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CLONING AND FUNCTIONAL EXPRESSION OF NOVEL CHOLESTEROL TRANSPORTERS ABCG1 AND ABCG4 IN GONADOTROPIN-RELEASING HORMONE NEURONS OF THE TILAPIA

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Abstract—In addition to reproduction, gonadotropin-releasing hormone (GnRH) has been postulated to control cholesterol metabolism via cholesterol transport, which is carried out partly by the members of ATP-binding cassette (ABC) transporters G1 (ABCG1) and G4 (ABCG4). However, there is yet to be evidence demonstrating the relationship between these transporters with reference to GnRH neurons. In the present study, we cloned two ABCG1 messenger RNA (mRNA) variants and one ABCG4 mRNA and examined their expression in the brain including GnRH neurons (GnRH1, GnRH2, and GnRH3) in the cichlid tilapia (*Oreochromis niloticus*). Comparison of nucleotide sequences of the tilapia ABCG1 and ABCG4 with that of other fish species showed that both of these genes are evolutionarily conserved among fishes. ABCG1 and ABCG4 were shown to have high mRNA expressions in the CNS, pituitary, and gonads. In the brain, real-time polymerase chain reaction (PCR) showed that ABCG4 mRNA was higher than ABCG1a in all brain regions including the olfactory bulb (ABCG1=13.34, ABCG4=6796.35; $P<0.001$), dorsal telencephalon (ABCG1=8.64, ABCG4=10149.13; $P=0.001$), optic tectum (ABCG1=22.12, ABCG4=13931.04; $P<0.01$), cerebellum (ABCG1=8.68, ABCG4=12382.90; $P<0.01$), and preoptic area-midbrain-hypothalamus (ABCG1=21.36, ABCG4=13255.41; $P=0.001$). Similarly, although ABCG1 mRNA level is much higher in the pituitary compared with the brain, it was still significantly lower compared with ABCG4 (ABCG1=337.73, ABCG4=1157.87; $P=0.01$). The differential pattern of expression of ABCG1 and ABCG4 in the brain versus pituitary suggests that the two transporters are regulated by different mechanisms. Furthermore, ABCG1 and ABCG4 mRNA expressions were found in all three types of laser-captured GnRH neurons with highly similar percentage of expressions, suggesting that cholesterol efflux from GnRH neurons may require heterodimerization of both ABCG1 and ABCG4.

Key words: ABCG transporters, teleost, GnRH, lipid, cholesterol.

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Abbreviations: ABC, ATP-binding cassette; AP, adapter primer; apoE, apolipoprotein E; AUAP, abridged universal amplification primer; BSA, bovine serum albumin; Cb, Cerebellum; cDNA, complementary DNA; dNTP, deoxyribonucleotide triphosphate; GFAP, glial fibrillary acidic protein; GnRH, gonadotropin-releasing hormone; HDL, high-density lipoprotein; HRAP, homologous-restraint accumulation primer; HRPCR, homologous-restraint PCR; LXR, liver X receptor; mRNA, messenger RNA; NBD, nucleotide-binding domain; OB, Olfactory bulb; OT, optic tectum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Pit, Pituitary; PMH, preoptic area-midbrain-hypothalamus; RACE, rapid amplification of cDNA ends; RT, Reverse transcription; SMART, simple modular architecture research tool; SREBP-2, sterol regulatory element binding proteins-2; Tel, dorsal telencephalon; TM, Transmembrane

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ATP-binding cassette (ABC) transporters belong to a superfamily of transporter proteins, which consists of eight subfamilies (ABCA–ABCH). These proteins are either full or half transporters that function to transport a diverse range of compounds including essential nutrients and drugs into or out of a given cell type (Pedersen, 2007). A full transporter is made up of two half transporters. Each half transporter typically comprises a hydrophilic ATP-binding domain (also known as a nucleotide-binding domain, NBD) and a hydrophobic transmembrane (TM) region. The former provides the site for ATP-binding and hydrolysis, whereas the latter consists of several α-helices that span the membrane, providing a pathway for molecules' passage through the membrane (Higgins, 1992). The ATP-binding domain contains three highly conserved motifs, named Walker A, B, and C, whereby the Walker C motif is characteristically located between the Walker A and B motifs (Dean, 2002).

All known members of ABCG subfamily are half transporters that require dimerization for proper functioning. A feature characteristic of the ABCG family is that the orientation of the two domains (TM and ATP-binding) is reverse of that of all other eukaryotic ABC transporters, with the ATP-binding domains located at the N-terminus, whereas the TM domains at the C-terminus (Higgins, 1992). Comparison of ABCG1 and ABCG4 amino acid sequences obtained from the databases (NCBI and ENSEMBL, see Table 2) shows that both transporters are highly conserved throughout all species. ABCG1 and ABCG4 are efflux transporters that play an important role in the transport of lipid, including sterol and cholesterol, across cell membranes (Schmitz et al., 2001; Wang et al., 2004; Vaughan and Oram, 2006). They have been found to form functional heterodimers (Cserepes et al., 2004) and act in concert to promote cholesterol efflux from cells in the brain (Wang et al., 2004; Vaughan and Oram, 2006).

However, several lines of studies have reported restricted tissue distribution and distinct differences in messenger RNA (mRNA) distribution pattern of ABCG1 and ABCG4 among the regions and cell types in the brain of various animals including humans (Klucken et al., 2000; Annilo et al., 2001; Engel et al., 2001; Langmann et al., 2003; Wang et al., 2004; Tarr and Edwards, 2008). These

studies thus suggest that heterodimerization between these two proteins (i.e. ABCG1-ABCG4) might not be critical for transporter functioning and a full transporter configuration could be easily satisfied by homodimerization (i.e. ABCG1-ABCG1 and/or ABCG4-ABCG4) (Tachikawa et al., 2005).

Regardless of this, the high-sequence similarity between ABCG1 and ABCG4 led to the belief that these two transporters play the same role in the CNS. Many studies have shown that ABCG1 mediates cholesterol efflux to partially lipidated apolipoprotein E (apoE) for high-density lipoprotein (HDL) formation (Nakamura et al., 2004; Kennedy et al., 2005; Karten et al., 2006). Other reported roles of ABCG1 include modulation of amyloid β production (Kim et al., 2007; Tansley et al., 2007) and induction of apoptotic cell death (Seres et al., 2008), both of which generate intense interest in this gene as a potential target in combating Alzheimer's disease and other associated neurodegenerative disorders.

The reason behind the differential level and patterns of expression of ABCG1 and ABCG4 are not yet fully known, these distinct differences point strongly at the inevitability of separate regulatory mechanisms for these two genes in the maintenance of cholesterol balance. Evidence has shown that the agonists of liver X receptor (LXR), a regulator of cholesterol efflux, potently stimulate ABCG1 expression in primary murine astroglia (Karten et al., 2006; Tarr and Edwards, 2008) while having no significant effect on ABCG4 expression (Tarr and Edwards, 2008). Conversely, the expression of sterol regulatory element binding proteins-2 (SREBP-2), which regulates cholesterol synthesis, was found to be greatly upregulated with the transient expression of either transporters in primary mouse neurons and astrocytes (Tarr and Edwards, 2008).

Cholesterol is inextricably linked to reproduction. In addition to being an essential component in the biosynthesis of sex steroids, recent evidences have revealed the critical role of cholesterol in gonadotropin-releasing hormone (GnRH) signaling by enabling the formation of lipid raft, which is essential for GnRH receptor function (Navratil et al., 2003; Robin et al., 2008).

GnRH is the primary driving force and regulator of reproduction. It is highly conserved throughout the vertebrate species, and all species examined so far have shown to express two or more types of GnRH (Somoza et al., 2002). There are three distinct types of GnRH (GnRH1, GnRH2, and GnRH3) in advanced teleosts, such as the Nile tilapia (*Oreochromis niloticus*) (Parhar et al., 2003). GnRH1 neurons are localized in the preoptic area and mainly project to the pituitary and are neurohypotrophic in function, whereas GnRH2 and GnRH3 neurons are localized in the midbrain and terminal nerve, respectively. The fibers of these two GnRH types project throughout the brain and are proposed to be neuromodulatory in function (Parhar et al., 2005). Moreover, GnRH2—the most conserved form of GnRH—has been implicated in the modulation of food intake (Kauffman et al., 2006). In the aforementioned study, GnRH2 treatment decreased food intake in female musk shrew, and food restriction in turn decreased GnRH2 mRNA level in the midbrain and GnRH2 peptide in other brain areas. These data are in agreement with that found in the goldfish (Matsuda et al., 2008) and strongly suggest that GnRH2 is involved in the control of energy metabolism (Matsuda et al., 2008). Whether the role of GnRH2 includes the control of cholesterol and whether this function is shared by GnRH3 remain to be established.

It has been documented, however, that three types of GnRH receptors (GnRHR1, GnRHR2, and GnRHR3) are widely distributed in the brain (Soga et al., 2005). The knowledge of the exact ligand for each type of GnRH receptor remains elusive, but it has been postulated that each GnRHR is activated by more than one type of ligand, albeit at a different potency (Illing et al., 1999; Okubo et al., 2001; Robison et al., 2001; Bogerd et al., 2002). As cholesterol biosynthesis is an energy-costly process, instead of constant production and break down, it follows reason that cholesterol is recycled and redistributed in the brain according to need. The widespread projections of GnRH neuronal fibers and expression of GnRHR make the GnRH network a potential system to modulate cholesterol redistribution to maintain cholesterol homeostasis in the brain via the ABCG1 and ABCG4 cholesterol efflux transporters. However, the exact link between GnRH and cholesterol transport has not been elucidated.

To gain deeper insights into the possible functions of ABCG1 and ABCG4, we investigate brain region-specific or cell type-specific expression of ABCG1 and ABCG4 mRNAs. The tilapia represents a valuable experimental model in the study of cholesterol regulation, as it is a mouth-brooding fish, and thus, it has a natural adaptation mechanism to survive ~12-day period of total food deprivation. Thus, in this study, we: (i) cloned the full-length complementary DNAs (cDNAs) for ABCG1 and ABCG4 from the brain of tilapia and determined the homology between their deduced amino acid sequences and those in other species, (ii) determined the levels of ABCG1 and ABCG4 mRNA expression in different tissue types as well as brain regions using Reverse transcription (RT)-polymerase chain reaction (PCR) and real-time PCR, and (iii) examined the expression of ABCG1 and ABCG4 mRNAs in the three types of single-cell laser-captured GnRH neurons in the brain to understand the relationship between ABCG1/ABCG4 and GnRH neuronal subtypes. This will then set the stage to further our understanding of the role of GnRH on the regulation of cholesterol homeostasis via cholesterol transport.

EXPERIMENTAL PROCEDURES

Animals

Mature male and female tilapia (~6 months old; standard length=15–17cm), housed individually, fed *ad libitum* twice a day, and maintained in fresh water at 27±1 °C with natural photo regimen (14/10-h light/dark cycle) were used in the present study. A minimum number of fish that was enough to enable statistical analysis were used and all animals were anesthetized by immersion into 0.01% benzocaine (Sigma-Aldrich, MO, USA) solution for 5 min before sampling to minimize suffering. All experimental