Socially induced brain differentiation in a cooperatively breeding songbird

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Birds living in social groups establish dominance hierarchies, and taking up the dominant position influences behaviour and physiological parameters. In cooperatively breeding white-browed sparrow weavers (*Plocepasser mahali*), the transition from subordinate helper to dominant breeder male induces the production of a new type of song. This song contains a large number of new syllables and differs in temporal pattern from duet songs produced by all other group members. Here we show that this change in social status of adult males affects the morphology of a behavioural control circuit, the song control system of songbirds that is composed of large neuron populations. The volume of the song control areas HVC and RA and their gene-expression levels depend on males' social status. Dominant males have several times larger testes than subordinates, which is not reflected in circulating androgen and oestrogen levels. Our findings suggest a remarkable differentiation of adult vertebrate brains in relation to changing social cues.

Keywords: song control system; vocal communication; social status; cooperative breeding; brain differentiation

1. INTRODUCTION

Changes in social status are known to induce profound changes in territorial and reproductive behaviours, presumably correlated with changes in hormone production and cellular properties of small groups of neuroendocrine neurons (Francis *et al.* 1993; Gross 1996; White *et al.* 2002; Burmeister *et al.* 2005). In the African cichlid *Astatotilapia burtoni*, males attaining breeding status undergo an increase in the size of gonadotrophin-releasing hormone (GnRH)-containing neurons, together with an increase in GnRH gene expression in the preoptic area of the brain (Francis *et al.* 1993; White *et al.* 2002). Moreover, the behavioural change is reflected in transiently increased neural activity as indicated by the induction of the immediate-early gene *egr-1* (Burmeister *et al.* 2005). In a recent study on naked mole-rats (*Heterocephalus glaber*), it was shown that dominance relationships can also influence the gross morphology of brain regions related to reproduction (Holmes *et al.* 2007). It is, however, unknown whether changes in social status affect the differentiation of a behavioural control circuit, such as the song control system of songbirds that is composed of large neuron populations.

In most social systems, individuals establish dominance hierarchies, and in many cooperatively breeding bird species, non-breeding group members serve as helpers and are subordinate to breeders (Stacey & Koenig 1990). White-browed sparrow weavers (*Plocepasser mahali*) are cooperatively breeding birds of eastern and southern Africa living in groups of 2–10 individuals in year-round territories with a dominant breeding pair and male and female subordinates (Collias & Collias 1978; Lewis 1982a). Two kinds of subordinates exist: former offspring of the breeding pair and unrelated individuals (Lewis 1982b). Once the dominant position is acquired,

individuals normally retain their status of life (Lewis 1982a). White-browed sparrow weavers possess an extraordinary vocal communication system with two types of song: male solo song and duet/chorus song of all group members. Temporal organization and repertoire composition of solo and duet songs are completely different, and mean repertoire sizes comprise 67 and 51 different syllable types, respectively (Voigt *et al.* 2006). Significantly, male solo singing is strictly status dependent. Only the dominant male of the group performs the solo song, i.e. it possesses two distinct repertoires (Ferguson 1988; Voigt *et al.* 2006). Solo song is produced at dawn during the breeding season and is thought to function mainly in inter-sexual communication (Ferguson 1988; Voigt *et al.* 2006). Duet and chorus singing occur year round and are strongly associated with territory defence (Wingfield & Lewis 1993).

The purpose of our study was to investigate the neuroanatomical and physiological correlates of status-dependent song behaviour by analysing the song control system and the plasma steroid hormone levels of birds observed and sampled in Zimbabwe. Neuroanatomical measurements focused on two forebrain song nuclei, HVC and RA (nucleus robustus arcopallii), involved in the production of learned vocal patterns (Nottebohm *et al.* 1976).

Changes in overall morphology of song areas are thought to depend on gonadal steroid hormones (Gahr & Metzdorf 1997; Tramontin *et al.* 2003; Ball *et al.* 2004). The nucleus HVC of most investigated songbirds contains both androgen and oestrogen receptors (Gahr & Metzdorf 1997) which play a key role in regulating testosterone-dependent seasonal plasticity (Tramontin *et al.* 2003). Here, we observe whether the HVC of both dominants and subordinates is a direct target of androgens and estrogens as reflected in the presence of androgen and oestrogen receptors. Further, we analyse

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the expression levels of these two genes to see if they would indicate hormone-dependent differences between the HVC of dominants and subordinates on the cellular level (Nastiuk & Clayton 1995; Gahr & Metzdorf 1997; Fusani *et al.* 2000). In association with that, we included two further genes that have been shown to delineate HVC and express differential steroid-hormone sensitivity, SNAP-25 and synaptopodin, which code for proteins associated with synapses (Voigt *et al.* 2004).

2. MATERIAL AND METHODS

(a) *Animals*

All birds used for histological analysis (dominant males, $n=8$; subordinate males, $n=8$) were sampled at our study site in southwestern Zimbabwe ($20^{\circ}08'S$ – $20^{\circ}14'S$; $28^{\circ}56'E$ – $29^{\circ}01'E$) during the rainy season (January to March in 2000 and 2001, respectively) and derived from groups of individually known birds. The breeding season of white-browed sparrow weavers coincides with the rainy season, which lasts from November until the end of March. Shortly after dusk (19.00–21.00) birds were captured inside their roosting nests. Immediately upon capture, a blood sample was taken by puncturing the wing vein, and after centrifugation with a mini-centrifuge (Bayer diagnostics), plasma was stored at -20°C until analysis. In an earlier study on this species, sampling birds at different times of the day had no effect on plasma steroid hormone levels (J. Wingfield 2006, personal communication). Each bird received a unique combination of aluminium and plastic rings. The sex was determined according to bill colour (Earle 1983). Afterwards all birds were released at their territories. Groups that had at least two males were selected for behavioural observations to identify the social status of the individual birds. Each group consisted of a dominant pair and several subordinates. We identified the dominant pair by breeding activities, their frequent duetting and chasing of other colony members. The dominant male was usually the last bird to enter the roosting nest in the evening, and was the only group member that performed the solo song at dawn (Voigt *et al.* 2006). We verified this by observing each group thrice at dusk and at dawn. Additional male group members, which were fully mature but did not show the behaviour of the dominant male, were considered subordinate.

(b) *Song recording and analysis*

Vocalizations were recorded with a Sony TCD-5M cassette recorder (Sony Corporation, Tokyo, Japan) equipped with a Sennheiser ME-88 directional microphone (Sennheiser Electronic, Wedemark, Germany). Male solo song was recorded in the morning between 05.00 and 05.45. In previous observations, we determined the approximate starting time, which was generally coincident with or slightly before first light. Recordings were obtained within a distance of 2–10 m from the bird. Solo song was usually produced only once a day at dawn as a single performance, which lasted 10–20 min. Details on sonographic analysis are described elsewhere (Voigt *et al.* 2006).

(c) *Neuroanatomical analysis*

Birds were killed with an overdose of chloroform and immediately perfused transcardially, first with 0.9% saline, followed by 4% phosphate-buffered formaldehyde (FPBS). Brains were removed and post-fixed in FPBS at 4°C until analysis. The gonads of each bird were removed and stored in

FPBS at 4°C . Brains were removed from FPBS and immersed in 15%, followed by 30% phosphate-buffered sucrose for several days at 4°C . Brains were then cut into $30\text{ }\mu\text{m}$ parasagittal sections and mounted onto Superfrost-Plus slides (Fa. Roth). One series of sections was used for Nissl staining; the others were processed with *in situ* hybridization for androgen receptor (AR), oestrogen receptor (ER), synaptosomal-associated protein (SNAP-25) and synaptopodin (SPO). The mRNA expression on brain sections was detected with antisense RNA probes of the zebra finch labelled with ^{35}S -CTP and followed a standard protocol with modifications (Whitfield *et al.* 1990). The cloning of a partial zebra finch AR, ER, SNAP-25 and SPO cDNA was done in our laboratory and has been described previously (Gahr & Metzdorf 1997; Voigt *et al.* 2004). Slides were analysed under brightfield and darkfield illumination using a Leitz Aristoplan microscope (Leitz Wetzlar, Germany). For volume measurements, the areas of the brain regions HVC and RA were video digitized using a PC equipped with an image analysis system (METAMORPH v. 4.6, Visitron Systems, Germany). Volumes were calculated from every fourth section as the sum of the area sizes multiplied by $120\text{ }\mu\text{m}$ (section interval $\times$ section thickness). Telencephalon volume was estimated by sampling every eighth section throughout the extent of the brain hemisphere. Total volume was the sum of the measurements from the right and the left hemispheres. Cell density in HVC and RA was estimated from Nissl-stained sections under high magnification with the help of the image analysis system METAMORPH v. 4.6. In each animal at the lateral, central and medial levels of the nucleus (see below), a counting frame of $10\,000\text{ }\mu\text{m}^2$ for HVC and $62\,000\text{ }\mu\text{m}^2$ for RA was analysed using the digitized images, and the average of these counts was calculated. We sampled a minimum of 160 cells in each nucleus per bird. We counted all profiles that contained one or two nucleoli throughout the entire depth ($30\text{ }\mu\text{m}$) of the section that fell within the boundaries of the counting frame. Density measurements are presented as $10^4\text{ cells mm}^{-3}$. The total number of cells in each nucleus was derived from multiplying the cell density by the volume of the nucleus. The mRNA expression in HVC was measured at the lateral, central and medial levels of the nucleus. These levels were estimated according to the Nissl-defined boundaries of HVC. We counted the number of HVC sections (N). *Central* was the section $N/2$. This section divided the HVC into a lateral and a medial portion. The *lateral* level was section $N_l/2$ and the *medial* level was section $N_m/2$ ($N_{l,m}$ = number of sections in the lateral and medial part, respectively). At each level, four areas ($13\,100\text{ }\mu\text{m}^2$ each) across HVC were analysed (in total $52\,400\text{ }\mu\text{m}^2$). To quantify the level of mRNA expression in an area, the image was converted to a greyscale image. A threshold level was then adjusted to separate the silver grains from the background. The above-threshold fraction of the area was calculated by a built-in function of the software. The mean of these measurements was named mRNA expression level. To correct for different amounts of background labelling, we measured the area covered by silver grains in a region of the same section lacking specific labelling. Correction was done by subtracting the value of background labelling from the value of HVC. ER mRNA expression was measured only at the medial level of HVC and for dominant male, data could be obtained only from six individuals.

(d) *Hormone analysis*

For the analysis of plasma steroid hormone, levels were obtained from blood samples from 27 dominant males and 14

Figure 1. Brightfield photomicrographs showing song control nuclei RA (robust nucleus of the arcopallium) and HVC (used as proper name) in Nissl-stained sections of dominant and subordinate males. Arrows indicate boundaries of the nuclei. Both brain areas were approximately 30% larger in dominant than in subordinate males. Dorsal is to the top and caudal is to the right in these parasagittal sections. Scale bar, 300 μ m.

subordinate males. The androgens 5α -dihydrotestosterone (DHT) and testosterone (T) and the oestrogen 17β -oestradiol (E2) were measured by radioimmunoassay (RIA) after extraction and partial purification on diatomaceous earth (celite) microcolumns using a modification of the methods described (Wingfield & Farner 1975). Antisera were obtained from Endocrine Sciences (Tarzana, USA): DT3-351 (DHT); T3-125 (T); and E17-94 (E2) and labelled steroids from Perkin Elmer Life Sciences. The detection limits (pg ml^{-1} plasma) of the steroid RIA were 19.0 for DHT, 12.5 for T and 4.0 for E2. The average recoveries were between 64 and 81%. The intra-assay variation was 1.5–2.5%; the inter-assay variation was 4.1–6.0%.

(e) Statistical analyses

Statistical analyses were carried out using SYSTAT v. 11.0 (SYSTAT Software, Point Richmond, CA, USA). Unless stated otherwise, data are presented as mean $\pm$ s.e.m. Morphological data were compared between groups with *t*-tests. The gene expression levels in HVC were analysed using ANOVA with social status as between-subjects factor, and cell density was included as covariate. There was a significant correlation between the cell density and the mRNA expression levels of SNAP-25 ($p=0.020$) and SPO ($p=0.049$). To remove this bias we used analysis of covariance. Hormone levels were analysed by non-parametric statistics. All tests were two-tailed, and the significance level was fixed at $p<0.05$.

3. RESULTS

(a) Morphological predictors of social status

Males of different status did not differ in morphological measurements, such as body weight ($t=1.04$, d.f.=14, $p=0.314$) and wing length ($t=1.51$, d.f.=14, $p=0.153$). However, testis size was a predictor of social status. Dominants had testes that were approximately thrice larger than that in subordinates ($t=3.88$, d.f.=14, $p=0.002$).

On the gross-morphological level of the brain, we compared the regional volume and cell properties of the

song control nuclei HVC and RA between both groups of males using Nissl-stained material (figures 1 and 2). Telencephalon volume was used to test for overall differences in brain size between dominants and subordinates but no significant differences were found ($t=1.66$, d.f.=14, $p=0.119$). Therefore, we applied no correction in further analyses. Delineation of the nuclei HVC and RA revealed approximately 30% larger volumes of both brain regions in dominant than in subordinate males (HVC: $t=4.53$, d.f.=14, $p=0.0001$; RA: $t=2.94$, d.f.=14, $p=0.011$; figure 2a). By the analysis of cell density, we verified for HVC that the gross anatomical differences were not the result of differences in cell spacing, as HVC cell density was similar in both groups of males ($t=0.15$, d.f.=14, $p=0.886$; figure 2b). Therefore, the volumetric difference of HVC resulted from a difference in cell number ($t=3.11$, d.f.=14, $p=0.008$; figure 2c). A different pattern was found in nucleus RA. Dominants had a lower cell density in RA than subordinates ($t=2.77$, d.f.=14, $p=0.015$; figure 2b), but RA cell number was similar in both groups of males ($t=0.66$, d.f.=14, $p=0.520$; figure 2c).

(b) Behavioural predictors of social status

All dominant males were observed singing a solo song at dawn as a continuous performance, lasting on average 12.3 ± 1.9 min (figure 3), whereas subordinate males never sang a solo song. The mean repertoire size of such a song was 68.9 ± 6.0 different syllable types. Solo song repertoire size of dominant males was neither significantly correlated with the volume of HVC ($r_s=0.072$, $p=0.882$) nor with RA ($r_s=0.539$, $p=0.171$).

(c) Gene expression in HVC reveals phenotype-related pattern

Both dominant and subordinate males expressed androgen (AR) and oestrogen receptors (ER) as well as the synaptic proteins SNAP-25 and synaptoporin in HVC. The expression level of each gene was higher in subordinates than in dominants (AR: $F_{1,13}=9.26$,

Figure 2. (a) Volumes, (b) cell density, and (c) cell number in song control nuclei HVC and RA of dominant and subordinate males. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.)

$p = 0.009$; ER: $F_{1,11} = 6.20$, $p = 0.030$; SNAP-25: $F_{1,13} = 9.57$, $p = 0.009$; SPO: $F_{1,13} = 14.36$, $p = 0.002$; figure 4a,b). Thus, status-dependent HVC size correlates negatively with status-dependent gene expression.

(d) Steroid hormone levels are similar in dominants and subordinates

To reveal whether these status-related differences within the song system correlate with differences in circulating blood hormones, we measured plasma steroid hormone levels in dominant and subordinate males. Our data do not support this hypothesis. The two groups of males did not differ significantly in plasma levels of testosterone

(T; median dominant males: 69.57 pg ml^{-1} , range: $12.5\text{--}1125.0 \text{ pg ml}^{-1}$, $n = 27$; median subordinate males: 12.50 pg ml^{-1} , range: $12.5\text{--}800 \text{ pg ml}^{-1}$, $n = 14$; $U = 233.0$, $p = 0.209$). Plasma levels of 5α -dihydrotestosterone (DHT) and 17β -oestradiol (E2) were basal and not different between groups (DHT: $U = 168.0$, $p = 0.253$, E2: $U = 202.5$, $p = 0.514$). Further, dominant males actively singing a solo song were not found to have higher testosterone levels than those that did not sing during the period of blood sampling (Fisher's exact test, $n = 22$, $p = 1.00$). There was no correlation between testosterone levels and androgen receptor expression in HVC (dominant males: $r_s = -0.072$, $p = 0.882$, $n = 8$; subordinate males: $r_s = -0.366$, $p = 0.360$, $n = 8$). Similarly, there were no correlations between testosterone levels and HVC cell number in either group of males ($p > 0.05$). In summary, although it is unclear whether such differences in gene expression translate into functional differences in the operation of HVC neuronal circuitry, the results suggest that the differences between dominants and subordinates are not driven by circulating sex steroid hormones.

4. DISCUSSION

This study, based on histological data, suggests that acquiring the dominant position induces large-scale differentiation of the adult song control system. Some songbird species are known to change their song behaviour seasonally such that males sing lengthier songs and more frequently during the breeding season (Catchpole & Slater 1995). In several such species, these behavioural changes correlate with pronounced modifications of the morphology and/or size of the forebrain song control circuit (Tramontin & Brenowitz 2000). In other species, however, activation of seasonal motor memories does not require gross anatomical differentiation. In island canaries (*Serinus canaria*), there is a seasonal change in the composition of the song syllable repertoire that is not paralleled by seasonal changes in HVC or RA morphology (Leitner *et al.* 2001).

Functionally, the increase in song nuclei size could relate to the acquisition of a new behaviour that is related to the motor activity of producing solo song. Such a correlation between 'brain space' and the capacity of a learned task was initially proposed by Nottebohm *et al.* (1981) to explain individual differences in syllable repertoire size in domesticated canaries, but subsequent studies on different species failed to produce consistent results (Gahr 2004; Jarvis 2004; Leitner & Catchpole 2004; but see Garamszegi & Eens 2004). Similarly, we found no significant correlations between solo song repertoire size and HVC or RA size among dominant male white-browed sparrow weavers. We propose that the production of the new type of song, the solo song, requires song system reorganization. For HVC, this is indicated by the different gene-expression patterns and the almost non-overlapping HVC sizes in both social groups of males (range of total HVC size in subordinate males, $1.807\text{--}2.894 \text{ mm}^3$; in dominant males, $2.839\text{--}4.482 \text{ mm}^3$). As an alternative scenario, the redifferentiation of forebrain song areas might be related to the physical demands of increased dominant male singing activity. In a study of European Starlings (*Sturnus vulgaris*) singing activity was reported to predict HVC size (Sartor & Ball 2005).

Figure 3. Sequence from the solo song of a male recorded in Zimbabwe. Solo song is exclusively produced by the dominant male of the group and only at dawn during the breeding season.

Figure 4. Expression levels of (a) androgen (AR) and oestrogen receptors (ER) in medial HVC and (b) the synaptic proteins SNAP-25 and synaptoporin (SPO) throughout HVC of dominant and subordinate males. For SNAP-25 and SPO we used means of lateral, central and medial measurements owing to no regional differences. (* $p < 0.05$, ** $p < 0.01$.)

However, such a correlation does not hold for other species (DeVoogd *et al.* 1995; Li *et al.* 2000; Boseret *et al.* 2006).

The underlying cellular mechanisms that drive growth of the song nuclei are speculative, but for HVC concern a large population of cells that need to be recruited (approx. 120 000). Recruitment of new neurons due to protracted neurogenesis is a common process in the adult avian brain and is one possibility for HVC (Goldman & Nottebohm 1983). Social status has been shown to influence neurogenesis in rats, with the most dominant males of the cohorts having higher numbers of new neurons in the hippocampus (Kozorovitskiy & Gould 2004). A higher survival rate of new neurons in forebrain song areas has also been described in zebra finches in response to a more complex social environment (Lipkind *et al.* 2002). However, in birds, there are large differences between species in the amount of neural recruitment into nucleus

HVC, and neural recruitment might also depend on the animals' physiology (Kirn *et al.* 1994; Scott *et al.* 2000); therefore the exact process of neurogenesis while becoming dominant still remains to be determined. As an alternative to the incorporation of newly born neurons, cell populations of the nidopallium ventral to HVC might be recruited. For RA, neuronal recruitment in adult birds has never been reported. Developmental and seasonal growth is achieved generally by an increase in soma size and neuronal spacing (Bottjer *et al.* 1986; Tramontin *et al.* 1998). Likewise, in our study, dominant male singing behaviour seems to be associated with larger RA neurons, as the total cell number remains constant, while cell density was found to be reduced.

The inductive physiological signal driving cellular changes in the song areas remains unclear. A previous field study on white-browed sparrow weavers showed that stress hormone levels are not related to social status

(Wingfield *et al.* 1991). Thus, removal of the inhibiting action of corticosteroids on neuronal recruitment, as suggested for the rodent hippocampus (Cameron & McKay 1999), is an unlikely explanation for status-induced differentiation of song control areas. Further, steroid hormones could directly act on song areas (via the presence of androgen and oestrogen receptors) but circulating androgens and oestrogens are similar in dominants and subordinates, and receptor expression is even higher in subordinates. In an earlier study on white-browed sparrow weavers, Wingfield *et al.* (1991) found significantly higher testosterone levels in dominant males compared with subordinates, but these were restricted to a period in the second half of the breeding season. Thus, a similar finding in our birds would not explain the differences between dominant and subordinate males since status-dependent singing behaviour is already present at the beginning of the breeding season (Voigt *et al.* 2006). Despite our larger sample size compared with the previous study (Wingfield *et al.* 1991), we confirm their observation that testosterone levels vary individually and show no relationship to any aspect of breeding behaviour (J. Wingfield 2006, personal communication). Our data are, however, insufficient to test for short-term changes in circulating blood hormone levels that may play a role in triggering brain and behavioural reorganization. Such short hormonal peaks might promote neuronal recruitment into HVC, while integrated neurons persist within the network despite subsequent low T levels (Alvarez-Borda *et al.* 2004). Alternatively, differences in de novo synthesis of steroid hormones in the brains of dominants and subordinates remain a possibility as drivers of neural differentiation locally (Schlinger & Brenowitz 2002). Yet another possibility is the presence of elevated plasma levels of the androgen precursor dehydroepiandrosterone, which can be converted into sex steroids within the brain. This hormone can drive song behaviour and song nuclei growth while plasma T levels are basal (Soma *et al.* 2002).

Polymorphism, as found in cooperatively breeding bird species, has been well studied in fishes in terms of male alternative reproductive strategies (Bass 1992; Scaggiante *et al.* 2004). Among higher vertebrates, including humans, intra-sexual variation in behaviour occurs in the context of sexual partner preference and is also reflected at the level of the brain (LeVay 1991; Roselli *et al.* 2004). Such polymorphic phenotypes are the probable result of ontogenetic differentiation. In contrast, our data suggest that in cooperative breeders such as the white-browed sparrow weavers, the transition from helper to breeder male is most likely due to adult brain redifferentiation and depends on the adult's environment. Whether activational effects of gonadal hormones modulate these brain structures once the dominant position is acquired, e.g. to induce seasonal plasticity, is currently not known, although solo song production is thought to be a seasonal trait (Ferguson 1988).

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NOTICE OF CORRECTION

The third sentence of §3(d) is now present in the correct form.

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