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The distributions of the duplicate oestrogen receptors ER-βa and ER-βb in the forebrain of the Atlantic croaker (*Micropogonias undulatus*): evidence for subfunctionalization after gene duplication

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Teleost fishes have three distinct oestrogen receptor (ER) subtypes: ER-α, ER-βa (or ER-γ) and ER-βb. ER-βa and ER-βb arose from a duplication of an ancestral *ER-β* gene early in the teleost lineage. Here, we describe the distribution of the three ER mRNAs in the hypothalamus and cerebellum of the Atlantic croaker to address two issues: the specific functions of multiple ERs in the neuroendocrine system and the evolution and fate of duplicated genes. ER-α was detected in nuclei of the preoptic area (POA) and hypothalamus previously shown to possess ER-αs in teleosts. AcER-βb, but not ER-βa, labelling was detected in the magnocellular neurons of the POA, nucleus posterior tuberis, the nucleus recessus posterior and cerebellum. By contrast, acER-βa, but not ER-βb, was detected in the dorsal anterior parvocellular POA and suprachiasmatic nucleus. Both ER-βs were found in posterior parvocellular and ventral anterior POA nuclei, the ventral hypothalamus, and periventricular dorsal hypothalamus. The differences we observed in ER subtype mRNA distribution within well-characterized brain nuclei suggest that ER-βa and ER-βb have distinct functions in the neuroendocrine control of reproduction and behaviour, and provide evidence that the teleost *ER-β* paralogues have partitioned functions of the ancestral *ER-β* gene they shared with tetrapods.

Keywords: oestrogen receptor; gene duplication; teleost fishes; neuroendocrine regulation; hypothalamus; brain

1. INTRODUCTION

Steroid hormone receptors are members of a large family of ligand-activated nuclear transcription factors that are critical to the reproduction, differentiation and development of vertebrates. All steroid receptors, including androgen, progesterone, glucocorticoid and mineralcorticoid receptors, are derived from an ancient oestrogen receptor (ER) that duplicated multiple times during early vertebrate evolution (Thornton 2001).

The primary physiological ligand for ERs is the sex steroid oestradiol-17β (oestradiol, E_2). Oestradiol, synthesized in the gonads or locally in the brain by aromatization of testosterone, plays an important role in the neuroendocrine control of reproduction and reproductive behaviour in vertebrates (Knobil & Neill 1994). Oestradiol acts in the pituitary and the brain to influence the secretion of gonadotropin-releasing hormone (GnRH) and other neuropeptides that control reproduction and behaviour (Gore 2002; Shupnik 2002). In addition to nuclear ERs, there is evidence for membrane localization of ERs and novel ERs in brain tissues (Blaustein 2004; Thomas *et al.* 2004; Toran-Allerand 2004).

Despite intense focus on oestrogen actions, the presence of more than one ER subtype escaped notice for decades. The discovery of a second ER subtype, (ER-β Kuiper *et al.*1996), in addition to the initially described ER-α, has expanded the potential mechanisms by which oestrogens can elicit their effects. Teleost fishes have two types of ER-β: ER-βa (formerly ER-γ; see Hawkins *et al.* 2000; Hawkins & Thomas 2004) and ER-βb (Menuet *et al.* 2002) as well as an ER-α. The two ER-β subtypes arose from the duplication of an ancestral *ER-β* gene early in the teleost lineage after the split of tetrapods and fishes (Hawkins *et al.* 2000).

The fate of genes after a duplication event is of fundamental importance in evolutionary biology because it is believed that this process is the predominant mechanism through which novel proteins evolve and new species arise (Ohno 1970; Amores *et al.* 1998). After a duplication event, genes are subject to mutations that result in three possible outcomes: gene silencing of one member of the pair (Mighell *et al.* 2000), the acquisition of a novel function by one copy (neofunctionalization; Ohno 1970) or the partitioning of the original functions between the two copies (subfunctionalization; Force *et al.* 1999). The two *ER-β* subtypes of teleosts

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provide an excellent system to investigate the fate of duplicated genes in vertebrate genomes, because ERs are such critical and extensively studied proteins (Postlethwait *et al.* 2004). Our sequence and phylogenetic analyses showed that after the duplication event, *ER-βa* underwent rapid change followed by strict conservation of certain amino acids (Hawkins *et al.* 2000). This suggested the acquisition of novel functions, or neofunctionalization, after the duplication event. In addition, ER-βa has unusual binding affinities for the endogenous steroid oestriol and other oestrogenic compounds, further suggesting novel functions for this teleost-specific subtype (Hawkins & Thomas 2004).

In this study, we describe the distribution of ER-α, ER-βa and ER-βb mRNA in the hypothalamus and cerebellum of the Atlantic croaker. This is an important first step toward characterizing the role of ER subtypes in the neuroendocrine regulation of reproduction in this well-established model species (Khan & Thomas 1999, 2001; Khan *et al.* 1999). In addition, comparing the distribution of ER-βa and ER-βb in teleosts to that of ER-β in tetrapods could provide insights into the functional evolution of duplicated genes.

In mammals and birds, the distribution of ER-β partially overlaps that of ER-α in the brain, but some nuclei express ER-β exclusively. These include the paraventricular nuclei of the preoptic area (POA), the suprachiasmatic nucleus, the tuberal hypothalamic nuclei and the cerebellum (Shughrue *et al.* 1997; Bernard *et al.* 1999; Foidart *et al.* 1999; Hileman *et al.* 1999; Mitra *et al.* 2003; Mercantheler *et al.* 2004). By contrast, ER-α mRNA is found exclusively in the ventromedial hypothalamus and the subfornical organ (Shughrue *et al.* 1997).

Little information is currently available on the distribution of the three ER subtypes in the teleost brain. ER-α protein and mRNA distribution have been mapped in the forebrain of rainbow trout (*Oncorhynchus mykiss*; Kah *et al.* 1997; Menuet *et al.* 2003), but the neuroanatomical distribution of a possible ER-βa or ER-βb has not been investigated in this species. Our preliminary studies showed that ER-α, ER-βa and ER-βb mRNA expression patterns differ in the suprachiasmatic nucleus of the POA of Atlantic croaker *Micropogonias undulatus* (Hawkins *et al.* 2000). Similarly, all three subtypes show partially overlapping distributions in the anterior preoptic area, the ventral hypothalamus and the posterior tuberculum of the zebrafish *Danio rerio* (Menuet *et al.* 2002). These differences in distribution suggest that the three ER subtypes have distinct roles in the reproductive physiology and behaviour of fishes. It also raises the possibility that the functions of *ER-βa* and *ER-βb* have diverged from their ancestral *ER-β* gene. A more detailed description of the distribution of the three subtypes will begin to address their specific functions in the neuroendocrine system as well as the more general issue of the evolution and fate of duplicated genes.

2. MATERIAL AND METHODS

(a) *Animal and tissue collection*

Adult female Atlantic croakers were collected from the waters surrounding Port Aransas, TX, USA, and maintained in tanks at the University of Texas Marine Science Institute under natural cycles of light and salinity. Six fishes with regressing gonads were injected intraperitoneally with E_2 at a concentration (1 mg kg^{-1}) that results in high physiological levels of E_2 and upregulation of ER in fishes (Hawkins *et al.* 2000). Fishes were deeply anaesthetized 48 h later in a seawater : phenoxy ethanol bath (1 : 2000) and quickly killed by severing the spinal cord. Brains were removed and frozen in dry ice-chilled isopentane within 2 min. Brains were stored at −70 °C until they were sectioned.

(b) *Creation of sequence-specific ER probes in Atlantic croaker*

The three ER constructs used as probes were designed and prepared in the same manner as described previously (Hawkins *et al.* 2000). The Atlantic croaker ER-α, ER-βa and ER-βb probes encompassed amino acids 177–293, 266–406 and 443–552, respectively. [^{35}S]CTP-labelled antisense riboprobes (New England Nuclear) were transcribed from each ER cDNA subclone.

(c) In situ *hybridization*

Frozen whole brains were embedded in optimal cutting temperature (OCT) compound (Tissutek) and cryosectioned at 20 μm (Frigocut, Reichert-Jung). Consecutive sections were transferred to a series of six poly-L-lysine-treated slides to localize the ER subtypes relative to each other in the same brain area. *In situ* hybridizations were conducted as described previously (Young *et al.* 1994; Hawkins *et al.* 2000).

After development, slides were visualized under dark field illumination to assess labelling in specific areas. In regions of low specific labelling or high background (cerebellum, NRL, PMm), positive labelling was confirmed via analysis with the grain counting software BRAIN as described previously (Young *et al.* 1994). Areas with silver grain density greater than 3× background were considered positively labelled. Four different animals were assessed for each area with the exception of the suprachiasmatic nucleus ($n=3$).

(d) *Nomenclature*

The terminology of Braford & Northcutt is followed in the text (Braford & Northcutt 1983), except in some cases where the equivalent terms of Peter & Gill are more specific (Peter *et al.* 1975). The terminology and abbreviations of both research groups are given in table 1.

3. RESULTS

(a) *The anterior hypothalamus/preoptic area*

The distribution of ER-α, ER-βa and ER-βb mRNA labelling in the croaker brain is summarized and compared with findings for other teleost species in table 1. Hybridization signals for the three forms of ER exhibited some overlap in the POA but also showed several areas with marked differences. Most rostrally in the anterior preopticus parvocellularis (PPa), ER-α showed strong labelling in both the ventral and dorsal POA regions, whereas ER-βb showed more restricted labelling and ER-βa demonstrated weaker labelling (figure 1*b–d*). Interestingly, ER-βb labelling was restricted to the ventral portion of the PP nucleus (PPa_v), while some ER-βa labelling was also observed dorsal to the ER-βb labelling (PPa_d; figure 1*c*,*d*). Proceeding caudally, all the ER probes

Table 1. The distribution of Atlantic croaker (ac)ER-α, acER-βa and acER-βb mRNA in the brain compared with the distribution of ERs and E2-concentrating cells in other teleosts. (Terminology of Peter & Gill (Peter *et al.* 1975) is given in italicized parentheses. The oyster toadfish (OTF; Fine *et al.* 1990), paradise fish (PF; Davis *et al.* 1977), goldfish (GF; Kim *et al.* 1979) and platyfish (PLF; Kim *et al.* 1979) tabular autoradiography data of E_2-concentrating cells were taken in part from Fine *et al.* (1990). Autoradiography data do not differentiate ER subtypes. The zebrafish (zf) ER mRNA data are from Menuet *et al.* (2002). The existence of few cells is labelled ( ). Superscript v indicates ventral labelling; superscript d indicates dorsal labelling; superscript 1 indicates rainbow trout (RT) ER-α mRNA (Kah *et al.* 1997); superscript 2 is (RT) ER-α antibody (Navas *et al.* 1995; Linard *et al.* 1996); superscript a means anatomical designation is not given in the original reference, but oestrogen-concentrating cells are shown in appropriate areas (Fine *et al.* 1990); superscript b means that no distinction was made between parvo and magnocellular (Fine *et al.* 1990); superscript c means designated n. saccus vasculosus in the original reference and a question mark identifies an area not investigated.)

	AcERs			ZfERs			RT	GF	OTF	PF	PLF
	α	βa	βb	α	βa	βb	α	E_2-concentrating cells			
preoptic area (POA)											
n. preopticus parvocellularis anterior (PPa) (=*n. preopticus periventricularis, NPP*)	$+^{v,d}$	$+^{(v)d}$	$+^{v}$	$+^{v}$	$+^{v,(d)}$	$+^{v,d}$	$+^{1,2}$	+	+	+	$+^{a}$
n. preopticus magnocellularis pars parvocellularis (PMp) (=*n. preopticus parvocellularis, NPOp*)	+	+	+	?	?	?	$(+)^{1}$	$+^{b}$	(+)	+	+
n. preopticus magnocellularis pars magnocellularis (PMm) (=*n. preopticus magnocellularis, NPOm*)	+	−	+	?	?	?	$(+)^{1,2}$	$+^{b}$	(+)	(+)	+
n. preopticus parvocellularis posterioris (PPp) (=*n. anterior hypothalami periventricularis, NAPv*)	+	−	−	?	?	?	$+^{1}$	−	(+)	−	−
n. suprachiasmaticus (SCN)	+	+	−	?	?	?	$-^{2}$	?	−	?	?
ventral hypothalamus (Hv):				+	+	−					
(*n. lateralis tuberis pars anterior, NLTa*)	+	+	+	?	?	?	?	(+)	(+)	(+)	−
(*n. lateralis tuberis pars posterior, NLTp*)	+	+	+	?	?	?	$+^{1,2}$	+	+	+	+
(*n. lateralis tuberis pars inferior, NLTi*)	+	+	+	?	?	?	$+^{2}$	+	+	+	+
(*n. lateralis tuberis pars lateralis, NLTl*)	+	+	+	?	?	?	$+^{1,2}$	?	?	?	?
n. anterior tuberis (TA)	+	+	+	?	?	?	$+^{2}$	−	(+)	−	−
n. lateralis hypothalami (LH)	+	+	+	+	+	−	?	−	+	−	−
dorsal hypothalamus (Hd):											
(*n. posterioris periventricularis, NPPv*)	+	+	+	?	?	?	$(+)^{1,2}$	+	+	(+)	$+^{a}$
(*n. recessus lateralis, NRL*)	+	−	(+)	?	?	?	$+^{1}$	−	+	+	+
caudal hypothalamus (Hc):											
(*n. recessus posterior, NRP*)	+	−	−	?	?	?	$(+)^{1,2}$	−	−−	−	−
posterior tuberculum											
n. tuberis posterior (TP)	+	−	+	+	−	+	$+^{2c}$	$-^{c}$	$+^{c}$	$-^{c}$	$+^{c}$
valvular cerebelli (VC)	−	−	+	?	?	?	?	?	?	?	?
corpus cerebellum (CC)	−	−	+	?	?	?	?	?	?	?	?

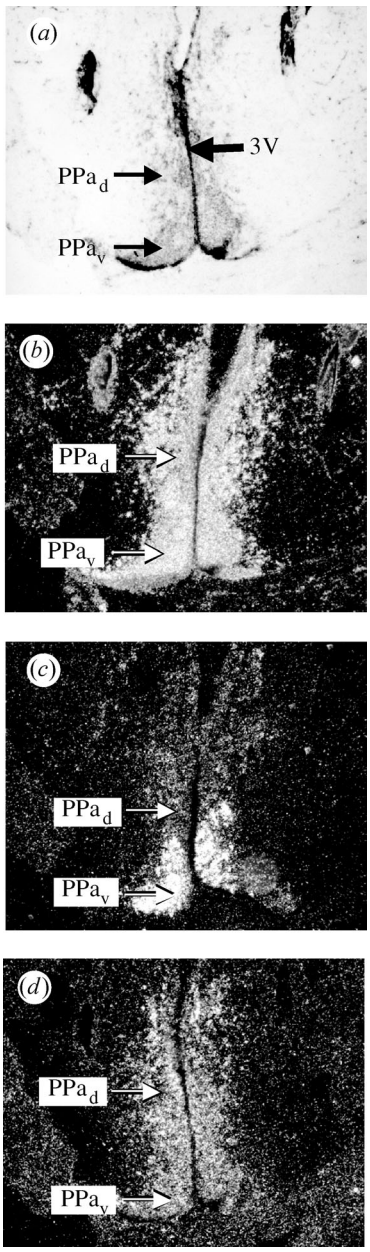

Figure 1. ER subtype mRNA expression patterns differ in the n. preopticus parvocellularis anterior (PPa) of the Atlantic croaker. (*a*) Arrows indicate the location of the dorsal PPa_d and ventral PPa_v in relation to the third ventricle (3V) in the most rostral portion of the preoptic area. (*b*) acER-α labelling is seen in both the PPa_d and PPa_v. (*c*) acER-βb labelling is detected only in the PPa_v. (*d*) acER-βa labelling is detected in the PPa_d, and there is some labelling in the PPa_v.

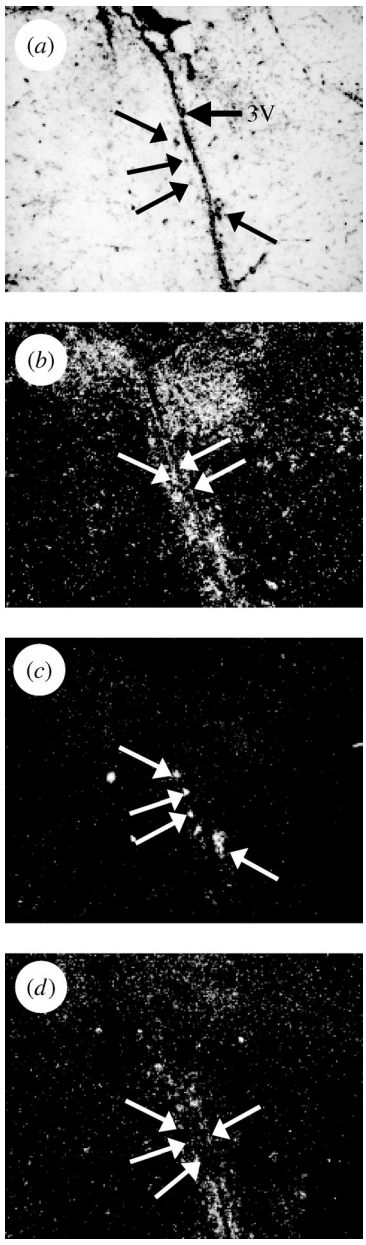

Figure 2. ER subtype mRNA expression patterns differ in the n. preopticus magnocellularis pars magnocellularis (PMm) of the Atlantic croaker. (*a*) Arrows indicate the location of large neurosecretory neurons along the third ventricle (3V). (*b*) acER-α labelling in the PMm is diffuse over and around large neurons. (*c*) acER-βb labelling is strong over magnocellular perikarya. (*d*) The acER-βa labelling is less than 3× background.

labelled the parvocellular portion of the magnocellular POA (PMp; table 1). More caudal and dorsal in the magnocellular nucleus (PMm), many large neurosecretory neurons were consistently and strongly labelled with the ER-βb probe, while ER-βa only very weakly labelled (less than 3× background) a few of these cells in some sections (figure 2*c*, *d*). In contrast to the patterns for ER-βb, the ER-α probe labelling over large neurons was more diffuse and was not discernibly stronger than that in the immediately surrounding tissue (figure 2*b*). The PPp is the most caudal nucleus of the POA and is arranged in laminae 2–5 cells thick. The PPp was labelled with ER-α probe but not with the ER-βa or ER-βb probe. ER-α and ER-βa labelling were seen in the suprachiasmatic nucleus as reported previously (Hawkins *et al.* 2000).

(b) *The hypothalamus and posterior tuberculum*

Most nuclei of the ventral and dorsal hypothalamus showed labelling for all three ERs (table 1). There were two notable exceptions: we found no ER-βa or ER-βb labelling in the NRP or the NRL. In the posterior tuberculum, the posterior tuberal nucleus (NPT) located dorsal to the ventricle was strongly labelled with the ER-βb probe (figure 3*c*). However, this nucleus was negative for ER-βa (figure 3*d*).

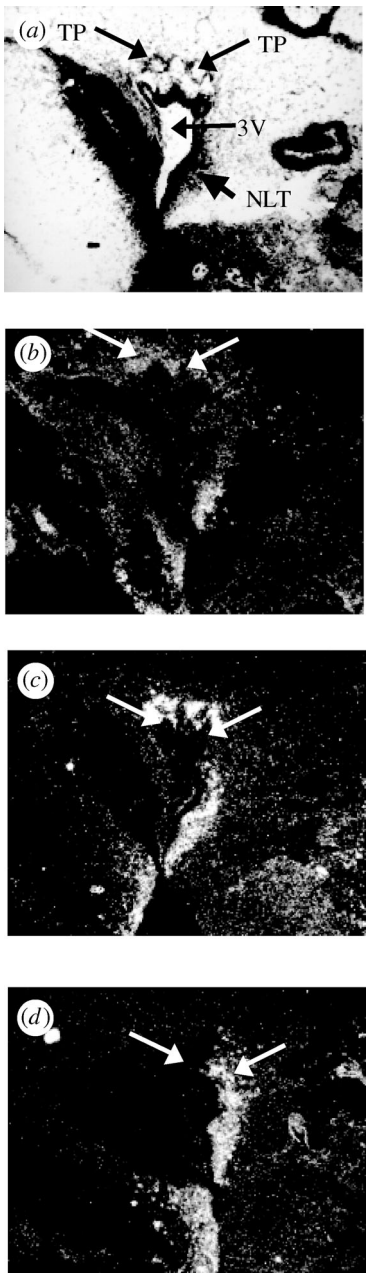

Figure 3. ER subtype mRNA expression patterns differ in the n. tuberis posterior (TP) of the posterior tuberculum of the Atlantic croaker. (*a*) Arrows indicate the location of the TP dorsal to the caudal portion of the third ventricle (3V) and the n. lateralis tuberis (NLT). (*b*) The acER-α probe diffusely labels the TP. (*c*) Strong acER-βb labelling is detected in the TP. (*d*) acER-βa labelling is not detected in the TP.

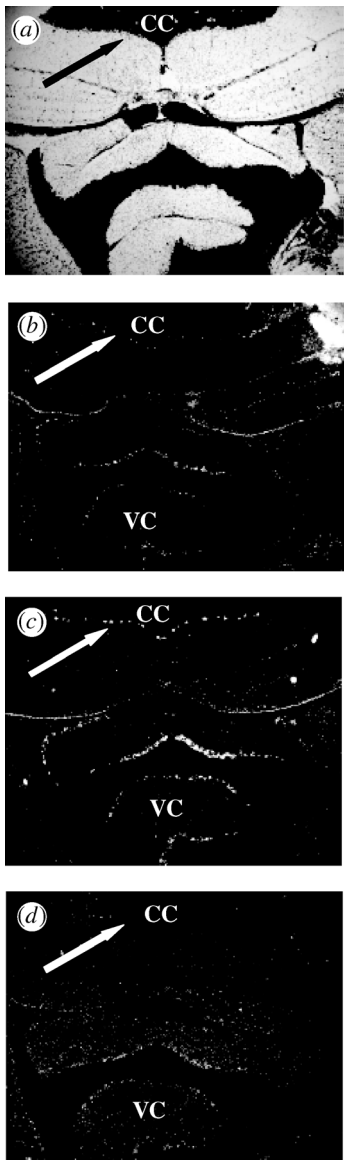

Figure 4. Distribution of acER mRNA in the corpus cerebellum (CC) of the Atlantic croaker. (*a*) Arrows indicate the location of the Purkinje cell layer. (*b*) acER-α labelling is less than 3× background. 'VC' is the valvular cerebelli. (*c*) The acER-βb riboprobe is specifically labelling Purkinje cells of the cerebellum. (*d*) acER-βa labelling is less than 3× background.

(c) *The cerebellum*

In the cerebellum, strong ER-βb labelling was observed in a bead-like arrangement in what appears to be the Purkinje cell layer (figure 4). Similar labelling was also observed in the valvular cerebelli. Neither the ER-α nor ER-βa probes labelled these regions.

4. DISCUSSION

Major differences were seen in the distribution of ER-βa and ER-βb subtypes in the parvo- and magnocellular preoptic areas, the suprachiasmatic nucleus, the posterior tuberis and the cerebellum of the Atlantic croaker brain. The PPa of the POA showed strong labelling with ER-βb in the ventral portion but no dorsal labelling, whereas ER-βa showed moderate labelling in both regions. There were also high levels of ER-βb expression in the PMm portion of the POA, but little or no ER-βa expression. In addition, ER-βb was expressed in the NPT and the cerebellum, while ER-βa was not detected in these areas. The only nucleus found to express ER-βa exclusively was the suprachiasmatic nucleus of the preoptic area. However, ER-βa distribution overlapped with that of ER-α and ER-βb in many areas (table 1).

The present study found that magnocellular neurons of the PM portion of the POA (PMm) express high amounts of ER-βb mRNA but little or no ER-βa or ER-α mRNA. This agrees with findings in mammals and birds, where

the magnocellular region of the paraventricular nucleus (PVN) expresses ER-β, but not ER-α (Shughrue *et al.* 1997; Bernard *et al.* 1999; Foidart *et al.* 1999; Hileman *et al.* 1999; Mitra *et al.* 2003; Mercantheler *et al.* 2004). ER-β is coexpressed in magnocellular neurons of the mammalian PVN that also produce and release arginine vasopressin (AVP) or oxytocin (OT) (Hrabovsky *et al.* 2004). These neurohypophyseal hormones are involved in modulating reproductive behaviours in vertebrates (Moore 1992). Oestrogen treatment alters AVP and OT mRNA levels in the PVN of mice but has no effect in ER-β knockout mice (Nomura *et al.* 2002; Patisaul *et al.* 2003). It is likely that some of the cells expressing ER-βb in croaker are also AVT or OT neurons.

Some neurons of the rat POA that express ER-β produce GnRH, a neuropeptidergic hormone which is central to the control of vertebrate reproductive function and behaviour and whose release and production is modulated by E_2 (Herbison & Pape 2001; Hrabovszky *et al.* 2001; Gore 2002). ER-α is not expressed in GnRH neurons, so until the recent discovery of ER-β in mammalian GnRH neurons (Herbison & Pape 2001), it was thought that in mammals and fishes, all of the oestrogenic effects on GnRH were indirect via interneurons that are regulated by E_2 and whose neurons possess ERs (Flugge *et al.* 1986; Linard *et al.* 1996; Herbison 1998; Senthilkumaran *et al.* 2001). However, the recent demonstrations that GnRH neurons express ER-β in mammals suggest that E_2 could have direct, as well as indirect, effects on GnRH regulation. The presence of ER-βa and ER-βb in the POA of croaker reopens the possibility that GnRH neurons in fishes may express an ER-β. This finding would suggest that ER expression in GnRH neurons is an ancient feature of vertebrate neuroendocrinology.

ER-α and ER-βb, but not ER-βa mRNA was detected in the NTP of the posterior tuberculum. These findings agree with those for zebrafish (Menuet *et al.* 2002). The TP as reported here appears to be at least in part the nucleus saccus vasculosus (SV) of Braford & Northcutt (1983). This nucleus is more appropriately named the TP because it does not innervate the SV organ as originally thought, but instead innervates the more rostral nucleus tuberis posterior of the posterior tuberculum. Oestradiol binding was detected here in oyster toadfish and platyfish (Kim *et al.* 1979; Fine *et al.* 1990). ER-α protein was also found in the nucleus SV of rainbow trout (Kah *et al.* 1997). This nucleus contains dopaminergic neurons and may be analogous to the nigrostriatal dopaminergic system of tetrapods (Rink & Wullimann 2001). In mammals, these dopaminergic neurons are responsive to oestrogen, and E_2 protects them from degeneration induced by the neurotoxins 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Arvin *et al.* 2000; Murray *et al.* 2003).

High levels of ER-βb mRNA were detected in the Purkinje cells of the cerebellum, while no ER-α or ER-βa labelling was observed. ER-βb was also detected in the goldfish and bird cerebellum by RT/PCR (Bernard *et al.* 1999; Foidart *et al.* 1999; Ma *et al.* 2000), and ER-β, but not ER-α, mRNA is present in Purkinje cells of the rat and mouse cerebellum (Price & Handa 2000; Mitra *et al.* 2003). In addition, ER-β may be involved in the E_2-induced changes in transcription of $GABA_A$ receptors in rats (Barami *et al.* 1993). The presence of an ER-β in the cerebellum of species in three different vertebrate classes suggests an ancient function for this subtype in motor learning and muscle coordination. GnRH and 5-HT fibres were also detected in the cerebellum of the Atlantic croaker, but their function here is unknown (Khan & Thomas 1993).

The distribution of E_2-concentrating cells in the brain is extremely conserved across species (Pfaff *et al.* 1988). In mammals, this conservation basically extends to ER-α and ER-β distribution (Shughrue *et al.* 1997; Hileman *et al.* 1999; Mitra *et al.* 2003; Mercantheler *et al.* 2004). In addition to our results presented here for croaker, there is one other study investigating the three ER subtypes' distribution in the brain of zebrafish (Menuet *et al.* 2002). Our results now provide a framework for comparing ER subtype distribution across fish species as well as across other vertebrate taxa. For example, in the PPa, we found ER-α and ER-βa in both the dorsal and ventral regions of the nucleus, while ER-βb was restricted to the ventral portion (table 1). In zebrafish, there is also a distinct, yet overlapping distribution of the three subtypes in the PPa. However, in this species ER-β1 (ER-βb) and ER-β2 (ER-βa) are found in both regions, whereas ER-α is restricted to the ventral region (Menuet *et al.* 2002). In the Hv we found all three subtypes in the nuclei investigated. However, zebrafish do not express ER-βb in the Hv. In the NPT, croaker and zebrafish have similar distributions of ERs. They show a more diffuse labelling with ER-α and very restricted labelling with ER-βb, while this region is negative for ER-βa in both species. Thus, while both species differentially express ER subtypes in many brain regions, the specific ER subtypes expressed in these areas are not conserved. A comparison incorporating more species is needed to assess the general conservation of ER subtype distributions among teleosts.

Since the ER subtypes were often found together in the same brain nuclei, it is possible that they colocalize to the same cells. If the ER subtypes are coexpressed, they could have either the same functions in these areas, or they could have different roles in the E_2-controlled regulation of physiology and behaviour. There is evidence that there are regulatory differences between ER-α and ER-β in mammals and fishes (Paech *et al.* 1997; Menuet *et al.* 2002; Schultz *et al.* 2002). Evidence from rats and humans indicate that ERs may form heterodimers, which may result in distinct interactions with DNA and transcriptional cofactors, and cause alternative gene expression patterns (Pace *et al.* 1997; Pettersson *et al.* 1997; Ogawa *et al.* 1998). The three croaker ERs have different affinities for E_2 and oestriol, which could allow for different levels of receptor activation in a given cell (Hawkins & Thomas 2004). Our recent findings have shown that the three ERs have distinct binding profiles for phytoestrogens and xenoestrogens (Hawkins & Thomas 2004, unpublished observations). Given the differential distribution of these receptor subtypes within the CNS, environmental exposure to oestrogenic substances may have profoundly different effects on neuroendocrine function.

ER-βa has the most restricted distribution of the three subtypes, suggesting that has very specific functions within the brain. ER-βa (as well as ER-βb) appears to have arisen in a duplication of the ancestral ER-β early in the teleost

lineage (Hawkins *et al.* 2000). Structural comparisons and phylogenetic analyses among teleost ER-βs indicate that ER-βa quickly diverged from ER-βb after the duplication, suggesting the acquisition of novel functions by ER-βa. The exclusive presence of ER-βa in a region lacking ER-βs in other species would lend further support to the hypothesis that neofunctionalization occurred after the duplication event. Alternatively, ER-βa and ER-βb may have partitioned the ancestral ER-β functions within tissues (subfunctionalization). In croaker, the only brain areas investigated that express ER-βa and not ER-βb are the SCN (table 1; Hawkins *et al.* 2000) and the dorsal PPa. Conversely, ER-βb, but not ER-βa, was found in the ventral PPa, the magnocellular neurons of the POA and the Purkinje cells of the cerebellum. In rats and mice, both the SCN and the ventral and dorsal portions of the anterior preoptic area (PPa) express ER-β (Shughrue *et al.* 1997; Mitra *et al.* 2003; Merchenthaler *et al.* 2004). Other regions that express ER-β in mammals and birds include the magnocellular region of the PVN (Shughrue *et al.* 1997; Foidart *et al.* 1999; Hileman *et al.* 1999; Mitra *et al.* 2003; Mercantheler *et al.* 2004) and the cerebellum (Shughrue *et al.* 1997; Bernard *et al.* 1999; Foidart *et al.* 1999; Mitra *et al.* 2003). Thus ER-βa and ER-βb are found in different regions that express ER-β in tetrapods, suggesting that each ER-β subtype has lost ancestral ER-β functions that their duplicated counterpart (paralogue) has retained. Therefore, our data indicate that after the gene duplication event, subfunctionalization may have occurred because the distributions of ER-βa and ER-βb in croaker appear to reflect a partitioning of the functions of the ancestral *ER-β* gene. It is important to note that homologies for brain nuclei both within the fishes and across vertebrates are somewhat uncertain, so these regions may not be comparable. In addition, the oestrogen treatments given to upregulate ER levels in this study may have differential effects on ER subtype expression patterns (Menuet *et al.* 2004). Alternatively, it is also possible that ER-βb and ER-βa are expressed in different subsets of neurons within brain nuclei and have acquired novel neuron-specific functions that have resulted in neofunctionalization.

We conclude that the striking differences in the distributions of the three subtypes within brain nuclei of the POA and hypothalamus in the Atlantic croaker indicate that ER-α, ER-βa and ER-βb have distinct functions in the neuroendocrine control of reproduction and behaviour. The differential expression of the ER subtypes in other brain regions, such as the cerebellum and the posterior tuberculum, indicate that ER multiplicity is important for additional neural pathways. The combined neuroanatomical distribution of ER-βa and ER-βb follows the distribution pattern of mammalian ER-β. This conserved distribution pattern reflects the origin of *ER-βa* and *ER-βb* as a duplication of *ER-β* early in the teleost lineage and suggests that ancestral ER-β functions were subfunctionalized between teleost ER-βa and ER-βb after the duplication event. However, our results indicate that the largely conserved distribution patterns seen between tetrapod ER-α and ER-β may not hold true for euteleost ER-α, ER-βa and ER-βb. Additional studies of ER subtype distributions in fishes are needed to resolve the differential patterns of the three ER subtypes in teleosts and ultimately interpret their roles in the evolution of vertebrate neural function.

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