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COCAINE MODULATES THE EXPRESSION OF TRANSCRIPTION FACTORS RELATED TO THE DOPAMINERGIC SYSTEM IN ZEBRAFISH

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Abstract—Nodal-related protein, Ndr2, and transcription factors such as Lmx1b, Otp, Nurr1 and Pitx3 are very important in the differentiation, function and maintenance of mesodiencephalic dopaminergic neurons, and are necessary for the activation of tyrosine hydroxylase (TH) and dopamine (DA) transporter expression. Hence, the aim of the present work was to evaluate the effects of cocaine on the expression of genes related to the embryogenesis development of the dopaminergic system. Zebrafish embryos were exposed to cocaine hydrochloride at 5 h post-fertilization (hpf), and collected at two important stages – 24 and 48 hpf – to study the effects of cocaine on the expression of *ndr2*, the *lmx1b.1*, *lmx1b.2*, *otpa*, *otpb*, *nurr1* transcription factors, and their target genes: TH and DA transporter expression. Our results by qPCR showed that cocaine affects the expression of these genes in different ways, depending on the stage of development. Furthermore by *in situ* hybridization we observed a change in the spatial distribution of *lmx1b.1* and *lmx1b.2* at both stages (24 and 48 hpf) due to exposure to cocaine. We also show the importance of Lmx1b and Otp in *th* expression through the knockdown of Lmx1b.1 and Lmx1b.2, and of Otpa and Otpb. Additionally, cocaine produced an increase and a decrease in TH levels at 24 and at 48 hpf, respectively, possibly due to the change in the expression of the transcription factors and *ndr2* expression. We conclude that cocaine alters the correct development of dopaminergic system affecting the expression of transcription factors, during the embryogenesis.

Key words: transcription factors, dopamine system, cocaine, zebrafish, embryogenesis.

INTRODUCTION

Cocaine produces alterations in the dopaminergic pathway and its consumption during pregnancy is related to adverse effects on brain structures and functions (Bhide, 2009). The specification of the region for dopaminergic neuron generation is an event that occurs early on in mesodiencephalic neuronal development, in which the signalling of fibroblast growth factor 8, sonic hedgehog and transforming growth factor beta (TGFβ) is involved (Smidt and Burbach, 2007). Nodal-related proteins belong to a subset of the TGFβ superfamily, and the number of nodal-related molecules differs among vertebrates, such as in mice, where a single gene encodes nodal protein. In zebrafish, there are three: Ndr1 (nodal-related 1 or squint), Ndr2 (nodal-related 2 or cyclops) and southpaw (previously known as Ndr3) (Rebagliati et al., 1998b; Weng and Stemple, 2003; Tian et al., 2008). It has been shown that a lack of Ndr2 produces the absence of diencephalic catecholaminergic neurons from the pretectum and posterior tuberculum (Holzschuh et al., 2003). Also, nodal signalling has been proposed as a positive regulator of Otpb expression in the posterior basal plate of the hypothalamus of zebrafish embryos (Del Giacco et al., 2006). Otp is a transcription factor that is essential for the development of specific subsets of dopaminergic neurons in the diencephalon of zebrafish and mice (Ryu et al., 2007). Zebrafish have two duplicates of the Otp protein, Otpa and Otpb, which have a homology score of 81% and 78% with the mouse counterpart, respectively (Del Giacco et al., 2006). Both Otpa and Otpb are required for the development of hypothalamic dopaminergic neurons and can specify aspects of the identity of dopamine (DA) neurons (Ryu et al., 2007). The Lmx1b transcription factor is one of the early factors involved in the generation and differentiation of mesencephalic and diencephalic dopaminergic (mdDA) neurons (Adams et al., 2000). Many authors have suggested that Lmx1b is important for the expression of Pitx3, that regulates tyrosine hydroxylase (TH) expression (Smidt et al., 2000; Wallen and Perlmann, 2003), but the importance of Lmx1b for the expression of *nurr1* and *th* is controversial (Smidt et al., 2000).

Midbrain dopaminergic neurons have been related to the generation of pleasure and to the development of addictive process by several drugs of abuse, such as cocaine (Ang, 2006). The enzyme TH is responsible for the biosynthesis of DA in the dopaminergic neurons

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Abbreviations: Ab, antibody; BSA, bovine serum albumin; cocaine-HCl, cocaine hydrochloride; DA, dopamine; hpf, hours post-fertilization; HPLC–MS, high-performance liquid chromatography–mass spectrometry; IHC, immunohistochemistry; ISH, *in situ* hybridization; mdDA, mesencephalic and diencephalic dopaminergic; MHB, midbrain–hindbrain boundary; MO, morpholino; PBS, phosphate-buffered saline; PBT, PBS + 0.1% Tween-20; PFA, paraformaldehyde; RT, room temperature; TBS, Tris-buffered saline; TGFβ, transforming growth factor beta; TH, tyrosine hydroxylase.

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(Ang, 2006). Since zebrafish has emerged as a new model organism for the study of the development of vertebrates (Lohi et al., 2012; Santoriello and Zon, 2012), we employed this organism to better understand the expression and function of TH during the embryonic development of this organism. The zebrafish present a duplicate of *TH* gene, named: *th1* and *th2*. In the present work we studied the expression of *th1*, since the Th1 showed a major homology to the mammalian TH (Yamamoto et al., 2010) (Th1 vs. TH, 69% and Th2 vs. TH, 60%). Additionally, the expression of *th1* was more abundant in the brain than *th2* (expressed preferably in the periphery) (Chen et al., 2009). It has been described that *th2* is also important in the development of the neurons in the brain of zebrafish embryos (Filippi et al., 2010). For convenience we designated *th* and TH (refers to *th1* and Th1) for mRNA expression and protein designation, respectively.

According to the above-mentioned investigations, the nodal-related protein (Ndr2), transcription factors Lmx1a/b, Otp and Nurr1 in vertebrates are mediating development, differentiation and function of mdDA neurons (Smidt et al., 2000; Holzschuh et al., 2003; Filippi et al., 2007; Ryu et al., 2007; Yan et al., 2011), Otp and Nurr1 being necessary for the activation (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Smidt et al., 2000) and development (Ryu et al., 2007) of TH expression. Likely, overexpression of Otp and knockdown of Lmx1b have been reported to induce an increment of TH expression in ectopic sites (Filippi et al., 2007; Ryu et al., 2007), indicating that these transcription factors are important in the generation of the dopaminergic neurons.

Since these transcription factors (*lmx1b.1*, *lmx1b.2*, *otpa*, *otpb* and *nurr1*) and Ndr2 are important for the development of the dopaminergic system, our aim was to determine the effects of cocaine in the expression of these transcription factors and Ndr2 during two important stages of embryogenesis of zebrafish: when the CNS is being formed (24 hpf) and during organogenesis (48 hpf). These data can help us to understand and to seek possible treatments to avoid damage to the embryos of pregnant women who consume cocaine.

EXPERIMENTAL PROCEDURES

Animals

Wild-type zebrafish embryos (*Danio rerio*, AB strains) were used in this study and stages were considered in hours post-fertilization (hpf) according to Kimmel et al. (1995). To obtain these embryos, adult zebrafish (AB strain) were maintained on a 12-h light:12-h dark (LD) cycle at 26 °C in a multi-tank system in our Fish Facilities, simulating their environmental condition. Fertilized embryos were selected using a stereomicroscope (Leica Z2000, Nussloch, Germany), raised at 28.5 °C, and maintained in dishes containing sterile E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM $MgSO_4$ in distilled water; Sigma, Madrid, Spain). All efforts were made to minimize the number of embryos used and their suffering. All procedures and experimental protocols were in accordance with the guidelines of the European Communities Council directive of 24 November 1986 (86/609/EEC), current Spanish Legislation (BOE 67/8509-12, 1998), and following the Guide for the Care and Use of Laboratory Animals as adapted and promulgated by the US National Institutes of Health. All experiments were performed at the University of Salamanca with the approval of the University of Salamanca Animal Care Committee.

Drug treatment

Zebrafish embryos at 5 hpf were exposed to 1.5 μM cocaine hydrochloride (cocaine-HCl) diluted in E3 medium (Shang and Zhdanova, 2007; Lopez-Patino et al., 2008). We studied the effects of cocaine on *ndr2*, the *lmx1b.1*, *lmx1b.2*, *otpa*, *otpb*, and *nurr1* transcription factors, and *th* and *dat* expression at 24 and 48 hpf.

The concentration of cocaine-HCl (1.5 μM) chosen is comparable to the concentration present in human maternal and foetal serum, placental fluid and foetal urine (Kesrouani et al., 2001) and in human umbilical cord in neonates (Dempsey et al., 1999). We used higher concentrations (data not shown), and no additional effects were observed as regards the expression experiments. Considering this, and the fact that Lopez-Patino et al. (2008) did not observe anaesthetic effects in adult zebrafish, we decided to use 1.5 μM cocaine HCl.

Determination of cocaine entering zebrafish embryos by high-performance liquid chromatography–mass spectrometry (HPLC–MS)

Zebrafish embryos were exposed to 1.5 μM cocaine-HCl from 5 to 24 hpf and from 5 to 48 hpf, after which they were harvested. Embryos at both developmental stages were washed three times for 5 min each in E3 medium and dechorionated. Dechorionation was performed in order to quantify the real cocaine concentration in embryonic tissue, since the chorion might prevent cocaine from entering the embryo. The embryos were kept at −20 °C. Samples were defrosted first at 4 °C (1 h) and then at room temperature (RT). After adding 1 ml of 10 mM ammonium formate, pH 9.3 (Dienes-Nagy et al., 1999), samples were homogenized mechanically on ice with a Polytron device. Homogenized embryonic tissue was centrifuged for 30 min at 4000*g* at 4 °C and the supernatants were collected and kept at 4 °C (Dienes-Nagy et al., 1999) until HPLC–MS analysis. Six samples per developmental stage (200 embryos per sample) were analysed by HPLC–MS. HPLC–MS analyses were performed as previously described (Dienes-Nagy et al., 1999) using a Waters ZQ 4000 device with an Alliance HT HPLC apparatus. HPLC conditions were as follows: column, Atlantis T3, 3 μm, 2.1 mm × 100 mm; solution A, 10 mM ammonium formate, pH 7.0, in H_2O; solution B, methanol. Initial conditions were 30% B and a gradient was performed over 11 min to reach 100% B. The 286 and 289 u.m.a. signals were recorded and integrated in SIM mode. Cocaine-D was used as an internal deuterated standard (Cerrilliant, Round Rock, TX, USA). With this methodology, 0.175 ± 0.0472 nM of cocaine was detected in each embryo, which represents approximately 12% of the initial concentration of the 1.5 μM cocaine used.

RNA extraction

Zebrafish embryos were divided into two groups, a control group and a cocaine-treated group, for each stage of study. Total RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), following the protocol recommended by the manufacturers. In all cases RNA samples were treated with DNase I (Roche Scientific, Madrid, Spain), following the protocol recommended by the manufacturers. RNA