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Research Report

Comparative expression of *p2x* receptors and ecto-nucleoside triphosphate diphosphohydrolase 3 in hypocretin and sensory neurons in zebrafish

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ABSTRACT

The hypocretin/orexin (HCRT/ORX) excitatory neuropeptides are expressed in a small population of lateral hypothalamic cells in mammals and fish. In humans, loss of these cells causes the sleep disorder narcolepsy. Identification of genes expressed in HCRT-producing cells may be revealing as to the regulation of sleep and the pathophysiology of narcolepsy. In this study, in situ hybridization analyses were performed to characterize the expression pattern of receptors and enzyme, which regulate ATP-mediated transmission in hypocretin cells of zebrafish larvae. The zebrafish cDNA encoding the ecto-nucleoside triphosphate diphosphohydrolase 3 (ENTPD3/NTPDase3) was isolated. This transcript was found to be expressed in zebrafish HCRT cells as previously reported in mammals. It was also expressed in the cranial nerves (gV, gVII, gIV and gX) and in primary sensory neurons (i.e., Rohon–Beard neurons) in the spinal cord. The expression of known zebrafish *p2rx* purinergic receptor family members was next studied and found to overlap with the *entpd3* expression pattern. Specifically, *p2rx2*, *p2rx3.1*, *p2rx3.2* and *p2rx8* were expressed in the trigeminal ganglia and subsets of Rohon–Beard neurons. In contrast to mammals, *p2rx2* was not expressed in HCRT cells; rather, *p2rx8* was expressed with *entpd3* in this hypothalamic region. The conservation of expression of these genes in HCRT cells and sensory neurons across vertebrates suggests an important role for ATP mediated transmission in the regulation of sleep and the processing of sensory inputs.

1. Introduction

Hypocretins/orexins (HCRT/ORX) are increasingly recognized as an important neuropeptide system regulating sleep, metabolic status and other behaviors in mammals (Beuckmann and Yanagisawa, 2002). These peptides (HCRT-1 and -2) are produced by a single gene primarily expressed in a discrete number of lateral, posterior hypothalamic neurons (~1,100

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Abbreviations: HCRT, hypocretin; ORX, orexin; ATP, adenosine triphosphate; ENTPD, ecto-nucleoside triphosphate diphosphohydrolas; gV, trigeminal ganglia; gVII, facial ganglia; gIX, glossopharyngeal ganglia; gX, vagal ganglia; RB, Rohon–Beard; REM, rapid eye movement; HLA, human leukocyte antigen; HPF, hours post-fertilization; DPF, days post-fertilization; ACR, apyrase-conserved regions; ISH, in situ hybridization; LH, lateral hypothalamus

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in rats and ~70,000 in humans; Peyron et al., 1998; Thannickal et al., 2000). These cells project widely in the brain and spinal cord and provide excitatory input to numerous downstream target systems such as monoaminergic and cholinergic cell groups (Peyron et al., 1998). In mammals, HCRT deficiency produces the sleep disorder narcolepsy, a neurological condition characterized by excessive daytime sleepiness and abnormal transitions into rapid eye movement (REM) sleep (Lin et al., 1999; Chemelli et al., 1999; Peyron et al., 2000). In humans, the disorder is human leukocyte antigen (HLA) DQB1*0602 associated (Juji et al., 1984; Mignot et al., 2001) and HCRT cells are missing, as demonstrated by the concomitant loss of HCRT with other known, co-localized markers (Crocker et al., 2005; Blouin et al., 2005). It is widely assumed that HCRT cells are the target of an autoimmune process (Chabas et al., 2003). We and others have recently characterized the HCRT system in zebrafish (Kaslin et al., 2004; Faraco et al., 2006; Prober et al., 2006). In this model, there are relatively few HCRT cells (~20 per larva), which are restricted to the lateral anterior hypothalamus and project to the spinal cord, hypothalamus and other brain areas. Given their translucent nature, the ease of genetic manipulation and the small number of HCRT producing cells, the zebrafish offers a simple and unique vertebrate model with which to study this important cluster of neurons.

The potential role of purinergic signaling in HCRT cells has been outlined by recent independent reports of ecto-nucleoside triphosphate diphosphohydrolase 3 (ENTPD3/NTPDase3) and P2X2, an ATP ligand-gated cation channel receptor, co-localization with HCRT in the rat brain (Belcher et al., 2006, Florenzano et al., 2006). Purinergic signaling is mediated by cell surface P2 (P2X and P2Y) receptors that bind extracellular nucleotides, while NTPDases control the extracellular concentration and accessibility of nucleotides through hydrolyzation. In mammals, P2X receptor transmission is involved in regulating a wide range of physiological processes, most notably sensory perception (Roberts et al., 2006; Khakh and North, 2006). Seven P2X subunits have been identified and cloned (North, 1996; North and Barnard, 1997). All family members, except P2RX7, are primarily expressed in the dorsal root ganglia and trigeminal ganglia (Xiang et al., 1998). E-NTPDases, an eight-member family of proteins, are expressed in a variety of tissues including the central nervous system (CNS; Zimmermann, 2006). NTPDase1-3 and 8 are cell surface molecules known to clear the extracellular space of ATP and ADP (Zimmermann, 2006; Fausther et al., 2006). NTPDase3 (also named HB6, Smith and Kirley, 1998; and CD39L3, Chadwick and Frischauf, 1998) was first cloned from human brain and is broadly expressed in the mammalian brain and pancreas (Smith and Kirley, 1998; Chadwick and Frischauf, 1998; Belcher et al., 2006). This enzyme has transmembrane domains on the C- and N-termini that flank an extracellular active domain, which include seven apyrase-conserved regions (ACR, Handa and Guidotti, 1996; Kirley et al., 2001). NTPDase3 hydrolyzes both ATP and ADP into ADP and AMP, respectively, but hydrolyzes ATP approximately three times faster than ADP (Lavoie et al., 2004; Zimmermann, 2006). Together, the co-localization of *p2x2* and *entpd3* in mammalian HCRT neurons suggests that purinergic signaling might modulate the known functions of the HCRT system, such as sleep and energy metabolism regulation.

Only recently has the purinergic system been studied in zebrafish. Nine *p2rx* receptor genes with high homology have been isolated: *p2rx1*, *p2rx2*, *p2rx3.1*, *p2rx3.2*, *p2rx4.1*, *p2rx4.2*, *p2rx5*, *p2rx7* and *p2rx8* (also called *p2rx514*; Kucenas et al., 2003). As in mammals, some of these receptors can form homo- or heterodimers with varying electrophysiological properties. In zebrafish embryos, several *p2rx* subunits are expressed predominantly in sensory neurons—cranial sensory ganglia and Rohon–Beard (RB) cells of the spinal cord (Boue-Grabot et al., 2000; Norton et al., 2000; Egan et al., 2000; Diaz-Hernandez et al., 2002; Kucenas et al., 2003, 2006). This discrete expression pattern offers a unique opportunity to study sensory neuron development and physiology (Kucenas et al., 2006). The presence of *p2rx* expression in other areas of the brain, such as the hypothalamus, has not been reported.

E-NTPDase family members have been reported in goldfish liver and torpedo electric organ (Alleva et al., 2002; Martin-Satue et al., 2007). The ecto-nucleosidase system has also received limited attention in zebrafish, first with the biochemical study of enzymatic activity in brain membrane extracts (Rico et al., 2003; Senger et al., 2004), then with the cloning and characterization of one *entpd1* and three *entpd2* paralogues (Rico et al., 2006; Senger et al., 2006). Importantly, however, the expression pattern of the E-NTPDase family has not been studied in this species, and only two members of this putatively large gene family have been isolated. In the present study, zebrafish *entpd3* was cloned and its expression pattern characterized. *P2rx* receptor family expression pattern was also studied and co-expression of various *p2rx* subtypes with *entpd3* in selected brain areas reported, with particular attention to the hypothalamic HCRT containing region.

2. Results

2.1. *Isolation and molecular characterization of entpd3*

The recent independent evidence of localization of the purinergic signaling components NTPDase3 and P2X2 (Belcher et al., 2006; Florenzano et al., 2006) in mammalian HCRT cells triggered an effort to study this system in the translucent and rapidly developing zebrafish embryo. At this stage, none of the *p2rx* receptor family members was reported to be expressed in the fish hypothalamus and zebrafish *entpd3* was not cloned. In an effort to identify the zebrafish *entpd3* gene, a bioinformatics analysis was carried out using the mouse mRNA sequence and Ensembl (Sanger Institute, Cambridge, UK) zebrafish whole genome assemblies. This search reveals the previously isolated zebrafish *entpds* (Rico et al., 2006) and the predicted sequences of two uncharacterized genes corresponding to *entpd8* (ENSDARG00000005565) and *entpd3* (ENSDARG00000035309). Expression and splicing of the predicted *entpd3* were tested by RT-PCR, cloning and sequencing. The cDNA sequence (GenBank accession no. EF446129) revealed that *entpd3* consists of 1554 bp,