Cell genesis in the hypothalamus is associated to the sexual phase of a hermaphrodite teleost

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The present study aimed to define sex differences in the genesis of hypothalamic and telencephalic cells in the adult brain of male, female and under sex reversal teleosts (*Sparus aurata*). Application of 5-bromo-2-deoxyuridine immunocytochemistry and quantification of the newborn cells using the disector method, determined a statistically significant difference in the dorsal hypothalamic ventricular area between sexual phases. Female brain exhibited a higher number of newborn cells, suggesting that estrogens possibly influence higher mitotic activity. In contrast, paraventricular organ and ventral nucleus of the ventral telencephalon did not show any significant changes, while cell proliferation differences in the preoptic area were correlated with age. The sex-specific cell proliferation pattern in the hypothalamus adds important information on the cellular mechanisms that underlie sex change in a protandrous hermaphrodite teleost. *NeuroReport* 12:2477–2481 © 2001 Lippincott Williams & Wilkins.

Key words: BrdU immunocytochemistry; Dorsal hypothalamic area; Preoptic area; Protandrous; Sexual dimorphism; *Sparus aurata*; Ventral telencephalon

INTRODUCTION

Neurons generated in the adult brain have been reported to show sex-specific differences in several brain regions of lower vertebrates and mammals. Sex differences in cell number in few neural structures have been suggested to develop through sex-steroid sensitive mechanisms that regulate cell proliferation, migration or apoptosis. Indeed, differential survival of newborn neurons has been reported in vocal control forebrain areas in zebra finch [1,2]. In addition, specific correlation of the rate of cell proliferation with respect to estrogen levels was made in the dentate gyrus of adult rats and meadow voles [3–5]. This brain region was found to be sexually dimorphic, with the female animals exhibiting higher numbers of newborn cells in both cases.

In contrast, males show higher numbers of cells than females in the preoptic area (POA), which in most vertebrates exhibits sexually dimorphic characteristics on neurochemical, structural and morphological features [6,7]. A difference in the time-course pattern of the neuroblasts proliferation has been suggested to account for the sexual dimorphism in the volume and cell number of preoptic area of adult rats [8]. In accordance, the ventricular zone on the plane of the preoptic area of young ring doves exhibited sex related cell proliferation differences [9]. Recently the number and size of preoptic GnRH-immunoreactive cells was correlated with the sexual phase in a protogynous (*Labrus berggylta*) and a protandrous (*Amphiprion melanopus*) teleost with males displaying higher cell numbers in both cases [10,11].

Hypothalamic and ventral telencephalic regions in teleosts are known to participate in the regulation of gonadotropin secretion and sex behavior [10–13]. A possible mechanism for the establishment of sexual dimorphism in these areas has been proposed to be differences in cell birth rate [14,15]. Therefore, quantitative comparisons of the proliferation rates between adult male, female and under sex reversal fish would provide important data in order to better understand possible central mechanisms that underlie sex reversal in teleosts. The gilthead sea bream (*Sparus aurata*) used in this study, is a protandrous hermaphrodite teleost [16]. Both male and female tissues co-exist in the gonad, thus providing a structural basis for sex reversal by sequential maturation first of the male and then the female germinal elements. Preliminary data on the cell proliferation pattern in these steroid-sensitive areas of the hypothalamus and ventral telencephalon between sexual phases was presented in an abstract form [17].

MATERIALS AND METHODS

All fish used were reared at the Institute of Marine Biology of Crete and animal experimentation was performed according to the National laws on Protection and Welfare of Animals and the European Communities Council direc-

tive (86/609/EEC) for the care and use of laboratory animals.

To label the mitotically active cells, nine mature fish (three males, three females and three sex changing individuals) were injected with a single dose of a BrdU–normal saline solution (Sigma, 0.2 mg/g, i.p.) and allowed to survive for 24 hours before the application of BrdU immunocytochemistry as described in detail in a previous report [15].

Briefly animals were anaesthetized in an ethylene glycol monophenyl ether solution (Merck, 0.4 ml/l), intracardially perfused with saline followed by 4% PFA in 0.01 M PBS and the brains were removed, post-fixed overnight at 4°C in the same fixative and rapidly frozen at −40°C. Free-floating coronal cryostat sections (50 μm, −24°C), spaced at 100 or 150 μm for animals weighing ~120 g and 320 g, respectively, were washed in 2 N HCl for 30 min at room temperature to denature DNA, rinsed with 0.01 M PBS, blocked with a Triton–horse serum solution, incubated overnight with anti-BrdU primary antibody (Becton Dickinson, 1:100, 10°C) followed by biotynilated anti-mouse secondary antibody (Vector Labs., 2 h, 1:200, room temperature) and an avidin–biotin–peroxidase solution (Vector Labs., 1 h, room temperature). Finally sections reacted with diaminobenzidine for peroxidase activity. Adjacent sections were incubated in the absence of primary or/and secondary antibody to indicate non-specific signal.

A Nikon microscope (Optiphot-2) connected to a PC via a color CCD SONY camera (DXC-950P) was used for the observation of the BrdU-labeled cells. The total number of BrdU-positive cells in the proliferation zones of the dorsal hypothalamic area (dHA), the preoptic area (POA), the paraventricular organ (PVO) and the ventral telencephalic nucleus of the ventral telencephalic area (Vv) was estimated using the disector method [18], in double blind analysis of the size and gender of the animals. Cell counting was confined within neuroanatomical borders of each area defined with the aid of histological preparations. All sections containing an area of interest were used for cell counts as well as the estimation of volumes according to the principles of the Cavalieri method [18]. An average of 1/5 of the total number of disectors, each with a volume of 0.0005 mm^3, occupying each area were chosen randomly and examined.

The sex of each animal was determined by the estimation of the volume fraction of male/total gonadal tissue through a systematic random sampling selection of digitized gonadal sections' images, with the aid of an image analysis program (Scion Image PC, version Beta 3b, 1998, Scion Corporation) as previously described [15]. Testicular tissue occupied at least $\geqslant 90\%$ of the gonad in males ($95 \pm 1\%$), a maximum of 10% in females ($7 \pm 1\%$) and 20–30% in sex changing animals ($25 \pm 2\%$). Analysis of variance verified the grouping of animals (one-factor ANOVA, $p < 0.01$).

Differences in cell numbers between sex and size groups were determined by ANOVA and ANCOVA (Statistica v. 5) and Tukey HSD *post-hoc* test ($p < 0.05$ were accepted as statistically significant). Simple regression analysis (Statistica *vs.* 5) was performed in order to indicate possible correlation between the body morphological characteristics and the estimated gonadal volume fractions and cell numbers.

RESULTS

All male and female fish were 1+ and 2+ years old respectively, whereas sex-changing fish were 2+, except one that was 1+ year old. The average body weight, standard length and brain weight of the younger animals were 120 ± 7 g, 19.5 ± 0.5 cm and 0.34 ± 0.01 g respectively, while the same characteristics for the older animals were 320 ± 22 g, 24.4 ± 1.2 cm and 0.54 ± 0.03 g.

Mitotic activity in the hypothalamus and ventral telencephalon was restricted in the ventricular zone. Labeling was pronounced throughout the rostro-caudal extent of dHA, which includes the nuclei recessus lateralis, anterioris and posterioris periventricularis as well as hypothalamus dorsalis (Fig. 1). In central and anterior hypothalamic levels a distinct population of BrdU-positive cells was confined within the neuroanatomical borders of PVO adjacent to the posterior tubercle. POA exhibited a continuous gradient of labeled cells with lower numbers in posterior nuclei, namely the magnocellular division of the pars gigantocellularis (PMg), pars parvocellularis (PMp) and parvocellularis posterioris (PPp) and higher numbers in the anterior parvocellular nucleus (PPa). Rostrally, a small gap with virtually no signs of proliferation in the ventricular area, was followed by a significant increase in the number of BrdU-positive cells that clearly indicated the borders of Vv.

Stereological analysis of the periventricular regions of dHA, PVO, POA and Vv was performed. Determination of the volumes of these areas showed that they were positively correlated to the body, brain weight and the age of each animal (simple regression analysis, adjusted $R^2 = 0.8$, $p < 0.01$), but not to the different sexual phases studied. Estimation of the total number of labeled cells using the disector method, in male, sex changing and female dorsal hypothalamic area, revealed statistically significant differences between sexual phases (one-way ANCOVA, $p < 0.01$). Specifically, the number of newborn cells in the female dHA was 3-fold higher than that in the male nucleus with the sex changing animals exhibiting intermediate levels of cell birth (Fig. 2a). An equivalent significant increase in the density of newborn cells (labeled cells/mm^3) in the female dHA (one-way ANCOVA, $p = 0.028$) further supported this difference (Table 1). Indeed, regression analysis showed no correlation between the body weight, length, brain weight or volume of the nucleus and the number of labeled cells. There was however, a clear association between the number of cells in dHA and the sexual phase and simple regression analysis correlates the cell number to the fraction of male/total gonadal tissue (adjusted $R^2 = 0.8$, $p = 0.0011$). No such differences in the numbers or densities of BrdU-positive cells were found between sexual phases in all other areas studied (Fig. 2b–d). Furthermore, neither the total numbers nor the densities of newborn cells in PVO and Vv change significantly with the size or the sex of the animals (Fig. 2b,d; Fig. 3b,d). However, the number of labeled cells in POA increased significantly with age (one-way ANCOVA, co-variate: %male/total gonadal tissue, $p < 0.05$; Fig. 3c). The fact that the labeled cell density in POA was similar in all the animals studied, clearly showed that this difference was size/age related since the size of the nucleus increased proportionally to the number of BrdU-

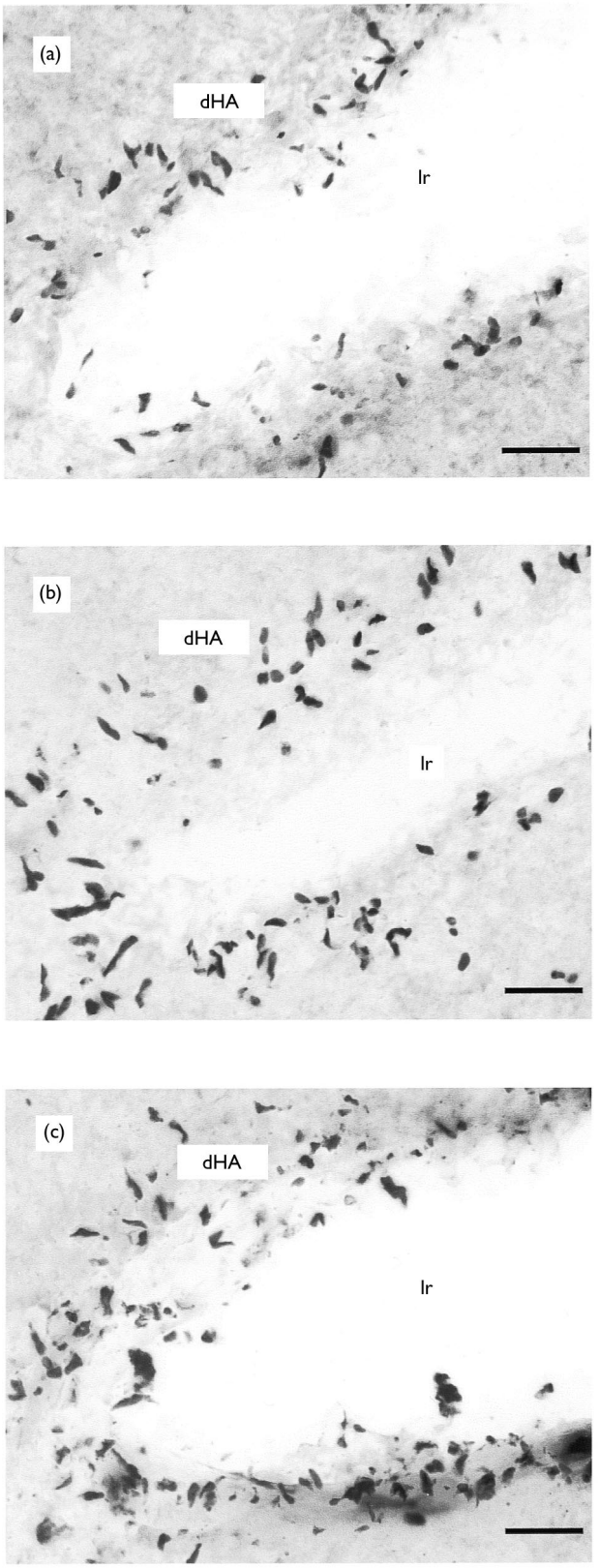

Fig. 1. Three-dimensional reconstruction of transverse sections of the dorsal hypothalamic area surrounding the lateral recess in a male (**a**), sex changing (**b**) and a female (**c**) fish brain. Bars = 30 μm. dHA, dorsal hypothalamic area; lr, lateral recess.

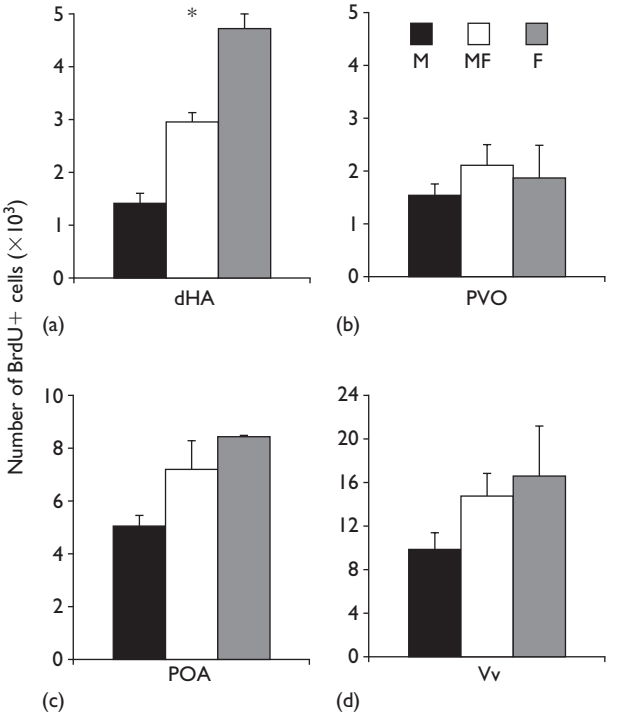

Fig. 2. Average numbers of BrdU-positive cells (± s.e.m.) in dHA (**a**), PVO (**b**), POA (**c**) and Vv (**d**) measured in male (M), sex changing (MF) and female (F) animals. *Statistically significant differences between all sexual phases (one-way ANCOVA, co-variate: either body, brain weight or dHA volume, $p < 0.05$).

positive cells while the rate of cell proliferation remained constant (Table 1).

DISCUSSION

The majority of young animals of this hermaphrodite species (*S. aurata*) are male and after the first reproductive period ~80% change sex and become female [16], rendering it almost impossible to ensure representation of all sexual states in each age group. For this, the animals used in this study were grouped based on their age, where two clusters of fish were apparent (body or brain weight, standard length and age were all strongly and positively correlated) and based on the percentage of male tissue in their gonads, revealing three clusters of animals (male, female and sex changing). The numbers of newborn cells in dHA showed significant differences both between age (one-way ANOVA, $p = 0.01$) and sex (one-way ANOVA, $p = 0.0001$) groups, but specific association to sexual phases was evident when one-way ANCOVA was employed (co-variate: either body, brain weight or dHA volume, $p < 0.01$). Application of ANCOVA showed that age groups had no differences (co-variate: % of male tissue in the gonad, $p = 0.68$). However, a possible influence of age on the increase of newborn cells in this area cannot be excluded. Analyses of the density of labeled cells in dHA verified that sexual phase was the source for the observed dimorphism (sex–cell density: one-way ANOVA, $p = 0.013$; one-way ANCOVA, co-variate: either body, brain weight or dHA volume, $p < 0.05$, age–cell density: one-

Table 1. Mean (± s.e.m.) densities of BrdU-positive cells/mm^3 in hypothalamic and telencephalic areas studied, in *Sparus aurata* teleosts.

	dHA	POA	PVO	Vv
Grouped by sex				
Male	526 ± 70	9720 ± 31	22638 ± 3319	24276 ± 3048
Sex changing	843 ± 125	8900 ± 441	23701 ± 4264	29143 ± 4232
Female	1335 ± 174*	9080 ± 200	19636 ± 5364	31390 ± 8145
Grouped by age/size				
1+ year old	667 ± 150	9665 ± 59	22887 ± 2360	27011 ± 3482
2+ years old	1088 ± 179	8840 ± 280	21685 ± 3910	28749 ± 4211

* $p < 0.05$, one-way ANCOVA; for details see Results and Discussion.

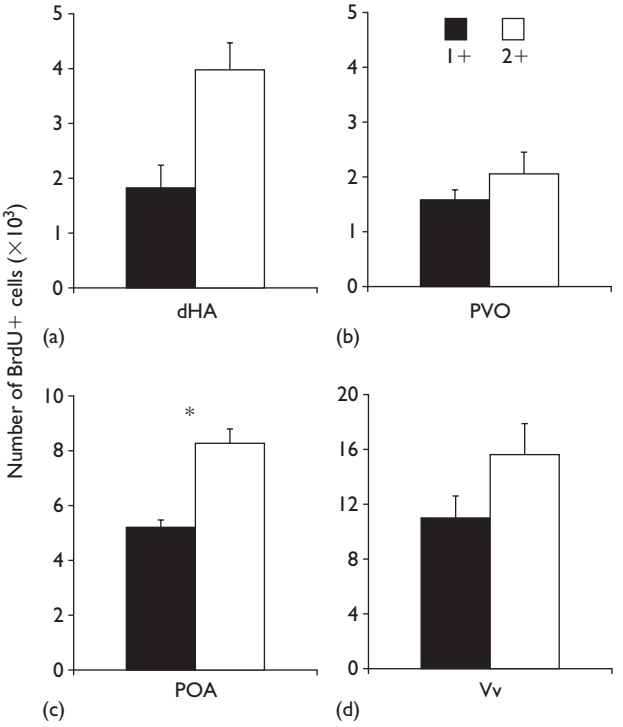

Fig. 3. Average numbers of BrdU-positive cells (± s.e.m.) in dHA (**a**), PVO (**b**), POA (**c**) and Vv (**d**) measured in different age groups (1 and 2+). *Statistically significant differences between age groups (one-way ANCOVA, co-variate: % male tissue in the gonad, $p < 0.05$).

way ANOVA, $p = 0.12$; one-way ANCOVA, co-variate: % of male tissue in the gonad, $p = 0.53$).

The present study showed that the gilthead sea bream preoptic area, PVO and Vv, areas with abundance of estrogen receptors [12], exhibited cell proliferation rates that did not show any significant sex differences. In contrast, posterior to the preoptic area, the dorsal hypothalamic area showed prominent and sexually dimorphic labeling, with higher proliferating activity in the female hypothalamus. Indeed, the transition from male to female was associated with a 3-fold increase in the number of newborn cells in dHA. This difference in the newborn cells population in dHA between the sexual phases could play an important role in the sex change process, particularly since sex determination through sex chromosomes or other factors is vague in most hermaphrodite teleost species. Indeed, the dorsal hypothalamic area is known to be involved in the regulation of gonadotropin secretion and sex behavior [12]. In addition, steroid receptor autoradiographic studies [12,19] have shown high densities of testosterone and estradiol receptors in some of the nuclei that comprise the dHA, which are the nucleus recessus lateralis and the nucleus posterioris periventricularis of the posterior hypothalamus. Moreover, this teleost hypothalamic area has been suggested to be equivalent to a subregion of the mammalian ventromedial hypothalamus [12], involved in the control of feminine reproductive behavior [20]. Our data support evidence that the higher mitotic activity in this region is associated to mature female gonads and higher levels of estrogen in sparidae teleost. In agreement, higher levels of estrogen are implicated in the increased cell proliferation rates in the dentate gyrus of female rats, meadow voles and prairie voles [3–5,21].

In teleosts, hypothalamic and telencephalic ventricular areas retain the ability to generate a large amount of new cells over long periods of their adult life [15,22] exhibiting a remarkable plasticity. Even though the fate and identity of those cells remains unknown, data on cell proliferation and migration in the brain of adult teleosts indicates that most of the newborn cells survive and are incorporated in functional neural or glial networks [22–25]. It is noteworthy that the observed sexual dimorphism in cell proliferation does not seem to coincide with an equivalent increase in the overall cell density in dHA (based on Nissl preparations), but parallels the increase in the volume of the nucleus in female sea bream brain. Similar differences in the volume or number of cells of other brain areas, such as the preoptic area and hippocampus have been observed in birds and mammals [3,4,7], leading to a number of hypotheses on the survival or apoptosis of the newly generated cells, suggesting that estrogen not only stimulates cell proliferation but also exerts important survival effects [3]. It is therefore difficult to determine the functional significance of this increased rate of cell proliferation in the female dHA. Studies on the fate, connectivity and survival of these sexual phase related new hypothalamic cells would further elucidate whether they are indeed incorporated in the neural circuits involved in sex change and/or female sexual maturation processes and give us insight on the mechanisms underlying the sexual differentiation of the brain.

CONCLUSION

Sexual phase regulates cell proliferation in the dorsal hypothalamic area of the adult hermaphrodire teleost *Sparus aurata*, with the female fish exhibiting three times more newborn cells than the males whereas, sex changing animals showed intermediate levels of cell birth. Other hypothalamic and telencephalic steroid sensitive areas studied (POA, PVO and Vv) did not exhibit such sex specific differences. The preoptic area was the only brain region in which cell proliferation rates increased with age, adding significantly higher numbers of newborn cells in older and bigger animals.

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