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SUBSTANCE P mRNA EXPRESSION DURING ZEBRAFISH DEVELOPMENT: INFLUENCE OF MU OPIOID RECEPTOR AND COCAINE

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Abstract—Zebrafish has emerged as an important vertebrate animal model for the study of human diseases and for developmental studies in mammals. Since there are few studies of the tachykinin 1 gene (*TAC1*), precursor of substance P (SP), in relation to embryonic development, we aimed to study the expression of SP transcript (mRNA) and determine the influence of cocaine and opioid receptors on the expression of this neuropeptide. In order to analyse the spatial and temporal SP mRNA expression in zebrafish, we cloned – based on human *TAC1* sequence – the sequence that originates SP. Phylogenetic analyses of the precursor of SP, revealed an alignment in the fish cluster, with a clear distinction from other species (amphibians, birds and mammals). Real time PCR (qPCR) results showed that SP mRNA was expressed in several stages of embryonic development, where it increased progressively from gastrula-8 hpf (hour post-fertilisation) to the end of the embryogenesis-72 hpf. SP mRNA was expressed mainly in the spinal cord in embryos at 20–30 hpf, whereas at 36, 42 and 48 hpf embryos SP mRNA was expressed mainly in the CNS telencephalon, diencephalon, hypothalamus, rhombomeres, epiphysis and in peripheral areas (heart and somites). Exposure of embryos to 1.5 μM cocaine altered the SP mRNA expression at 24 (increasing) and 48 hpf (decreasing). We also report that knockdown of μ-opioid receptor induced an increase of SP mRNA expression while the knockdown of the two delta opioid receptors did not produce changes in SP mRNA expression. In conclusion, SP mRNA in zebrafish is expressed during embryonic development in the CNS and peripherally, suggesting that SP would play a critical role during embryogenesis. Furthermore, cocaine exposure and the knockdown of μ-opioid receptor affect the SP mRNA expression. These observations can be important in the pain and addiction field where SP is involved.

Key words: TAC1, substance P, development, cocaine, opioid receptors.

INTRODUCTION

Substance P (SP), neurokinin A (NKA), neurokinin B (NKB), endokinins (EKs) and hemokinin 1 (HK-1) are the best known members of closely related peptides that belong to the tachykinin (TK) family (Regoli et al., 1987; Pennefather et al., 2004). There are three genes that encode *TAC* precursors, *TAC1*, *TAC3* and *TAC4* (for a review, see Harrison and Geppetti, 2001; Pennefather et al., 2004). The human precursor of *TAC1* gene, also commonly known as preprotachykinin-A *(PPT-A)*, shows alternative RNA splicing, which results in four isoforms: α*TAC1*, β*TAC1*, γ*TAC1* and δ*TAC1*. All isoforms contain the region that encodes the SP sequence (Pennefather et al., 2004). The typical mammalian tachykinin member is SP, which – together with NKA, neuropeptide γ (NPγ) and HK-1 – shares the conserved C-terminal sequence -FXGLM-NH_2 (Maggi, 1995; Lin and Peter, 1997; Pennefather et al., 2004; Van Loy et al., 2010). SP can bind to neurokinin receptors; NK_1, NK_2 and NK_3 (also named TACR1, TACR2 and TACR3), but has a higher affinity for the TACR1 (affinity 0.05–0.5 nM) (Regoli et al., 1987, 1994; Maggi, 1995; Harrison and Geppetti, 2001). SP and its receptors are involved in different processes such as pain (Lin et al., 2010; Teodoro et al., 2012), vasodilatation (Patacchini and Maggi, 1995; Wong and Minson, 2006), neurogenic inflammation (Geppetti et al., 1995; Ang et al., 2011), smooth muscle activity, such as gastrointestinal tract (Cipriani et al., 2011; Yik et al., 2011) and bladder muscle contractions (Kamata et al., 1993; Chien et al., 2003; Shaffer et al., 2011). Likewise, different studies have reported that SP is found in the GABAergic projection neurons of the nucleus accumbens (NAc) (Napier et al., 1995) and use similar mechanisms than cocaine in the synapses of the NAc (Kombian et al., 2003, 2009). It has also been described that SP acts in the ventral tegmental area (VTA) (West and Michael, 1991). A decrease in SP can increase anxiety and alcohol preference while SP receptor

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Abbreviations: ANOVA, analysis of variance; CCV, common cardinal vein; Cq, cycle quantification; CRH, corticotropin-releasing hormone; EDTA, ethylenediaminetetraacetic acid; EKs, endokinins; HCl, hydrochloride; HK-1, hemokinin 1; hpf, hour post-fertilisation; MEGA, Molecular Evolutionary Genetic Analysis; MHB, midbrain–hindbrain boundary; ML, Maximum Likelihood; MOs, morpholino oligonucleotides; NAc, nucleus accumbens; NJ, Neighbour-Joining; NKA, neurokinin A; NKB, neurokinin B; NPγ, neuropeptide γ; ORF, open reading frame; PBS, phosphate saline buffer; PFA, paraformaldehyde; PPT-A, preprotachykinin-A; SEM, standard error of the mean; SP, substance P; TBS, Tris-buffered saline; TK, tachykinin; VTA, ventral tegmental area.

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activation reduces anxiety and response to alcohol (Yang et al., 2009). Accordingly, the SP neuropeptide located in the VTA and NAc plays an important role in regions related to reward and addiction. Furthermore, several studies have reported that the tachykinin and opioid systems are closely related. TAC1–DOR (Minami et al., 1995; Guan et al., 2005) and TACR1–MOR can functionally interact (Pfeiffer et al., 2003; Yu et al., 2009), where the activation of TACR1 can modulate the endocytosis of MOR (Pfeiffer et al., 2003; Yu et al., 2009) while TAC1 would modify the sensitivity of nociceptive afferents to opioids (Guan et al., 2005).

Several investigations have established that the zebrafish is a valuable vertebrate model for modelling human diseases and development studies in vertebrates (Chakraborty et al., 2009; Lohi et al., 2012; Santoriello and Zon, 2012). Recently, the *tac1* (Ogawa et al., 2012), *tac3* (*tac3a* and *tac3b*) (Biran et al., 2012; Zhou et al., 2012) (*tac3*, also named *tac2*; *tac2a* and *tac2b* by Ogawa et al., 2012) and *tac4* (*tac4a* and *tac4b*) (determined by bioinformatics predictions) (Biran et al., 2012; Zhou et al., 2012) precursors have been cloned. In a similar way the tachykinin receptors: Tacr1s (Tacr1a and Tacr1b) (López-Bellido et al., 2013), Tacr2 (determined by bioinformatics prediction) and Tacr3s (Tacr3a: Tacr3a1 and Tacr3a2, and Tacr3b) (Zhou et al., 2012) (also named Tac3rs; Tac3ra, Tac3rb and Tac3rc by Biran et al., 2012), have been cloned in recent times. Even when there are few studies about the expression of SP during the embryogenesis, they suggest that SP plays an important role in the development of the CNS and peripheral tissues (Gilbert and Emson, 1979; St John and Stephens, 1992; St John et al., 1997). Taking the above into consideration, we aimed to study the expression of SP during embryogenesis using the zebrafish as a research model. Likewise, we propose to study the interrelationship between opioid receptors and SP, since there is evidence of a close relationship between the opioid and tachykinin systems (Minami et al., 1995; Pfeiffer et al., 2003; Guan et al., 2005; Yu et al., 2009). Finally, since SP is also related to the addiction process (Kombian et al., 2003, 2009; Commons, 2010) we studied the effects of cocaine on the level of SP mRNA expression.

EXPERIMENTAL PROCEDURES

Animals

Adult zebrafish (AB strain) were raised in a 14-h-light:10-h-dark (LD) cycle at 26 °C at our Fish Facilities at the University of Salamanca. Embryos were raised at 28.5 °C and maintained in E3 medium Petri dishes (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM $MgSO_4$ in distilled water; Sigma, Madrid, Spain). Embryonic ages were expressed as hours post-fertilisation (hpf). The fish were handled according to 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 Institute of Health.

Drug treatment

Zebrafish embryos at 5 hpf were exposed to 1.5 μM cocaine hydrochloride (HCl). The 1.5 μM cocaine HCl concentration was used since this concentration is comparable to the concentration present in the human umbilical cord in neonates (Dempsey et al., 1999) and also in the placental fluid, foetal serum and urine (Kesrouani et al., 2001). Moreover, this concentration did not show anaesthetic effects in adult zebrafish (Lopez-Patino et al., 2008).

Nomenclature used in this study

The nomenclature of the genes and proteins used in this study are based on the human data base HUGO Gene Nomenclature Committee (HGNC), Rat Nomenclature Database (RGD), Mouse Genome Informatics (MGI) and Zebrafish Nomenclature Guidelines (ZFIN) (Table 1).

Cloning of *tac1* and SP from the zebrafish

Polymerase chain reaction (PCR) primers, designed using PCR Primer Stats (SMS), were obtained from Isogen, Life Science. Primers and annealing temperatures are shown in Table 2. The PCR programme used for TAC1 and SP amplification (NM_001256391.1) was 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C; 45 s at 64 °C, and 45 s at 70 °C, and a final extension temperature of 70 °C for 10 min. PCR products were visualised on agarose gel, and the fragments corresponding to the *tac1* and SP genes were cut from the agarose gel and purified with the QIAquick PCR Purification Kit, (QIAGEN) and eluted in DNase-free water. Then, the desired *tac1* (564 bp) and SP (299 bp) amplicons were subcloned using the pCR®II vector (Invitrogen). TOP 10́F cells (Invitrogen) were transformed with the constructs, and miniprep (ZYMO) and midiprep (Sigma) were performed to obtain large amounts of the construct of *tac1* and SP. These constructs were digested with XhoI and HindIII for 1 h at 37 °C and sent for sequencing using the T7 and SP6 sequencing primers, which flank the inserted DNA. All DNAs obtained were sequenced as least from three independent clones. DNA sequences were analysed

Table 1. Gene and protein nomenclature used in this study

Species	Gene	Protein	Database
Human	*TAC1*	TAC1	HGNC[a]
Rat	*Tac1*	TAC1	RGD[b]
Mouse	*Tac1*	TAC1	MGI[c]
Zebrafish	*tac1*	Tac1	ZFIN[d]

[a] http://www.genenames.org/guidelines.html.
[b] http://rgd.mcw.edu/nomen/rules-for-nomen.shtml.
[c] http://www.informatics.jax.org/mgihome/nomen/.
[d] https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines.