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Research paper

The effects of migratory stage and 11-ketotestosterone on the expression of rod opsin genes in the shortfinned eel (*Anguilla australis*)

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ABSTRACT

The androgen 11-ketotestosterone (11KT) can induce many of the changes associated with silvering, i.e., the transformation of a non-migrating 'yellow' eel into a migrating 'silver' eel. We posited that plasticity in spectral sensitivity of the eye, accompanied by expression of different opsins in the retina during silvering, is controlled by 11KT. To test this hypothesis, mRNA levels of freshwater (*fwo*) and seawater (*swo*) opsins and of the two androgen receptors (*ara* and *arb*) in retinas of wild-caught female shortfinned eels, *Anguilla australis* were compared. *Swo* expression was much higher (3–4 orders of magnitude) and *fwo* expression substantially lower in silver than in yellow eels, whereas mRNA levels of both *ars* did not differ between stages. Yellow eel retinas exposed to 11KT *in vitro* exhibited a robust dose-dependent increase in *swo*, but weak decreasing effects on *fwo* transcript abundance were inconsistent. Similarly, increased retinal *swo* expression was seen after *in vivo* treatment of yellow eels with 11KT implants, whereas expression of *fwo* remained unaffected. Lastly, co-treatment with 11KT and the androgen receptor blocker flutamide was undertaken to determine whether 11KT exerts its effects through nuclear androgen receptors. Flutamide did not block 11KT-affected expression of any target gene, neither *in vivo* nor *in vitro*. We conclude that 11KT greatly increases the abundance of *swo*, identifying the androgen as an important regulator of the opsin switch during silvering in freshwater eels.

1. Introduction

Freshwater eels have an extra-ordinary life history, characterized by fascinating, long-distance migrations, by movements between fresh- and seawater environments and by transformations that make these activities possible (Aoyama and Miller, 2003; Bruijs and Durif, 2009; Schmidt, 1923; Lokman, 2016). In brief, adults spawn in the ocean and eggs hatch and develop into leafshape-like, leptocephalus larvae that travel to distant freshwater habitats on ocean currents (Van Ginneken and Maes, 2005). Prior to entry into fresh water, larvae metamorphose into glass eels, and then start acquiring pigmentation to become so-called "yellow" or non-migrant eels (Bruijs and Durif, 2009). Upon reaching adult size, they transform – an event known as "silvering" – into a "silver" or migrant eel that is pre-adapted morphologically, behaviorally and physiologically to life in the ocean (Aoyama and Miller, 2003) – oceanic life is a prerequisite for successful return to their natal area where the eels will spawn to complete their life cycle (Bruijs and Durif, 2009; Tesch and Rohlf, 2003).

Silvering involves a suite of changes that typically include color changes to the pectoral fins and skin (e.g., Todd, 1981; Okamura et al., 2007; Jessop, 1987), flattening of the head, and slimming and lengthening of the lower jaw (c.f., Lokman et al., 2003). One of the most distinctive changes that further occurs during silvering is an increase in eye size (Durif et al., 2005; Sudo et al., 2011; Hagihara et al., 2012; Todd, 1981; Jessop, 1987). There are also changes in retinal function, reflected in the expression of different visual pigments (e.g., Archer et al., 1995).

Visual pigments, located on photoreceptor cells (rods and cones), are responsible for light absorbance in the retina. In rods, the pigments are complexes composed of an opsin, which is a G-protein linked membrane receptor, and a vitamin A-like chromophore, either Vitamin A1 or A2. The resulting pigments, known as rhodopsin and porphyropsin, display sensitivity to shorter and longer light wavelengths, respectively (Beatty, 1975; Bridges, 1972), photic spectral sensitivity ultimately being determined by

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the ratio of both in the retina. Yellow eels utilize more porphyropsin (λ_{max} = 523 nm) than rhodopsin (λ_{max} = 501 nm) to yield an overall retinal λ_{max} ranging between 515 and 518 nm in the American eel, *A. rostrata* (Beatty, 1975). It has been suggested that this provides high sensitivity to yellow/green wavelengths in the freshwater habitat (Wood et al., 1992).

In most teleosts, only a single rod opsin is typically expressed in the retina and the vitamin A1/A2 ratio is used to alter spectral specificity. However, many *Anguilla* spp. can further alter spectral specificity by producing a novel, deep-sea opsin in preparation for migration (Beatty, 1975; Hope et al., 1998; Zhang et al., 2000; Wang et al., 2014). The retina of the yellow eel is dominated by the freshwater opsin and following an 'opsin switch' the silver eel retina becomes dominated by the deep-sea opsin in the American eel (Beatty, 1975) and the Japanese eel (Zhang et al., 2000). Similarly, artificially matured European eels experience this switch from high freshwater opsin expression to high deep-sea opsin expression in the retina during development (Hope et al., 1998). Accordingly, with transition from the yellow to silver stage, there is a shift in retinal spectral sensitivity from λ_{max} = 523 nm to 482 nm in the European eel (Carlisle and Denton, 1959; Hope et al., 1998), and from λ_{max} = 515 nm to 484 nm in the American eel (Beatty, 1975). The change in visual pigments in the rod photoreceptor results in a change from green-light to blue-light sensitivity, which reflects the wavelengths that eels will encounter during migration from green-lit fresh water to a blue-lit deep-sea environment (Archer et al., 1995).

In several fish species, including rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*), opsin gene expression can be affected by thyroid hormones (TH) (Suliman and Flamarique, 2014); however, no changes in levels of TH were found in European eel in relation to silvering or migration (Aroua et al., 2005). In stickleback (*Gasterosteus aculeatus*), the androgen 11-ketoandrostenedione up-regulated the expression of a red-sensitive opsin, associated with a change in red-light sensitivity during the breeding period (Shao et al., 2014). Likewise, a change in opsin expression was induced by treatment with gonadotropin, which mediates its effects, in part by steroid hormones, in European eel (Archer et al., 1995; Hope et al., 1998; Wood and Partridge, 1993).

Interestingly, plasma levels of the steroid hormone 11-ketotestosterone (11KT) are much higher in silver than in yellow eels (Lokman et al., 1998; Sudo et al., 2011). Moreover, experimental exposure of yellow eels to 11KT results in dramatic changes in appearance, similar to those seen during silvering – notably, it caused a slimming of the snout, blackening of the pectoral fins and an increase in eye size, alongside a suite of other changes (Rohr et al., 2001). Consequently, 11KT appears to be a key driver of silvering in eel. This prompted us to hypothesize that 11KT will control the 'opsin switch' that pre-adapts the silver eel to an oceanic existence.

To test this hypothesis, we sampled wild yellow and silver New Zealand shortfinned eels, *A. australis*, to provide a baseline on expression of the freshwater (*fwo*) and seawater opsins (*swo*) in the retina. Subsequently, we determined the effect of exogenous treatment with 11KT on the expression of both opsins in the retina using *in vivo* and *in vitro* approaches. Additionally, we determined the expression of the presumed mediators of 11KT action, i.e., androgen receptor-a (Ara/*ara*) and -b (Arb/*arb*) (Ikeuchi et al., 1999), in the retina. Lastly, we employed the androgen receptor blocker flutamide (c.f., Kwon et al., 2005) to further characterize the androgen-dependence of regulation of opsin gene expression in the retina. Our findings provide compelling evidence for a role of 11KT in opsin switching during silvering of freshwater eels, principally by up-regulating retinal *swo* gene expression.

2. Materials and methods

2.1. Animal collection and experimental design

Female shortfinned eels were supplied by a commercial fishery (Mossburn Enterprises) and sourced from Lake Ellesmere, New Zealand during early autumn. Fish were either sampled soon after capture (Section 2.1.1) or transported to our laboratory facilities at the University of Otago, Dunedin, for experimental use (Sections 2.1.2 and 2.1.3). Eels kept in captivity were maintained in tanks with recirculating water at ambient temperature (10–13 °C) and a salinity of 12 ppt. Prior to experimentation, water temperature was gradually increased to 16 °C, with light cycles set to a regime of 12 light:

12 dark hours. Fish were not fed during this time. Collection and manipulation of eels were approved by the University of Otago Animal Ethics Committee in accordance with the guidelines of the Australian & New Zealand Council for the Care of Animals in Research and Teaching.

2.1.1. Field survey: Rod opsin and androgen receptor mRNA levels in the retina of wild-caught shortfinned eels in yellow and silver stages

Eels harvested from fyke nets set overnight were assigned, based on size and migratory status, to one of three categories designed to represent successive stages of ontogenetic development. Categories (N = 7 in each) utilized were small-sized (body weight, BW, 300–600 g) non-migrant 'yellow' eels (YS), medium-sized (BW 800–1800 g) non-migrant 'yellow' eels (YM), and migrant 'silver' eels (S; BW 800–1400 g). Silver eels were distinguished from yellow eels using previously established morphological differences in coloration and head shape (Todd, 1981).

Eels were euthanized with benzocaine (0.3 g/L) and physical parameters, i.e., BW, length and eye diameter (vertical and horizontal), were measured; the latter two measures were used to calculate the eye index (eye surface area standardized over body length) after Pankhurst (1982). The tail was transected and blood collected for steroid measurement by radioimmunoassay (Section 2.2). Thereafter, ovaries were dissected and weighed to allow the calculation of the gonadosomatic index (GSI: ovary as a percentage of body weight). Eyecups, without lens and iris, were snap-frozen on dry ice, stored at −70 °C and utilized for quantification of mRNA levels of *swo, fwo, ara, arb,* and the reference gene, ribosomal protein l36 (*l36*) (see Section 2.3).

2.1.2. In vitro effects of 11KT on mRNA levels of rod opsins and androgen receptors in retinal tissue of shortfinned eels

Effects of a suite of compounds on rod opsin gene expression in eel retinas were preceded by several preliminary trials in order to optimize incubation conditions. Thus, eyes were retrieved from the eel head, placed in Ringer solution, and connective tissue was carefully removed using scissors and forceps. Fine scissors were used to separate the black eyecup from the front part of the eye by cutting along the edge of the gold-iridescent iris. The iris, lens and vitreous humor were discarded and the empty eye cup was then cut open and the pigment layer was *very gently* scraped off, leaving a thin layer of tissue < 200 µm thick (Suppl Fig. 1, Fig. 1). The tissue was carefully cut into small pieces (around 15 mm^2) and these were placed in wells of a 24-well plate in the presence or absence of the compounds of interest.

All incubations were maintained under agitation and set up by submerging the tissues in eel ringer containing 0.1 g/L streptomycin and 100,000 IU/mL penicillin; the use of an agarose support on which a piece of PVDF membrane was placed to enable retinal tissue to be incubated at the interface between incubation medium

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