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NEW INSIGHTS INTO OPIOID REGULATORY PATHWAYS: INFLUENCE OF OPIOIDS ON Wnt1 EXPRESSION IN ZEBRAFISH EMBRYOS

F. M. SANCHEZ-SIMON,[a1] A. S. LEDO,[b1] R. AREVALO[b] AND R. E. RODRIGUEZ[a]*

[a]*Department of Biochemistry and Molecular Biology, Institute of Neuroscience of Castilla y León, University of Salamanca, Spain*

[b]*Department of Cellular Biology, Institute of Neuroscience of Castilla y León, University of Salamanca, Spain*

Abstract—Opioids are the most potent analgesics known today, but their prolonged administration produces severe adverse effects such as constipation, bradycardia, besides addiction, a concept not fully understood at present, which represents one of the most important challenges of modern bioscience. Wnts constitute an important family of vertebrate genes that encode secreted signaling proteins implicated in various developmental processes (patterning of the neural tube, neuronal differentiation), and are extensively conserved through evolution. In this study we have focused on Wnt1, an essential signal in axis polarity, as well as in proliferation and the development and differentiation of the CNS, roles shared by opioid receptors. Our previous studies in zebrafish show that morphine, the most potent analgesic known today, increases cell proliferation and induces neuronal protection and dopaminergic differentiation by activating the opioid receptors. The aim of the present study is to determine whether these effects are a consequence of an interaction between Wnt1 and the endogenous opioid system, which may act as a transcription regulator of Wnt1. Hence, we have exposed embryos to morphine, the endogenous delta opioid agonist Met-Enkephalin-Glu-Tyr (MEGY) (it binds with high affinity to both zebrafish delta opioid receptors, ZfDORs), and SNC80, a highly specific delta agonist, which displays low affinity towards the ZfDORs. Although at earlier stages, all opioids reduced the expression level of Wnt1, further on development, mainly during the differentiation of the CNS (24–48 h post fertilization (hpf)), morphine and MEGY increased Wnt1 expression. Our results point to the possibility that opioid signaling controls the transcription of Wnt1 and that through Wnt1, the opioid system regulates cell proliferation and neuronal differentiation. The present work opens a door to the discovery of new mechanisms that regulate opioid activity and its adverse effects, and hence, it might provide a good target to design new drugs that prevent or avoid these effects.

Key words: opioid system, Wnt1, development, zebrafish.

The endogenous opioid system is the best analgesic system known, and besides, it is implicated in the development of tolerance and dependence to drugs (opioids, cannabinoids, nicotine, alcohol) (Waldhoer et al., 2004; Corbett et al., 2006). The opioid system is constituted by the opioid receptors (mu, delta, and kappa) and the opioid peptides (enkephalins, dynorphin, endorphins), whose 3D structure is similar to that of morphine, the most potent analgesic used to control chronic pain. These receptors belong to the seven transmembrane domain receptors coupled to G proteins family. The endogenous opioid system has been implicated in developmental processes such as neurogenesis and neuronal differentiation *in vitro* (Hauser et al., 1990; Narita et al., 2006; Kim et al., 2006) and *in vivo* (Sanchez-Simon et al., 2010a), which may be related to the two main effects that opioids have, pain control and the development of addiction to the different drugs.

The Wnt1 gene encodes a signaling protein that plays important roles in the development of the central nervous system: it regulates neurogenesis, neuronal differentiation, and is involved in adult neuronal plasticity (Amoyel et al., 2005; Beaumont et al., 2007). Wnt1, as the other wnt genes, exert their action when bound to the Frizzled receptors and they can activate different signaling pathways (through β-catenin, cyclic AMP, etc; review by Montcouquiol et al., 2006). During early stages Wnt1 has a posteriorizing effect (Grove, 2002), establishing the hindbrain metameric pattern (Riley et al., 2004; Amoyel et al., 2005). The β-catenin-dependent cascade also regulates the regionalization of the forebrain promoting diencephalic fates (review by Wilson and Houart, 2004). Wnt1 has been described as a positive regulator of mitosis (Dickinson et al., 1994) and a key regulator in the establishment of the cerebellum and the optic tectum (OT) (Houart et al., 2002; Lekven et al., 2003). Also, Wnt1 has been proven to be involved in the differentiation of dopaminergic neurons (Castelo-Branco et al., 2003), possibly sharing this role with the opioid system (Sanchez-Simon et al., 2010b).

Considering that Wnt1 and the opioid system are implicated in similar developmental processes (proliferation, neuronal differentiation) it is possible that Wnt1 may be a target gene of opioid activity. Hence, in our study we analyze the effects of morphine, which binds to the three opioid receptors, SNC80, a potent delta agonist, and MEGY (Met-Enkephalin-Glu-Tyr), a novel peptidic endogenous opioid agonist from zebrafish, on the gene expression levels of Wnt1, to determine whether the Wnt signaling pathway may be regulated by the opioid system. We have used the zebrafish in our study, given the advantages it presents as an experimental model in both developmental and disease research (Fishman, 2001; review by Trede et al., 2004). The present work opens a door to the estab-

[1] Both authors contributed equally to this work.

*Corresponding author. Tel: (+34) 923294626; fax: (+34) 923294750.
E-mail address: requelmi@usal.es (R. E. Rodriguez).

Abbreviations: hpf, hours post fertilization; MEGY, Met-Enkephalin-Glu-Tyr; MHB, midbrain-hindbrain boundary; OT, optic tectum.

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lishment of new mechanisms and intracellular pathways through which opioids, endogenous and exogenous, are implicated in their various roles, besides their well known involvement in pain and addiction, that is, proliferation, neuronal differentiation, and neuronal protection.

EXPERIMENTAL PROCEDURES

Zebrafish embryos

Adult zebrafish from the AB strain bred in our own facility (Institute of Neuroscience of Castilla y Leon) were kept and raised according to standard protocols (Westerfield, 1995). Zebrafish embryos were incubated at 28.5 °C until their use. Animals were handled according to the guidelines of the European Community Council directive of November 24, 1986 (86/609/EEC), the current Spanish Legislation (RD 1201/2005, BOE 252/34367-91, 2005) and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institute of Health.

Drug treatment

Zebrafish embryos were divided into four experimental groups: control embryos (untreated), exposed to 10 nM morphine, exposed to 10 nM MEGY, and exposed to 10 nM SNC80. We have previously reported that a concentration of 10 nM morphine is the highest concentration that can be used without a toxic effect on the embryos (Sanchez-Simon et al., 2010a). Morphine was acquired from the Spanish Ministry of Health. MEGY is a zebrafish endogenous delta agonist, synthesized in the laboratory of Dr. Valencia, from the CSIC (Barcelona). SNC80 is a synthetic delta agonist acquired from Tocris. We have also used the universal opioid antagonist naloxone 1 μM (Tocris, Biogen Cientifica S.L., Madrid, Spain) to inhibit the opioid effects. Embryos were exposed to each substance at 5 h post fertilization (hpf) until their sacrifice at 8 hpf, 16 hpf, 19 hpf, 24 hpf, 48 hpf, and 72 hpf.

Real time PCR (qPCR)

Quantification of expression levels of Wnt1 was determined in embryos from the four experimental groups (control, morphine exposed, MEGY exposed, and SNC80 exposed) at the six developmental stages explained earlier in the text. Total RNA was extracted using Trizol ®Reagent (Invitrogen S.A., Madrid, Spain). cDNA synthesis was carried out by reverse transcription of total RNA to cDNA using the Promega reverse transcription KIT as previously described (Sanchez-Simon and Rodriguez, 2008).

The concentration of cDNA was determined by measuring the absorbance at 260 nm with a SmartSpec™Plus spectrophotometer (Bio-Rad Laboratories, Inc.). The quantification of the PCR products was performed using the SYBR-Green method as previously described (Sanchez-Simon and Rodriguez, 2008). The oligonucleotides used to amplify Wnt1 were ZfWnt1-F: ATGTAGGCTGATGCTTTG; ZfWnt1-R: GTCGGCTCGGAAACTGCA. PCR products were amplified in an ABI Prism 7300 detection system (Applied Biosystems, Madrid, Spain), with the following conditions: 10 min at 95 °C followed by 35 cycles of 10 s at 95 °C and 1 min at 60 °C. Three different samples have been used in the qPCR experiments and each experiment has been repeated three times. EF1α was used as internal control.

Statistical analysis. qPCR results are expressed as mean±SEM. The mean of the number of transcripts from each experimental group was compared with the mean of the number of transcripts from the control group using an ANOVA followed by Dunnett's post hoc test to strengthen the statistical analysis ($P<0.05$ and $P<0.01$, Prism; GraphPad Software, Inc., La Jolla, CA, USA).

In situ hybridization

Wnt1 and shh riboprobe plasmids were generated and gently donated by S. Wilson (University College London). It has been already tested (Cavodeassi et al., 2005). Anti-sense RNA probes were synthesized using a digoxigenin RNA labelling kit (Roche Applied Science, Madrid, Spain) and following the protocol recommended by the manufacturer.

Embryos at 24 hpf and 48 hpf were dechorionated, fixed 60 min in 4% paraformaldehyde, and conserved in methanol at −20 °C until their use. Whole mount *in situ* hybridization was performed as previously described (Shanmugalingam et al., 2000). Briefly, after rehydration, embryos were treated with proteinase K (Sigma-Aldrich, Madrid, Spain) and refixed with 4% paraformaldehyde. After 2 h of prehybridization, they were incubated overnight at 68 °C to hybridize with the probe. Embryos were washed several times with SSC during 3 h, they were blocked during 2 h and incubated over night with an anti-digoxigenin antibody conjugated with alkaline phosphatase (1:6000; Roche) at 4 °C. Embryos were washed with PBS during 2 h and the hybridization was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrates (Roche).

RESULTS

In order to make a better comparison of the changes induced by exposure to opioid agonists we have analysed the gene expression level and distribution of Wnt1 during zebrafish development. Our results show that Wnt1 is expressed from the beginning of development, 0.5 hpf, in which the number of copies almost reaches half a million copies per 25 ng of cDNA (Fig. 1). A decrease in the gene expression level of Wnt1 can be observed during midblastula transition (3 hpf), and Wnt1 is upregulated again at 8 hpf (midgastrula), when the highest peak of Wnt1 developmental expression is detected (700,000 copies per 25 ng of cDNA). Although Wnt1 transcription level decreases from 8 to 16 hpf, an expression enhancement is observed during the segmentation period (16–24 hpf), increasing progressively until 24 hpf, when the number of Wnt1 transcripts is reduced. From this stage onwards, the expres-

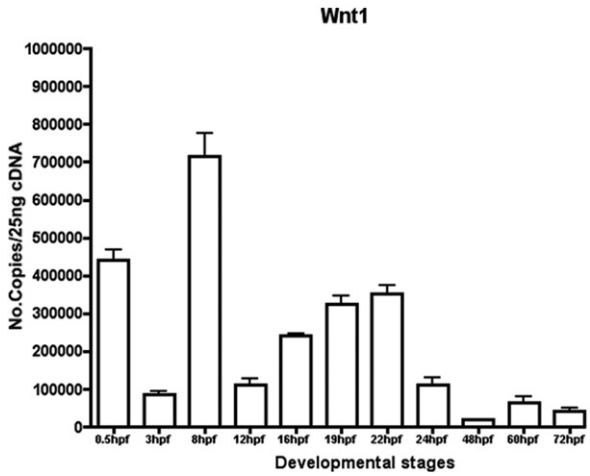

Fig. 1. Gene expression profile of Wnt1 throughout zebrafish development, from the stage of 0.5 hpf (hours post fertilization) to the last embryonic stage, that is, 72 hpf. Results are expressed as number of copies per 25 ng of cDNA.