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Review

The time enzyme in melatonin biosynthesis in fish: Day/night expressions of three aralkylamine *N*-acetyltransferase genes in three-spined stickleback

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ABSTRACT

In vertebrates, aralkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87) is a time-keeping enzyme in melatonin (Mel) biosynthesis. Uniquely in fish, there are several AANAT isozymes belonging to two AANAT subfamilies, AANAT1 and AANAT2, which are encoded by distinct genes. The different substrate preferences, kinetics and spatial expression patterns of isozymes indicate that they may have different functions. In the three-spined stickleback (*Gasterosteus aculeatus*), there are three genes encoding three AANAT isozymes. In this study, for the first time, the levels of *aanat1a*, *aanat1b* and *aanat2* mRNAs are measured by absolute RT-qPCR in the brain, eye, skin, stomach, gut, heart and kidney collected at noon and midnight. Melatonin levels are analysed by HPLC with fluorescence detection in homogenates of the brain, eye, skin and kidney. The levels of *aanats* mRNAs differ significantly within and among organs. In the brain, eye, stomach and gut, there are day/night variations in *aanats* mRNAs levels. The highest levels of *aanat1a* and *aanat1b* mRNAs are in the eye. The extremely high expressions of these genes which are reflected in the highest Mel concentrations at this site at noon and midnight strongly suggest that the eye is an important source of the hormone in the three-spined sticklebacks. A very low level of *aanat2* mRNA in all organs may suggest that AANAT1a and/or AANAT1b are principal isozymes in the three-spine sticklebacks. A presence of the isozymes of defined substrate preferences provides opportunity for control of acetylation of amines by modulation of individual *aanat* expression and permits the fine-tuning of indolethylamines and phenylethylamines metabolism to meet the particular needs of a given organ.

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1. Overview

The aralkylamine *N*-acetyltransferase also known as serotonin *N*-acetyltransferase (AANAT; EC 2.3.1.87) belongs to the acetyltransferase enzymes superfamily. In vertebrates, it is a well-recognized enzyme controlling the daily rhythm of melatonin synthesis (Falcón et al., 2009). Melatonin (Mel; *N*-acetyl-5methoxytryptamine) is a well-known component of the endogenous circadian clock and calendar in vertebrates, including fish (Reiter et al., 2010). This time-keeping hormone is synthetized from serotonin by successive action of two enzymes: AANAT and acetylserotonin *O*-methyltransferase (ASMT; EC 2.1.1.4.) at two major sites: pinealocytes in the pineal gland and photoreceptor cells in the retina, mostly during the night (Klein et al., 1997). It is established that the daily rhythm of Mel production is regulated by diurnal changes in AANAT capacity to acetylate serotonin and therefore AANAT has been named the "Timezyme" (Klein, 2007). In animals living in temperate and arctic regions, an annual pattern of Mel synthesis serves as an "endocrine calendar": there are short-night periods of increased synthesis in summer and long-night periods of increased synthesis in winter (García-Allegue et al., 2001; Sokołowska et al., 2004). A pattern of Mel synthesis in fish, as ectothermic, is regulated not only by photoperiod but also temperature which fluctuates on a daily and seasonal basis (Zachmann et al., 1991, 1992; Falcón et al., 1994). In several fish species as rainbow trout (*Oncorhynchus mykiss*), white sucker (*Catostomus commersoni*) and goldfish (*Carassius auratus*), Mel synthesis increases with rising water temperature but only within the optimal temperature ranges which are different for each species (Max and Menaker, 1992; Zachmann et al., 1992; Iigo and Aida, 1995). This effect has been explained by influence of temperature on kinetic properties of the enzymes in Mel biosynthesis pathway; among them is AANAT (Cazaméa-Catalan et al., 2012, 2013).

Teleost fish are unique among vertebrates because they possess several AANAT isozymes belonging to two AANAT subfamilies, AANAT1 and AANAT2, while in most vertebrates only a single AANAT has been identified to date. In terms of sequence, teleostean AANAT1 subfamily is more closely related to AANAT of other vertebrates, while AANAT2 subfamily has no counterpart (Coon and Klein, 2006; Cazaméa-Catalan et al., 2014). Quite recently Cazaméa-Catalan et al. (2014) and Li et al. (2016), in in silico studies, have presented multiple AANAT isoforms in several fish species, but the first data on different characteristic of AANAT isozymes in fish in the retina and pineal have been provided by Falcón et al. (1996), Coon et al. (1999) and Benyassi et al. (2000). For instance, Falcón et al. (1996) have been the first to consider the presence of a different AANAT in the pineal organ and retina in the northern pike (*Esox lucius*), and Coon et al. (1999) have shown a comparable affinity of AANAT1 for indolethylamines and phenylethylamines in the retina and a preferential affinity of AANAT2 for indolethylamines in the pineal in the same species. The later reports on substrate preferences, kinetics and spatial expression patterns of AANAT1 and AANAT2 have demonstrated that AANAT2 is dedicated to Mel synthesis and AANAT1 covers wider range of activities (Zilberman-Peled et al., 2004, 2006). For example, AANAT1 acetylating aralkylamines besides serotonin (Nisembaum et al., 2013; Paulin et al., 2015) can be involved in metabolism of dopamine and preventing toxic reactions (Iuvone et al., 2005; Zilberman-Peled et al., 2006). There are several findings in fish indicating that AANAT1 in the retina has a much wider role beyond "timezyme". Besseau et al. (2006) have shown that the activity of AANAT1 in the rainbow trout retina is low throughout the night and increases during the light period, in contrast to activity of AANAT2 in the pineal organ. They proposed that the function of AANAT1 in the retina is related to aromatic amine detoxification rather than keeping daily rhythm of Mel synthesis (Besseau et al., 2006). It may explain high Mel production in the retina during the photophase that was reported earlier by Gern and colleagues in this species (Gern et al., 1978).

Regarding the genes encoding AANAT in fish, as early as 1998 Bégay and co-workers have detected AANAT transcripts in the pineal organ of the rainbow trout and pike and in the retina and pineal organ of the zebrafish (*Danio rerio*) (Bégay et al., 1998). Coon with co- workers thereupon has distinguished two different AANAT genes (*aanat*) in the pike, *aanat1* expressed in the retina and *aanat2* in the pineal gland (Coon et al., 1999). Three different AANAT genes, two related to AANAT1, *aanat1a* and *aanat1b,* and third related to AANAT2, *aanat2*, have been shown by in silico analysis in the genome of the pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*) and medaka (*Oryzias latipes*) by Coon and Klein (2006). Later on, Isorna et al. (2011) have cloned two *aanat* cDNAs corresponding to the *aanat1a* and *aanat1b*, and analysed the relative expression of these genes by RT-qPCR in the retina of the sole (*Solea senegalensis*) (Isorna et al., 2011). Authors have postulated the different functions of two isoforms, because the profiles of *aanat1a* and *aanat1b* expression changed during sole development: *aanat1a* mRNA predominated before metamorphosis and did not exhibit rhythmicity and the *aanat1b* mRNA predominated after metamorphosis and displayed a daily rhythm. Quite recently Paulin et al. (2015) have demonstrated in the sea bass (*Dicentrarchus labrax*) that *aanat1a* and *aanat1b* are expressed in different brain regions, retina and peripheral organs/tissues, i.e. *aanat1a* and *aanat1b* in gonads, intestine, liver and muscle, and *aanat1b* in gills and heart whereas *aanat2* only in pineal and gonads. The findings of rhythmic expression of *aanat2*, lower at midday and higher at midnight, in the turbot (*Scophthalmus maximus*), zebrafish and sole (*Solea senegalensis*) at different stages of development (Vuilleumier et al., 2007; Isorna et al., 2009) are in agreement with the role of AANAT2 in timekeeping (Falcón et al., 2009).

In our study, for the first time, the levels of *aanat1a*, *aanat1b* and *aanat2* mRNAs are analysed by absolute quantitative real-time polymerase chain reaction preceded by reverse transcription (RT-qPCR) in the whole brain, eye, skin, stomach, gut, heart and kidney of the three-spined stickleback (*Gasterosteus aculeatus*). A quantitative comparison of three *aanat*s expression in various organs of the three-spined stickleback at daytime and night is the first step to recognize physiological status of AANAT isozymes at different locations in fish. There is a lot of evidence that AANAT role extends far beyond that of the time enzyme in Mel biosynthesis, thus AANAT as a multi-faceted enzyme merits consideration and extensive studies.

1.1. Why the absolute quantitative RT-qPCR?

In real-time PCR, there are two strategies: genes mRNA levels can be quantified by relative or absolute RT-qPCR. So far, in most studies of expression of *aanat*s the relative method has been applied. In such a case, one or more housekeeping genes are recommended for data normalization and the expressions of genes of interest in different organs/tissues represent the relative values versus the reference (organ or tissue) (Livak and Schmittgen, 2001; Pfaffl, 2001). However, such scientific approach where arbitrarily chosen organ/tissue is used as a reference has its limitation because results strongly depend on this choice and may mislead. What is more, there is no justification from either physiological or anatomical point of view for the choice of any particular organ/tissue as a reference. The pitfalls associated with using the reference genes in qPCR technique and the relative method in calculation of mRNA levels are extensively discussed by Kozera and Rapacz (2013). Also Paulin et al. (2015) have found the comparison between relative amounts of *aanat* mRNAs in the retina and pineal problematic. The absolute quantitative RT-qPCR method where mRNA levels are calculated on the basis of a standard curve helps us to deal with the problem and enables us to compare the expressions of genes encoding AANATs in different organs.

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