experiments were performed in triplicates and repeated at least six times. Statistical analysis was performed using Graph Pad Prism software.

CpG islands determination

A 3000 bp promoter sequence (between −3000 bp and −1 bp) for each gene was retrieved from the zebrafish reference genome ENSEMBL GRCz10.85 database (https://www.ensembl.org/Danio_rerio/Info/Index). CpG island prediction was assessed with MethPrimer (https://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (Li and Dahiya, 2002) and EMBOSS CpG plot (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) (Madeira et al., 2019). The following criteria were established: size of the island = 100–500 bp, GC content > 50% and observed/expected ratio > 0.6. The UCSC Genome Browser database (https://genome.ucsc.edu/) was also searched turning on the *"GpC island"* feature on the *"expression and regulation"* track.

Methylation analysis

gDNA was obtained using the Quick-DNA Miniprep Plus Kit (Ref. D4068, Zymo) following the manufacturer's instructions. According to the manufacturer's instructions, global methylation, expressed as % 5-meC/total cytosine, was determined using 5-mC DNA ELISA Kit (Ref. D5325, Zymo). ODs were measured at 405 nm using a Labtech LT-4000 Plate Reader (Labtech). Samples were assayed in triplicates, and a standard curve was included in each experiment. Statistical analysis was performed using Graph Pad Prism.

RESULTS

Transcriptomic changes elicited by drugs of abuse in zebrafish embryos

Zebrafish embryos were exposed to 10 μM morphine and 15 μM cocaine from 5 hpf until 72 hpf to determine the effect of drugs of abuse on gene expression during zebrafish development. No significant changes were observed in embryonic growth, in the yolk sac volume, nor in the time for yolk sac absorption (Suppl. Fig. S1). Besides, no gross morphological malformations were observed for the drug-treated groups. RNA was extracted from a pool of 20 embryos per sample, and RNAseq was performed. More than 30 bp million reads were obtained for each sample (Suppl. Table S2). All samples passed QC (Suppl. Fig. S2). In total, 31,541 genes were found to be expressed at 72 hpf, 2885 genes were deregulated in the samples from morphine-treated embryos (1387 upregulated and 1498 downregulated genes), and 2814 genes in the samples from cocaine-treated embryos (1225 upregulated and 1589 downregulated genes). For the morphine treatment, the 60 most representative genes were selected for further analysis, and 41 for the cocaine treatment (Suppl. Tables S3 and S4); 16 of them were deregulated in both treatments (Fig. 1).

Functional enrichment analysis

GO Terms were used to classify the differentially regulated genes into the three categories -Biological Process, Molecular Function, and Component- and ranked according to their Log2 Fold enrichment value (Fig. 2(A) for morphine and Fig. 2(B) for cocaine). Additional functional enrichment analysis was performed with the REACTOME database to find the overrepresented biological processes (Suppl. Fig. S3), yielding similar results to GO enrichment analysis. Pathways related to lipid metabolism (triglyceride catabolism, regulation of fatty acid biosynthesis, cholesterol absorption, and homeostasis or lipid transport) appear to be overrepresented for both treatments. In contrast, terms related to detoxification (e.g., cytochrome C oxidase pathway) have also been found in the case of morphine and calcium signaling pathways in the case of cocaine.

ShinyGo Gene Ontology Enrichment Analysis (Ge et al., 2020) was performed to determine the overrepresented high-level GO categories in our samples, and it was observed that response to stress, catabolic pro-

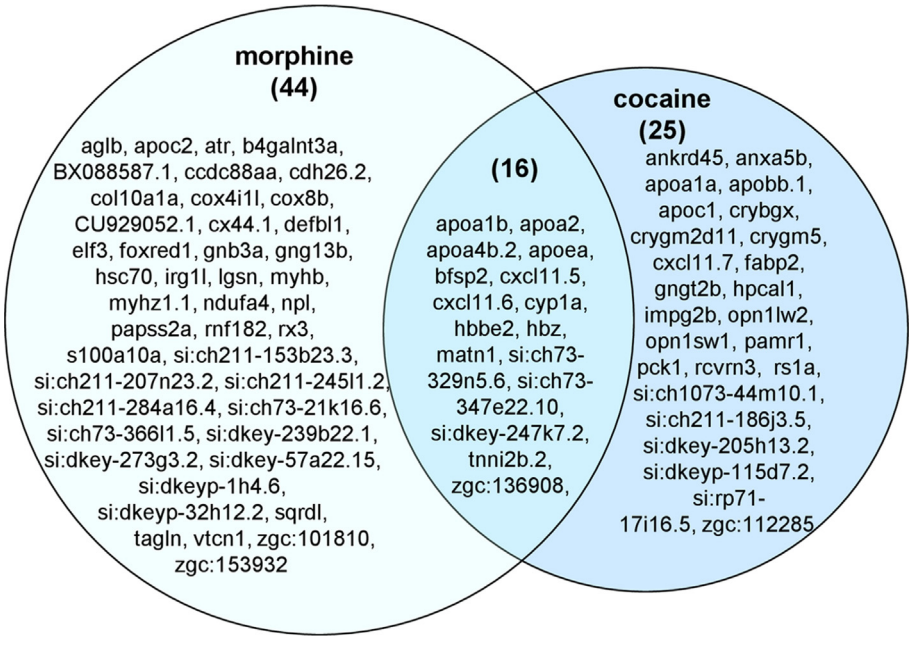

Fig. 1. Venn diagram of deregulated transcripts in zebrafish embryos exposed to 10 μM morphine or 15 μM cocaine.

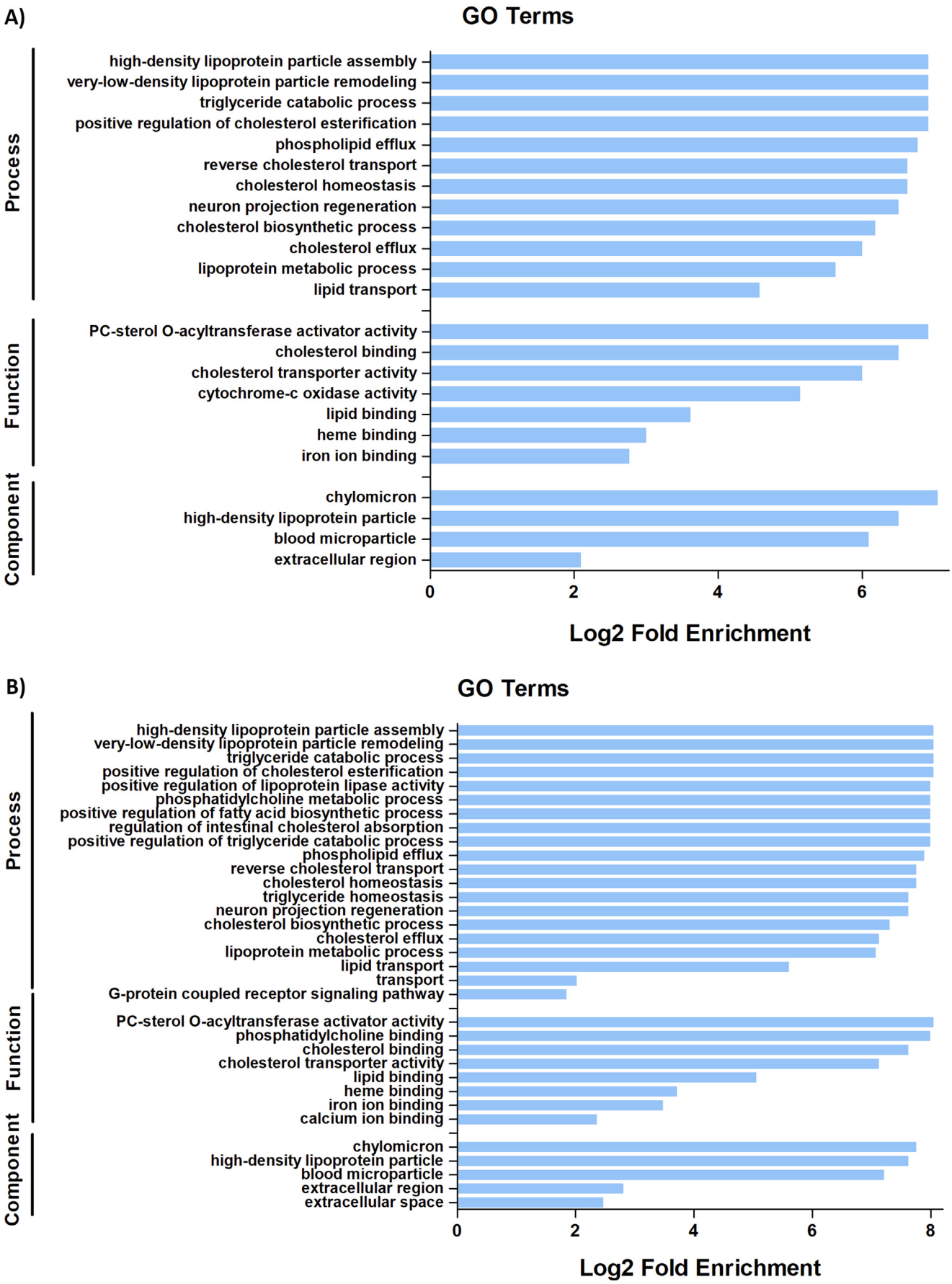

Fig. 2. GO enrichment analysis and functional characterization of differentially regulated genes. Analysis of overrepresented GO Terms for (**A**) morphine and (**B**) cocaine exposure. GO Terms have been classified based on the three categories (Biological Process, Function, or Location Component), and the Log2 Fold enrichment value is represented on the x-axis.

cesses, cellular component organization or biogenesis, developmental processes are affected by morphine exposure (Table 1 and Suppl. Table S5). Likewise, response to chemicals, stress or external stimulus, cellular components organization or biogenesis, catabolic process, and establishment of localization are found for cocaine (Table 2 and Suppl. Table S6). Panther GO Slim analysis was used to analyze the evolutionary conserved overrepresented GO Terms. Morphine exposure alters the expression of those genes related to catalytic activity and binding (nucleic acid binding (GO:0003676), lipid binding (GO:0008289), calcium ion binding (GO:0005509), nucleotide-binding (GO:0000166), and protein binding (GO:0005515)). Cocaine is also related to catalytic activity and binding, but signal transduction and lipid transport are significantly overrepresented (Fig. 3 and Suppl. Fig. S4).

APID software was used to assess the interactions among the deregulated genes, showing that they are organized in a functional network with more connections between nodes in the case of morphine and three interconnected networks in the case of cocaine (Fig. 4).

Several genes were selected (Suppl. Table S7) to confirm the results of the RNAseq assay and to study

Table 1. Shiny GO ontology enrichment analysis for overrepresented high level GO categories Biological Process in morphine treatment

N	High level GO category	Genes
15	Cellular component organization	ZGC:136908 APOA4B.2 MATN1 HBAE5 HBBE2 COL10A1A CCDC88AA ATR CDH26.2 FOXRED1 APOA1B APOEA ZGC:101810 BFSP2 APOA2
15	Localization	ZGC:136908 HSC70 APOA4B.2 CCDC88AA CXCL11.5 CXCL11.6 APOA1B APOEA COX4I1L GJA8B HBAE5 HBBE2 NDUFA4 APOC2 COX8B
15	Cellular component organization or biogenesis	ZGC:136908 APOA4B.2 MATN1 HBAE5 HBBE2 COL10A1A CCDC88AA ATR CDH26.2 FOXRED1 APOA1B APOEA ZGC:101810 BFSP2 APOA2
13	Establishment of localization	ZGC:136908 HSC70 APOA4B.2 CCDC88AA APOA1B APOEA COX4I1L GJA8B HBAE5 HBBE2 NDUFA4 APOC2 COX8B
10	Developmental process	LGSN MATN1 ENSDARG00000074919 ELF3 CDH26.2 RX3 MYHZ1.1 APOA2 COL10A1A ATR
10	Anatomical structure development	LGSN MATN1 ENSDARG00000074919 CDH26.2 RX3 ELF3 MYHZ1.1 APOA2 COL10A1A ATR
9	Response to stress	ZGC:136908 HSC70 IRG1L ATR CXCL11.5 CXCL11.6 DEFBL1 ELF3 COL10A1A
9	Cellular component biogenesis	APOA4B.2 HBAE5 HBBE2 CDH26.2 FOXRED1 APOA1B APOEA ZGC:101810 ATR
8	Catabolic process	ZGC:136908 APOA4B.2 HBAE5 HBBE2 AGLB APOA1B APOEA APOC2
8	Biosynthetic process	APOA4B.2 PAPSS2A ELF3 APOA1B APOEA LGSN RX3 AGLB
7	Response to chemical	ZGC:136908 HSC70 CXCL11.5 CXCL11.6 CYP1A HBAE5 HBBE2
6	Anatomical structure morphogenesis	LGSN MATN1 CDH26.2 MYHZ1.1 APOA2 ATR
6	Regulation of biological quality	APOA4B.2 ATR APOA1B APOEA SI:CH211-153B23.3 ZGC:101810
5	Response to external stimulus	CXCL11.5 CXCL11.6 BX088587.1 DEFBL1 IRG1L
5	Regulation of metabolic process	APOA4B.2 ELF3 APOA1B APOEA RX3
5	Macromolecule localization	ZGC:136908 APOA4B.2 APOA1B APOEA APOC2
5	Positive regulation of biological process	APOA4B.2 VTCN1 APOA1B APOEA ZGC:101810
5	Regulation of molecular function	APOA4B.2 APOC2 CXCL11.6 APOA1B APOEA
4	Immune system process	VTCN1 CXCL11.5 CXCL11.6 DEFBL1
4	Immune response	VTCN1 CXCL11.5 CXCL11.6 DEFBL1
4	Response to biotic stimulus	CXCL11.5 CXCL11.6 DEFBL1 IRG1L
4	Multi-organism process	CXCL11.5 CXCL11.6 DEFBL1 IRG1L
4	Response to other organism	CXCL11.5 CXCL11.6 DEFBL1 IRG1L
3	Cell adhesion	CDH26.2 SI:DKEY-239B22.1 SI:CH73-329N5.6
3	Biological adhesion	CDH26.2 SI:DKEY-239B22.1 SI:CH73-329N5.6
3	Regulation of localization	APOA4B.2 APOA1B APOEA
3	Locomotion	CXCL11.5 CXCL11.6 CCDC88AA
3	Cell motility	CXCL11.5 CXCL11.6 CCDC88AA
3	Localization of cell	CXCL11.5 CXCL11.6 CCDC88AA
3	Regulation of plasma lipoprotein particle levels	APOA4B.2 APOA1B APOEA
2	System process	TNNI2B.2 GNG13B
2	Response to abiotic stimulus	HSC70 BX088587.1
2	Growth	MATN1 COL10A1A
2	Taxis	CXCL11.5 CXCL11.6
2	Regulation of response to stimulus	VTCN1 CXCL11.6
2	Developmental growth	MATN1 COL10A1A
2	Leukocyte migration	CXCL11.5 CXCL11.6
2	Cellular localization	ZGC:136908 CCDC88AA
2	Detoxification	HBAE5 HBBE2
2	Cellular detoxification	HBAE5 HBBE2

Table 2. Shiny GO ontology enrichment analysis for overrepresented high level GO categories Biological Process in cocaine treatment

N	High level GO category	Genes
14	Localization	ZGC:136908 APOA1A ANXA5B APOA4B.2 SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706 APOA1B APOEA APOBB.1 HBAE5 HBBE2 APOC1
11	Response to chemical	ZGC:136908 PCK1 SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706 FABP2 APOBB.1 CYP1A HBAE5 HBBE2
10	Cellular component organization	ZGC:136908 APOA1A APOA4B.2 MATN1 HBAE5 HBBE2 APOA1B APOEA BFSP2 APOA2
10	Cellular component organization or biogenesis	ZGC:136908 APOA1A APOA4B.2 MATN1 HBAE5 HBBE2 APOA1B APOEA BFSP2 APOA2
8	Catabolic process	ZGC:136908 APOA1A PCK1 APOA4B.2 HBAE5 HBBE2 APOA1B APOEA
8	Establishment of localization	ZGC:136908 APOA1A APOA4B.2 APOA1B APOEA APOBB.1 HBAE5 HBBE2
7	Response to external stimulus	PCK1 SI:RP71-17I16.5 OPN1LW2 CXCL11.5 CXCL11.6 ENSDARG00000094706 OPN1SW1
6	Response to stress	ZGC:136908 PCK1 SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706
6	Biosynthetic process	APOA1A PCK1 APOA4B.2 SI:RP71-17I16.5 APOA1B APOEA
6	Macromolecule localization	ZGC:136908 APOA1A APOA4B.2 APOA1B APOEA APOBB.1
6	Locomotion	ANXA5B SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706 APOC1
6	Cellular component biogenesis	APOA1A APOA4B.2 HBAE5 HBBE2 APOA1B APOEA
6	Cell motility	ANXA5B SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706 APOC1
6	Localization of cell	ANXA5B SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706 APOC1
6	Regulation of biological quality	APOA1A PCK1 APOA4B.2 SI:RP71-17I16.5 APOA1B APOEA
6	Regulation of molecular function	APOA1A APOA4B.2 CXCL11.6 ENSDARG00000094706 APOA1B APOEA
5	Regulation of localization	APOA1A ANXA5B APOA4B.2 APOA1B APOEA
4	Immune system process	SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706
4	System process	TNNI2B.2 OPN1LW2 OPN1SW1 IMPG2B
4	Regulation of metabolic process	APOA1A APOA4B.2 APOA1B APOEA
4	Developmental process	PCK1 MATN1 APOA2 APOC1
4	Taxis	SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706
4	Positive regulation of biological process	APOA1A APOA4B.2 APOA1B APOEA
4	Anatomical structure development	PCK1 MATN1 APOA2 APOC1
4	Leukocyte migration	SI:RP71-17I16.5 CXCL11.5 CXCL11.6 ENSDARG00000094706
4	Regulation of plasma lipoprotein particle levels	APOA1A APOA4B.2 APOA1B APOEA
3	Immune response	CXCL11.5 CXCL11.6 ENSDARG00000094706
3	Response to biotic stimulus	CXCL11.5 CXCL11.6 ENSDARG00000094706
3	Response to abiotic stimulus	OPN1LW2 OPN1SW1 RCVRN3
3	Anatomical structure morphogenesis	MATN1 APOA2 APOC1
3	Multi-organism process	CXCL11.5 CXCL11.6 ENSDARG00000094706
3	Response to other organism	CXCL11.5 CXCL11.6 ENSDARG00000094706
2	Cell adhesion	SI:DKEYP-115D7.2 SI:CH73-329N5.6
2	Biological adhesion	SI:DKEYP-115D7.2 SI:CH73-329N5.6
2	Regulation of signaling	CXCL11.6 ENSDARG00000094706
2	Regulation of response to stimulus	CXCL11.6 ENSDARG00000094706
2	Detection of stimulus	OPN1LW2 OPN1SW1
2	Detoxification	HBAE5 HBBE2
2	Cellular detoxification	HBAE5 HBBE2

their expression levels by qPCR, obtaining similar results as those previously found in the transcriptomic analysis (Fig. 5).

Methylation assays

An *in silico* analysis was conducted within 3000 bp of the proximal promoter of those genes whose expression is modified by morphine and cocaine to determine the existence of putative GpC islands. At least one CpG island was predicted for eight of those genes (Table 3). Besides, global methylation levels were determined by ELISA, and it was found that both morphine and cocaine treatment produced a statistically significant increase in DNA global methylation levels at 72 hpf (Fig. 6).

DISCUSSION

Nowadays, Next Generation Sequencing (NGS) has changed the research paradigm, introducing a holistic view in Molecular Biology. These techniques have successfully been employed in toxicological assays with zebrafish embryos to analyze the effect of several compounds in the whole organism. It has been shown that bisphenol A and its analogs bisphenol F and S cause toxicity in zebrafish embryos exposed until 120 hpf, and an RNAseq assay has found changes in the

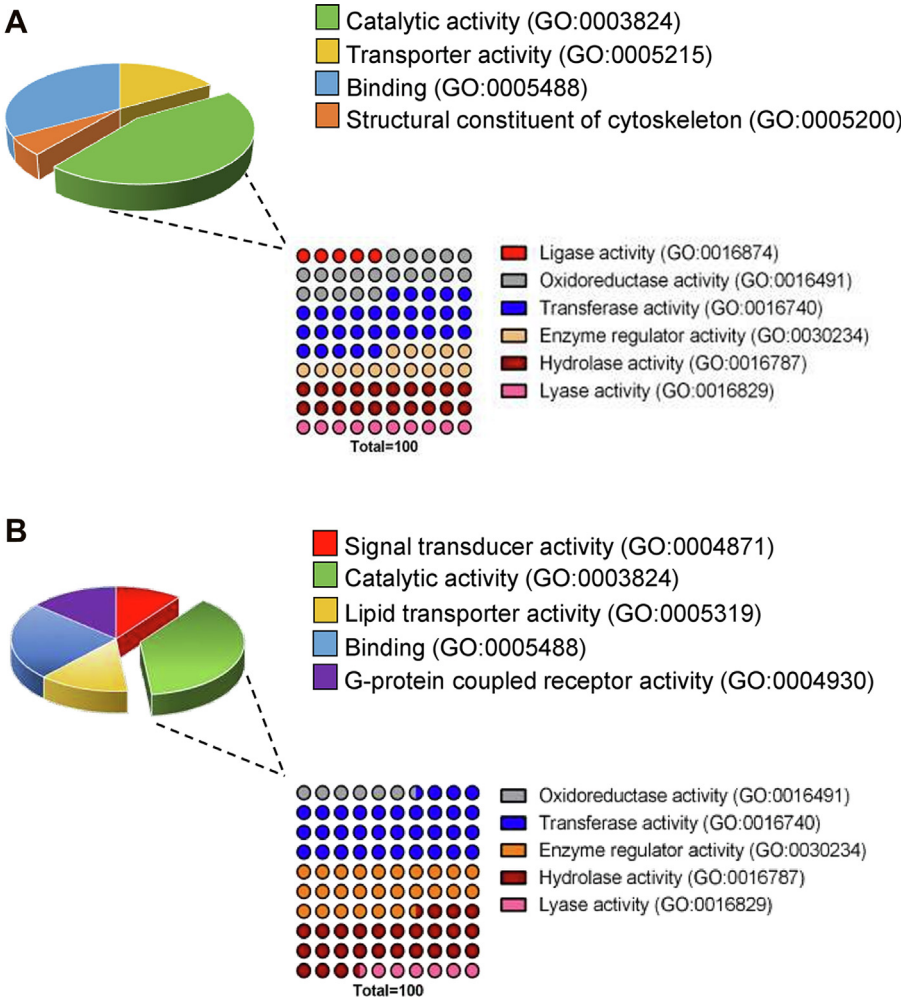

Fig. 3. Schematic representation of overrepresented GO Terms for the category Molecular Function in the transcriptomic analysis of zebrafish embryos exposed to (**A**) morphine and (**B**) cocaine. The 10x10 dot plots represent the lower level category GO Terms distribution in the catalytic activity category.

expression of 190 genes; 19 of them were validated by qPCR (Qiu et al., 2019). An RNAseq assay has been performed using liver tissue from adult zebrafish exposed to FeS nanoparticles stabilized with carboxymethyl cellulose for 96 h, showing changes in the expression of genes related to the immune and inflammatory response, detoxification, oxidative stress, and processes related to DNA repair and damage (Zheng et al., 2018). Following a similar protocol to that described in these two studies, a transcriptomic assay has been performed with zebrafish embryos exposed to drugs of abuse until 72 hpf; the bioinformatic outcome has been experimentally validated by qPCR with selected genes from different overrepresented pathways. We have found changes in the expression levels of genes related to lipid metabolism (*apoa1b*, *apoa2*, *apoa4b*, *apoc1,* and *apoea*); chemokine receptor ligands involved in the immune response (*cxcl11.5* and *cxcl11.7*); *rx3*, a transcription factor expressed in the retina and that participates in the development of the visual system; hemoglobins and cytochrome p450 (*cyp1a*) involved in detoxification processes. Some of these genes had their expression modified by both morphine and cocaine, while in other cases, the changes were only found for one of the two treatments. Functional enrichment analysis has determined that the deregulated metabolic pathways by both drugs are related to lipid metabolism: triglyceride catabolism, regulation of fatty acid biosynthesis, absorption, and homeostasis of cholesterol or lipid transport. Detoxification pathways, calcium-binding, and GPCR signaling are also overrepresented.

By using the PANTHER classification system, it has been found that catalytic activity is the most overrepresented molecular function by both treatments, being the transferase and hydrolase activities the most abundant. This outcome can be explained by considering that these two compounds are biotransformed by detoxification pathways in the liver, based on oxidoreductases, hydrolases (phase I enzymes), and transferases (phase II enzymes). Interaction studies have shown that the interaction map found for morphine deregulated genes has more interactions between nodes than that obtained for cocaine. This outcome may indicate that morphine-altered pathways might be functionally linked: crystallines, which are part of a protein family related to eye lens and with broad expression patterns, are connected to apolipoproteins; in its turn, ApoA1 is linked to hemoglobins. In the case of cocaine, the topology of the interaction map is formed by two or three functional modules with several interactions among nodes and a few edges connecting to other neighborhood modules.

The cytochrome oxidase *cyp1a* is involved in the hepatic detoxification of xenobiotics, and its expression is modified by several drugs that bind the Aryl hydrocarbon receptor (AhR) (Kais et al., 2018), as well as by several endogenous compounds as retinoids (Murphy et al., 2007; Stevison et al., 2019). The expression of CYP genes is regulated by multifactorial mechanisms, including epigenetic mechanisms such as DNA methylation and histone acetylation (Zanger and Schwab, 2013). Our RNAseq assay showed that both drugs downregulate cyp1a expression, and this result has been validated by qPCR. Besides, we have also found changes in the expression of chemokine receptor ligands, a class of signaling molecules related to immune and inflammatory responses. It has been reported that cocaine use disorder (CUD) correlates with altered

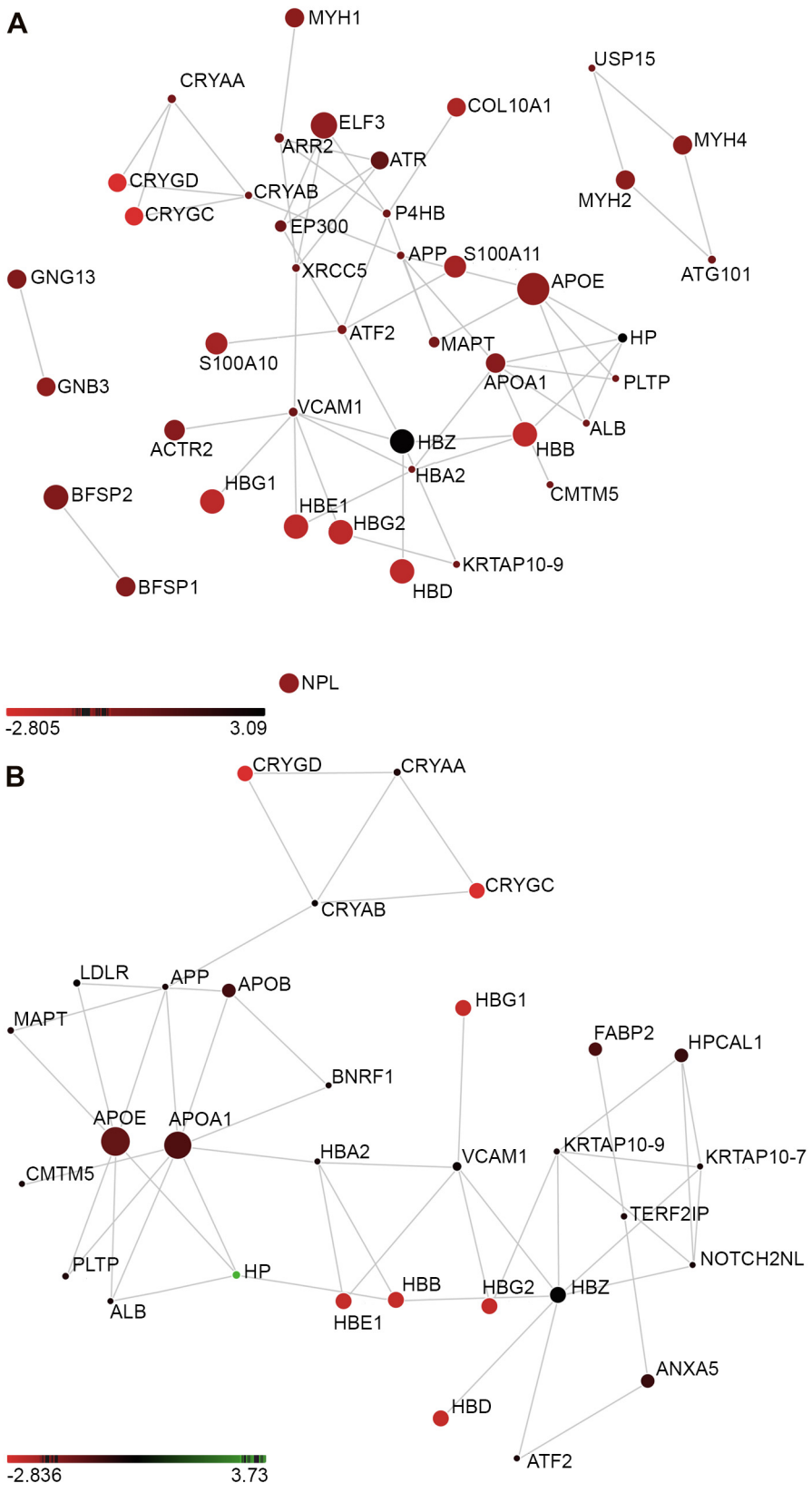

Fig. 4. Network analysis of deregulated genes identified in the transcriptomic analysis for (**A**) morphine and (**B**) cocaine. APID software was used to determine the functional interactions, and visualization of the results was performed with RJSplot software. The color and the size of the nodes represent the changes in the gene expression level between the control and treated groups.

plasma levels of circulating chemokines and interleukins, such as IL-17α, MIP-1α, and TGFα, which can reflect an increased pro-inflammatory state in the brain. Thus, these cytokines may be used as potential biomarkers of cocaine use (Maza-Quiroga et al., 2017). Also, we have found that the expression levels of several genes related to the visual system are downregulated. There is evidence that prenatal exposure to drugs leads to changes in the development of the visual system, and more specifically, cocaine abuse during pregnancy has been correlated with optic nerve abnormalities, eyelid edema, and delayed visual maturation in infants (Good et al., 1992). There is evidence of an association between cocaine abuse and hemoglobins and iron metabolism. A longitudinal study showed that the rate of iron-deficiency anemia in four-year-old children was higher among prenatally cocaine-exposed infants, and this fact correlated with a significant decrease in Full-Scale IQ (Nelson et al., 2004). Besides, it has been reported that one-third of people who use crack presented low levels of hemoglobin and hematocrit, which can correlate with protein-energy malnutrition and anemia, which, in turn, may be caused by an unbalance diet poor in proteins, micronutrients, and vitamins (Escobar et al., 2018).

Other of the affected pathways by cocaine were calcium ion binding, G protein-coupled receptors (GPCRs), ligand binding, and signaling by GPCRs. It has been shown that D2 receptor availability was reduced by 15–20% after one week of initiating self-administration of cocaine in monkeys and that this reduction remained approximately by 20% during one year of exposure (Nader et al., 2006). Interestingly, our results show that cocaine affects signal transduction activity and lipid transport more than morphine. This fact mentioned above can be explained if we consider that cocaine directly affects dopaminergic signaling in the reward system compared to morphine.

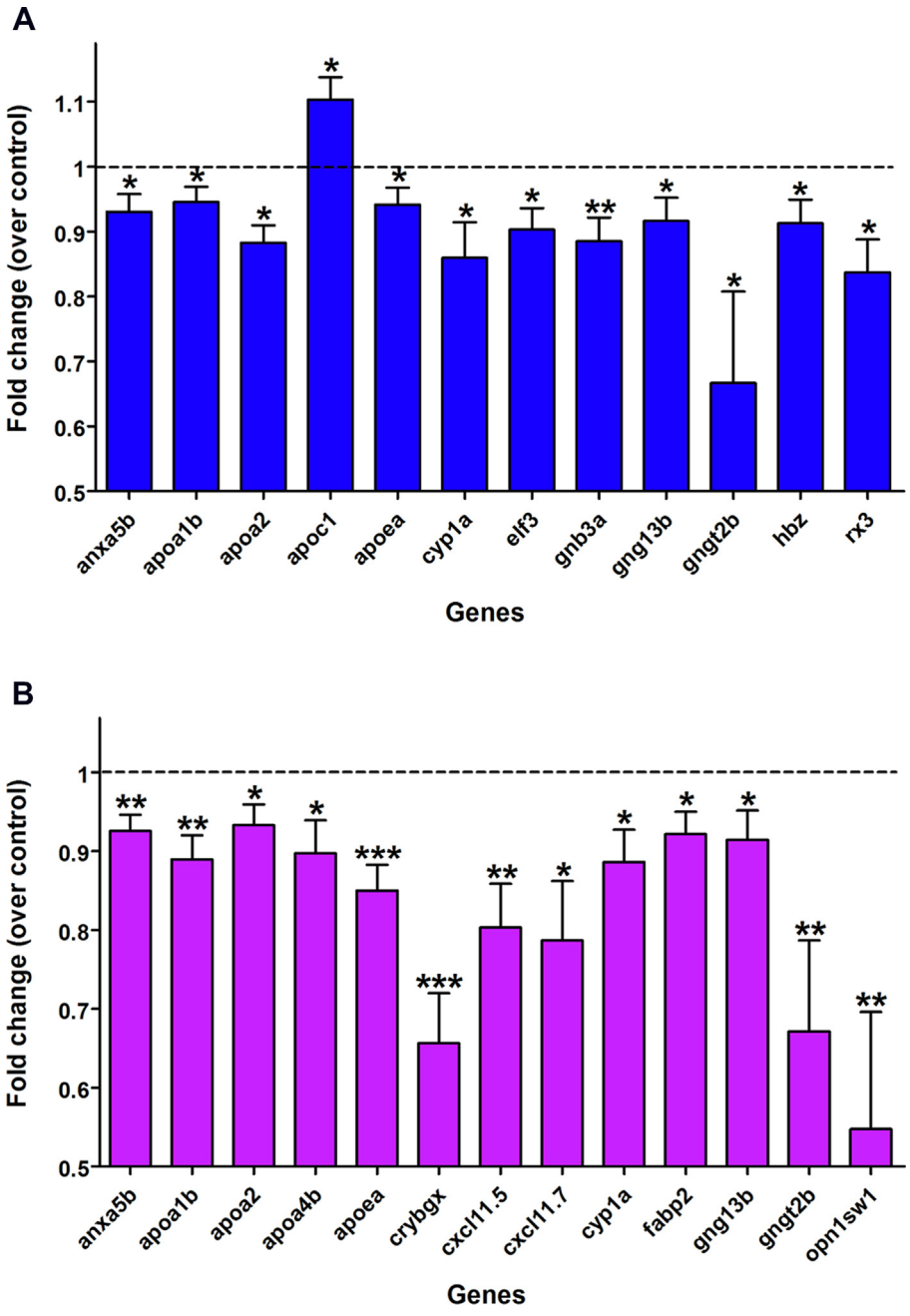

Fig. 5. Qpcr results of selected genes from the rnaseq assay. expression levels were compared between control embryos of 72hpf and exposed to (**A**) 10 μM morphine or (**B**) 15 μM cocaine. Data represent the mean ± SEM of at least seven independent assays performed in triplicate. Results have been analyzed with one-way ANOVA (control, morphine, and cocaine treatment), followed by a Tukey post-test. Legend: **p*-value < 0.05; ***p*-value < 0.01; ****p*-value < 0.001.

Several studies are related to the metabolic effects of drugs of abuse, but the obtained results are contradictory and depend on the length of exposure, animal model, type of drug, and tissue or organ analyzed. A microarray assay performed on the prefrontal cortex of 42 subjects with cocaine-, cannabis and phencyclidine-use disorder revealed that these drugs increased the expression of genes related to cholesterol biosynthesis and transport (*FDFT1, APOL2,* and *SCARB1*), as well as genes associated with the endoplasmic reticulum and Golgi apparatus (Lehrmann et al., 2006). There have also been reported changes in the expression of 25 genes in the lateral hypothalamus of mice after chronic administration of morphine (Befort et al., 2008), a brain region that mediates the hedonic properties of natural stimuli and drugs of abuse. These authors reported changes in the expression of several genes related to signal transduction pathways: *RGS4*, a GTPase acting as a negative regulator of G-proteins, or *SGK*, a Ser/Thr protein kinase involved in the cellular stress response. Besides, apolipoprotein D, aquaporin 4, and prostaglandin synthase were also transcriptionally deregulated; these genes are related to different metabolic pathways, but all of them are considered to be general indicators of modifications in brain physiology.

LXR type Nuclear receptors are the primary regulators of cholesterol homeostasis in the CNS. An increase in cholesterol levels in astrocytes triggers LXR activation, which induces the transcription of genes involved in cholesterol trafficking and efflux: apolipoprotein E, cytochrome P450 enzymes, and different ABC transporters. In transgenic mice that express Tat protein (a transactivator required for HIV replication), cocaine exposure has been shown to decrease LXRβ receptor levels, as well as the expression of its target genes; as a consequence, the bioavailability of cholesterol in the brain of these mice is increased (Cotto et al., 2018). Changes in lipid metabolism in the CNS may be related to the instatement of HIV-associated neurocognitive disorders (HAND), and the illicit use of drugs, such as cocaine, is one of the main risk factors related to the development of HAND (Larrat and Zierler, 1993). Besides, it has been shown that components of the plasma membrane as cholesterol can affect morphine signaling in the brain (Levitt et al., 2009; Qiu et al., 2011) and that morphine induces modifications in the lipid rafts in the prefrontal cortex of rats, with an up-regulation of caveolin-1 and cholesterol (Ujcikova et al., 2017).

At present, the effect of drugs of abuse on lipid metabolism and their systemic effects has not been well established yet. It is known that lipid signaling, either by sex steroids or prostaglandins, is a crucial constituent

Table 3. CpG island prediction. MethPrimer, EMBOSS and the UCSC genome browser search engine were used to *in silico* analyze 3000 bp of the proximal promoter of those genes with differential expression after morphine or cocaine exposure and to predict the existence of CpG islands. The putative islands indicated here were predicted with at least one of the three softwares. The location of the islands is given from the transcription start site

Gene	ENSEMBL ID	CpG Island Position
anxa5b	ENSDARG00000016470	−2949 a −2785
		−1960 a −1860
		−1221 a −1111
apoa1b	ENSDARG00000101324	−2506 a −2403
crybgx	ENSDARG00000036140	−2227 a −2126
cyp1a	ENSDARG00000098315	−2604 a −2308
		−2294 a −2187
elf3	ENSDARG00000077982	−1451 a −1321
		−1112 a −887
gnb3a	ENSDARG00000004358	−2597 a −2029
		−1670 a −1499
gng13b	ENSDARG00000037921	−2655 a −2510
hbae5	ENSDARG00000045142	−2001 a −1897

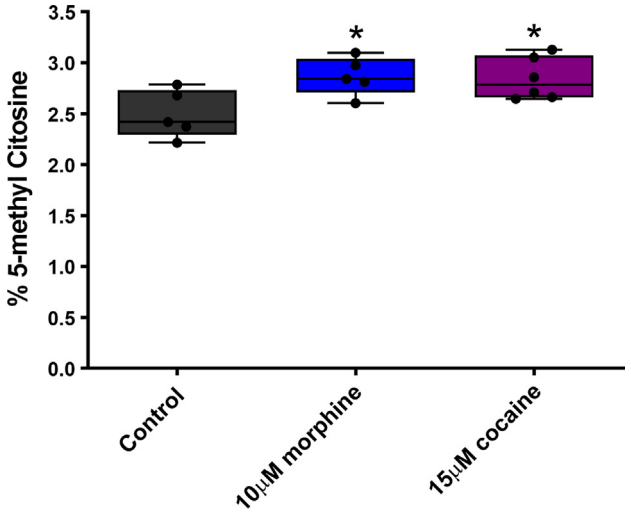

Fig. 6. Global methylation levels (expressed as % of 5-methyl cytosine) in zebrafish embryos of 72hpf, control and treated with 10 μM morphine and 15 μM cocaine. Data correspond to the mean ± SEM of 5 independent experiments performed in duplicate. Results have been analyzed with one-way ANOVA, followed by Tukey post-test. Legend: **p*-value < 0.05.

for developing and maintaining addiction (Leishman et al., 2013), and lipidomic techniques have helped to understand the mechanism of cocaine hepatoxicity (Shi et al., 2012). Cocaine use and abuse affect eating behavior, leading to appetite suppression and altered metabolic and neuroendocrine regulation through multifactorial mechanisms. It has been reported that the increase in dopaminergic neurotransmission caused by cocaine suppresses dietary food intake but increases the ingestion of fat- and carbohydrate-rich foods due to enhanced stimulation of D2R receptors (Thanos et al., 2011). Cocaine use also correlates with uncontrolled eating patterns due to losing control over food intake (Ersche et al., 2013). On the other hand, cocaine blocks serotonin reuptake by blocking the serotonin transporter Sert, and as a consequence, it induces a leptin-dependent anorexic effect (Yadav et al., 2009). A decrease in appetite has been reported in rats after cocaine administration, although the weight loss observed in those people with cocaine use disorder cannot be explained by a global suppression of appetite and is instead a consequence of an imbalance between fat intake and storage (Ersche et al., 2013).

Cocaine abuse is correlated with altered serum lipid levels, as a significant percentage of people who smoke crack had low levels of HDL cholesterol and high levels of triglycerides, and some individuals also presented high total cholesterol and glucose (Escobar et al., 2018). Thus, chronic exposure to cocaine can result in an increased risk for cardiovascular disease and metabolic disorder (Kim and Park, 2019). These conditions are usually associated with changes in serum lipid levels, so it is difficult to establish whether these changes are a manifestation of the disease itself, or are due to the direct action of cocaine, or to changes in eating patterns. Our study exposed zebrafish embryos to morphine and cocaine until 72 hpf, a stage at which embryos consume their yolk and do not show independent feeding. Thus, the changes in the expression of several genes related to lipid metabolism are instead caused by morphine or cocaine exposure, and the potential contribution of changes in the eating patterns can be ruled out. Besides, both drugs produced a decrease in cholesterol levels in embryos of 72 hpf, and cocaine also induces a significant decrease in triacylglycerol levels (preliminary data not shown), which agrees with previously published data from human studies.

It has been studied whether an impaired nutritional status in hospitalized patients with substance use disorder can be related to different factors, such as the type of drug, the intensity or length of the addiction, hepatitis B infection, or anorexia with poor food and drink consumption (Santolaria-Fernandez et al., 1995). This study concludes that most of these patients suffer from severe malnutrition, 92.4% of them being underweight and that this malnutrition is related to multifactorial aspects such as gender, type of drug, and disturbance of the social and family bonds.

One of the critical mechanisms for global regulation of gene expression is DNA methylation, which appears in CpG islands located in the regulatory promoter. Methylation of the CpG islands located in the proximal promoter usually results in gene silencing, whereas methylation within the gene body promotes gene expression (Vaillancourt et al., 2017). We have predicted the existence of CpG islands in the proximal promoter of eight genes deregulated by morphine or cocaine treatment, and these two drugs produced a statistically significant increase in DNA global methylation levels at 72 hpf. Thus, CpG islands hypermethylation could cause a decrease in their expression, as found in the transcriptomic assay. It has been shown that chronic drug exposure causes changes at the epigenetic level, such as DNA methylation, acetylation, and histone (de)methylation, which lead to chromatin remodeling and altered gene expression (Skinner, 2011; Joanna et al., 2017). Cocaine

administration causes an increased expression of DNA methyltransferases in NAc, as well as hypermethylation of DNA (Wright et al., 2015) and increased binding of the MeCP2 protein to the ppc1 promoter (the catalytic subunit of protein phosphatase-1), which induces the silencing of PP1c in NAc (Anier et al., 2010). There are few studies related to the effects of drugs on global methylation in the brain and even fewer on the whole organism. The effects of cocaine and heroin on global DNA methylation levels in the whole brain and liver of mice have been examined, and no statistically significant differences were found in both tissues; however, these authors do not rule out the possibility of finding differences among regions that would be offset in the global analysis (Fragou et al., 2013).

Our studies performed in whole zebrafish embryos show that exposure to drugs of abuse leads to changes in the expression of several genes related to different metabolic and signaling pathways that might affect the metabolic status and even developmental processes. This outcome may be due to the drug's direct action or to changes in global regulatory mechanisms, including DNA methylation. Epigenetic changes may also imply that these systemic effects can persist during the adult age and are potentially transmitted to the offspring.

AUTHOR CONTRIBUTIONS

ACG: performed the experiments, data collection, analysis, and interpretation of data, and participated in manuscript writing. MPF and DCA: performed the experiments and participated in data collection and analysis. MSB: participated in study design and data analysis; IRM: participated in data interpretation and manuscript writing; VGN: participated in study design, performed the experiments, data collection, analysis and interpretation of data, financial support, and manuscript writing. All authors have approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2022.10.017.

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