1. GENERAL INTRODUCTION

1.1. Sexually dimorphic behaviour and brain

Since a long time it has been known that males and females differ in their behaviours. Such sex differences, named sexual dimorphisms, occur in two forms. Sex-specific behaviours are those exclusively shown by one sex and include female life-birth and male intromission. More often behaviours are sex-typical, which means the pattern is more frequently shown by one sex but can be produced under certain conditions by the other sex as well. Examples are clasping in frogs, singing and copulation-solicitation display in songbirds and mounting in many mammalian species (Beach 1961; Kelley 1988; Gahr 1994).

Sexually dimorphic behaviour patterns have been well studied in many groups of animals since the beginning of the last century, especially in the context of reproduction. At the same time, it was recognised that sex differences in circulating androgens and oestrogens account for sex differences in behaviour patterns (Beach 1961). By the 1960s, it was known that steroid hormones exert their effects on behaviour by acting on specific brain regions and distinct areas were identified that accumulate steroid hormones (Hutchison 1967; Pfaff 1968; Stumpf 1968).

However, it was not until the 1970s that the first sex differences in brain structure of vertebrates had been discovered (Raisman & Field 1971; Raisman & Field 1973). These studies focused on reproductive behaviour in rats and revealed a sex difference in the type of synaptic input to the preoptic area of the rat brain, an important region for the control of male and female sexual behaviour (Pfaff 1980). These studies were followed by the discovery of the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is about eight times larger in males than in females (Gorski et al. 1978; Gorski et al. 1980). These sex differences were so prominent that one could detect them without a microscope. Soon after the initial report of sexually dimorphic neural structures in mammals, Nottebohm & Arnold (1976) described sex differences in the size of the song control nuclei in songbirds. They found in zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*) that several telencephalic song nuclei were many times larger in the male than in the female brain and it is only the male that normally sings in these species. Later on in other birds and mammals and in all other classes of vertebrates neural sexual dimorphisms were reported, e.g. in the goldfish (*Carassius auratus*) (Rao et al. 1996), in the African clawed frog (*Xenopus laevis*) (Kelley & Dennison 1990), in a species of whiptail lizards (*Cnemidophorus inornatus*) (Crews et al. 1990), in Japanese

quail (*Coturnix japonica*) (Adkins-Regan & Watson 1990), in the guinea pig (Hines et al. 1985) and in humans (Swaab & Fliers 1985).

The traditional view about the development of these sex differences in brain structure is that genetic sex determines gonadal sex and gonadal sex determines phenotypic sex (for review, see Arnold 2002). Early experiments on mammals confirmed that manipulations of steroid hormone levels at certain periods during ontogeny permanently affect structural sex differences in the brain and let Phoenix et al. (1959) to propose that steroid hormones act in two fundamentally different modes: organisational and activational. Sex steroids act early in life to *organise* neural structures and these effects are permanent. In adulthood, sex steroids *activate* differentiated brain structures to mediate behaviours. Therefore, female guinea pigs prenatally exposed to androgens will show less feminine behaviour in adulthood because androgen had organised their brains in a masculine manner (Phoenix et al. 1959). Conversely, males deprived of androgen during ontogeny will develop a feminine brain (Young et al. 1964). Many of the examples of neural sex differences, which were discovered in the 1970s, were conform to the organisational hypothesis. For example, exposure of newborn female rats to testosterone masculinises the size of the SDN-POA whereas castration of newborn males results in a feminine appearance of this region (Gorski et al. 1978). However, the sexual differentiation of brain and behaviour in the zebra finch fits only partly the hypothesis. There, exposure of juvenile females to steroid hormones, especially oestrogen, masculinises the song control system and song behaviour, but depriving young males of steroids does not prevent masculine development (Arnold 1997). In birds, there is now increasing evidence that sexual differentiation is controlled not only by gonadal steroid hormones but also by brain-intrinsic, genetically determined mechanisms (Wade & Arnold 1996; Agate et al. 2003; Gahr 2003).

1.2. Polymorphic behaviour and brain

From earlier studies on alternative reproductive strategies, e.g. in fish and amphibians, it was recognised that the adult phenotype not only comes in two forms, male and female, but also differs between individuals of the same sex (Howard 1978; Gross 1982). Such polymorphism is found for example in the plainfin midshipman (*Porichthys notatus*), a sound-producing fish with two distinct male phenotypes, which differ in reproductive and vocal behaviour. Whereas type I males engage in nest-building and egg-guarding and generate a mate song during the breeding season, type II males are non-territorial, sneak spawn and do not vocalise (Bass 1992). Furthermore, the differences in vocal

behaviour are paralleled by differences in the cellular structure of the motor neurons innervating the sonic muscles, which are responsible for sound generation (Bass & Andersen 1991). Among humans, homosexuality and transsexuality represent well-studied examples of behavioural polymorphism. There, similar to reports from fish, the phenotypic within-sex differences were found to be reflected in differences in brain structure (Swaab & Hofman 1995; Zhou et al. 1995). For instance, the volume of the suprachiasmatic nucleus of the hypothalamus is about twice as large in homosexual then in heterosexual men (Swaab & Hofman 1990).

With respect to the organisational-activational concept (see 1.1.), which predicts the differentiation of sexually dimorphic behaviour and brain structure, the existence of polymorphic phenotypes raises the question about the general applicability of this hypothesis. According to it, an ‘organising’ effect would mean that the adult phenotype is predetermined during a critical period of development and remains fixed throughout life, whereas an ‘activational’ effect would induce the different phenotype in adulthood and should be reversible. A first attempt to solve this problem has been made by Moore (1991), who proposed the ‘relative plasticity hypothesis’. He distinguished between two different forms of polymorphism, a system with *plastic* phenotypes, in which adult individuals change from one type to another, and a system with *fixed* phenotypes, in which individuals attain one phenotype before sexual maturity and do not change it in adulthood. From this, the hypothesis states that differences between individuals of the fixed phenotype should be organised by early steroid hormone action whereas differences between individuals of the plastic phenotype are due to the activational action of steroid hormones. Data obtained from studies on mating systems and endocrine profiles in several vertebrate taxa were in support of the hypothesis (reviewed in Moore 1991). For example, in tree lizards (*Urosaurus ornatus*), which exhibit the fixed alternative phenotype regarding throat colour, castration or testosterone treatment of male hatchlings results in significant alterations of adult phenotype, which is not obtained when applying the similar treatment to adult individuals (Hews et al. 1994; Hews & Moore 1995). On the other hand, cooperatively breeding bird species such as the pied kingfisher (*Ceryle rudis*), the Florida scrub jay (*Aphelocoma coerulescens*) or the white-browed sparrow weaver (*Plocepasser mahali*) were regarded as having plastic alternative phenotypes because helpers may eventually become breeders. There, the hormone profiles of adults differed in respect to phenotype supporting an activational effect of steroid hormones. However, not all available data could be fit to the predictions of the hypothesis (Moore 1991) and studies exploring the neural basis underlying behavioural polymorphism are so far mainly conducted in a few species of fish and in

humans. Nevertheless, extensive studies on one of these fish species, the African cichlid *Haplochromis burtoni*, revealed the remarkable result that social behaviour exerts profound effects on the reproductive axis and on the brain structures controlling reproduction (Fernald 2002). This pattern is not restricted to maturation but also occurs in adult fish. Males of ascending social status experience an increase in gonad size and an increase in the size of gonadotropin-releasing hormone containing neurons of the preoptic area. These changes revert when the dominant position is lost. Such a system allows the fish to respond quickly to changes in social environment and mating opportunities (Fernald 2002).

1.3. The study of birdsong

For studying the neural mechanisms of behaviour, birdsong represents an attractive model, which enables an integrative approach of behavioural and neurobiological studies because of the following reasons: 1) the brain pathways controlling song learning and song production are well characterised, 2) the circuitry is sensitive to steroid hormones both during development and in adulthood, 3) in most species the song control system is sexually dimorphic, 4) the behaviour can be clearly measured and quantified and 5) birdsong is a sexually dimorphic, learned behavioural pattern.

Birds use their song for inter- and intra-sexual communication. The song of a male may signal to a female the presence of a potential mate whereas to another male, song may convey information about his location and territory ownership (Catchpole & Slater 1995). The hormonal basis of birdsong was inferred early from observations on the relationship between seasonal occurrence of song and reproduction (Armstrong 1963).

Songbirds (*Passeriformes: Oscines*), which account for about half of all bird species, acquire the stereotyped song pattern typical of adult birds by vocal learning. Song learning occurs in two steps during ontogeny, in a sensory phase and in a sensorimotor phase. During the sensory phase, birds build up an auditory template of songs they hear during this period, thereby showing innate preference for conspecific song. Later on during the sensorimotor phase, birds match their vocal output to the acquired template, a process requiring auditory feedback. In some species these two periods overlap, such as in the zebra finch and in the canary, while in others they can be separated by several months, during which the bird needs not to rehear the song, e.g. in the swamp sparrow (*Melospiza georgiana*). When vocal learning is restricted to a critical period at the juvenile stage, these species are referred to as “age-limited” learners, such as the chaffinch (*Fringilla coelebs*) and the white-crowned sparrow (*Zonotrichia*

leucophrys). In contrast, others, like the canary, the starling (*Sturnus vulgaris*) and the red-winged blackbird (*Agelaius phoeniceus*) can modify and expand their song repertoires in adulthood and are called “open-ended” learners (Konishi 1985) (Nottebohm 1999). However, the ability to acquire new auditory memories persists in adult birds independently of a sensitive period and exists even in females that never produce any song. This allows birds to show preference to songs of their mate or to discriminate between songs of territorial neighbours (Nottebohm 1999).

Song behaviour in temperate zone birds is a seasonal trait, highly correlated with breeding and is mainly confined to males though there are species where song is produced in the non-breeding season (Schwabl & Kriner 1991; Rost 1992; Leitner et al. 2001a) and where females occasionally sing (Gerber 1955; Pesch & Guettinger 1985; Kriner & Schwabl 1991). In sharp contrast is the pattern found in the tropics (23.5° N - 23.5° S), where about 80 % of all passerine species live. Due to less variability in day length, climate and food availability compared with the temperate zone, breeding seasons are prolonged and many species are residents and year-round territorial. Furthermore, song is a common characteristic of both males and females and occurs intensively in the context of territory defence (Stutchbury & Morton 2001). Duetting, a primarily tropical phenomenon, represents a special form of singing, where both members of a mated pair vocalise in precise temporal coordination (Farabaugh 1982). This type of song production has evolved independently in distinct taxonomic groups leading to a great diversity among duetting species regarding song characteristics.

Several years after the initially described sex differences in the song control system of canaries and zebra finches, in which females do rarely sing or not at all (Nottebohm & Arnold 1976), duetting songbirds became an interesting subject in this field of research because they represent the other extreme in terms of song behaviour (Brenowitz et al. 1985). Comparative studies on three neotropical duetting wrens of the genus *Thryothorus*, the bay wren (*T. nigricapillus*), the rufous and white wren (*T. rufalbus*) and the buff-breasted wren (*T. leucotis*) and on the African duetter, the white-browed robin chat (*Cossypha heuglini*), which all differ in the degree of sexually dimorphic song production, confirmed previous results showing that the sex differences in song repertoire size correlated well with the sex differences in the volume of song nuclei in the brain (Brenowitz et al. 1985; Brenowitz & Arnold 1986; Brenowitz 1997). However, in a recent study by Gahr et al. (1998) this view was challenged. Their study species, the slate-coloured boubou shrike (*Laniarius funebris*) exhibits no sex difference in duet repertoire size despite a pronounced sex difference in song nuclei volume.

1.4. The neural control of birdsong production and learning

The song control system consists of a network of interconnected nuclei of the fore-, mid- and hindbrain that control song learning and song production. The brain nuclei of these two main pathways have been identified by means of lesion and tract-tracing studies (Nottebohm et al. 1976; Nottebohm et al. 1982). Within the descending motor pathway that controls song production, HVC (nucleus (n.) hyperstriatalis ventrale, pars caudale or high vocal center) of the neostriatum projects to RA (n. robustus archistriatalis), which sends projections to the midbrain nucleus DM (n. dorsomedialis of n. intercollicularis) and to the brainstem motor nucleus nXIIts (n. hypoglossus, pars tracheosyringealis) that innervates the muscles of the sound-producing organ, the syrinx (Fig. 1.1, blue arrows)[1]. When the motor neurons of the syrinx are stimulated, the syringeal muscles contract and the expiratory air stream leads to vibrations of the medial and lateral labia, which results in sound production (Goller & Larsen 1997). Lesions of any of the nuclei within this pathway prevent the bird from singing. Nucleus RA also projects to brainstem nuclei (Ram, rVRG), which send their projections to regions of the spinal cord, which in turn innervate respiratory muscles (Wild 1997). Electrophysiological studies revealed a hierarchical organisation of the nuclei within the motor pathway (McCasland 1987; Vu et al. 1994; Yu & Margoliash 1996).

Within the pathway that controls song learning, also known as anterior forebrain pathway (AFP, Fig. 1.1, green arrows), HVC projects rostrally to Area X, which projects via the thalamic nucleus DLM (n. dorsolateralis thalami, pars medialis) to lMAN (lateral n. magnocellularis of the anterior neostriatum) and finally to RA. Neurons of lMAN also send projections to Area X, providing the potential for feedback. Lesions of any of the AFP nuclei do not interrupt adult song production but interfere with vocal learning in juveniles. Nucleus HVC appears to be a major site for the integration of information because it is connected with both pathways and receives auditory (from field L), visual and somatosensory input (from Uva, n. uvaeformis, and from Nif, n. interfacialis, for review, see Margoliash 1997).

Several nuclei of the song control system have been shown to exhibit steroid hormone sensitivity, i.e. express steroid hormone receptors (for review, see Gahr 2001). Androgen receptors (AR) can be found in all nuclei of the descending motor pathway as well as in some nuclei of the AFP (lMAN, mMAN, DLM; Fig. 1.1, red areas). Oestrogen receptors (ER) are restricted to nucleus HVC (Fig. 1.1, orange area), which is therefore the only nucleus that contains both AR and ER. However, cells that do not express the receptor could

[1] see Appendix for revised nomenclature.

still be ‘sensitive’ because steroids cannot only act via genomic but also via non-genomic mechanisms (McEwen 1994). The spatio-temporal expression pattern of steroid hormone receptors is probably genetically controlled. For example, the AR expression in HVC of zebra finches is already sexually dimorphic at posthatching day 9 when the receptors first appear (Gahr & Metzdorf 1999). After the initial induction of the receptors during development, the expression might be retained throughout life as in the case of most songbirds studied so far. Alternatively, the expression is restricted to a short period as in the female zebra finch and is lost afterwards due to the pronounced neuronal death, which subsequently leads to the many times smaller song nuclei compared to males.

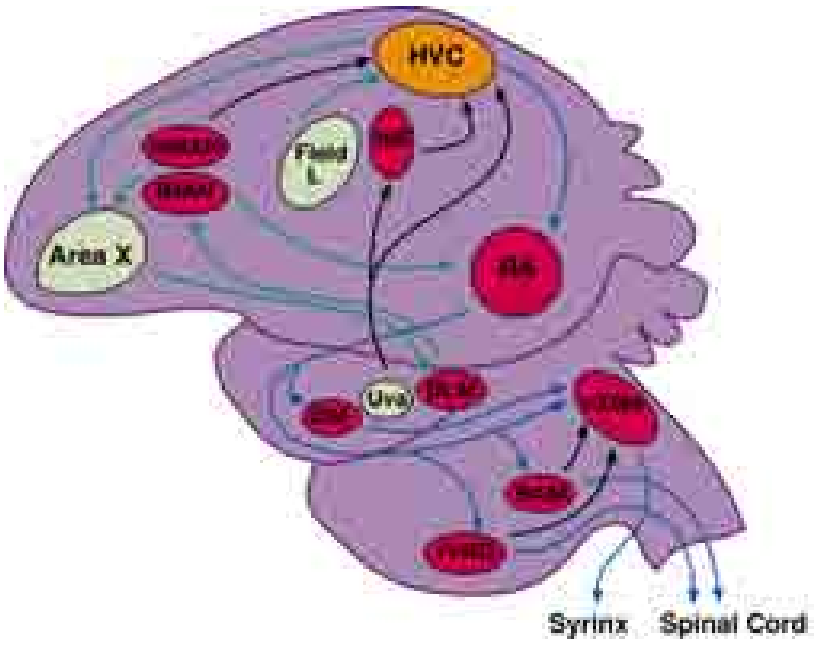

Fig. 1.1: Schematic parasagittal view of the vocal control system of songbirds showing the song nuclei and their projections (arrows). Song production is controlled by the descending motor pathway (blue arrows), which includes the nuclei HVC, RA, DM, nXIIts, Ram and rVRG. Vocal learning involves the nuclei of the anterior forebrain pathway (green arrows), consisting of HVC, Area X, DLM, lMAN and RA. HVC receives input from field L, an auditory region, as well as from nuclei Uva, Nif and mMAN. Vocal areas that express androgen receptors are in red, those that express both, androgen and oestrogen receptors (HVC only) are in orange and those that express neither are in grey. *Abbreviations*: DLM, nucleus (n.) dorsolateralis thalami, pars medialis; DM, n. dorsomedialis of n. intercollicularis; HVC, n. hyperstriatalis ventrale, pars caudale; lMAN, lateral n. magnocellularis of the anterior neostriatum; mMAN, medial n.magnocellularis of the anterior neostriatum; Nif, n. interfacialis; nXIIts, n. hypoglossus, pars tracheosyringealis; RA, n. robustus archistriatalis; Ram, n. retroambigualis; rVRG, rostroventral respiratory group; Uva, n. uvaeformis. (After Gil & Gahr 2002).

1.5. Aim of the study

In the current view of brain-behaviour differentiation of songbirds it is thought that the degree of sex differences in song nuclei size correlates with the degree of sex differences in the complexity of song behaviour, expressed in repertoire size (for review, see Ball & MacDougall-Shackleton 2001); Schlinger & Brenowitz 2002). However, there are a number of studies showing that female song nuclei differ from those of males in far more features beyond brain area size, e.g. neuron density, neuron size, synaptic properties, steroid hormone target cells (reviewed by Balthazart & Adkins-Regan 2002). Moreover, studies on several species, in which females sing, such as white-crowned sparrows (*Zonotrichia leucophrys*) (Baker et al. 1984; Baptista et al. 1993), white-throated sparrows (*Zonotrichia albicollis*) (DeVoogd et al. 1995) and slate-coloured boubou shrikes (Gahr et al. 1998) together with results obtained from testosterone-induced singing in female domesticated canaries (Nottebohm 1980; Appeltants et al. 2003) do not support such a relationship between song nuclei size and song complexity. For example, two white-throated sparrow morphs show the same degree of sex difference in HVC size although in one morph both males and females sing and have repertoires of similar size whereas in the other morph only the male sings (DeVoogd et al. 1995). Rather these data show that female song control is accomplished in a different way than it is in males. So far, functional interpretations about the observed sex differences within the song system are rare (Ball & MacDougall-Shackleton 2001). Clearly, studies are needed investigating brain-behaviour relationships at a more detailed structural level. Duetting species are interesting subjects in this regard due to their great diversity in song behaviour (Farabaugh 1982).

The aim of the present study was to investigate the neural basis of male and female song behaviour in duetting white-browed sparrow weavers (*Plocepasser mahali*). This species was chosen because it exhibits behavioural polymorphism in terms of song production, which allows conducting a comparative study. White-browed sparrow weavers are cooperatively breeding birds of eastern and southern Africa that live in groups of 2 to 10 individuals, with a dominant breeding pair and male and female subordinates (Collias & Collias 1978; Lewis 1982). Song production can be divided into two major types, duet song (when produced by more than two individuals called chorus song) and solo song. Whereas duet and chorus songs are common among all group members and are performed throughout the year, the solo song is restricted to the dominant male and only produced during the breeding season (Ferguson 1988a; Wingfield & Lewis 1993). The existence of two adult male phenotypes, dominants and subordinates, which differ in the production of solo

song, enables fruitful inter- and intrasexual comparisons of brain structure and behaviour. The focus for analysing the structure of the song control system, particularly of nucleus HVC was to go beyond brain area size by using a cytochemical approach in addition to the commonly used characterisation by cytoarchitecture. Such an approach allows identifying differences within functionally defined neuron subpopulations.

The thesis is divided into five experimental chapters. At the beginning, I give a detailed description of the song behaviour of males and females (chapter 4). Thereby I focus on the song output of dominant pairs recorded in Zimbabwe and these data are completed by studies on captive birds. The following chapter characterises the song nucleus HVC in both sexes by means of cytoarchitecture, e.g. volume, total number of cells and cell density of the nucleus (chapter 5). In the next chapter (chapter 6.1), I use the cytochemical approach and focus on the steroid hormone sensitivity of nucleus HVC by analysing the expression pattern of androgen and oestrogen receptors. In chapter 6.2, I introduce synaptic proteins as a new group of neurochemical markers for the characterisation of the song system. The basic expression pattern of these proteins and their steroid hormone sensitivity was characterised in the zebra finch because these birds are less precious than white-browed sparrow weavers and represent a model species for the study of birdsong. In chapter 6.3, I apply this new type of marker to the song system of male and female white-browed sparrow weavers. In chapter 7, I investigate the gonadal activity of males and females by measuring circulating levels of androgens and oestrogens and explore their influence on the steroid hormone sensitivity of the song system and on the expression of song. Chapter 8 covers a preliminary experiment studying the influence of exogenous testosterone on the song behaviour in females, which should reveal whether sex differences in adult song production could be attributed to ‘organisational’ or ‘activational’ effects of steroid hormones. At last, the results from all chapters are integrated and discussed (chapter 9).