Brain expression of tyrosine hydroxylase and its regulation by steroid hormones in the European eel quantified by real-time PCR

F.-A. Weltzien[1,2], C. Pasqualini[2], N. Le Belle[1], B. Vidal[1], P. Vernier[2], S. Dufour[1]*

[1] USM 0401, UMR 5178 CNRS/MNHN/UPMC Biologie des Organismes Marins et Ecosystèmes, Département des Milieux et Peuplements Aquatiques, Muséum National d'Histoire Naturelle, 75231 Paris Cedex 05, France; [2] DEPSN, UPR CNRS 2197, Institut de Neurobiologie Alfred Fessard, CNRS, 91198 Gif-sur-Yvette Cedex, France

*Corresponding author (Tel: +33 1 40793612; Fax: +33 1 40793618; E-mail: dufour@mnhn.fr)

ABSTRACT

In the eel, dopamine inhibits pubertal development. To investigate the regulatory mechanisms involved, we developed a quantitative real-time RT-PCR assay for measurement of brain expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine. TH expression was highest in the olfactory bulb, followed by the di-/mesencephalic areas, and the telencephalon/preoptic area. TH expression in the optic lobes and in the hindbrain was low or below the detection limit. In vivo treatment with testosterone, but not estradiol, resulted in increased TH expression in the forebrain, except optic tectum, but not in the hindbrain. The results were confirmed by in situ hybridization.

Keywords: dopamine, Elopomorpha, puberty, steroid feedback, teleost.

INTRODUCTION

In many adult teleosts, dopamine (DA) plays an inhibitory role on the control of luteinizing hormone (LH) and on ovulation/spermiation. Whether DA plays a role also in the regulation of puberty and the early stages of gametogenesis is not well established. The European eel is a

useful model to investigate the role of DA in pubertal development because of its unique life cycle with a long prepubertal stage and a blockage of further development until the reproductive oceanic migration takes place. Earlier results from our lab indeed showed that DA plays a key inhibitory role in the pubertal development of the eel [1]. To investigate the molecular regulation of this DAergic inhibition of puberty, we developed a quantitative real-time RT-PCR (qrtRT-PCR) assay for the brain expression of eel tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, and previously characterized by our group [2]. A careful validation of the qrtRT-PCR assay included comparison of different reference genes, and comparison of expression patterns with those obtained using in situ hybridization. The validated assay was then used to study the distribution of TH in the eel brain and the effects of steroid hormones on the expression of this enzyme.

MATERIALS AND METHODS

Female freshwater European eels (Anguilla anguilla L.) were netted in the Loire River during their downstream migration (prepubertal "silver" stage). For in vivo steroid treatments, eels received weekly perivisceral injections of 2µg/g BW of estradiol (E2), testosterone (T) or saline as control for 6-8 weeks according to [3]. At the end of the experiments, eels were killed and the brain dissected into different regions. Total RNA was extracted using the FastRNA Pro Green kit (Qbiogene, CA USA), followed by DNase I treatment (Ambion, UK). Reverse transcription was performed using 2 µg total RNA. The assay for eel TH was set up using the Light Cycler system (Roche, France) with CYBRgreen non-specific detection. As reference genes, we used either eel cytochrome b (cytb), or eel acidic ribosomal phospholipoprotein P0 (ARP). Except for cytb, which contains no introns, primer sequences were designed in exon-exon borders to avoid amplification of genomic DNA. PCR was performed in a total volume of 15 µl using 40-45

cycles at 94°C for 15 s, 58°C for 5 s, and 72°C for 10 s. Before analyzing the unknown samples, serial cDNA dilutions from each brain part were set up and run in duplicate both of target gene and reference genes to assess PCR efficiency and which dilutions to use for the unknown samples. In situ hybridization was performed according to [4].

RESULTS AND DISCUSSION

In this work, we have established and validated a qrtRT-PCR assay for relative quantification of eel TH, using as reference genes either eel cytb or eel ARP. The TH qrtRT-PCR assay gave highly reproducible results, amplifying unique and specific products of 100-200 bp in length for the three genes (TH, cytb, and ARP). Melting curve analysis, gel electrophoresis, and sequencing assessed the identity of the products. Using cytb or ARP as reference genes gave similar results on the relative expression of TH, indicating that although cytb contains no introns, it can still be reliably used as a reference gene in expression studies.

Results showed a distinct spatial distribution of TH mRNA in female silver eels: Highest relative levels were found in the olfactory bulbs, followed by the di-/mesencephalic areas and telencephalon (including preoptic area). TH was detectable but at low levels in the optic tectum and medulla oblongata, while TH expression was undetectable in the cerebellum. This spatial distribution of TH expression is in agreement with earlier analysis using Northern blot, RT-PCR, or immunohistochemistry [1, 2, 4], and by in situ hybridization, confirming the specificity of the assay.

In vivo treatment with T resulted in an increased expression of TH in all parts of the forebrain except the optic tectum, but not in the hindbrain. In situ hybridization analyses support these data by showing more intense staining after T treatment, particularly in the nucleus preopticus anteroventralis (NPOav), the region from where the DAergic neurons innervating the

gonadotropes originate. The stimulatory effect of T was specific as E2 treatment had a slight inhibitory effect on brain TH expression. Earlier studies in the silver eel have showed differential effects of T and E2 on the gonadotropic axis: on FSH, LH,mGnRH and cGnRH-II both in vivo (FSH/LH/GnRH) and in vitro (LH) [3, 5]. The positive effect of T, but not of E2, on TH is different from that reported in adult teleosts (e.g. trout), where E2 is thought to increase the DAergic tone during vitellogenesis [6]. In the eel, androgens are believed to play a key-role in the silvering process in both sexes. Our data suggest that T may be also responsible for the early up-regulation of the DAergic tone in the prepubertal silver eel

ACKNOWLEDGEMENTS

We thank Sophie Coudouel for cloning of ARP and Fréderic Moret for helpful advice. Supported by European projects MCFI-2002-01609 to FAW and Q5RS-2001-01836-EELREP to SD.

REFERENCES

1. Vidal, B. et al. 2004. Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: A neuroendocrine lock for the onset of puberty. Biol. Reprod. 71: 1491-1500.

2. Boularand, S. et al. 1998. Tyrosine hydroxylase in the european eel (*Anguilla anguilla*): cDNA cloning, brain distribution, and phylogenetic analysis. J. Neurochem. 71: 460-470.

3. Montero, M. et al. 1995. Differential regulation of the two forms of gonadotropin-releasing hormone (mGnRH and cGnRH-II) by sex steroids in the European female silver eel (*Anguilla anguilla*). Neuroendocrinology 61: 525-535.

4. Kapsimali, M. et al. 2000. Distribution of the mRNA encoding the four dopamine D1 receptor subtypes in the brain of the European eel (Anguilla anguilla): comparative approach to the function of D1 receptors in vertebrates. J. Comp. Neurol. 419: 320-343.

5. Schmitz, M. et al. 2004. Differential feedback by sexual steroids is involved in the opposite regulation of luteinizing hormone and follicle-stimulating hormone expression during ovarian development in the European eel. (submitted).

6. Saligaut, C. et al. 1998. Release of pituitary gonadotrophins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*): modulation by estradiol and catecholamines. Gen. Comp. Endocrinol. 109: 302-309.