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Anatomical Organisation, Auditory Processing of Communication Signals and Role of Auditory Experience in the Auditory Midbrain of the Zebra Finch

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy, The University of Auckland, 2011.
Abstract

Zebra finch males learn their vocalizations from a tutor that is usually the father. The song is biologically important for both reproduction and social interactions, making song perception a key parameter in songbird biology. Song selectivity and auditory processing in songbirds has been primarily studied in the forebrain and only a few studies have addressed the mechanisms of auditory processing in the brainstem. Since most auditory information reaching forebrain structures passes through the midbrain nucleus mesencephalicus lateralis, pars dorsalis (MLd), this nucleus is a prime candidate for early tuning to complex sounds, including conspecific signals. The focus of this thesis, therefore, was to describe the anatomical organisation of MLd with respect to auditory projections from lower brainstem nuclei and to describe the processing of both simple and complex auditory signals by MLd neurons.

In contrast to non-singing species, calcium-binding protein staining patterns in the zebra finch MLd showed two distinct subdivisions that did not match the terminal fields of ascending auditory projections. Moreover, although the nuclear expression of the IEG ZENK in MLd in response to simple (tones, white noise) and complex (conspecific and heterospecific songs) stimuli was positive, the expression pattern after tone presentation did not match the known frequency representation in the nucleus, thus questioning the validity of this widely used method.

Responses of single-units in MLd of birds reared by their conspecifics (ZF-ZF) to the same categories of simple and complex stimuli were recorded. MLd neurons were classified into units that responded to all types of stimuli and units that showed a higher level of selectivity. As a population, MLd units were also able to discriminate conspecific from heterospecific signals. These selective neurons could represent a first stage in the processing of complex song. Moreover, recordings of single units in MLd of birds reared by Bengalese finches showed the same general tuning properties but the units were not able to discriminate conspecific signals as well as those recorded in the MLd of ZF-ZF birds. That is, the rearing environment was found to influence the response properties of neurons even at this early stage of the auditory pathway.
To my grandparents,

To my parents who always supported me.

To Lola, Malheo, Agathe and Alyssa that I hope to meet soon...

Un verdadero revolucionario está guiado por grandes sentimientos de amor. Amor a la humanidad, amor a la justicia y a la verdad...

Ernesto « Che » Guevara

A moral being is one who is capable of reflecting on his past actions and their motives - of approving of some and disapproving of others.

Charles Darwin
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List of abbreviations

**AFP**  Anterior forebrain pathway
**Bas**  Nucleus basalis
**BOS**  Bird’s own song
**CaBP**  Calcium-binding protein
**CB**  Calbindin
**CB-LI**  Calbindin-like immunoreactivity
**Ce**  Central nucleus of the torus semicircularis (reptiles)
**Cec**  Core region of the central nucleus of the torus semicircularis (reptiles)
**Cep**  Peripheral area of the central nucleus of the torus semicircularis (reptiles)
**CLM**  Caudal lateral mesopallium
**CM**  Caudomedial (extension of MLd)
**CMM**  Caudal medial mesopallium
**CON**  Conspecific
**CR**  Calretinin
**CR-LI**  Calretinin-like immunoreactivity
**DC**  Dorsal cortex (mammals)
**DLM**  Medial nucleus of the dorsolateral thalamus
**DM**  Dorsomedial nucleus of the intercollicular complex
**DVR**  Dorsal ventricular ridge
**FM**  Frequency modulation
**HET**  Heterospecific
**HVC**  Abbreviation used as a proper noun
**IC**  Inferior colliculus
**ICC**  Central nucleus of the inferior colliculus
**ICo**  Intercollicular complex
**ICX**  External nucleus of the inferior colliculus (owl)
**IEG**  Immediate-early gene
**ILD**  Interaural level difference
**ITD**  Interaural time difference
**L**  Laminar and superficial nuclei of the torus semicircularis (reptiles)
**LC**  Lateral cortex (mammals)
**LLD**  Dorsal nucleus of the lateral lemniscus
**LLDa**  Anterior part of the dorsal nucleus of the lateral lemniscus
**LLDp**  Posterior part of the dorsal nucleus of the lateral nucleus
LLI  Intermediate nucleus of the lateral lemniscus
LLV  Ventral nucleus of the lateral lemniscus
LMAN Lateral magnocellular nucleus of anterior nidopallium
MLd  Nucleus mesencephalicus lateralis, pars dorsalis
MLd.I Inner region of MLd
MLd.O Outer region of MLd
NA  Nucleus angularis
NCM Caudal medial nidopallium
NIf Nucleus interfacialis
NM  Nucleus magnocellularis
NL  Nucleus laminaris
Pam Nucleus parambighalis
PV  Parvalbumin
PV-LI Parvalbumin-like immunoreactivity
OS  Superior olive
Ov  Nucleus ovoidalis
RA  Robust nucleus of the arcopallium
Ram Nucleus retroambigualis
SAM Sinusoidally amplitude modulated tones
STRF Spectro-temporal receptive field
TI  Laminar nucleus of the torus semicircularis (amphibians)
Tmc Magnocellular nucleus of the torus semicircularis (amphibians)
Tp  Principal nucleus of the torus semicircularis (amphibians)
Uva Nucleus uvaiformis
WN  White noise
Chapter I

Introduction
Songbirds have become one of the most useful models to study the ecology, evolution and neurobiology of behaviour. Not only can they be bred and domesticated fairly easily, they also present many characteristics that make them attractive for scientific studies. Songbirds (as well as hummingbirds and parrots) are vocal learners and very few species in the animal kingdom possess the ability to learn their vocalizations - apart from the obvious human example, only a few bat and cetacean species are able to learn and communicate using vocal imitation. Finally, birdsong is fairly easy to record, quantify and manipulate for playback experiments. Birdsong is used by birds in a variety of social contexts that can be reproduced in the laboratory, allowing both an analysis and quantification of behaviours that can be observed only with difficulty in the natural environment.

This chapter is devoted to these varied aspects of songbirds. The first part of this chapter focuses on the anatomical description of the avian brain: how it compares to the organisation of the mammalian brain, the song system, and the avian auditory pathway, particularly the auditory midbrain. The second part highlights the learning and structure of the complex and crucial signal that is birdsong and its functions in avian ecology and behaviour.

1 - The Avian Brain and the Evolution of Vertebrates

Among the chordates, vertebrates are thought to have diverged less than 500 million years ago (MYA; Figure 1.1) and then tetrapods diverged from the bony fish ~ some 416 MYA (Carroll, 1988). The class Amphibia was the first to branch off from the tetrapods ~360 MYA, later followed by the Reptilia ~330 MYA. The bird lineage diverged in the late Jurassic ~160 MYA. Birds are thought to have evolved from the theropod dinosaurs (for review, see Padian & Chiappe, 1998), the alvarezsauroid theropod *Haplocheirus sollers* being the oldest possible common member discovered so far (Choiniere et al., 2010) and dated between 158 and 161.2 MYA. Of the ~9000 species of living birds, about 5300 species constitute the order Passeriformes, to which the zebra finch belongs. The Order Passeriformes diverged from the
Figure 1.1

Cladogram of vertebrates
other birds some 80 MYA in the late Cretaceous (for review, see Clayton, D. F. et al., 2009) and is divided into two main groups: the suboscines (composed of about 1150 species) and the oscines (the songbirds, with ~ 4580 species). The radiation of oscine birds started around 77 MYA and, ~65 MYA, the basal oscine passerine divergence lead to the 2 major oscine clades of Passerida and Corvida. The group Passerida, that includes the zebra finches, branched off around 45 MYA and includes some 3500 species.

1.1 - Avian and mammalian brain homology

Despite the aforementioned vertebrate radiations observed, there is a common developmental and anatomical organisation of the central nervous system (Farries, 2004). There are four basic subdivisions of the embryonic vertebrate central nervous system: the spinal cord, the rhombencephalon (or hindbrain), the mesencephalon (or midbrain) and the prosencephalon (or forebrain), the last being differentiated into a diencephalon and a telencephalon. The basic pattern of organisation of the spinal cord, rhombencephalon and mesencephalon is highly conserved. The organisation of the telencephalon shows the most anatomical variation and relative size differences among vertebrates. In mammals, the telencephalon consists of a 6-layered isocortex, as well as a hippocampus, an amygdala, a pirifom cortex and a claustrum that is separated from the basal ganglia by the white matter. In contrast, the avian telencephalon is largely nuclear and pseudolaminar, with the pallium being divided into the hyperpallium (or Wulst), the mesopallium, the nidopallium and the arcopallium (Farries, 2004; Reiner et al., 2004; Striedter, 2005).

Identification of homologous structures within the adult mammalian and avian brains is controversial. Two major hypotheses have been formulated (for review, see Jarvis et al., 2005; Striedter, 2005). The Claustroamygdalar Hypothesis states that the dorsal ventricular ridge (DVR; composed of the ventral hyperstriatum, neostriatum and archistriatum) is homologous to parts of the mammalian amygdala and/or the claustrum and endopiriform nucleus. The
Neocortical Hypothesis states that certain nuclear clusters from the Wulst and the DVR are homologous to parts of the mammalian neocortex based on shared similarities in functionality and connectivity. Most recently Wang et al. (2010) reported on the presence of an interlaminar radial and columnar organization of the auditory telencephalon of the chick brain, an organisation typical of the mammalian brain.

1.2 - The Song System

A particular feature of the avian brain is seen in those lineages where song learning takes place (songbirds, parrots and hummingbirds). The song system consists of a set of interconnected nuclei, distinct from the auditory pathway (Nottebohm et al., 1976), that is involved in both vocal learning and vocal performance (Bottjer et al., 1984; Nottebohm, et al., 1976). It is observed in songbirds and, albeit with a slightly different organisation, in some hummingbirds and parrots (Brauth et al., 1997; Durand et al., 1997; Gahr, 2000; Jarvis & Mello, 2000; Jarvis et al., 2000; Paton et al., 1981; Striedter, 1994). Despite the phylogenetic distance between these three avian orders, they all evolved the capacity for vocal learning, together with a distinct set of specialized nuclei within the brain, absent or rudimentary in non vocal learning species. The song system also presents some sexual dimorphism in species where only one of the sexes sings (Arnold, 1997; Nottebohm & Arnold, 1976). In the zebra finch, for instance, only the male sings and possesses a well-developed song system, while the size of the nuclei in the non-singing female is much reduced. The song system is generally considered to be composed of two groups of interconnected nuclei: those making up a posterior premotor pathway and those making up an anterior forebrain pathway.

1.2.1 - The premotor pathway

Details of interconnections in the song premotor pathway are represented in green on Figure 1.2. The premotor pathway originates in the forebrain with the dorsal nidopallial nucleus HVC.
Figure 1.2

The avian song system

The green nuclei and arrows represent the premotor pathway involved in vocal output.
The orange nuclei and arrows represent the Anterior Forebrain Pathway involved in song learning.
(abbreviation used as a proper noun) that sends descending projections to the robust nucleus of the arcopallium (RA), which then projects directly upon vocal motor neurons in the medulla (tracheosyringeal part of the hypoglossal motor nucleus nXIIIts). RA also projects upon the dorsomedial nucleus of the intercollicular complex (DM) of the midbrain (present also in non-vocal learning species), which also projects upon vocal motor neurons, and both RA and DM project upon the respiratory-related premotor nuclei parambighualis (PAm, inspiratory) and retroambighualis (RAm, expiratory) in the hindbrain for the coordination of breathing and singing. The thalamic nucleus Uvaeformis (Uva) and the nidopallial nucleus Interfacialis (NIf) are involved in the ascending projections to HVC. These two nuclei have in common that they share connections with both the song system and the ascending auditory pathway (see section 1.3).

Forebrain nuclei of the premotor pathway play different roles in song production (for review, see Margoliash, 1997) and their neurons respond selectively and preferentially to the Bird’s Own Song (BOS) (for review, see Margoliash, 1997; Theunissen et al., 2004). These responses are recorded in anesthetized birds but can also be observed, to a much lesser extent, in the HVC of awake birds.

1.2.2 - The Anterior Forebrain Pathway

Details of interconnections in the Anterior Forebrain Pathway (AFP) are depicted in orange in Figure 1.2. The Anterior Forebrain Pathway, like the premotor pathway, originates from the forebrain nucleus HVC. HVC neurons different from those projecting to RA project upon Area X of the basal ganglia, which is composed of both striatal and pallidal components, both of which project upon the thalamus. The medial nucleus of the dorsolateral thalamus (DLM) projects back upon the lateral magnocellular nucleus of the anterior nidopallial (LMAN), which then projects, like HVC, upon RA, but with very different functions (Bottjer et al., 1989).
Chapter I: Introduction

The AFP is not required for singing but is required for song learning and recognition (Bottjer, et al., 1989; Doupe & Solis, 1997; Margoliash, 1997) and nuclei of the AFP contain BOS selective neurons (Doupe, 1997; Doupe & Konishi, 1991; Solis & Doupe, 1997; Vicario & Yohay, 1993).

1.3 - The avian ascending auditory pathway

In songbirds, as in humans, the auditory pathway mediates auditory input emanating from both other singers/speakers and from self (auditory feedback). Songbirds deafened or raised in isolation produce abnormal songs when adult; that is, auditory feedback is essential for the maintenance of normal song structure (see section 2.1.2 this chapter on auditory feedback). Therefore, a full and detailed knowledge of the routes by which the auditory information is relayed and transmitted to the highest auditory centres is necessary to investigate the emergence of selectivity through the different steps of auditory processing, from hindbrain to forebrain.

The avian auditory pathway has been fairly well studied and the main set of connections has been identified. The following sections first describe the brainstem projections, through which neuronal responses in the auditory midbrain are shaped, and then the thalamic and telencephalic projections are summarized. Details of the projections and interconnections within the ascending auditory pathway are shown on Figure 1.3.

1.3.1 - Brainstem to Thalamus Projections

The general pattern of ascending auditory projections through the brainstem of songbirds, e.g. zebra finch, parallels that observed in non-songbirds (Krützfeldt et al., 2010a, 2010b; Wild et al., 2010). Auditory information arriving over the eighth nerve from the cochlea reaches the cochlear nuclei angularis (NA) and magnocellularis (NM) (Boord & Rasmussen, 1963). NM then projects bilaterally to the third order nucleus laminaris (NL) [pigeon: (Boord, 1968; Wild, 1995); chicken: (Parks & Rubel, 1975); barn owl: (Takahashi & Konishi, 1988a); zebra finch: (Krützfeldt, et al., 2010a)]. NL and NA send efferent projections to the superior olive (OS), predominantly
Figure 1.3

The avian ascending auditory pathway

Auditory projections are represented with orange arrows. For clarity, some of the telencephalic projections are not represented. The two green nuclei are nuclei from the premotor pathway of the song system. Note that auditory nuclei CM, L1 and L3 project to the “shelf” and “cup” of these nuclei.

The blue arrow represents the direct projection from LLI in the brainstem to Bas in the forebrain.
ipsilaterally (mallards: Arends, 1981; chicken: Conlee & Parks, 1986; zebra finch: Krützfeldt, et al., 2010b; pigeon: Leibler, 1975; barn owl: Takahashi & Konishi, 1988a). NA and NL also project upon the nuclei of the lateral lemniscus. NA projects to the ventral and intermediate nuclei of the lateral lemniscus (LLV and LLI, respectively) (chicken: Conlee & Parks, 1986; pigeon: Leibler, 1975) and to the posterior part of the dorsal nucleus of the lateral lemniscus (LLDp) (barn owl: Takahashi & Konishi, 1988a). NL projects to the contralateral LLV, LLI (chicken: Conlee & Parks, 1986; pigeon: Leibler, 1975) and the anterior part of the LLD (LLDa) (barn owl: Takahashi & Konishi, 1988a). Finally, both NA and NL send projections to the contralateral midbrain nucleus mesencephalicus lateralis pars dorsalis (MLd) [pigeon: (Correia et al., 1982; Wild, 1995); chicken: (Conlee & Parks, 1986); mallard: (Arends, 1981); barn owl: (Takahashi & Konishi, 1988a)], the avian homolog of the central nucleus of the inferior colliculus of mammals (Karten, 1967).


The nuclei of the lateral lemniscus have different patterns of projections and therefore are described separately. First, LLV projects upon the two other lemniscal nuclei LLI and LLDp (Wild, et al., 2010) as well as upon MLd (pigeon: Leibler, 1975; zebra finch: Wild et al., 2009). LLV also sends projections to the thalamic nuclei Ov (pigeon: Wild, 1987) and Uva (reported in Coleman, M. J. et al., 2007) Second, LLI targets without a thalamic relay the forebrain nucleus basorostralis (Bas) (pigeon: Arends & Zeigler, 1986; finch: Hall, W. S. et al., 1993; Wild, 1997; Wild & Farabaugh, 1996; barn owl: Wild et al., 2001a). Third, LLD sends projections to its counterpart as well as to MLD (chicken: Conlee & Parks, 1986; barn owl: Adolphs, 1993; Takahashi & Konishi, 1988a; Takahashi & Konishi, 1988b; Takahashi et al., 1989). The projections of LLDa and LLDp onto MLd differ in their terminal fields depending on the
species. While these terminal fields are segregated in the non-songbirds, they substantially overlap in the zebra finch, presumably with different implications for auditory processing (Krützfeldt, et al., 2010a). LLDp also projects upon the contralateral LLDp, as seen in the barn owl (Manley, G. A. et al., 1988; Takahashi et al., 1995; Takahashi & Keller, 1992) and LLDa projects upon the contralateral LLDa in zebra finches (Wild, et al., 2010), a connection that has not been being documented so far in any other avian species. LLD also sends projections to Ov (pigeon: Wild, 1987; zebra finch: Wild, et al., 2010), which create a route that bypasses the midbrain. Finally, MLd sends projection to its counterpart (Takahashi, et al., 1989; Wang & Karten, 2010) as well as to Ov bilaterally (pigeon: Wild, 1987).

1.3.2 - Thalamic and telencephalic projections

Ov and Uva are the two thalamic nuclei that receive projections from the brainstem and that relay auditory information to the forebrain. As mentioned above, Uva is the recipient of LLV projections (Coleman, M. J., et al., 2007) and constitutes the first auditory input into the song system, projecting to both the interfacial nucleus of the nidopallium (Nif) and HVC in the forebrain (Nottebohm et al., 1982; Wild, 1994; Williams, 1989). Ov, in contrast, has no involvement in the song system, although Lei and Mooney (2010) showed recently that electrical stimulation in Ov disrupts the song. It receives its main input from MLd, with additional inputs from LLV, LLDa, LLDp, and then projects massively upon the forebrain. Karten (1968) suggested that Ov was homologous to the ventral nucleus of the medial geniculate body of mammals. The pattern of Ov projections to the forebrain has been examined in both non-songbird (guinea fowl: Bonke et al., 1979; ring dove: Durand et al., 1992; pigeon: Karten, 1967; Karten, 1968; Wild, 1987, 1993) and songbird species (canary: Kelley, D. B. & Nottebohm, 1979; zebra finch: Vates et al., 1996). Ov’s main projection is to field L2 in the caudomedial hemisphere, which Karten (1968, 1969) suggested was homologous to layer IV of mammalian auditory cortex (see also Wang & Karten, 2010). Ov also has some projections to regions
flanking L2, namely L1 and L3 and the caudomedial nidopallium (NCM) (Vates, et al., 1996). Periovoidal nuclei, which do not receive MLd afferents, nevertheless possess auditory neurons and project upon other regions of the auditory telencephalon, such as NCM and CLM (Durand, et al., 1992). Much current work on the auditory forebrain is concerned with the analysis of auditory processing through the various field L laminae (L2, L1, L3), their connections with the adjacent caudomedial and caudolateral mesopallium (CMM and CLM) and NCM, and with song recognition, song memory and auditory-vocal integration. Detailed documentation of this work is beyond the scope of this thesis and is not considered further.

1.4 - Focus on the avian auditory midbrain nucleus MLd

The auditory midbrain of tetrapods is referred to either as the inferior colliculus (IC), MLd or torus semicircularis. There is an accepted core/shell (belt) organization of this nucleus, with the core being exclusively auditory and the shell or belt receiving multisensory, including auditory, information. The core is characterized by a tonotopic organization resulting from topographic projections from lower brainstem auditory nuclei and it conveys auditory information to the thalamus. Auditory information in the belt is composed of minor ascending auditory projections, projections from the core itself, as well as from descending projections from higher auditory centres (Mello et al., 1998).

In the present thesis, the MLd of songbirds is defined as the midbrain region receiving ascending projections from the auditory brainstem, as previously described (Krützfeldt, et al., 2010a; Wild, et al., 2010). In the midbrain of the zebra finch, as in other avian species, MLd is surrounded by the intercollicular complex (ICo) (see schematic in Figure 2.4, Chapter II). Its boundaries can be defined on the basis of its cytoarchitecture, although its ventral and ventrolateral borders can prove difficult to differentiate from the adjacent ICo. In the transverse plane MLd has an ovoid shape and is oriented obliquely along a ventrolateral to dorsomedial axis. At its caudal and rostral poles MLd abuts the medial edge of ICo, but at intermediate rostrocaudal levels, where it
Chapter I: Introduction

reaches its maximum size, it expands laterally from this edge towards the tectal ventricle, from which it is separated only by a thin periventricular lamina (Puelles et al., 1994). At caudal levels MLd presents a dorsomedial extension called CM (caudomedial, Puelles, et al., 1994), which has been identified in greenfinches (Wild, 1997), pigeons (Wild, 1995) and chickens (Puelles, et al., 1994). At more rostral levels the dorsomedial border of MLd is straight-edged, where it abuts a core nucleus of the intercollicular complex (Puelles, et al., 1994), commonly known as the dorsomedial nucleus (DM). Unlike in pigeons (Karten, 1967) or chickens (Puelles et al., 2007), MLd in zebra finches lacks a clear hilar region on its medial aspect, (Karten, 1967; Puelles, et al., 2007) so that its afferents from auditory brainstem nuclei enter the nucleus over a wide dorsoventral extent (Boord, 1968; Conlee & Parks, 1986; Konishi, 2003; Krützfeldt, et al., 2010a; Wang & Karten, 2010; Wild, 1995; Wild, et al., 2010). MLd was originally shown by Karten (1967) (see also Wild, 1987) in pigeons to project to Ov in the thalamus, and later also confirmed in ring doves (Durand, et al., 1992), barn owls (Proctor & Konishi, 1997) and zebra finches (Krützfeldt, et al., 2010a).

As mentioned previously in section 1.3.1, a major difference in songbirds arises in the organisation of MLd, by virtue of the afferent projections from the lower auditory brainstem nuclei. While the terminal fields of NA and NL projections are segregated within MLd in chickens (Conlee & Parks, 1986; Wang & Karten, 2010), pigeons (Leibler, 1975; Wild, 1995) and barn owls (Takahashi & Konishi, 1988a), they extensively overlap in zebra finches (Krützfeldt, et al., 2010a). In barn owls, core and shell regions are the recipients of NL and NA projections, respectively, and have specific neurophysiological properties that originate from separate time-coding and intensity-coding pathways (referred as ITD and ILD, respectively). These pathways eventually converge in the lateral shell, from where projections are sent to the external nucleus, ICX, where a map of auditory space emerges (for review, see Konishi, 2003). This organisation in barn owls is consistent with their nocturnal habits and other anatomical specialisations of the auditory apparatus. Therefore, the specific differences in organisation
reported in songbirds could have evolved to serve different functional needs arising from the motor and perceptual complexities associated with vocal learning.

Neurons of the auditory midbrain have been shown to be sensitive to species-specific temporal and spectral features in mammals (for review, see Covey & Carr, 2005; Portfors & Sinex, 2005), amphibians (for review, see Bass et al., 2005; Feng et al., 1990; Wilczynski & Ryan, 2010) and fish (Bass, et al., 2005). The encoding of species-specific properties is, of course, crucial for any individual of a species to perceive and respond to its conspecifics. Because of its position along the auditory pathway, the auditory midbrain is a perfect candidate to compute these varied inputs and encode complex features of species-specific vocalisations. Given that conspecific and heterospecific sounds are defined by their spectral and temporal components, the convergence of the ascending information is a necessary step in the integration of the features used for the discrimination of conspecific and heterospecific signals. Conspecific sounds usually share a common spectral component (i.e. the frequency range of the vocalisations) and vary in their temporal aspects (amplitude and frequency modulations). It has been shown that the auditory midbrain of anurans presents filtering properties sensitive to the spectro-temporal characteristics of conspecific calls. For instance, neuronal responses encode not only frequency modulations and combination of tones but also duration of notes, amplitude modulations and pulse repetition (for review, see Feng, et al., 1990; Rose, G. J. & Gooler, 2006; Wilczynski & Ryan, 2010). In the midshipman fish auditory midbrain units are tuned to frequencies within the spectral range of conspecific calls but also encode the difference between two frequencies (dF) making up the beats generated by the hums (fish vocalisation) of two neighbouring males. The degree of synchronisation of individual units (measured as vector strength) to varying values of dFs could not be explained by either their spike rate to dFs or by their frequency tuning to each of the two frequencies (for review, see Bass & McKibben, 2003; Bass, et al., 2005). Similarly, in mormyrids (that use repetitive pulses to vocalise) auditory midbrain neurons exhibit selective responses to inter-click intervals (for review, see Bass & McKibben, 2003; Bass, et al., 2005).
mammals, encoding of and selectivity to echolocation signals in the auditory midbrain have been extensively studied in bats. Bats emit either frequency-modulated (FM) or constant frequency-frequency modulated (CF-FM) ultrasonic calls. Midbrain neurons show a sharp tuning to frequencies near the predominant frequency of the call, respond to FM sweeps with selectivity to direction, and encode duration of the stimulus. Responses of midbrain neuron to calls are shaped by both excitatory and inhibitory processes, in contrast to lower brainstem nuclei where responses are evoked solely by excitation (for review, see Covey & Carr, 2005; Portfors & Sinex, 2005).

The few studies in the finch MLd reported a dorsoventral tonotopic organization of the nucleus, with low frequencies being represented dorsally and high frequencies ventrally (Woolley, S. M. N. & Casseday, 2004), consistent with that described in other avian species and tetrapods (Bass, et al., 2005; Cant, 2005; Covey & Carr, 2005). MLd neurons respond to features specific of the complex signal that is birdsong, such as frequency modulated (FM) sweeps and sinusoidally amplitude modulated (SAM) tones (Woolley, S. M. N. & Casseday, 2005). Neurons of MLd were also shown to be broadly tuned to frequencies and exhibit onset responses to stimuli that would make them well suited for temporal processing (Woolley, S. M. N. & Casseday, 2004, 2005). Spectro-temporal receptive field (STRF) analysis also characterised different functional groups of neurons in MLd and showed that they are well suited to encode elements of songs such as amplitude envelope or syllable onsets. Schneider and Woolley (2010) investigated neural discrimination to conspecific songs in the zebra finch midbrain. They established that while the firing rate and spike train duration increased discriminatory ability of single neurons temporal and spectral tuning did not. They also demonstrated that discrimination between conspecific songs was better when the output of pooled single neurons was considered, especially when these units presented similar tuning properties.


2 - Birdsong

2.1 - Song learning and the importance of auditory feedback

2.1.1 - Song learning

In songbirds, song is a learned vocalization and the learning process in young birds follows a similar pattern in different species (Zann, 1996). Before a young bird, typically a male, starts singing, he will first listen and memorize the song of the tutor, typically the father, during the sensory learning period (that ends ~ 65 days of age; see Figure 1.4 below). A young male will copy elements present in the tutor’s song and rearrange them to form his own song (Figure 1.5). However, in some cases and frequently in aviaries due to the proximity of the cages and the availability of many tutors, a young male will copy chunks of song from more than one tutor (Williams & Staples, 1992). Still, each male will end up singing a unique song (Morris, 1954). In zebra finches between ~ 35 to ~ 50 days of age, the young bird will produce a subsong that parallels babbling in infants. The male starts producing vocalizations, and calibrating the output to the memorized tutor template. From ~ day 50 up to ~ 80 days of age, the young male goes through a plastic song phase. He will elaborate a plastic song that starts resembling the adult song and by around 60 days of age most of the elements and the organisation of the mature song are observed. By 90 days of age the bird enters the crystallisation phase: the song is now in its adult, stereotyped form. The boundaries between the different phases are quite plastic and show some overlapping and their length can even be extended if the bird is denied a tutor initially but is presented with one later (Eales, 1985; Funabiki & Funabiki, 2009; Jones et al., 1996; Morrison & Nottebohm, 1993). The length and timing of these stages also varies according to the species and whether the birds are seasonal breeders or not. It has also been shown that although birds will take their father for a tutor in the wild, songbirds bred in aviaries can readily learn from playbacks (Houx & ten Cate, 1999; Marler & Peters, 1988; Phan et al., 2006; White, 2001) and will develop normal songs using those playbacks as models. However, zebra finches that are
deprived of a tutor altogether will develop abnormal songs (Price, 1979), with alterations in note structure, while the temporal pattern is largely retained.

**Figure 1.4**

Phases of song learning

![Phases of song learning diagram](image)

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2.1.2 - **Auditory feedback and song maintenance**

Auditory feedback is necessary for songbirds in order to maintain normal song and singing behaviour, as has been shown in deafening experiments (for review, see Konishi, 2004). Birds deafened by cochlea removal before they have started to sing produce songs that are much more abnormal than the songs of birds raised in isolation, and more abnormal than birds deafened at different times (between 7-10 days, ~20 days, first year bird, adults) during song development. Therefore, it has been proposed that birds possess an innate song template but need to be in contact with a tutor to develop a normal song. Deafening in adult songbirds also provokes a degradation of the song, but the time course and degree of degradation seem to be species-related, with Bengalese finch songs degrading much faster than zebra finch songs (Lombardino & Nottebohm, 2000; Nordeen & Nordeen, 1992; Okanoya & Yamaguchi, 1997; Woolley, S. M. N. & Rubel, 1997, 2002). When a bird is deafened, introductory notes in the bird’s own song retain a normal structure, but significant changes can be seen first in the structure of syllables and later in syllable order. Also, deafening experiments in zebra and Bengalese finches have provided important insights into which features of song are used by the birds in maintaining their songs. It appears that proper hearing of low-frequencies below 1500Hz is actually sufficient for
**Figure 1.5**

Song learning: copying from a tutor and structure of the crystallised song

Example of crystallized songs of a male and the three males he tutored (here a father and his three genetic sons). Note how the young males copied several elements of their father’s song, that they arranged in a different order, and also created some new elements.

*i* = introductory notes; *a-g* = elements of the father’s song; *new* = new note created by the young tutored males; *Motif* = assemblage of elements in a defined sequence
the bird to maintain normal singing behaviour. However, it is still not known what aspect of information (spectral or temporal) contained within that frequency range is sufficient for song maintenance. In reversible deafening experiments, the song matched the pre-deafening song in both the order and structure of syllables after normal hearing was restored. This also indicates that the birds retained their memorized song template stored while singing the degraded song.

2.2 - Birdsong structure

2.2.1 - The Zebra finch song

Zebra finches are closed-ended learners, in contrast to other species such as the canaries, which learn new song elements each year. Close-ended learners learn the one song they will sing within the first 120 days after hatching, while open-ended learners continue to add elements into their songs every season. One consequence of this is the long-term stereotopy of the zebra finch song. Once crystallised (see section 2.1.1 for learning stages), it will be delivered in a very specific fashion, the order of the syllables as well as their acoustic features being sung with very little variability. A zebra finch sings only one song, the “canonical song” with a preferred and invariant order of syllables. The “song” or “bout” usually consists of 2 to 8 introductory notes (Price, 1979) followed by the repetition, within one rendition, of an assemblage of several elements in a defined sequence which is called a “motif” or “song-phrase” (for review, see Williams, 2004; Zann, 1996).

The song of a zebra finch will possess 3 to 15 “notes” or “elements” and a motif will be composed of all or most of them with 5 to 10 ms of silence between elements. These elements are divided into two categories: call-like and non call-like, with the call-like elements representing ~ 67 % of the sung elements. The non-call-like elements provide most of the variability that can be observed in a song.

Birdsong usually has a function in territory defence and female attraction. Estrildid finches are not territorial and, therefore, the song is used in courtship and in male-female interactions (for
Zebra finch males sing one song but they can modify both the quality and quantity of song rendition when singing to a female. This directed song is longer, faster and less spectrally variable than the undirected song, which is the song a male sings alone (Cooper & Gooler, 2006; Kao & Brainard, 2006; Ölveczky et al., 2005; Teramitsu & White, 2006).

The directed song has two principal functions. First, it is used as a pre-copulatory signal (Clayton, N. S. & Pröve, 1989) and not only do females prefer song of conspecifics but they can also recognize and usually prefer songs of males from their own subspecies (Clayton, N. S., 1990; Clayton, N. S. & Pröve, 1989). Second, it is both an individual and a kin recognition signal that is used by females to recognize the songs of their mates (Miller, 1979a) and by daughters used to recognize the song of their father (Miller, 1979b). Both of these signals, mate’s and father’s songs are also preferred by females. The undirected song has been found to be a signal more related to attraction of females and as an indicator of quality of the male. It also appears to be used when a pair is building their nest and laying eggs (see Zann, 1996).

2.2.2 - Cross-fostering

In the experiments carried out in this thesis, some of the birds used were zebra finches that had been cross-fostered with Bengalese finches. Those birds are different from hybrids, in that they are not the offspring of a zebra and a Bengalese finch. Bengalese finches are estrildid finches, related to zebra finches. Like zebra finches, they require auditory feedback for the maintenance of song in adulthood (Okanoya & Yamaguchi, 1997; Woolley, S. M. N. & Rubel, 1997) and their songs share with those of zebra finches the same frequency range (Woolley, S. M. N., 2004) and notes composed of harmonic stacks. Bengalese finches sing stereotyped songs with sequences of syllables, but their songs differ from those of zebra finches in that they are not linear (some variation in note order can be observed, for review, see Okanoya, 2004), and they contains trills that are composed of fast repeated short notes.
Zebra finches that are reared by Bengalese finches sing different songs than normal zebra finches or isolate zebra finches: they will incorporate into their songs notes that they copy from their Bengalese foster-father (Clayton, N. S., 1989; Eales, 1987). Takahasi et al. (2006) also reported that, even though cross-fostered zebra finches learn Bengalese finch specific song elements well, the element structure is actually incomplete. Cross-fostered zebra-finches will sing a song that will contain more elements and that will also be longer than the normal zebra finch song (although not as long as the Bengalese finch song) (Clayton, N. S., 1989; Eales, 1987; Takahasi, et al., 2006). That zebra finches can incorporate features of the Bengalese finch song shows that the zebra finch auditory system can not only process the Bengalese finch song but can also categorise those elements as ‘song’. This feature is necessary to test the role of experience in shaping auditory responses.

3 - Aim of the thesis:

In light of the scant information about the organisation of the songbird MLd, the aims of this thesis are: a) to provide an anatomical and electrophysiological characterisation of MLd, b) to examine the role of MLd in the processing of species-specific signals and c) to examine the role of experience in shaping the electrophysiological responses of MLd neurons.

The recent report that the organisation of the auditory projections to MLd from the brainstem in the zebra finch differs from that observed in non-songbirds (Krützfeldt, et al., 2010a) underscores the need to re-evaluate the anatomical description of the subdivisions of MLd. Chapter II, therefore, describes patterns of calcium-binding protein immunohistochemistry in the MLd of songbirds and shows how these relate to the ascending auditory projections.

To investigate the auditory processing within MLd to both simple and complex stimuli, two approaches were used. Chapter III examines the expression of Immediate-Early Genes (IEGs), with the aim of obtaining an initial overview of MLd neuronal responses to simple (tones and
noise) and complex stimuli (songs of conspecific and heterospecific birds) with respect to the subdivisions delineated in Chapter II. Chapter IV provides a detailed electrophysiological characterisation and description of tuning properties of single MLd units in response to the simple and complex stimuli.

The role of experience in shaping the response properties of MLd neurons is examined in Chapter V. The electrophysiological properties of MLd neurons in normal zebra finches are compared to those of MLd neurons from zebra finches reared by Bengalese finches. The songs of cross-fostered zebra finches exhibit some zebra finch characteristics, but also introduce some Bengalese finch specific traits. What is examined here, then, is the extent to which this exposure, and its consequent effects on song output, is accompanied by changes in the processing properties of MLd neurons.
Chapter II

Subdivisions of the Auditory Midbrain in Zebra Finches using Calcium-Binding Protein Immunocytochemistry
1 - Introduction

The nucleus mesencephalicus lateralis pars dorsalis (MLd) has been extensively studied both anatomically and electrophysiologically in several avian species, e.g., chicken (Coles & Aitkin, 1979; Conlee & Parks, 1986; Puelles, et al., 1994; Wang & Karten, 2010; Zeng, S et al., 2008a), guinea fowl (Scheich et al., 1977), mallard (Arends, 1981), barn owl (Knudsen, E. I., 1983; Knudsen, E. I. & Konishi, 1978; Konishi, 2003; Kubke et al., 1999; Takahashi et al., 1987; Takahashi & Konishi, 1988a; Wagner et al., 2003; Wagner et al., 1987) and pigeon (Correia, et al., 1982; Leibler, 1975; Wild, 1995). Fewer data are available for the MLd of songbirds, which comprise roughly half the number of avian species. In birds generally the nucleus is a major relay in the ascending auditory pathway to the forebrain but in songbirds it also has the special function of mediating auditory feedback for the learning and maintenance of song. The ascending projections to MLd have been recently described (Krützfeldt, et al., 2010a; Wild, et al., 2010), while the present study describes the regional organization of MLd in zebra finches based on the different staining patterns resulting from the application of antibodies to three calcium binding proteins.

Most of our knowledge of the anatomical organization of MLd in songbirds derives from histochemical and immunohistochemical studies in zebra finches (Braun et al., 1991; Braun et al., 1985; Montagnese et al., 1993; Watson et al., 1988), and from studies that have examined auditory and somatosensory connections of MLd and adjacent structures in various finches (Krützfeldt, et al., 2010a; Wild, 1997; Wild, et al., 2010; Zeng, S. et al., 2004). In the present study, MLd is defined as the midbrain region receiving ascending projections from the auditory brainstem, as previously described (Krützfeldt, et al., 2010a; Wild, et al., 2010). Antibodies against calbindin (CB), calretinin (CR) and parvalbumin (PV) were chosen to describe the internal structure of the nucleus, these three calcium-binding proteins (CaBPs) being expressed in different neuronal subpopulations in both the peripheral and central nervous systems, with
only a partial overlap (Andressen et al., 1993; Baimbridge et al., 1992). They belong to the EF-hand family of CaBPs (Persechini et al., 1989) and generally bind to calcium ions (Grabarek, 2006). The expression of these three proteins has been used for many years to describe anatomically various sensory pathways and nuclei (for review, see Andressen, et al., 1993), including the auditory torus of amphibians (Endepols et al., 2000; Zeng, S. et al., 2008b), reptiles (Belekhova et al., 2010; Belekhova et al., 2004; Yan, 2008; Yan et al., 2010), birds (Braun, et al., 1991; Braun, et al., 1985; Kubke, et al., 1999; Puelles, et al., 1994; Takahashi, et al., 1987; Wagner, et al., 2003; Zeng, S, et al., 2008a) and mammals (Celio, 1990; Coleman, J. R. et al., 1992; Friauf, 1994; Glezer et al., 1998; Idrizbegovic et al., 1999; Kelley, P. E. et al., 1992; Lohmann & Friauf, 1996; Paloff et al., 2004; Resibois & Rogers, 1992; Rogers & Resibois, 1992; Seto-Ohshima et al., 1990; Sharma et al., 2009; Tardif et al., 2003; Vater & Braun, 1994; Zeng, S. et al., 2009; Zettel et al., 1991; Zettel et al., 1997). CaBP immunocytochemistry has been used previously to describe MLd in zebra finches (Braun, et al., 1991; Braun, et al., 1985), although these studies mainly examined the patterns of expression in male birds (both adults and juveniles) and were performed in the absence of knowledge of the pattern of ascending inputs to MLd in this species. The present study, therefore, complements these previous descriptions by examining the patterns of expression in adult male and female zebra finches, in the context of our knowledge of the ascending inputs to MLd (Krützfeldt, et al., 2010a; Wild, et al., 2010) and the electrophysiological in vivo data now available (Hsu et al., 2004; Woolley, S. M. N. & Casseday, 2004, 2005; Woolley, S. M. N. et al., 2009; Woolley, S. M. N. et al., 2006)).

2 - Materials and Methods

2.1 - Animals

The experimental procedures were carried out according to the guidelines of the Animal Ethics Committee of the University of Auckland. Birds were obtained from a local breeder, housed in a
large flight aviary, provided with food and water ad lib, and maintained under a constant light/night cycle in the University of Auckland animal facility.

2.2 - Immunocytochemistry

Each of the 7 birds was deeply anaesthetized with an intramuscular injection of equal parts of ketamine (Parnell Laboratories, Auckland, New Zealand; 166 mg/kg) and xylazine (Rompun Bayer; 33 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB 0.1M, pH 7.4) as per University of Auckland Animal Ethics Committee permits. The brains were post-fixed in 4% paraformaldehyde (PFA) before being cryoprotected in 30% sucrose in phosphate buffered saline (PBS 0.01M, pH 7.4). They were then cut coronally on a freezing microtome and serial, free-floating 35\(\mu\)m thick sections were collected in PBS in four columns. Sections in one column were mounted on subbed slides and stained with cresyl violet for the identification of cytoarchitecture. Sections in the three other columns were immediately processed for immunocytochemistry. After three 10 minutes rinses (all rinses in this protocol lasted 10 min) in PBS, endogenous peroxidase activity was blocked using 50% methanol and 1% hydrogen peroxide in distilled water for 10 min at room temperature. Sections were then rinsed 3 times in PBS before being incubated in a primary antibody against PV, CB or CR (monoclonal anti-parvalbumin antibody: SWANT clone 235, Basel, Switzerland for three males and three females; monoclonal anti-calbindin D28k: SWANT clone 300, Basel, Switzerland for three males and three females; polyclonal anti-calretinin antibody: SWANT 7699/4, Basel, Switzerland for three males and three females; monoclonal anti-calretinin antibody: SWANT 6B3, Basel, Switzerland for one male) at a final dilution of 1:5000 in PBS with 0.4% Triton X-100 (PBS-T) and 2% Normal Horse Serum (NHS) overnight at room temperature. After 3 rinses in PBS, sections were transferred to a biotinylated donkey anti-mouse secondary antibody (711-065-150, Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in PBS-Triton with 2% NHS at a dilution of 1:300 for 90 minutes at room temperature and rinsed 6 times in PBS before being incubated in avidin-biotin peroxidase.
complex (PIE 31001, Global Science and Technology Ltd, Auckland, New Zealand) at 1:1000 in PBS-T for 1h at room temperature. Following 6 rinses in PBS, sections were finally incubated in a chromagen-solution consisting of 0.025% 3,3’-diamino-benzidine (DAB), 0.005 % H2O2 and 0.015% CoCl2 in PBS. The reaction was stopped by several washes in PBS. Sections were subsequently mounted on subbed slides, dehydrated in successive ethanol baths, cleared in xylene, and coverslipped using DePeX (Serva, Heidelberg, Germany).

2.3 - Analysis

All of the material was examined using light microscopy. Comparisons between different staining patterns were performed on adjacent sections, and the overall staining patterns and their boundaries were mapped onto a single, serially cut, cresyl violet-stained zebra finch brain for inter-individual comparisons. Outlines of the nucleus and visible subdivisions were drawn using a camera lucida and then scanned into a personal computer for digital representation. Sections were also digitally photographed using a Nikon 80i light microscope (5 megapixel camera). Levels were then adjusted using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA).

3 - Results

3.1 - Parvalbumin-LI

The general parvalbumin staining pattern in MLd described by Krützfeldt et al. (2010a) was shown to overlap closely the area in receipt of ascending brainstem auditory afferents, thereby defining the boundaries of MLd. The present study describes regional differences in the pattern of staining in more detail. Two regions within MLd were defined by the PV-LI: inner (MLd.I) and outer (MLd.O) (Figure 2.1A-D). MLd.O was characterized by a PV-LI positive neuropil and by the presence of stained fibers and stained and unstained somata (Figure 2.1F). The neuropil in this region showed a dense punctate staining pattern, whereas many of its neurons presented
**Figure 2.1**

**Patterns of parvalbumin-like immunoreactivity (PV-LI)**

**A-D:** caudal (top) to rostral (bottom) coronal sections showing PV-LI through the right MLd of a female zebra finch delineating two regions within the nucleus: MLd.O and MLd.I. The asterisk in **B** indicates the small nucleus within MLd.O that shows a different staining pattern.

**E-G:** high magnifications views of areas boxed in **A** and **B. E:** Photomicrograph of MLd.I (inset **E** in panel **A**) showing the specific neuropil staining and low punctate density of this region. **F:** Photomicrograph of MLd.O (inset **F** in panel **A**) depicting the neuropil staining, high punctate density and somata with visible processes specific to this region. **G:** high magnification view of the region marked by an asterisk in **B.** Note the darker neuropil staining than that of MLd.O and the absence of punctate staining. **H:** Photomicrograph of a Nissl stained section also shown in Figure 2.4B, showing the dorsomedial MLd subnucleus marked with an asterisk in **B**

Scale bar: A-D = 100μm; E-G = 50μm
stained processes and those along the lateral edge of MLd exhibited elongated somata concentric with the overlying ventricle (not shown). At the level of DM, a small region on the dorsomedial aspect of MLd that can be identified in Nissl stained sections (Figure 2.1B-asterisk, G, H-asterisk) exhibited a staining pattern distinct from that of the MLd.O. This small region had a darker PV-LI positive neuropil, stained fibers and stained somata and processes, but was characterized by an absence of punctate staining.

The inner region (MLd.I) could be distinguished by an apparent lower density of stained puncta (Figure 2.1E; cf Figure 2.1F). Like MLd.O, MLd.I also showed an immunopositive neuropil, stained and unstained somata, and some punctate staining. The processes of the stained neurons were, however, not visible in most cases. Incoming fibers - presumably mostly afferents from lower auditory nuclei - were seen mainly through the caudal half MLd. Caudally they were found widely dispersed along the ventromedial edge of the nucleus, while rostrally these were found more restricted to the most ventral edge (data not shown, but see Krützfeldt, et al., 2010a; Wild, et al., 2010).

3.2 - Calretinin-LI

Brains of three males and three females were immunostained using a polyclonal anti-calretinin antibody and one other brain from a male was immunostained using a monoclonal anti-calretinin antibody. The staining patterns resulting from use of the monoclonal antibody were better delineated than those from use of the polyclonal, but were otherwise identical and are thus described together. Incubation with antibodies raised against calretinin resulted in a pattern of staining that, as in the case of parvalbumin, revealed two distinct regions within MLd: inner (MLd.O) and outer (MLd.I) (Figure 2.2A-D). Caudally, MLd.O occupied a ventral position within the ICo, expanding towards the tectal ventricle at intermediate rostrocaudal levels (Figure 2.2A). MLd.O lay on ventrolateral to dorsomedial axis, finally to be located in a dorsal position, underlain by the ICo at more rostral levels. The outer region presented a relatively unstained
Figure 2.2

Patterns of calretinin-like immunoreactivity (CR-LI)

**A-D:** caudal (top) to rostral (bottom) coronal sections showing CR-LI through the right MLd of a male zebra finch delineating MLd.I and MLd.O regions within the nucleus and the ICo. The white arrow in A points to the presumptive ascending afferent fibers. The asterisk in B indicates the small nucleus within the MLd.O that shows a different staining pattern.

**E-G:** high magnifications views of areas boxed in A, B and C. **E:** Photomicrograph of the MLd.I and MLd.O (inset E in panel A) showing the specific neuropil staining of these regions as well as the trajectory of presumptive ascending auditory fibers. **F:** high magnification view of the region marked by an asterisk in B. Note the lighter neuropil staining. **G:** Photomicrograph of MLd.I (inset G in panel C) showing the intense CR-LI staining of the neuropil. **H:** Photomicrograph of the MLd.O (inset H in panel C) showing the absence of neuropil staining and the presence of a few stained somata and fibers.

Scale bar: A-D = 100μm; E-G = 50μm
neuropil, within which some stained somata as well as numerous fibers were observed (Figure 2.2E and H). Within this region, the same small, dorsomedial part found to be PV-positive (see above and Figure 2.1B and G) was either devoid of staining or presented a very light neuropil staining. (Figure 2.2B-asterisk, F-asterisk).

MLd.I was readily distinguished from MLd.O by its darkly-stained neuropil (Figure 2.2E and G). Some stained somata, as well as perikaryal ‘ghosts’ and terminal boutons, were observed. MLd.I occupied a central position caudally (Figure 2.2A), extended along a ventrolateral to dorsomedial axis at intermediate levels where MLd is at its largest (Figure 2.2B and C), and occupied a dorsal position rostrally (Figure 2.2D). CR-LI fibers were seen running along the external surface of MLd.I, and some CR-LI fibers were also seen traversing it (Figure 2.2E). The trajectory of presumptive ascending afferent fibers was seen mainly through the caudal half of the nucleus. As with the fibers showing PV-LI, CR-LI fibers entered MLd caudally from its internal edge over a wide extent (Figure 2.2A, white arrow), while rostrally they were restricted more ventrally.

The outer and inner regions defined by CR-LI correspond to the outer and inner regions defined by PV-LI. Outside but immediately adjacent to MLd, an external CR-LI positive region was identified (Figure 2.2A-D), characterized by an immunopositive neuropil, within which numerous small stained somata were observed; a few of these also showed stained processes.

Caudally and rostrally, this region surrounded MLd (Figure 2.2A, C and D) and at intermediate levels it was restricted to a ventrolateral position (Figure 2.2B). Since this region does not receive ascending auditory inputs (Krützfeldt, et al., 2010a; Wild, et al., 2010) it is considered part of ICo (Krützfeldt, et al., 2010a; Wild, et al., 2010).

**3.3 - Calbindin-LI**

Calbindin-LI was also different in the inner (MLd.I) and outer (MLd.O) regions of MLd, although it did not reveal the two subdivisions as clearly as the other two antibodies (Figure 2.3A-D) but changes in the homogeneity of the staining of neuropil and somata were observed.
Figure 2.3

Patterns of calbindin-like immunoreactivity (CB-LI)

A-D: caudal (top) to rostral (bottom) coronal sections showing CB-LI through the right MLd of a female zebra finch delineating MLd.I and MLd.O within the nucleus and the ICo. The asterisk in B indicates the small nucleus within MLd.O. Note that it is not as clearly visible as with the PV- and CR-like staining.

E-G: high magnification views of areas boxed in A, B and C. E: Photomicrograph of MLd.I (inset E in panel A) showing the specific high density of labeled terminals in this region (some are indicated by arrow heads) and the absence of neuropil staining in intermediate sections. F Photomicrograph of MLd.I (inset F in panel C) showing the staining pattern in a more rostral section. Note the high density of labeled terminals and the difference in neuropil staining as well as the number of stained somata. G: Photomicrograph of ICo (inset G in panel B) showing the CB-LI staining of this region. H: Photomicrograph of PV-LI staining in ICo in an adjacent section (inset H in panel B - Figure 2.1) showing the absence of neuropil staining.

Scale bar: A-D = 100μm; E-G = 50μm
For instance, at its most caudal and rostral levels, MLd showed a CB-LI positive neuropil and stained somata, some of which had immunostained processes and terminal boutons in both MLd.I and MLd.O (Figure 2.3C and D). At intermediate levels, MLd.O showed light or no staining of neuropil, with some stained somata. The lateral and dorsal parts of MLd.O had a lightly stained neuropil, while its medial aspect appeared devoid of it (Figure 2.3B). Also, the whole region presented some stained somata as well as a low density of terminal boutons. The subnucleus at the dorsomedial corner of MLd that shows distinct PV-LI and CR-LI and is also visible in Nissl stained-sections (Figure 2.3B asterisk), showed a light neuropil staining and some small stained somata with stained processes. In contrast to calretinin and parvalbumin staining, CB-LI did not stain presumptive afferent fibers to MLd. MLd.I did not present a homogeneous staining throughout the whole nucleus; at intermediate levels it was devoid of neuropil staining but was filled with a considerable number of terminal boutons and a few CB-LI positive large somata, which were less frequently observed than in MLd.O (Figure 2.3A, B and E). Caudally and rostrally, however, the neuropil of MLd.I was stained and had greater density of somata (Figure 2.3C, D and F). As in the CR-LI stained material, CB-LI was observed surrounding MLd caudally and rostrally, while occupying a ventrolateral position at intermediate levels (Figure 2.3A-D). It was characterized by a CB-LI positive dark neuropil, some stained fibers and terminal boutons, and numerous small stained somata, some with stained proximal processes (Figure 2.3G). This region is not the recipient of ascending auditory inputs and is therefore considered part of ICo rather than of MLd.

3.4 - Summary of staining patterns

PV staining was commensurate with the whole of MLd, defined as that region of the torus receiving direct and indirect ascending auditory afferents (Krützfeldt, et al., 2010a; Wild, et al., 2010). Within the PV-LI region, two regions based mainly on the apparent abundance of puncta could be differentiated. An inner region, which had less punctate staining, corresponded to a region that also exhibited a CR-LI-positive neuropil and matched the part of MLd where most of
the terminal boutons showing CB-LI were concentrated. This region was termed the inner region (MLd.I). An outer region of MLd (MLd.O), which surrounded the inner region, was characterized by PV-LI exhibiting a higher density of punctate staining than that of MLd.I. In contrast, MLd.O exhibited a CR-LI negative neuropil, but was rich in CR-positive fibers and also presented a few CB-LI-positive somata and a light CB-LI positive neuropil. Surrounding MLd is a CB-LI- and CR-LI- positive region (Figures 2.2 and 2.3) which is not stained with PV (Figure 2.3H), does not receive ascending auditory projections and is, therefore, considered part of ICo. It is interesting that the set of CaBPs used in the current study clearly delineate two regions within MLd, as well as the ICo (Figure 2.4E-H), whereas these boundaries do not always appear as easily identifiable in Nissl stained material (Figure 2.4A-D) especially at the most caudal and rostral levels (Figure 2.4A and D). CaBP staining patterns in the midbrain revealed no differences between males and females, except for differences in PV staining patterns of DM (compare Figure 2.1 A-D with Figure 2.5). In females, only few DM somata were immunostained (Figure 2.5B), while in males DM showed a strong PV-LI neuropil with few stained somata (Figure 2.5F-H), as shown previously in the context of song system anatomy (Wild et al., 2001b). CaBP staining patterns revealed no differences between males and females in the organization of the auditory midbrain, except for differences in PV staining patterns of DM (Figure 2.5). In the present study, cell numbers in MLd in the various cases were not quantify, but no striking differences were apparent either between males and females (see Figure 2.5) or between left and right MLds.
**Figure 2.4**

**Subdivisions of MLd in Nissl stained material**

**A-D:** caudal (top) to rostral (bottom) coronal Nissl-stained sections through the right MLd. The asterisk in **B** indicates the small nucleus within MLd.O showing a different staining pattern in PV- and CR-LI adjacent sections. Note that it is clearly visible in Nissl-stained sections.

**E-G:** schematic drawings of the different regions based on PV-, CR- and CB-LI staining. The asterisk in **F** indicates the subnucleus delineated by the PV- and CR- LI staining and by Nissl staining. Note how the boundaries can be identified by naked eye for more intermediate sections (**F** and **G** and corresponding panels **B** and **C** respectively) while they are obscure in the more caudal and rostral ones (**E** and **H** and corresponding panels **A** and **D** respectively).

Scale bar = 100μm
Figure 2.5

Parvalbumin-like immunoreactivity in males and females

A-D: caudal (top) to rostral (bottom) coronal sections showing PV-LI through the right MLd delineating MLd.I and MLd.O within the nucleus in a female zebra finch.

E-G: caudal (top) to rostral (bottom) coronal sections showing PV-LI through the right MLd delineating MLd.I and MLd.O within the nucleus in a male zebra finch. Note the absence of sexual dimorphism in the staining of nucleus MLd and the presence of sexual dimorphism in the staining of nucleus DM (see text).

Scale bars = 100µm
4 - Discussion

CaBP immunostaining was used to identify the regional organization of MLd within the context of its ascending projections recently described (Krützfeldt, et al., 2010a; Wild, et al., 2010). An inner and an outer region of MLd (MLd.I and MLd.O, respectively) can be distinguished based on the patterns of CaBP immunoreactivity, which also distinguish MLd proper from the surrounding ICo. The patterns of staining in the areas examined in the midbrain were similar in males and females, with the exception of DM. This is interesting because zebra finches – and songbirds in general – are known to present sexual dimorphism of certain brain areas. Adult females, for instance, do not possess a song system (interconnected nuclei, distinct from the auditory pathway, involved in both vocal learning and vocal motor output) as developed as that seen in males (Nottebohm & Arnold, 1976). DM receives direct descending projections from the forebrain robust nucleus of the arcopallium (Fukushima & Aoki, 2000; Nottebohm, et al., 1982; Vicario, 1991; Wild, 1993) in both sexes, but only in males do projection neurons in RA and their terminal fields in DM stain with PV (Wild, et al., 2001b). Sexual dimorphism has also been shown in the higher auditory center NCM, where Pinaud et al., (Pinaud et al., 2006) found that males had twice as many CB-LI positive cells as females.

4.1 - Comparison with other avian species

Most of what is known about anatomical subdivisions of the avian MLd, based on cytoarchitecture and/or neurochemistry, ascending projections from the brainstem or neurophysiological properties, applies to non-songbirds [chicken: (Coles & Aitkin, 1979; Conlee & Parks, 1986; Puelles, et al., 1994; Wang & Karten, 2010; Zeng, S, et al., 2008a); guinea fowl: (Scheich, et al., 1977); mallard: (Arends, 1981); barn owl: (Knudsen, E. I., 1983; Knudsen, E. I. & Konishi, 1978; Konishi, 2003; Kubke, et al., 1999; Takahashi, et al., 1987; Takahashi & Konishi, 1988a; Wagner, et al., 2003; Wagner, et al., 1987) and pigeon: (Leibler, 1975; Wild,
In comparison, data available for songbirds are fewer (Braun, et al., 1991; Braun, et al., 1985; Hsu, et al., 2004; Krützfeldt, et al., 2010a; Montagnese, et al., 1993; Poirier et al., 2009; Watson, et al., 1988; Wild, et al., 2010; Woolley, S. M. N. & Casseday, 2004, 2005; Woolley, S. M. N., et al., 2009; Woolley, S. M. N., et al., 2006; Zeng, S. et al., 2007). Recent studies (Krützfeldt, et al., 2010a; Wild, et al., 2010) have shown that the projection patterns of the ascending inputs to the auditory torus in zebra finches differ from those described for non-songbirds, thereby challenging the validity of a general schema for MLd subdivisions (Puelles, et al., 1994; Takahashi & Konishi, 1988a; Wagner, et al., 2003), as well as the generally assumed subdivisions of MLd in songbirds (Braun, et al., 1991; Braun, et al., 1985; Zeng, S., et al., 2007). While the terminal fields of NA and NL projections are segregated within MLd in chickens (Conlee & Parks, 1986; Wang & Karten, 2010), pigeons (Leibler, 1975; Wild, 1995) and barn owls (Takahashi & Konishi, 1988a), they extensively overlap in zebra finches. Thus, segregated terminal fields within MLd cannot be used in the zebra finch (and presumably not in the closely related Bengalese finch) to differentiate what in other species are called core and shell. Braun et al. (1991; 1985) and Zeng et al. (2007) have attempted to identify the regional organization of MLd in zebra and Bengalese finches, respectively. Braun and her colleagues described the complementary staining patterns of PV and CB (and other markers) in the brain of young male zebra finches and implied that the PV positive staining was restricted to a core of MLd, while a shelf (sic; = shell) or marginal zone of MLd was characterized by CB-positive immunoreactivity. This differentiation, which has also been referred to (and presumably accepted) by other authors, blurs the real boundary of MLd. Specifically, the present work suggests that the CB positive region that they seem to consider part of their shell is actually part of ICo, since it corresponds to a similar CB-positive region defined in the present study, but one that is now known not to receive ascending auditory afferents and therefore not part of MLd. If this is correct, the ‘core’ defined by PV-positive staining in the studies of Braun et al. would comprise the entirety of MLd.
Zeng et al. (2007), using antibodies to Met-enkephalin, substance P and serotonin, suggested that the MLd of Bengalese finches was equivalent to MLd of barn owls. Thus, an MLd core and shell in Bengalese finches was considered analogous to the core and both lateral and medial shells of the central nucleus of the inferior colliculus of barn owls (Takahashi & Konishi, 1988a). Zeng et al. also considered that ICo was equivalent to the external nucleus of the inferior colliculus of mammals. In their study, the core of MLd can easily be identified by a lack of substance P, as was reported for pigeons (Wild, 1995), but the boundary between shell and ICo remained quite unclear (see Figure 8, p.11 in Zeng, S., et al., 2007). The results of Zeng et al., when compared to ours, suggest that their subdivisions are congruent with ours, but a re-examination of their neurochemical boundaries with regards to known projection patterns needs to be reassessed. The complementarity of the CB and PV staining patterns characteristic of ICo and MLd, respectively, is, however, strikingly clear in their study on the neurogenesis of core and shell areas in the chick brain (Zeng, S, et al., 2008a). Still, neither of their two studies included the use of an antibody against calretinin, which Puelles et al. (1994) suggest is the appropriate marker for the core region of the central nucleus in chickens, in which a dark CR-positive plexus appears to match the projections of NL onto MLd in this species (Conlee & Parks, 1986; Wang & Karten, 2010), as it does in barn owls (Kubke, et al., 1999; Takahashi, et al., 1987; Wagner, et al., 2003). Since the ascending projections to MLd have now been traced and their terminal fields described within the nucleus in zebra finches (Krützfeldt, et al., 2010a; Wild, et al., 2010), these results can be tentatively correlated with the subdivisions observed using CaBPs in the present study. In doing so, it appears that the PV-positive staining demarcates the terminal fields of ascending projections of both NA and NL and the nuclei of the lateral lemniscus, and should therefore include all MLd subdivisions. Based on this hodological criterion, the CB-positive external zone should not be considered part of MLd, but rather of ICo, which is in agreement with the organization proposed by Zeng and his colleagues for Bengalese finches (Zeng, S., et al., 2007). Moreover, within the PV-positive region in the present study of zebra finch MLd, two distinct
Chapter II: Subdivisions of the auditory midbrain

regions could be delineated based on the density of puncta. Also, the central zone with less puncta anatomically matched the dark CR-positive central neuropil within MLd. The two MLd regions, inner and outer, may, or may not, represent two functionally different zones. For instance, in barn owls, core and shell regions are the recipients of NL and NA projections, respectively, and have specific neurophysiological properties that originate from separate time and intensity pathways (referred as ITD and ILD, respectively). These pathways eventually converge in the lateral shell, from where projections are sent to the external nucleus, ICX, where a map of auditory space emerges (for review, see Konishi, 2003). As mentioned earlier, NA and NL terminal fields in zebra finch MLd are not strictly segregated, but rather overlap substantially. These inter-species differences may reflect different evolutionary demands on the auditory system in these two lineages. Barn owls, as nocturnal hunters, excel at sound localization (Knudsen, E. I. et al., 1979; Payne, 1971), while zebra finches, as songbirds, perform rather poorly in sound localizing tasks (Park & Dooling, 1991) (but see also Nelson & Suthers, 2004). This difference, in itself, could suggest that the functional organization of MLd in songbirds is different from that in barn owls. In any case, exhaustive neurophysiological studies in the zebra finch midbrain are required to establish proper functional subdivisions. The few studies that have investigated electrophysiological properties of neurons in the finch MLd have reported a dorsoventral tonotopic organization of the nucleus, with low frequencies being represented dorsally and high frequencies ventrally (Woolley, S. M. N. & Casseday, 2004), as seen in other avian species and in the mammalian central nucleus of the inferior colliculus (Covey & Carr, 2005). By comparing the recording loci of Woolley and Casseday (Figure 2, p.139, Woolley, S. M. N. & Casseday, 2004) with the staining patterns of the present study, it seems that these recording loci fall outside the external edges of both the inner CR-positive region and our PV-positive region with less puncta described here, suggesting that they were located in what in the present study was called the outer region of MLd. However, this conclusion must remain tentative without double labeling of recording loci and CR-IR. Thus,
whether MLd.I and MLd.O exhibit different physiological properties remains to be established. Unlike the case in barn owls, these regions in finches are probably unrelated to the encoding of ITDs and ILDs, but rather to the processing of biologically relevant vocalization signals, given the differences in the organization of ascending inputs (Krützfeldt, et al., 2010a; Wild, et al., 2010). Woolley and Casseday (Woolley, S. M. N. & Casseday, 2004, 2005) and Logerot et al. (2009), described neurons in zebra finch MLd with tuning properties indicative of the processing of complex acoustic signals, especially their temporal aspects. Woolley et al. (2009) reported four functional groups in the midbrain of the zebra finch, each of them being involved in the extraction of different features of vocal sounds. They identified broadband neurons, narrow-band temporal neurons, wideband neurons and two-band excitatory based on the spectrotemporal receptive fields of these neurons. Thus, in songbirds, feature extraction from songs might actually be a more important function than sound localization, and could be necessary for accurate song recognition (Nelson & Suthers, 2004), although the likelihood of single units coding for songs seems remote. Rather, synchronized responses of populations of neurons are proposed to “create a neural representation of the temporal patterns of the song” (Woolley, S. M. N., et al., 2006 p.2510). Precise recording localizations of Woolley and Casseday (2004, 2005) and Logerot et al. (2009) units in respect to the boundaries defined in the present study would be a valuable addition in understanding the role of MLd subdivisions in the finch. In terms of population encoding, Poirier et al. (2009) investigated MLd’s responses to the Bird’s Own Song (BOS), conspecific (CON) and heterospecific (HET) vocalizations. They found lateralized processing to BOS and CON, with BOS selectivity in the right MLd and CON selectivity in the left MLd. No differences were found in the present study between the staining patterns of left and right MLd. Using IEG immunocytochemistry, Woolley and Doupe (2008) found no differences in ZENK expression in response to familiar or unfamiliar songs in MLd. This further adds to the necessity of investigating neuronal properties in MLd within the context of the anatomical subdivisions and how these relate to ascending inputs.
4.2 - Comparison with other tetrapods

The present study deliberately eschewed a core and shell divisional schema for MLd of the zebra finch, because of potential interpretive problems associated with comparisons across species and authors: what is shell for some workers is core for others, and vice versa. Nevertheless, a core/shell (or belt) organization of the auditory midbrain is frequently assumed in the literature and is thought to be a conserved feature of tetrapods. The core is exclusively auditory by way of the topographic projections from lower brainstem auditory nuclei which result in a characteristic tonotopic organization of the nucleus. Moreover, the core conveys auditory information to the thalamus over lemniscal pathways. The belt is the site of multimodal sensory, including auditory, information. It receives minor ascending auditory projections, projections from the core itself, as well as from descending projections from higher auditory centers.

In mammals, the core or central nucleus of the inferior colliculus (ICC) can be differentiated from the belt area (composed of dorsal (DC) and lateral (LC) cortices) on the basis of cytoarchitecture, auditory and somatosensory projections (for review see Huffman & Henson Jr, 1990; Oliver, 2005) or neurophysiological properties (for review see Ehret & Schreiner, 2005). Numerous studies have shown the complementarity of parvalbumin and calbindin as well as calretinin staining, with PV-LI structures predominantly in the ICC and CR-LI and CB-LI structures predominant in the shell (rat: Celio, 1990; Coleman, J. R., et al., 1992; Friauf, 1994; Lohmann & Friauf, 1996; Resibois & Rogers, 1992; Rogers & Resibois, 1992; mice: Idrizbegovic, et al., 1999; Zeng, S., et al., 2009; Zettel, et al., 1997; chinchillas: Kelley, P. E., et al., 1992; cat: Paloff, et al., 2004; gerbils: Seto-Ohshima, et al., 1990; humans: Sharma, et al., 2009; Tardif, et al., 2003; bats: Vater & Braun, 1994; Zettel, et al., 1991).

Similar observations have been reported in reptiles and amphibians, although the number of studies and species investigated are fewer. The torus semicircularis (auditory midbrain) of reptiles is composed of a central nucleus (Ce)–surrounded by laminar and superficial (L) nuclei. The cytoarchitecture, hodology and electrophysiological properties of these regions have been
characterized (Browner, 1983; Browner et al., 1981; Browner & Rubinson, 1977; Butler & Bruce, 1981; Drakontides & Browner, 1986; Foster & Hall, 1978; Kennedy & Browner, 1981; Khachunts, 1983; Künzle, 1986; Künzle & Woodson, 1982; Manley, J. A., 1971; Pritz, 1974; Vergne et al., 2009). The laminar nucleus has been proposed to correspond to part of the intercollicular nucleus of birds (Zeng, S., et al., 2007). Belekhova et al., (2010; 2004) reported calcium-binding protein staining in the turtle midbrain. As is seen in mammals and birds, Ce was highly PV immunostained and CB-LI and CR-LI structures were mainly seen in the laminar nucleus. Interestingly, the staining patterns they observed in Ce were not homogeneous: the core region of the central nucleus (Cec) presented PV-LI staining while PV-, CB- and CR-LI structures were seen in the peripheral area of the central nucleus (Cep). Yan (2008) and Yan et al. (2010) investigated the pattern of staining in the midbrain torus of the gecko that revealed the presence of PV-, CB- and CR-LI structures in both the central and laminar nuclei. The staining patterns of these three CaBPs within the central nucleus showed distinct distributions, indicating subdivisions that could delineate some segregation of the ascending auditory projections. Whether a tonotopic organization of the gecko’s torus exists is unknown, which makes it difficult to relate to the present findings in zebra finch. Clearly, detailed electrophysiological studies are needed before any clear conclusion can be drawn as to a similar organization of the reptilian torus and the mammalian and avian auditory midbrains.

In amphibians, three subdivisions of the torus semicircularis are recognized: principal nucleus (Tp), laminar nucleus (Tl) and magnocellular nucleus (Tmc). The morphology, ascending projection patterns and neurophysiological properties of these three areas are well documented (Bass, et al., 2005; Cant, 2005; Edwards & Kelley, 2001; Endepols & Walkowiak, 2001; Feng & Lin, 1991; Hermes et al., 1981; Lowe, 1986; Luksch & Walkowiak, 1998; Mohneke, 1983; Potter, 1965a, 1965b; Simmons et al., 2000; Walkowiak & Luksch, 1994; Will et al., 1985a, 1985b). As seen in sauropsids and mammals, there is an exclusively auditory area, Tp, and multisensory areas Tl and Tmc. Very few studies have reported patterns of calcium-binding
protein immunoreactivity in the midbrain of amphibians and only the results for parvalbumin staining are available (Endepols, et al., 2000; Zeng, S., et al., 2008b). In their study, Endepols et al. observed a clear cut boundary between Tp, which was strongly PV-positive and Tl, which was completely devoid of PV-LI. In Tmc, however, some PV-positive cell bodies were visible. Zeng et al. (2009) reported that Tp neuropil showed a stronger parvalbumin staining than did Tl or Tmc.

These studies in various tetrapods show that the tonotopically organized, central nucleus is characteristically strongly immunostained and delineated with CaBPs. However, as was pointed out for birds in the present study, the existence of similar “anatomical” subdivisions cannot be taken to imply functional parallels between different species. First, as mentioned earlier, the pattern of auditory ascending projections in the zebra finch (Krützfeldt, et al., 2010a; Wild, et al., 2010) appears to be closer to that seen in mammals, in which there are topographically organized but overlapping terminal fields of brainstem projections (for review see Cant, 2005). But the regions defined by different CaBPs seen in the present study are somewhat closer to those described in the barn owl and chicken (Kubke, et al., 1999; Puelles, et al., 1994; Takahashi, et al., 1987; Wagner, et al., 2003), or in reptiles (Belekhova, et al., 2010), where there are two distinct subdivisions within the central nucleus. These apparent mismatches between MLd subdivisions and the pattern of ascending auditory inputs raise the question of the functional significance of these differences in the zebra finch MLd. As stated above, NA and NL show overlapping terminal fields in both the inner and outer regions of MLd (Krützfeldt, et al., 2010a). Contralateral and ipsilateral ascending projections of the LLV may, however, differentiate between these two regions (Figure 2.6). Ipsilaterally, LLV projections can be seen in both the inner and outer regions of MLd, while contralaterally, they appear to be restricted to the outer MLd (Figure 2.6). Biotinylated dextran amine injections into nucleus ovoidalis (Ov) also delineate both the inner and outer regions of MLd: retrogradely labeled somata are mainly present within MLd.I (Figure 2.7) but are also seen in MLd.O. Moreover, CB-LI and CR-LI
structures defined a region just outside MLd that may correspond to the belt of other tetrapod ICs. However, previous studies (Krützfeldt, et al., 2010a; Wild, et al., 2010) clearly showed that this region is not an apparent recipient of any ascending auditory brainstem projections and therefore this region was classified as part of the ICo in the present study. Whether the outer region of MLd could be homologous to the belt region of other vertebrates cannot be ascertained because of the general lack of studies focusing on somatosensory projections to the songbird auditory torus (Wild, 1997) which could reveal an area in which multisensory inputs take place.
**Figure 2.6**

**Pattern of LLV projections onto MLd**

**A-D:** caudal (top) to rostral (bottom) transverse sections showing projections to the contralateral MLd after BDA injection in right LLV. Note the presence of ascending auditory fibers and terminal fields mainly in the outer region of MLd.

**E-H:** caudal (top) to rostral (bottom) transverse sections showing projections to the ipsilateral MLd after BDA injection in right LLV. Note that the ascending auditory fibers and terminal fields can be observed in both the inner and outer region of MLd.

Scale bars = 100μm

Courtesy of Dr. Nils Krutzfeldt and Prof. Martin Wild
Figure 2.7

Retrograde labelling in MLd from a BDA injection into Ov

**A-D:** caudal (top) to rostral (bottom) transverse sections showing the retrograde staining pattern in the ipsilateral MLd after BDA injection in Ov. Note that retrogradely labeled somata are mainly visible and located within the inner MLd, and more specifically its lateral part. Also note the absence of retrograde labelled structures in the ICo.

Scale bar = 100μm

**E-H:** schematic drawings of the different regions based on the PV-, CR- and CB-LI stainings from the present study.

Courtesy of Prof. Martin Wild
Chapter III

From anatomy to auditory processing: overview of sound-evoked Immediate-Early Gene expression in the zebra finch MLd
1 - Introduction

While CABP immunoreactivity allows us to anatomically describe MLd and its subdivisions, it does not give us any information about how auditory processing of vocal signals occurs in this nucleus. In response to stimulation, neurons respond over time courses that are as fast as a few milliseconds up to several hours.

Immediate-Early Genes (IEGs) are characterized by their rapid and transient activation that is induced within minutes after stimulation (Bartel et al., 1989; Greenberg & Ziff, 1984; Morgan & Curran, 1989) in contrast to the late response genes whose expression is modified over a time frame of hours (Armstrong & Montminy, 1993; Bliss & Collingridge, 1993; Comb et al., 1987; Goelet et al., 1986) through transcription factors for which the early genes code. There are different families of IEGs that have been identified, among which the most studied are Fos [cfos (Bravo et al., 1985; Cochran et al., 1984; Greenberg & Ziff, 1984; Kruijer et al., 1984; Rollins & Stiles, 1988), Fos-B (Zerial et al., 1989), Fra-1 (Cohen & Curran, 1988), Fra-2 (Nishina et al., 1990), Jun (c-jun (Lamph et al., 1988), jun-B (Lau & Nathans, 1987), jun-D (Hirai et al., 1989; Ryder et al., 1989)] and Krox (krox-20 and krox-24 Lemaire et al., 1988) the latter also being known as zif 268, egr-1 NGFI-A (Christy et al., 1988; Milbrandt, 1987; Sukhatme et al., 1988) and therefore often referred to by its acronym ZENK. Though they all bind to DNA, they differ in the sequence they recognise and are, therefore, classified into two different categories based on their binding domains: the Leucine zippers for the Fos and Jun families and the Zinc fingers for the Erg family (to which ZENK belongs).

The analysis of the expression of immediate early genes (IEGs) has been proven an efficient, fast and practical technique to measure neuronal activation (for review, see Hughes & Dragunow, 1995), and has therefore become widely used, including in the avian brain. Recently, up to 33 regulatory genes have been identified in the zebra finch brain, during, or as a result of, the act of singing (Wada et al., 2006) and at least a 100 are thought to be implicated in singing. This first,
very complete, study by Wada and his colleagues focusing on the cascade of genes involved in singing might led to further experimental approaches; so far studies have mainly focused upon the expression of two major IEGs: ZENK and FOS (only two other studies tested the IEGs Arc and UCHL1, (Lombardino et al., 2005; Velho et al., 2005). Therefore, the expression patterns of these IEGs associated with singing (Jarvis & Nottebohm, 1997; Jarvis et al., 1998; Jarvis et al., 1997; Jin & Clayton, 1997; Kimpo & Doupe, 1997) or song perception (for review see Mello et al., 2004) in the bird brain are quite well documented, but, as it is often the case in songbirds, most of the studies were aimed at describing forebrain nuclei. Thus, very little is known about what kind of expression patterns of IEGs occur at the level of the brainstem. Apart from three studies mentioning some staining visible in MLd, but not investigating it further (Jarvis, et al., 1998; Mello & Clayton, 1994; Mello & Ribeiro, 1998), only one study so far has described more specifically the pattern of ZENK activation detectable in MLd after stimulating the birds with familiar vs unfamiliar and directed vs undirected conspecific songs (Woolley, S. C. & Doupe, 2008). Only one group has examined IEG activity (FOS and JUN) at the level of the hindbrain in the cochlear nucleus magnocellularis and in the third order nucleus laminaris in the chicken (Alladi et al., 2005), a non-songbird avian species.

Auditory–evoked expression of IEGs represents an interesting intermediate step to approach anatomical correlations of song processing, which could potentially give an overview of sound processing at the level of the whole nucleus MLd. In the present study the expression of IEGs (FOS, JUN and ZENK) were examined after presenting birds with both simple (tones and WN) and complex (conspecific and heterospecific songs) stimuli in an attempt to identify differential activation within the anatomical subdivisions previously identified.
2 - Material and methods

2.1 - Animals and ethical consideration

The experimental procedures were carried out according to the guidelines and approval of the Animal Ethics Committee of the University of Auckland.

Thirteen zebra finches (six males, six females), *Taeniopygia guttata*, were used in the ZENK study. Twelve birds (seven males and five females) were used in the FOS/JUN experiment. Birds were either obtained from a local breeder or reared in our conspecific-only aviary (full description of rearing protocol in chapter IV). The normally reared birds (ZF-ZF) were bred in an indoor single species aviary at the Department of Psychology, University of Auckland. Daily light was provided through a compact Arcadia fluorescent bird lamp (2.4% UVB and 12% UVA) so as to imitate outdoor conditions. The mean temperature in the aviary was kept at 21 ± 3°C, daily humidity between 35-65% and care was taken to keep a constant airflow. Pairs of zebra finches (*Taeniopygia guttata*) were first housed together in cages and, once the chicks hatched, families were transferred to separate cages. After reaching adulthood (>100 days), birds were transferred to a holding aviary in the Faculty of Medical and Health Sciences. At all times, birds were held at a constant light/dark cycle of 14h/10h in the aviary and birds were provided with food and water ad lib.

2.2 - Experimental procedure

Each bird were anesthetized with an intramuscular injection of a 0.04-0.05 ml of a mixture consisting of four parts of distilled water, one part of ketamine (Parnell Laboratories, Auckland, New Zealand; 55 mg/kg) and one part of xylazine (Rompun, Bayer; 11 mg/kg). Once the bird was anaesthetized, it was placed in a soundproof box, in dim light conditions for an hour in order to reduce the basal levels of IEG activity and avoid any interference due to prior sensory or motor activation. Birds were then exposed to one of the five stimuli tested in this study. Birds were presented with different stimuli: either a tone, a noise (brown or white), a conspecific song
(zebra finch song) or a heterospecific song (Bengalese finch song) for 1½ hr. Control birds were kept in the same conditions, except that they did not receive any auditory stimulation for the 1½ hour stimulation period. Following the stimulation period, the birds were left in the dark for another 30 minutes. The timing of these experiments was adjusted experimentally in an attempt to maximise the appearance of staining in the nucleus and reduce staining in the cytoplasm. The starting point for these tests was initially based on the results of Mello and Ribeiro (1998), in which they reported some activation in nucleus MLd and showed that the maximum level of expression of ZENK in the forebrain area NCM was achieved between one and two hours after exposure.

2.3 - Stimulus generation and presentation

Noise (brown or white), pure tones, conspecific and heterospecific songs were used as stimuli in this experiment. Brown noise (more energy in the lower frequencies), white noise and tones (2, 3 or 4 kHz) were generated using Adobe Audition 3.0 software. Pure tone stimuli were of 1s duration with a built-in rise and fall ramps of 150 ms each at onset and offset. These ramps were necessary to avoid stimulus clicking. The noise stimuli were also 1 sec in duration and contained frequencies ranging from 0 to 10 kHz. Noise and tone stimuli were presented at 75 dB SPL, as measured with a RadioShack SPL meter 30 cm from the speaker. The songs used in this experiment were a conspecific (zebra finch) song and a heterospecific (Bengalese finch) song. Both these songs were used in the set of experiments presented in chapters IV and V and were, therefore, familiar to the conspecific-only reared birds. However, as the point of this study was to obtain the closest comparison possible between ZENK auditory-evoked expression and single unit recordings in MLd, it appeared important to use identical stimuli in all the different experiments (Woolley, S. C. & Doupe, 2008 showed there was no effect of familiarity/unfamiliarity of conspecific songs on ZENK auditory–evoked expression in MLd). The conspecific song was chosen amongst the birds raised in the same aviary as the ZF-ZF birds. The conspecific song tested was obtained by recording each bird overnight using Sound Analysis.
Pro software and a RadioShack microphone. The song was subsequently bandpass filtered (300 Hz – 12 kHz) using Raven 1.3 software. The heterospecific song was chosen amongst three Bengalese finch songs kindly provided by Dr Sarah Woolley from Columbia University, New York. Songs were calibrated for presentation with an average power of 75 dB (as calibrated using a ½-inch free-field microphone, Brüel and Kjaer and Raven 1.3 software). Stimuli were played to the bird through a multifunction processor (Tucker-Davis Technology, TDT System 3), routed through an attenuator (TDT) that allowed presentation of all stimuli at equal intensities (75 dB SPL average power). The signal was then processed through a TDT Stereo Amplifier connected to a free-field magnetic loudspeaker (TDT) placed 30 cm in front of the bird. Before an experiment, the output of the loudspeaker was checked using a RadioShack SPL meter so to ensure constancy of the average power at which stimuli were delivered.

2.4 - Immunocytochemistry

Immediately after the experiment, the birds were deeply anaesthetized with an intramuscular injection of equal parts of ketamine (Parnell Laboratories, Auckland, New Zealand; 166 mg/kg) and xylazine (Rompun Bayer; 33 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB; pH 7.4). The brains were post-fixed in 4% paraformaldehyde for 24 hours before being cryoprotected overnight in a 30% sucrose solution in phosphate buffered saline (PBS; pH 7.4). They were then cut coronally on a freezing microtome and serial, free-floating 35μm thick sections were collected in PBS and immediately processed for immunocytochemistry. After 3 rinses of 10 minutes (all rinses in this protocol lasted 10 min) in PBS, endogenous peroxidase activity in the sections was blocked using 50% methanol and 1% hydrogen peroxide in distilled water for 10 min at room temperature. Sections were then rinsed 3 times before being incubated in a blocking solution made of 5% Normal Horse Serum (NHS) in PBS with 0.4% Triton-X (PBS-T) for 20 minutes at room temperature. They were then immediately incubated with a rabbit polyclonal primary antibody (anti-Erg-1 SC-189 or anti c-fos SC-52 or anti c-jun SC-44 Santa Cruz Biotechnology, Inc., Santa Cruz, CA)
at dilution 1:10,000 for 24 hours at room temperature (a series of trials at different dilutions was previously performed to determined the optimal dilution. Dilutions tested were 1:800; 1:1000; 1:5000; 1:10,000 and 1:20,000). After 4 rinses, sections were then transferred in a biotinylated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS-T at dilution 1:300 for 1h at room temperature and rinsed 3 times before being incubated in avidin-biotin peroxidase complex (S-911, Invitrogen, Eugene, OR) at 1:1000 in PBS-T for 1h at room temperature. Following the 3 rinses, sections were finally incubated in a chromogen-solution (0.025% 3,3’-diamino-benzidine [DAB], 0.005 % H2O2 and 0.015% CoCl2 in PBS). The reaction was stopped by several washes in PBS. Either all or half the sections were subsequently mounted, dehydrated in successive ethanol baths, cleared in xylene, and then coverslipped using DePeX (Serva, Heidelberg, Germany). The second half of brain was in some cases immunostained with a primary antibody against calbindin following the same protocol used for the CaBPs study (see chapter II for details).

2.5 - Analysis

In all cases, a close inspection of HVC, RA and DM was performed. These nuclei are all involved in the motor pathway and, as has been shown previously, show IEG expression when the bird sings. The experiment took place in a sound-attenuated box and a bird that sang during the experiment, as the anaesthesia wore off, could not be heard. IEG expression in HVC, RA and DM was therefore a good indicator of singing activity.

Sections were analyzed and digitally photographed, at 10x magnification, using a Nikon 80i light microscope (5 megapixel camera). Levels were then adjusted using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA). Schematics of sections where drawn using CorelDRAW.
3 - Results

3.1 - Staining patterns in the auditory brainstem using Fos, Jun and ZENK

No auditory-evoked Fos or Jun expression was observed in the auditory nuclei NA, NM, NL, OS, nLL or MLd in the auditory brainstem of the zebra finch, at any of the tested dilutions. These antibodies were not used any further. Similarly, no ZENK auditory-evoked expression was observed in NA, NM, NL and OS. However, some staining was detectable in MLd, consistent with previous reports. The following results therefore describe auditory-evoked expression patterns of ZENK in the auditory midbrain.

3.2 - Control birds: “silence” condition

Two birds were tested with no sound stimulation and used to determine the baseline levels of expression of ZENK to be used as controls (Figure 3.1). These cases showed substantial variability in ZENK expression. In one case very little IEG expression within both subdivisions of MLd, MLd.I and MLd.O was observed while the ICo, exhibited numerous stained nuclei. The second case appeared to show more stained nuclei in both MLd and ICo, with ICo exhibiting a higher apparent density of stained nuclei than MLd.

3.3 - ZENK expression patterns in MLd in response to tones

Three birds (three females) were tested for one of each of the tones of: 2 kHz, 3kHz and 4 kHz. Under the three conditions, ZENK expression in ICo was high (as expected from the controls). Some ZENK expression was also detectable in MLd with the majority of the stained cells located within MLd.O (Figure 3.2A and B). MLd.I showed less ZENK activity. Interestingly, the
Figure 3.1

IEG expression pattern in the control condition

Coronal sections through the left MLd of two birds that were not exposed to any auditory stimulus (controls).

These cases show the variability in staining encountered in MLd in the control condition. In the left panel, very little stained nuclei can be observed compared to those in the right panel.
localization of the stained nuclei in MLd.O appeared to be quite similar, with many cells occupying a dorso-lateral position and no obvious evidence of tonotopic organisation (Woolley, S. M. N. & Casseday, 2004).

### 3.4 - ZENK expression in MLd in response to noise

Three birds (two males and one female) were tested in this condition, two of them with brown noise, one with white noise. The same frequency range was represented in the brown noise as in the white noise, but brown noise possesses more power in the lower frequencies. As was seen when tones were presented stained cell nuclei were visible in both ICo and MLd. Within MLd, the number of stained cell nuclei still appeared quite small but more nuclei were stained within the MLd.I than in the cases of tone stimulation. For instance, the dorso-lateral localization of most cells that was seen in the tone cases was not as marked (Figure 3.2C).

### 3.5 - ZENK expression in MLd in response to songs

Four birds (two males and two females) were presented with a conspecific (CON) song and two birds (one male and one female) were presented with a heterospecific (HET) song. One of the birds that were presented with HET song showed strong ZENK expression within HVC and DM, suggesting that he sang during the experiment, and this bird was removed from the analysis. The ZENK staining patterns observed in response to songs were similar to those seen for noise, and there were no obvious differences between the staining patterns in birds presented with either HET or CON song. Both subdivisions of MLd, showed cell nuclei expressing ZENK. Cells were also dispersed within MLd as was seen with noise and not aggregated on the dorso-lateral part of the nucleus (Figure 3.2D), as seen in response to tones.

### 4 - Discussion

The aim of this experiment was to give an overview of sound processing at the level of MLd, correlated with the subdivisions described in chapter II.
**Figure 3.2**

**IEG expression patterns to simple and complex stimuli**

A: Coronal section through the left MLd of a bird stimulated with a 2 kHz frequency.

B: Coronal section through the left MLd of a bird stimulated with a 4 kHz frequency.

Note how the IEG expression patterns in both cases do not reflect the known tonotopy existing in the avian MLd with a dorsal (lower frequencies) to ventral (higher frequencies) organisation. The white arrows point to the dorsolateral part of the nucleus where most of the stained nuclei are encountered.

C: Coronal section through the left MLd of a bird stimulated with a white noise (WN).

D: Coronal section through the left MLd of a bird stimulated with a conspecific song (CON).

Note how more cell nuclei appear to be stained in response to both these stimuli. The IEG expression pattern evoked by the CON song shows stained nuclei throughout the whole nucleus while the IEG expression evoked by the WN shows stained nuclei mainly in the dorsolateral and lateral part of the nucleus as shown by the white arrows.
Staining patterns following auditory stimulation were compared to those obtained for birds that had received no auditory stimulation. While in one of the control birds very little staining was observed in MLd, a second bird had numerous stained nuclei despite the absence of any auditory stimulus. The reason for this variability is not clear, but sets a high threshold for the interpretation of staining patterns in experimental conditions. In both control cases, the ICo, was filled with stained cell nuclei, although its significance is beyond the scope of this study.

Birds that were presented with tones showed puzzling results. The tonotopy of MLd/IC is well known and therefore a dorso-ventral gradation of the ZENK staining expression was expected for birds that were presented with tones of different frequencies, with a stronger expression dorsally in response to lower frequencies and stronger expression ventrally in response to higher frequencies. However, the ZENK expression pattern within MLd of the three cases appeared to be quite similar, with most stained nuclei clustered dorsolaterally in MLd. The staining patterns obtained in response to noise and songs differed from those obtained in response to tones. In the former cases, stained cells were seen within both divisions of MLd, with a greater dispersion than that observed in the auditory midbrain of birds presented with tonal stimuli.

This raises question of the validity of the use IEGs in auditory experiments. The total absence of any visible tonotopic representation in the patterns of ZENK expression is unexpected: the levels of expression of IEGs do not match the expected levels of neuronal activity that should be elicited with tone presentations based on other studies (Figure 2 in Woolley, S. M. N. & Casseday, 2004).

Even more puzzling is that the regional patterns of staining obtained with tones are not similar to those obtained with white noise or songs. This difference would suggest that ZENK activity is reflecting some aspect of auditory processing, but what those aspects may be cannot be determined from these studies. Further, the relatively low level of ZENK expression after auditory stimulation would suggest that the neuronal responses to auditory stimuli would be
expected to be low, but as shown in chapters IV and V MLd neurons readily and vigorously respond to auditory stimulation.

Previous studies have reported ZENK auditory-evoked expression in MLd (Jarvis, et al., 1998; Mello & Clayton, 1994; Mello & Ribeiro, 1998; Woolley, S. C. & Doupe, 2008) and many cells in these cases appeared to be stained in response to auditory stimulation [see Figure 10 p. 6663 in (Mello & Clayton, 1994), Figure 9A p. 435 in (Mello & Ribeiro, 1998), Figure 7A p. 781 in (Jarvis, et al., 1998) and Figure 5 p.0531 in (Woolley, S. C. & Doupe, 2008)], more than in the present study. A plausible explanation for these density differences could arise from the arousal state of the birds tested. In most studies available, birds were presented with songs while awake, while in the present study they were anaesthetized in order to correlate the findings with those of the electrophysiology experiments. While one could argue that the levels of arousal might influence neuronal activation (and hence ZENK expression), this argument is hard to reconcile with the high levels of activity (both spontaneous and evoked) encountered in MLd neurons during physiological experiments. Whether there is a direct anaesthetic effect on the ZENK activation pathway that could explain these incongruities is not known. Never the less, these pilot studies showed that ZENK staining patterns could not be used to guide the electrode penetrations in these studies.
Chapter IV

Auditory processing in the midbrain: single unit responses
1 – Introduction

Immediate-Early Gene expression revealed that MLd neurons respond to both simple (pure tones and white noise) and complex stimuli (conspecific and heterospecific songs) even though the sound-evoked expression patterns to tones did not match the already known characteristic of nucleus MLd that is the tonotopical organisation of frequencies (Woolley, S. M. N. & Casseday, 2004). The next step is therefore to record electrophysiologically from single units within MLd in birds raised by both their parents and in contact with conspecifics. This chapter describes the tuning properties of these single units to the two categories of stimuli (simple and complex) and then determines if the zebra finch MLd shows a species-specific encoding bias as seen in other vertebrates.

Acoustic communication plays a key role in the behaviour of many vertebrates because it mediates biologically relevant information. Vocalizations are usually used in the context of reproduction, species-specific interactions and in territorial defence (Catchpole & Slater, 1995). Thus, conspecific sounds must be appropriately represented in the central auditory system for an animal to be able to identify and discriminate them from other complex stimuli (e.g., heterospecific vocalisations) so that appropriate behaviour can be produced. A sharper tuning to features of conspecific sounds is expected and is usually present in the auditory system in general and in the midbrain in particular [mammals: for review, see (Portfors & Sinex, 2005); amphibians and fish: for review, see (Bass, et al., 2005); birds: (Woolley, S. M. N. & Casseday, 2004, 2005; Woolley, S. M. N., et al., 2009)].

The auditory midbrain receives inputs from all the lower brainstem auditory nuclei (see chapter I), and is thus a centre of convergence of auditory information processed by other brainstem auditory nuclei. This convergence of information has been shown to contribute to the processing of auditory features that lead to the emergence of, for example, auditory space maps in barn owls (for review, see Konishi, 2003), coding of vocalisation in bats (for review, see Portfors & Sinex,
In songbirds, the encoding of communication signals has been largely studied in the forebrain, and mainly in the song system with a minority of studies on field L. In the song system (HVC, LMAN, area X) neurons respond more strongly to the bird’s own song (BOS) than to any other simple (e.g., tones or broadband noises) or complex (e.g., conspecific songs) stimuli (Doupe & Solis, 1997; Lewicki, 1996; Margoliash, 1983, 1986; Solis & Doupe, 1997; Volman, 1996). Since these song-selective neurons also show a preference for BOS over BOS either played in the reverse direction (BOS-rev) or BOS in which the order of syllables has been reversed (BOS-ro), they are evidently sensitive to the temporal order of song components (Doupe & Solis, 1997; Lewicki & Arthur, 1996; Margoliash, 1983; Margoliash & Fortune, 1992; Solis & Doupe, 1997). By modifying spectral parameters of song in BOS and synthetic versions of BOS, these neurons have also been shown to be sensitive to spectral parameters of the stimuli (Margoliash, 1983, 1986; Theunissen & Doupe, 1998).

However, as mentioned above, coding in the song system of birds is biased towards the perception and encoding of BOS. In forebrain areas that are not part of the song system, however, no selectivity to BOS over other kind of stimuli is observed and conspecific vocalizations evoke strong neuronal responses [(Field L: Amin et al., 2004; Janata & Margoliash, 1999; Lewicki & Arthur, 1996; Margoliash, 1986); (CM: Amin, et al., 2004) and (NCM: Chew et al., 1995; Chew et al., 1996; Stripling et al., 2001; Stripling et al., 1997)], although BOS is still preferred over BOS-rev. However, little or no difference is found between responses to BOS and to BOS-ro in both field L and CM, indicating that these areas are sensitive to the spectro-temporal arrangements within syllables and not to the order of syllables within the song. Sensitivity to conspecific features has also been reported by Hauber et al. (2007b) in field L of female zebra finches where greater neuronal responses to forward conspecific songs over reverse versions (that share the same power spectrum but different spectro-temporal
characteristics) were observed. They also demonstrated that this preference in females was independent of the rearing condition, either socially within a conspecific colony or isolated from their father.

Grace et al. (2003) showed that, in both field L and CM of male zebra finches, subsets of neurons responded preferentially to conspecific vocalizations over synthetic stimuli that matched the acoustical features of the zebra finch song. These neurons could be perfect candidates to detect species-specific vocalizations. Interestingly, Hauber et al. (2007a) showed in adult female zebra finches that, while the selectivity toward conspecific vocalizations over synthetic stimuli, including a reversed version of conspecific song, is present in field L (Hauber, et al., 2007b), this characteristic is not observed in CLM.

The selectivity toward conspecific stimuli has also been reported in NCM by Chew et al. (1996). In their study, all conspecific stimuli (calls and songs of male zebra finches and calls of female zebra finches) elicited greater responses than heterospecific stimuli (songs of canaries, Bengalese finches and silver bills). Also, all call and song reverse versions were treated as new stimuli and elicited a response as great as that of the forward versions. Their results did not show any sex differences. Therefore, auditory forebrain areas differ in what aspect of songs they encode (for review, see Knudsen, D. P. & Gentner, 2010). For instance, NCM is suggested to be involved in the discrimination of novel songs versus familiar songs, based on the fact that responses of neurons within this area decrease with repetition of a conspecific stimulus (Chew, et al., 1995; Chew, et al., 1996; Gentner et al., 2004; Mello et al., 1995; Stripling, et al., 2001; Stripling, et al., 1997). NCM responses also adapt to repeated presentations of a heterospecific stimulus in adults but the persistence of the habituation is less long lasting (Chew, et al., 1995; Stripling, et al., 1997). In juveniles, the habituation is more obvious to forward conspecific songs than to heterospecific songs and reverse conspecific songs (Stripling, et al., 2001). CMM neurons also respond to novel songs but they also appear to be involved in familiar song recognition and do not show habituation when repeatedly presented with these familiar songs (Gentner, et al., 2004).
Lesions in CMM of female zebra finches have been shown to abolish the discrimination between conspecific and heterospecific songs (MacDougall-Shackleton et al., 1998). Moreover, it has been shown in European starlings that learned to recognize songs, that CMM neurons will respond selectively to some complex acoustic features within motifs if these motifs were part of the songs the birds learnt (Gentner & Margoliash, 2003). Therefore, it seems more appropriate to think that the encoding, perception and selectivity to conspecific signals takes place in the forebrain nuclei of the central auditory system.

At levels lower than the forebrain, however, data are rather scarce. In the thalamus, Amin et al. (2010) showed that Ov neurons present spectro-temporal receptive fields (STRFs) with characteristics similar to those observed in field L and/or MLd but also a greater proportion of neurons sensitive to frequency sweeps than that observed in field L and not observed in MLd. Considering that the auditory pathway is providing several inputs to the song system (through Nif and HVC shell and RA shell, see Figure 1.3 in chapter I), it appears important to study how the selectivity toward conspecific signals emerges. It is therefore rather surprising that so few studies have investigated the properties auditory neurons in thalamic or brainstem nuclei such as MLd in songbirds (Ov: Amin, et al., 2010; Bigalke-Kunz et al., 1987; Brainstem: Konishi, 1970; Sachs & Sinnott, 1978; Woolley, S. M. N. & Casseday, 2004; Woolley, S. M. N. et al., 2005; Woolley, S. M. N., et al., 2009; Woolley, S. M. N., et al., 2006; Woolley, S. M. N. et al., 2010).

The zebra finch song is a complex signal composed of a succession of broadband sounds and notes with temporal and spectral modulations. Woolley and Casseday (2004, 2005) investigated the properties of zebra finch MLd neurons and characterized their frequency tuning and responses to song features such as frequency and amplitude modulations. They found that MLd neurons are well suited to encode zebra finch vocalizations: they are broadly tuned to frequencies at behavioural sound levels and they appear to be adapted to encode temporal processing, since many units exhibit onset response patterns (Woolley, S. M. N. & Casseday, 2004). Many units also responded strongly to noise stimuli and to species-characteristic
frequency modulations (Woolley, S. M. N. & Casseday, 2005) but did not seem to differentiate between upward and downward frequency modulations. Woolley et al. (2009) went further and described functional groups of neurons in MLd based on the unit’s STRF and showed that they are well suited to encode elements of songs such as amplitude envelope or syllable onsets. Finally, Woolley et al. (2010) investigated the responses of MLd neurons to both conspecific and heterospecific vocalizations and reported that they were not able to differentiate between these two types of signals. In the present study, the experiment carried out was designed to describe the response properties of MLd neurons to both simple and complex stimuli, which has not been shown before in this nucleus. Results confirm the broadband tuning of zebra finch MLd neurons to frequencies and suggest the presence of at least two neuronal populations: one apparently processing temporal information and another probably responding to particular spectral features in the complex songs. Using a combination of neuronal measurements, results also reveal that, contrary to the findings of Woolley et al. (2010), MLd neurons can discriminate between conspecific and heterospecific vocalizations and that their preferences are stimulus dependant, that is, some songs appear to be preferred more than others. However, discrimination between conspecific and heterospecific signals also appears to be a property of the neuronal population rather than of single units.

2 – Material and Methods

2.1 - Animals and ethical consideration

The experimental procedures were carried out according to the guidelines and approval of the Animal Ethics Committee of the University of Auckland. Birds were bred in an aviary at the Department of Psychology, University of Auckland. Daily light was provided through a compact Arcadia fluorescent bird lamp (2.4% UVB and 12% UVA) as to imitate outdoor conditions. The mean temperature in the aviary was kept at 21 ± 3°C, daily humidity between 35-65% and care
was taken to keep a constant airflow. Pairs of zebra finches (*Taeniopygia guttata*) were first housed together in cages and, once the chicks hatched, families were transferred to separate cages. Six adult zebra finches (>100 days old), that were raised having been exposed only to their conspecifics were used in this study (2 males and 4 females). After reaching adulthood (>100 days), birds were transferred to a holding aviary in the Faculty of Medical and Health Sciences. At all times, birds were held at a constant light/dark cycle of 14h/10h in the aviary and birds were provided with food and water ad lib.

### 2.2 – Surgery

Each bird was deeply anesthetised with an intramuscular injection of 0.04-0.05 ml of a mixture consisting of four parts of distilled water, one part of ketamine (Parnell Laboratories, Auckland, New Zealand; 55 mg/kg) and one part of xylazine (Rompun, Bayer; 11 mg/kg). Additional small doses of anaesthetic were given during the course of the experiment, as required. Once anaesthetised, the bird was placed in a custom-made stereotaxic apparatus (Herb Adams) with a head tilt of 28º from the vertical plane, with ear bars and a beak holder defining the rotation axis. The skin was opened to expose the skull and a custom-made metal head holder was then fixed to the surface of the skull using dental cement (Land Dental Manufacturing Co., Inc., Wheeling, IL). This head holder was used to hold the head at the chosen angle after the ear bars were removed in order to present the auditory stimulation. A small portion of skull was then removed above the bifurcation of the mid-sagittal sinus (Y sinus) and the coordinates of interest were measured with reference to that point. A small opening in the skull and dura mater made over the midbrain coordinates allowed electrode penetration.

### 2.3 – Stimulus generation and presentation

White noise, pure tones, conspecific and heterospecific songs were used as stimuli in this experiment. Both white noise and tones were generated using Adobe Audition 3.0 software and taking into account described hearing ranges for zebra finches (see Okanoya & Dooling, 1987).
Pure tone stimuli were of 1s duration with a built-in rise and fall ramps of 150 ms each at onset and offset. These ramps were necessary to avoid stimulus clicking. Tone stimulus frequencies were 1, 2, 3, 4, 5 and 6 kHz. The white noise stimulus was also 1 sec in duration and contained frequencies ranging from 0 to 10 kHz. Noise and tone stimuli were presented at 75 dB SPL, as measured with a SPL meter (RadioShack), 30 cm from a free-field speaker.

The songs used in this experiment were conspecific (zebra finch) and heterospecific (Bengalese finch) songs. The conspecific songs were chosen amongst birds raised in the same aviary as the birds used in this experiment and were therefore familiar to the tested birds. They were obtained by recording vocalizations of each individual bird overnight using Sound Analysis Pro software and a RadioShack microphone. They were then bandpass filtered (300 Hz – 12 kHz) using Raven 1.3 software. Heterospecific songs were kindly provided by Dr Sarah Woolley from Columbia University, New York. Care was taken to choose conspecific and heterospecific songs with similar lengths. Song lengths ranged between 1.816s to 2.691s for the conspecific songs and 1.940s to 3.091s for the Bengalese finch songs. All songs were calibrated to be presented with an average power of 75 dB (as calibrated using a ½-inch free-field microphone, Brüel and Kjaer and Raven 1.3 software). Songs were played in the forward and backward directions. Each stimulus (white noise, tones and songs) was constructed using Adobe Audition 3.0 to contain 500 ms of silence preceding and following the actual stimulus.

Each stimulus was presented to the bird 15 consecutive times, with a randomised order of presentation. The interval between the 15 consecutive presentations of a single stimulus type was kept constant within a sequence, but these intervals were randomised for each separate sequence (between 0.5 to 1.5 s, 0.1 s steps). Each unit was therefore stimulated with 19 stimuli: white noise, 6 pure tones and 12 songs (three conspecific songs in their forward and reverse directions and three heterospecific songs in their forward and reverse directions). At the end of each set of stimuli presentation, 30 s of the baseline activity of each unit was also recorded at least two
minutes after the last stimulation (so as to ensure that the unit had returned to a basal level of activity) and was used to calculate the spontaneous firing rate.

Stimuli were played to the bird through a multifunction processor (Tucker-Davis Technology, TDT System 3), routed through an attenuator (TDT) that allowed presentation of all stimuli at equal intensities (75 dB SPL average power). The signal was then processed through a TDT Stereo Amplifier connected to a free-field magnetic loudspeaker (TDT) placed 30 cm in front of the bird. Before an experiment, the output of the loudspeaker was checked using a RadioShack SPL meter so to ensure constancy of the average power at which songs were delivered.

2.4 – Electrophysiological recordings

The location and identity of MLd was determined using a variety of search stimuli (white noise, hand claps, clicks, vocalizations), so as to avoid missing cells that would only respond to one but not other types of stimuli. Tones were not used as search stimuli, so the data set may contain an underrepresentation of units responsive only to specific tones. All recordings were made in a sound attenuation chamber (Microbooth, All-DUCT Fabrication, PTY.Ltd, Melbourne, Australia), using tungsten or platinum backed electrodes (FHC Inc., Bowdoin, ME; impedances around 10 MΩ at 1 kHz).

The signal was filtered (300 Hz low/5 kHz high pass) and amplified (gain 100x) using an AC amplifier (A-M systems 1800) and digitised using the TDT multifunction processor RX6. Threshold and spike discrimination were achieved using the OpenController interface of the TDT OpenEx Suite (but see also below). Raster plots and peri-stimulus time histograms (PSTH) could be visualized in real-time (using the OpenScope interface) allowing us to qualitatively assess the auditory selectivity of isolated units prior to recording. Song presentations and unit recordings were made using a custom-made program designed using the TDT RPvdsEx control. A collection of songs was built into the program so that any song within that collection could be called upon as a stimulus through the OpenController interface during an experiment. The order in which songs in this collection were presented was determined randomly for each series of
presentations. The interface also allowed us to enter the number of times a stimulus was presented, the interstimulus interval and the attenuation. Each stimulus was attenuated so that the output of the speaker was 75 dB measured at the position of the bird’s head. The epoch store tracked the onset of each stimulus repeat within a stimulation sequence. This was then used to align sequential presentations to construct the peri-stimulus time histograms, raster plots and to calculate the latencies.

Small electrolytic lesions (40 μA, DC for 10 s) were made at sites of interests in order to later identify and reconstruct the recording sites. Lesion sizes turned out to be very variable. In some birds, lesions were small enough to determine the recording location while in others lesions were too large to be used for detailed placement of the electrode, but, combined with electrode tracks, suitable to confirm placement of the electrode in MLd.

2.5 – Analysis

Units were analysed off line using TDT’s OpenSorter and OpenExplorer packages of the OpenEx suite. OpenSorter was used to manually eliminate outlier spikes and to determine whether recordings were actually of single units. When there was doubt the data were discarded. PSTHs of 20ms bins (or 5 ms bins in case of latency determination) were constructed using TDT Openexplorer.

The auditory activity of a unit within each bin was assessed by comparing the number of action potentials in a 20 ms bin in the PSTH to a set threshold. The threshold corresponded to the mean + 5 s.d. of the baseline firing rate (Prather et al., 2009). The baseline firing rate was defined as the firing rate during the 500 ms of silence preceding the sound stimulus. A unit was classified as ‘auditory’ when the number of action potentials exceeded the threshold in at least one bin for at least one of the stimuli. The spontaneous firing rate of the unit (spikes/s) was calculated using the first 20 s of the 30 s baseline activity recorded at the end of the session.
**Determination of latency:**

The latency of a unit was determined using its response to the white-noise (WN) stimulus. The latency was estimated to the nearest 5 msec from PSTH of 5 msec bins (Solis and Doupe, 1997). The mean baseline firing rate was calculated from the 500 ms of silence preceding the sound stimulus. Threshold was set at twice the mean baseline firing rate (Solis and Doupe, 1997). Latency was determined as the time of onset of the first of two consecutive bins with spike rates that exceeded threshold. Latencies were only calculated for units that exhibited a sustained response to the WN stimuli. Units that presented a chopper-like response were not used for this analysis.

**Tuning Curve Bandwidth:**

Tuning curves were constructed using unit responses to tones. Responses to tones above threshold (5 s.d. above the mean) were normalised against the power of each stimulus, and were plotted against the frequency of the tones. The bandwidth of the tuning curve was calculated by measuring the width of the curve at the halfpoint of the maximal response (preferred frequency) at 75 dB (See Figure 4.5A). In many cases, the bandwidth could not be confidently determined and those units were classified as undetermined bandwidth (Figure 4.5B and C).

**Response Strength (RS):**

Response strength is used as a measure of comparison between the evoked responses using different stimuli. It measures the amount of evoked activity above spontaneous rate. The response strength is sensitive to changes in levels of evoked activity in response trains that are sustained during the presentation of the stimuli. It is not well suited, however, to evaluate responses of units that are locked to specific features of the stimulus. Indeed, strong locking to a few specific features does not necessarily translate into robust changes in firing rates above
threshold. RS was measured for all song stimuli (conspecific, heterospecific, forward and reverse) for all units that met criteria to be classified as auditory (see above). Response strength was calculated for each song by subtracting the spontaneous activity (in spikes/sec, SSR) from the evoked spike rate (spikes/sec, ESR) for each of the 15 consecutive presentations and averaging these values:

\[ RS = \frac{\sum_{i=1}^{N} ESR_i - SSR_i}{N} \]

**Temporal Sparseness Index (TSI):**

This index is sensitive to the timing of spikes within the train. TSI values range from zero to 1. TSIs close to zero are indicative of spike trains that are sustained throughout the stimulus presentation, whereas TSI values close to 1 indicate that spikes are occurring sparsely at times during stimulus presentation.

TSIs were calculated by determining the fraction of the bins in the PSTH that reached spike rates above threshold (over mean +5s.d. from prestimulus firing rate), as per:

\[ TSI = 1 - \frac{\text{number of bins above threshold}}{\text{total number of bins}} \]

**d'**

This parameter measures the difference in response (or preference of a unit) to a pair of stimuli, A and B. If the value of d’ (of responses to A compared to B) is greater than zero, it indicates that stimulus A elicited a greater response in the unit that did stimulus B. A d’ smaller than zero indicates that stimulus B elicited a larger response than stimulus A, and values equal to zero indicate that the unit responded equally to both stimuli. Differences in the responses to two stimuli (A and B) are calculated by comparing the RS of the unit to each of the two stimuli (RSA and RSB, respectively).
Chapter IV: Auditory processing in normal MLd

The $d'$ is calculated as:

$$d'_{A \rightarrow B} = \frac{2(RS_A - RS_B)}{\sqrt{\sigma^2 RS_A + \sigma^2 RS_B}}$$

One advantage of the $d'$ value is that the differences between the means of the RS for each stimulus is weighted against the variance of their distributions. A second advantage is that the $d'$ is insensitive to sample size.

**Selectivity Index (SI):**

This index compares the responses of a neuron to two different stimuli (A and B) but does not take into account response variability. The selectivity index is calculated as:

$$SI = \frac{RS_A}{RS_A + RS_B}$$

The selectivity index is expressed in values between zero and 1, with SI’s close to 1 and zero showing preference for one or the other stimulus, and values close to 0.5 indicating that there is no preferential response to either of the two stimuli.

Unlike the comparisons based on the selectivity index, the $d'$ does not provide information regarding the strength of the responses themselves. Thus, both SI and $d'$ are usually used in conjunction.

**Criteria used for SI and $d'$:**

Which values of SI and $d'$ would be considered as the criterion for selectivity was determined in a way similar to that of Solis and Doupe (1997). For SI, a unit is considered to be selective when the mean RS of the unit to one stimulus is at least twice that to the other stimulus. Thus, neurons that are considered selective had SI=$< 0.33$ or SI=$> 0.66$. The criterion for $d'$ was established in a way similar to Solis and Doupe (1997). An interval of values of $d'$ (non-selectivity zone) was
chosen in which most neurons showed non-selectivity based on SI criterion. A criterion of $|d'|=2$ was established since most neurons with $|d'| \geq 2$ were selective (as per SI) whereas most neurons with $|d'| \leq 2$ were non-selective (as per SI). (See Figures 4.17 and 4.18)

3 – Results:

3.1 - Spontaneous activity and latency

Responses of 58 units to all stimuli were recorded (from six birds, 4-14 units/bird). 65.5% of these units showed no spontaneous activity (<1 spike/s), 27.6% showed low spontaneous activity (<7 spikes/s) and only 6.9% had spontaneous activity between 7 and 15 spikes/s (Figure 4.1).

Latency was calculated based on sustained responses of units to the WN stimulus presented at 75 dB; 18 cells met the criteria for latency calculation. Latencies in these units ranged from 30 to 75 ms, with a mean of $46.94 \pm 25.03$ ms.

3.2 - Characterization of responses to all stimuli

To characterize as thoroughly as possible what is encoded by units within MLd, responses of each unit to all stimulations have to be investigated in order to see if any specific unit pattern stands out. Using the normalized responses above threshold of units to tones, WN and songs, we were able to identify 4 main patterns (Table 4.1).
**Figure 4.1**

Distribution of spontaneous rate of the units recorded in the “normal” MLd
Table 4.1: Different patterns of units when considering all 3 types of stimulation: Tones, WN and songs

<table>
<thead>
<tr>
<th>Response Pattern</th>
<th>% Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responsive only to songs</td>
<td>1.72</td>
</tr>
<tr>
<td>Responsive to tones and songs</td>
<td>12.07</td>
</tr>
<tr>
<td>Responsive to WN and songs</td>
<td>6.9</td>
</tr>
<tr>
<td>Responsive to all 3 stimuli</td>
<td>79.31</td>
</tr>
<tr>
<td>Tones &gt; WN</td>
<td>52</td>
</tr>
<tr>
<td>WN &gt; Tones</td>
<td>48</td>
</tr>
</tbody>
</table>

One of the 58 units (1.72%) responded neither to WN nor tones but did respond to at least some of the song stimuli (Figure 4.2).

Seven units (12.07%) did not show a response to WN but responded to one or several pure tones and to songs. Five of these responded to one tone only and two other units showed more broadband tuning.

Four units (6.9%) did not respond to tones but responded to WN and songs.

Forty-six units (79.31%) responded to all three types of stimulation. Amongst these, 24 (52%) showed a greater response to tones than to WN and 22 (48%) had a stronger response to WN than to tones. Of the units that had a stronger response to tones than to WN, 15 (62.5%) also had a normalized response level to songs close to that of the response to WN. The remaining 37.5%, on the contrary, had a level of response to songs that was similar to their responses to their best frequency. Out of the 22 units that responded stronger to WN than to tones, 11 (50%) showed responses to songs that were close to the responses to WN. Most of these units showed very different levels of response to tones. The other 11 units had a level of response to songs different from that of response to WN. Their responses were lower than that to WN.

3.3 - Characterisation of responses to tones and WN

Of the 58 recorded units, 49 (84.5%) showed responses to WN (Table 4.2), 53 (91.4%) responded to at least one tone and 47 (81%) responded both to WN and at least one tone
Figure 4.2

Summary of responses of each of the 58 individual units to each stimulation.

The left column of the table indicates the individual units (black: units recorded from males; red: units recorded from females). Each row then represents the response of each unit to the 19 stimuli used in the experiment: WN, 6 pure tone stimuli, 6 conspecific songs (forward and reverse versions) and 6 heterospecific songs (forward and reverse versions).

The response of a unit to each stimulus is represented using a colour code: if the unit was inhibited by the stimulus its response is indicated in blue; an excitatory response to a stimulus is indicated in green. Light green represents a light to moderate response; dark green represents a strong response from the unit elicited by the stimulation. No colour indicates that the unit failed to respond to the stimulation.

The preferred frequency of a unit is indicated by a crossed box.
stimulus. Of the 49 units that responded to WN, two units (4.1%) did not show significant responses to any tone stimulus. Of the 53 units that responded to at least one tone stimulus, 19% responded to one tone only, 39.7% responded to two tones, 15.5% of cells responded to three tones, 13.8% responded to four tones and 3.4% responded to five tones (Figure 4.3). None of the recorded cells responded to all six pure tone stimuli.

The percentage of units that responded to each individual tone stimulus is shown in Table 4.2.

Table 4.2: Percentages of units responding to each pure tone and WN stimuli

<table>
<thead>
<tr>
<th></th>
<th>WN</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>3 kHz</th>
<th>4 kHz</th>
<th>5 kHz</th>
<th>6 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of units</td>
<td>84.48</td>
<td>67.24</td>
<td>67.24</td>
<td>48.28</td>
<td>22.41</td>
<td>8.62</td>
<td>3.45</td>
</tr>
</tbody>
</table>

At the population level, responses to lower frequencies (primarily 1 and 2 kHz and to a certain degree 3 kHz) were overrepresented compared to other frequencies. Tones of 1, 2 and 3 kHz (as well as white noise) also evoked higher firing rates in MLd than did higher frequencies. Units responded to tones either by increasing their spontaneous rate above threshold (excited by the tone) or by reducing their spontaneous rate with respect to threshold (inhibited). These differences were not explored in detail in this study.

3.3.1 - Preferred Frequency and Tuning Curve Bandwidth

The neuron’s best frequency (BF) is hereby derived from the response curves of each unit, given that the temporal constraints of the experimental design prevented the use of threshold curves. BF is hereby defined at that frequency at which the unit showed maximal response at 75 dB SPL. Out of the 53 units that showed an above threshold response to at least one tone, 28.3% had a BF of 1 kHz, 37.7% had a BF of 2 kHz (Figure 4.4), 26.4% had a BF of 3 kHz, 5.7% a BF of 4 kHz and 1.9% a BF of 5 kHz. No unit showed a BF of 6 kHz.

The bandwidth of the response curves were calculated for 33 units. Bandwidth could not reliably be obtained for some units, e.g. those with BFs of 1 kHz (our lower stimulation frequency) (see
Percentages of units responding to either 0, 1, 2, 3, 4, 5, or 6 tones.
Figure 4.4

Percentages of units per preferred frequency
**Figure 4.5**

**Determination of tuning curve bandwidth and related issues**

A: The tuning curve of a unit was based on its normalized responses above threshold. Then the width of the curve was measured at halfpoint of the maximal response, at the preferred frequency.

B and C: The bandwidth could not be determined in some cases because of the responses of units to 1 kHz, our lowest stimulation.
examples in Figure 4.5) and these units were excluded from this analysis. Bandwidth values ranged from 1 to 2.84 kHz. Figure 4.6 suggests that there may be a correlation between BF and bandwidth, but the size of the population is too small to determine that with confidence.

### 3.3.2 - Temporal response patterns

Four different response patterns have been previously observed in avian auditory neurons and these response patterns were also found in our study. Units in this study could be classified as onset, sustained, primary-like or primary-like with notch response patterns based on the shapes of the PSTHs (Figure 4.7). An example of an onset unit is shown in Figure 4.7A exhibiting the typical strong response at the onset of the stimulus. An example of a sustained unit is shown in Figure 4.7B, a unit that fires quite constantly throughout the whole stimulation period. A primary-like responding unit is shown in Figure 4.7C, with a clear initial high rate of spikes followed by a sustained, but lower rate of firing throughout the remainder of the stimulus. Figure 4.7D shows a primary-like response with notch, similar to the primary-like, except that a dip in firing rate follows the onset peak of response. Forty of the 58 units (69%) could be assigned to one of these categories based on their temporal response pattern. Of these, 20% were classified as onset units, 60% as sustained units, 10% as primary-like units and 10% as primary-like with notch units.

Neurons could be classified into the 4 response types above when their responses to WN were analysed. Only 8 of all the units recorded did not show a response to WN. Of these 50 units, only 22 could be confidently assigned to any one category. From these 22 units, 18% showed an onset pattern, 59% a sustained pattern, 14% a primary-like pattern and 9% a primary-like with notch pattern, a relative distribution similar to that obtained from the analysis following tone stimulation. Of these 22 units, two did not show responses to tones, but in 90% of the remaining units (18/20) there was no difference in the categorisation when the tone-evoked PSTH or the WN-evoked PSTH was used.
Comparison of tuning curve bandwidths of units (dots), at 75dB SPL, and their preferred frequencies
Examples of the four response patterns found in the normal zebra finch MLd

Panel A: PSTH of a unit representing an onset response pattern with a typical strong response at the onset of the stimulus (yellow line in the underlying spectrogram) followed by either no or little response during the rest of the stimulation.

Panel B: PSTH of a unit representing a sustained response pattern where the evoked response is constant throughout the stimulation (yellow line in the underlying spectrogram).

Panel C: PSTH of a unit representing a primary-like response pattern. This pattern is characterized by an onset component followed by a constant but less vigorous firing rate up to the end of the stimulation (yellow line in the underlying spectrogram).

Panel D: PSTH of a unit representing a primary-like with notch response pattern. This pattern strongly resembles that of the primary-like response pattern with the exception of a dip in firing rate after the onset response.
3.3.3 - Inhibition

In the IC, inhibition is thought to play a role in shaping the selectivity of units towards species-specific sounds (Yang et al., 1992; Hall, 1999; Klug et al., 2002; Portfors, 2004; Xie et al., 2005). As we did not present our range of frequencies at different intensities, sideband inhibitions of the units cannot be described. However, at 75 dB SPL, a few units still were inhibited by some of the frequencies presented. While the analysis used in this study does not identify inhibitory changes in firing patterns, the observed reduction in firing rate associated with the presentation of certain stimuli suggests that at least some aspects of inhibition are retained within the circuit (See Figure 4.8 for example) under the anaesthetic regime used in this study.

3.4 - Characterization of responses to songs

Birds were presented with stimuli consisting of conspecific and heterospecific songs. The aim of these experiments was to determine whether in MLd there is any detectable bias toward either of these categories of sound stimuli. Songs are complex stimuli and it is not intuitively obvious which features of the auditory stimulus will elicit a response (but see Gill et al., 2006; Woolley, S. M. N., et al., 2005; Woolley, S. M. N., et al., 2006). Songs contain both complex power spectra and complex time-related features. In order to determine the role of time-related features of the songs in eliciting a response, all songs were played in the forward and backward directions. It is expected that differences in response to forward versus reverse would only be present in units that code specific aspects of the temporal features of song, but not when they are primarily responding to the power spectrum (which is identical in forward and reverse song). This section provides first a description of the types of responses evoked in MLd units by song stimuli and then an examination of whether some selectivity toward conspecific vocalizations can already be found at the level of MLd units.
Figure 4.8

Example of inhibition evoked by the presentation of a stimulation (here a pure tone)

The spectrogram of the stimulus (yellow line) on this figure has been shifted so as to account for the latency and for a better visualization of the response.

The red arrow below the PSTH marks the beginning of the sound stimulus proper (red arrow under the spectrogram).
Figure 4.9

Example of the response of two units to song stimuli: responses to all components

A: Example of a unit that responded to conspecific song #1 (left panel) and heterospecific song #1 (right panel), shown over underlying spectrograms, followed the temporal amplitude modulation pattern of the stimuli. Note how the unit responded to most if not all components.

B: Example of a unit that had a sustained response to the same song stimuli as that used in panel A. Here the temporal pattern of response to each separate component of the songs is not as visible as that in panel A.

The spectrogram under each corresponding PSTH has been shifted so as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
3.4.1 - Characterization of the pattern of responses to conspecific and heterospecific songs

Both conspecific (CON) and heterospecific (HET) songs elicited robust responses from MLd neurons. However, units differed in their patterns of responses to the different song stimuli. Many units showed strong responses to most (if not all) of the components of both CON and HET songs, and most responses occurred in bursts that followed the temporal amplitude modulation pattern of the song stimulus (i.e., showed responses that were locked to the temporal patterns of the song, Figure 4.9A). In only one case was a neuron found to respond in a more sustained manner throughout the song (Figure 4.9B).

Some of the units appeared to be locked to all elements of the song, while others appeared to lock primarily to particular features within the song structure. The unit shown in Figure 4.10 provides a good example of the type of response variability encountered after presenting different song stimuli. This unit shows a component of the response that appears to be strongly locked to the harmonic stacks presented within CON songs #1 and #3 (Figure 4.10A and B). However, in the absence of those harmonic stacks (as in CON song #2, Figure 4.10C and HET song #1 Figure 4.10D), the unit appears to more readily lock to most of the features of the song. (In the case of the HET song, the unit appears to only lock to the first part of the trill train, but reduces its response thereafter (Figure 4.10D, arrow). Determining what aspects of the song elicit these different types of responses is beyond the scope of this study, but these results suggest that there is some degree of feature extraction at the level of MLd. A second example of the type of variability in responses encountered in MLd units is shown in Figure 4.11. This unit responded to several elements in CON song #2, showed a smaller response to CON song #3, but did not respond at all to CON song #1. While the unit shown in Figure 4.10 showed similar responses to CON songs #1 and #3, the unit shown in Figure 4.11 showed more similar responses to CON songs #2 and #3. This suggests that the differences in responses are not due to degrees of song similarity but rather reflect different properties of the neurons themselves.
**Figure 4.10**

**Example of responses showing some degree of locking to the stimuli**

This figure represents the evoked responses of a unit to four song stimulations.

Panels A and B show the responses of the unit to conspecific songs #1 (A) and #3 (B). Note the strong component of the responses that locks to harmonic stacks. Also note that not all harmonic stacks evoke such a strong response (green arrow on Panel B in the PSTH and under the corresponding spectrogram).

Panels C and D show responses of the same unit to two songs that do not contain harmonic stacks, conspecific #2 (C) and heterospecific #1 (D). Note how the responses in these cases are locked to many elements within the songs with still a few interesting exceptions such as the second set of trills in heterospecific song #1 (D, blue arrow on Panel D in the PSTH and under the corresponding spectrogram).

The spectrogram under each corresponding PSTH has been shifted as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
This figure shows the variability in responses of a unit to three conspecific songs presented in the forward direction.

Note how the unit responded to specific features of conspecific song #2 (Top right PSTH and spectrogram) and, to a lesser degree, of conspecific song #3 (Bottom PSTH and spectrogram) but did not respond to conspecific song #1 (Top left PSTH and spectrogram).

The spectrogram under conspecific song#2's PSTH has been shifted so as to account for latency. The red arrow below the PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
Example of encountered limitations with units inhibited by stimulation

This figure shows a unit whose firing rate dramatically diminished during song presentation.

Panel A: response of the unit to heterospecific song #1. No response above threshold would be detected for such a unit although its auditory activity is visible. Note that the spectrogram of the song has been shifted, accounting for latency, so as to facilitate visual representation of the unit’s response to the stimulus. Red arrows under both the PSTH of the unit and the spectrogram of the song indicate the beginning of the sound stimulus.

Panel B: response of the same unit to conspecific song #2. Note that although a locked response to precise features within the song is observed, it is barely above threshold. While the spike rate during stimulation actually approaches that of the spontaneous rate, only 2.29 spikes are detected above threshold.

As in panel A, the spectrogram of the song has been shifted so as to account for latency and red arrows mark the beginning of the sound presentation proper.
Figure 4.12 shows the responses of a unit that exemplifies the type of limitations in the analysis used in this study, and that must be considered when drawing conclusions about the population behaviour of MLd neurons. This unit is one that shows a spontaneous activity of 2.87 spikes/sec, but during the stimulus period the firing rate falls either under or just over the threshold line. In response to HET song #1 (Figure 4.12A), the firing rate of the unit falls to 1.82 spikes/sec and in response to CON-reverse song #2 (Figure 4.12B) is 2.54 spikes/sec. However, a close inspection of the PSTH shows that while there are no detectable increases in firing rate, a clear response is evident in changes in firing pattern as the unit’s activity becomes locked to the patterns of the auditory stimulus. These types of changes are not quantified in the current analysis, except for the RS value, which detects reduction in firing rate.

3.4.2 - Role of recording site and unit properties

In three of the recording sites two single units could be isolated and recorded from simultaneously. This provides useful information as to what types of units are in proximity to each other. These three instances showed that units that are in sufficient proximity to be recorded from with a single electrode, could exhibit different response characteristics (see Figure 4.13).

3.4.3 - Response strength to songs

The response strength measures the amount of activity within the total firing rate that can be directly attributed to the presentation of the stimulus. The response strength (RS) of the units recorded in this study ranged from -3.58 to 41.37 spikes/s, with most of the units being characterized by low values of RS (Figure 4.14). In 29 instances RS values are below 0, indicating that presentation of the stimulus resulted in inhibition of the unit. The mode of the distribution of RS values is 0, but the tail of the distribution reaches an RS value of 41.37 spikes/s.

RS values close to 0 could represent units that did not respond to some songs, but could also represent units where responses were highly locked to a small number of features within the
Figure 4.13

Example of responses of two units simultaneously recorded

This figure represents the responses to three different stimulations of two single-units whose responses were recorded simultaneously at the same site. Note how the responses of the two units to each stimulation differ showing that units with different properties and extracting different features from the song stimuli can be in proximity within MLd.

Top PSTHs: responses to WN, conspecific song #2 and heterospecific song #1 reverse of one of the units.

Bottom PSTHs: responses to the same three stimulations of the second unit.

The spectrogram under each PSTH has been shifted so as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
Figure 4.14

Distribution of all RS values from all 58 units to all song stimuli

Note that most instances are concentrated around the lowest values.
song; i.e. low values of RS could indicate both a lack of response as well as a highly feature-specific response. This is a limitation of the RS, in that it does not take into account the temporal features of the responses, but these temporal features are better accounted for by other metrics used in this study (see below). Nevertheless, RS values obtained from responses to CON songs were generally higher than those obtained in response to HET songs (5.76±0.60 spikes/s and 4.49±0.48 spikes/s respectively; Figure 4.15A, C) and this was true for both males and females (Table 4.3).

**Table 4.3:** Comparison of mean ± SEM RS (spikes/sec) values in response to forward CON songs and forward HET songs

<table>
<thead>
<tr>
<th></th>
<th>Forward CON songs</th>
<th>Forward HET songs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole neuronal population</td>
<td>5.76 ± 0.60</td>
<td>4.49 ± 0.48</td>
</tr>
<tr>
<td>Male neuronal population</td>
<td>5.34 ± 0.76</td>
<td>3.61 ± 0.50</td>
</tr>
<tr>
<td>Female neuronal population</td>
<td>5.94 ± 0.80</td>
<td>4.88 ± 0.65</td>
</tr>
</tbody>
</table>

No obvious differences are seen when RS are compared from forward versus reverse presentation of a song stimulus (Figure 4.15A and Figure 4.15B). When the RS value obtained for the forward and reverse presentation of a given song stimulus in a single unit are plotted against each other, most data points (with the exception of a few) fall on or near the equality line (Figure 4.15B). The values of RS for the different forward and reverse stimuli are shown in Figure 4.15A (means and SEM) and summarized in Table 4.4.
Comparisons of RS values in the zebra finch midbrain

Panel A: Mean ± SEM values of RS to each song stimulus in forward (black bars) and reverse (gray bars) directions.

Panel B: Plot of mean RS value of each individual unit for a song versus its reverse counterpart. The diagonal line represents the equality line where the response of a unit to both songs is identical.

Panel C: Mean ± SEM values of RS to forward conspecific songs and forward heterospecific songs.

Panel D: Mean ± SEM values of RS to forward songs and reverse songs.

The asterisk indicates statistical difference (p < 0.05)
Chapter IV: Auditory processing in normal MLd

Table 4.4: Mean ± SEM values of RS for each forward and backward song stimulus

<table>
<thead>
<tr>
<th></th>
<th>CON 1</th>
<th>CON 2</th>
<th>CON 3</th>
<th>HET 1</th>
<th>HET 2</th>
<th>HET 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOR</td>
<td>5.41±0.99</td>
<td>5.8±1.02</td>
<td>6.06±1.12</td>
<td>4.54±0.79</td>
<td>4.9±0.88</td>
<td>5.36±1.04</td>
</tr>
<tr>
<td>REV</td>
<td>5.77±1.10</td>
<td>5.77±0.93</td>
<td>6.71±1.19</td>
<td>5.15±0.79</td>
<td>5.36±1.04</td>
<td>4.02±0.82</td>
</tr>
</tbody>
</table>

A Wilcoxon Signed Rank Rest comparison of RS values in response to forward and backward songs suggests that these differences are actually significant (p < 0.0001).

3.4.4 - Temporal Sparseness Index

The temporal sparseness index (TSI) is a good indicator of how sustained a train of spikes is. The more locked to a stimulus feature the response is, the closer to 1 the TSI will be. There were no obvious differences in the values of the TSI for any of the stimuli used in this study as shown on Figure 4.16A and summarized in Table 4.5.

Table 4.5: Mean ± SEM values of TSI for each forward and backward song

<table>
<thead>
<tr>
<th></th>
<th>CON 1</th>
<th>CON 2</th>
<th>CON 3</th>
<th>HET 1</th>
<th>HET 2</th>
<th>HET 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOR</td>
<td>0.80±0.03</td>
<td>0.79±0.03</td>
<td>0.76±0.03</td>
<td>0.81±0.02</td>
<td>0.82±0.03</td>
<td>0.85±0.02</td>
</tr>
<tr>
<td>REV</td>
<td>0.79±0.03</td>
<td>0.78±0.03</td>
<td>0.75±0.03</td>
<td>0.80±0.03</td>
<td>0.81±0.03</td>
<td>0.83±0.03</td>
</tr>
</tbody>
</table>

When the TSI value for a song is plotted against the TSI value for its reverse, most data points fall on or near the equality line (with a few interesting exceptions, Figure 4.16B). Average values of TSI FOR were higher for HET than for CON (0.79± 0.02 for CON and 0.83 ± 0.01 for HET) and the comparisons between forward and reverse were significantly different (0.81 ±0.01 for FOR and 0.79 ± 0.01 for REV; Wilcoxon Signed Rank test, p=0.015) (Figure 4.16C and D, see chapter V for full statistical analysis).

Table 4.6 shows the distribution of TSI value ranges for the different stimulus types, showing the percentage of responses for each stimulus type that showed TSIs over 0.75 or below 0.25.
**Figure 4.16**

Comparisons of TSI values in the zebra finch midbrain

Panel A: Mean ± SEM values of TSI to each song stimulus in forward (black bars) and reverse (gray bars) directions.

Panel B: Plot of mean TSI value of each individual unit for a song versus its reverse counterpart. The diagonal line represents the equality line where the response of a unit to both songs is identical.

Panel C: Mean ± SEM values of TSI to forward conspecific songs and forward heterospecific songs.

Panel D: Mean ± SEM values of TSI to forward songs and reverse songs.

The asterisk indicates statistical difference (p < 0.05)
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Considering that a unit that fails to respond to a stimulus will be attributed a TSI value of 1, any value of exactly 1 was removed from the calculations for each stimulation (though it could also represent an inhibitory response). For all stimulus types, usually more than half of the presentations of the stimulus resulted in responses with values of TSI over 0.75, indicating a bias against sustained responses to these stimuli. In fact, sustained responses (defined as values below 0.25) were seen in less than 5% of the cases for all stimuli (except for song CON #3 Reverse, in which this value was 5.17%).

Table 4.6: Proportion of TSI values > 0.75 and < 0.25 for each song stimulation. Values of 1, indicating 0 spikes above threshold, were removed from the analysis. The number of units considered per stimulation was: CON1 48; CON1r 52; CON2 53; CON2r 56; CON3 49; CON3r 51; HET1 53; HET1r 52; HET2 54; HET2r 49; HET3 51; HET3r 52

<table>
<thead>
<tr>
<th></th>
<th>CON1</th>
<th>CON1r</th>
<th>CON2</th>
<th>CON2r</th>
<th>CON3</th>
<th>CON3r</th>
<th>HET1</th>
<th>HET1r</th>
<th>HET2</th>
<th>HET2r</th>
<th>HET3</th>
<th>HET3r</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI &gt; 0.75</td>
<td>75.00</td>
<td>71.15</td>
<td>67.92</td>
<td>62.50</td>
<td>71.43</td>
<td>66.67</td>
<td>75.47</td>
<td>82.69</td>
<td>75.93</td>
<td>83.67</td>
<td>80.39</td>
<td>82.69</td>
</tr>
<tr>
<td>TSI &lt; 0.25</td>
<td>4.17</td>
<td>3.85</td>
<td>3.77</td>
<td>1.79</td>
<td>2.04</td>
<td>5.88</td>
<td>1.89</td>
<td>1.92</td>
<td>1.85</td>
<td>4.08</td>
<td>1.96</td>
<td>1.92</td>
</tr>
</tbody>
</table>

3.4.5 - d’ and Selectivity Index

Two methods, d’ and selectivity index (SI), were used to compare the differences in responses of a unit to a pair of songs. Both the d’ value and the SI can be used to assess a unit’s selectivity (or ‘preference’) of one stimulus over another in the pair. Both quantifiers are based on the unit’s mean RS values for each of the stimuli. In the present study, a d’_{A→B} > 2 indicates a neuron selective for stimulus A over stimulus B while a d’_{A→B} < -2 indicates a neuron selective for stimulus B over A (as described in the Material and Methods section).

The preference towards a song above any other becomes apparent by plotting the cumulative distribution of the d’values for that song against all the others for all instances in which that song was presented. Figures 4.17 and 4.18 show these distributions of d’ for all instances when each
Figure 4.17

Cumulative distributions of $d'$ values for conspecific songs

Top Panel: cumulative distributions of $d'$ values to conspecific song #1 versus its reverse version and other forward song stimulations of each individual unit.

Middle Panel: cumulative distributions of $d'$ values to conspecific song #2 versus its reverse version and other forward song stimulations of each individual unit.

Bottom Panel: cumulative distributions of $d'$ values to conspecific song #3 versus its reverse version and other forward song stimulations of each individual unit.

In all plots, black-filled circles represent non-selective instances as determined by SI value (see Material and Methods)
Unfilled circles represent selective instances as determined by SI value.
The vertical black lines delimit the non-selectivity zone where $d' < |2|$
**Figure 4.18**

**Cumulative distributions of d' values for heterospecific songs**

Top Panel: cumulative distributions of d' values to heterospecific song #1 versus its reverse version and other forward song stimulations of each individual unit.

Middle Panel: cumulative distributions of d' values to heterospecific song #2 versus its reverse version and other forward song stimulations of each individual unit.

Bottom Panel: cumulative distributions of d' values to heterospecific song #3 versus its reverse version and other forward song stimulations of each individual unit.

In all plots, black-filled circles represent non-selective instances as determined by SI value (see Material and Methods this chapter).

Unfilled circles represent selective instances as determined by SI value.

The vertical black lines delimit the non-selectivity zone where d' $< |2|$.
CON song and each HET songs were compared against all others. These data show that there is no preference for an individual song stimulus at the population level. Most responses fall within the non-selectivity interval, but many responses are found outside of this interval. Thus, MLd units are able to discriminate between stimuli. For example, in Figure 4.17B it can clearly be observed that for those instances showing responses that fall outside the no preference interval, most prefer CON song #2 over its reverse or other forward songs, with a smaller proportion showing negative preference towards it (i.e., preferring the other stimulus used in the comparison). Heterospecific song #3 (Figure 4.18C), on the contrary, shows that in most instances where the selectivity criterion is met, there is also a negative preference for that stimulus. Figure 4.19 shows the proportion of instances where a stimulus elicited positive or negative preference with respect to all instances.

Figure 4.20 shows the degree of selectivity for individual units for specific stimulus comparisons. When considering all possible comparisons, only two units did not show in any instance of a preference for at least one stimulus over another (Figure 4.20). This suggests that the great majority of MLd neurons are able to discriminate at least one feature within the stimulus landscape.

**4 - Discussion**

The auditory midbrain nucleus MLd is the centre of convergence of inputs originating from audition brainstem nuclei (and from the contralateral MLd) and provides the most substantial ascending auditory input to the forebrain. A secondary projection from LLI to n. basalis in the telencephalon is also present, but its function is not well understood (Arends & Zeigler, 1986; Wild & Farabaugh, 1996; Wild, et al., 2001a), and connections from LLV to auditory thalamic areas have also been recently described (Coleman, M. J., et al., 2007). Because of the degree of convergence of ascending auditory inputs, MLd is well positioned to serve as a major centre where selectivity to specific complex sounds, such as conspecific signals, could arise. For
**Figure 4.19**

**Proportion of selective instances where selectivity was observed for each song stimulus**

Black bars represent the proportion of instances where the stimuli against which the song in abscissa was plotted was the preferred stimulation (Negative preference, see data points for which \(d' < -2\) on Figures 4.17 and 4.18)

Gray bars represent the proportion of instances where the song in abscissa was preferred over the other stimuli it was plotted against (Positive preference, see data points for which \(d' > 2\) on Figures 4.17 and 4.18)

Instances that were in the non-selective zone are not represented here.
Figure 4.20

Examples of d' values distributions at the level of single unit

Panel A: Distribution of d' values of the 58 individual units recorded for conspecific song #2 against other forward song stimuli.

Panel B: Distribution of d' values of the 58 individual units recorded for heterospecific song #3 against the other forward song stimuli.

In both panels, the horizontal black lines delimit the non-selectivity zone with d' < |2|. Data points above the non-selectivity zone indicate instances in which the song was preferred over the stimulus it was tested against (blue arrows in panel A show three instances of units that preferred conspecific song #2 over heterospecific song #3). Data points below the non-selectivity zone indicate instances in which the stimulus the song was tested against was preferred (green arrows in panel B show three instances of units that preferred conspecific song #3 over heterospecific song #3).
A  
\( d' \) values Conspecific song #2 vs FOR songs

B  
\( d' \) values Heterospecific song #3 vs FOR songs
example, in the midbrain of both mammals and amphibians, neurons show tuning to both spectral and temporal characteristics of conspecific vocalisations and have been shown to act as feature detectors (mammals: for review, see Portfors & Sinex (2005); amphibians: for review, see Feng et al. (1990); Wilczynski & Ryan (2010)). In male zebra finches, MLd neuronal responses to tones, white noise and specific features found in zebra finch song, such as frequency or amplitude modulations, and songs have been described by Woolley and Casseday (2004, 2005) and Woolley et al. (2005; 2009; 2006).

The goal of the present study was to characterize the responses of MLd units to both simple (tones and white noise) and complex (songs) stimuli and in both male and female zebra finches in order to determine the role of experience in shaping response properties (see chapter V). Particular interests were to determine whether some degree of feature extraction occurs at the level of the auditory midbrain and whether a bias toward the processing of conspecific vocalizations was already present at the level of the midbrain, as is the case for other vertebrates that do not have a dedicated ‘song system’.

Almost 2/3 of the MLd units recorded in this study showed no spontaneous activity. This compares with Woolley and Casseday’s (2004) figure of 90% showing no spontaneous activity. For those units that did show spontaneous activity, Woolley and Casseday’s values are also lower than those reported in the current study. Woolley and Casseday report that few units showed a spontaneous discharge rate under 7 spikes/s whereas the current study shows units firing up to 14.4 spikes/s. This discrepancy is not surprising considering that Woolley and Casseday used urethane anaesthesia, whereas a combination of ketamine/xylazine anaesthesia was used in the present study and is consistent with previous reports on the effect of anaesthesia on spontaneous activity in the inferior colliculus of the guinea pig (Astl et al., 1996).

MLd units in this study were found to have latency values, at 75 dB SPL, from 30 to 75 ms, with a mean of 46.94 ± 25.03 ms. This is higher than that found by Woolley and Casseday (2004) in
the zebra finch MLd. Coles and Aikin (1979) did not provide the entire range of latencies they observed in the chicken MLd, but reported a minimum latency of 4-5 ms, which is consistent with that reported by Woolley and Casseday. There could be several reasons for the difference between the latency values found in the present study and those of Woolley and Casseday (2004) and Coles and Aitkin (1979). First, the role of the anaesthetics used has to be considered, as units recorded under ketamine anaesthesia have proven to exhibit longer latencies (Astl, et al., 1996; Semple & Kitzes, 1985). Second, white noise was the stimulation used to determine latency in the present study, while other studies examined latencies to the presentation of the BF tone. Third, latencies are influenced by sound intensity. Given the variability exhibited by the units in this study in response to different stimuli, this parameter was excluded from the analysis.

In this study, very few units failed to respond to any tone and most units actually responded to several frequencies. At 75 dB SPL, the units were mainly tuned to the lower frequencies: 2 kHz being the most represented best frequency, followed by 1 and 3 kHz. Thus our data set is biased towards low frequency tuned units in MLd. Woolley and Casseday (2004) found many units with characteristic best frequencies of either 4 kHz, 5 kHz or 6 kHz, in addition to lower frequency tuned neurons. An examination of the recording sites displayed by Woolley and Casseday (figure 2, p.139), shows that units tuned to frequencies above 4 kHz are found most ventrally in the largest cross section of MLd, i.e., in the middle of the rostrocaudal extent of the nucleus. However, based on the analysis of electrode tracks and available lesions, the recording sites in the present study tend to occur more frequently in the caudal half of MLd, which could explain the limited number of units tuned to frequencies above 4 kHz. Despite this bias, the tonotopic organization of MLd was still visible, especially when examining the responses of units recorded during the same electrode track (not shown). Tonotopy is a well known characteristic of the auditory midbrain in birds (Calford et al., 1985; Coles & Aitkin, 1979; Scheich, et al., 1977; Takahashi & Konishi, 1988a; Woolley, S. M. N. & Casseday, 2004), mammals (for review, see
Ehret & Schreiner, 2005), reptiles (Manley, J. A., 1971), amphibians (for review, see Wilczynski & Endepols, 2006) and fish (Echteler, 1985).

Another characteristic is the presence of both V-shaped and more complex tuning curves in birds (Coles & Aitkin, 1979; Wagner et al., 2002; Woolley, S. M. N. & Casseday, 2004), mammals (for review, see Cant, 2005) and amphibians (for review, see Feng, et al., 1990). For those units where tuning curve bandwidths could be obtained (see Figure 4.5), bandwidths were narrow with values that did not exceed 2.84 kHz. These values are narrower than those observed by Woolley and Casseday (2004), even in the comparable frequency range. At 80dB SPL, cells they recorded had tuning curve bandwidths ranging from 0.5 to 7 kHz. Even if smaller, the values of the present study are still consistent with their finding that units tuned to the lower frequencies had narrower bandwidths. The narrower bandwidths obtained in this study compared to those reported by Woolley and Casseday may also be a result of the choice of anaesthetic. In the chinchilla, Kaltenbach (1987) in the dorsal cochlear nucleus and Nuding et al. (1999) in the inferior colliculus, showed that a greater number of sharply tuned units were recorded under ketamine anaesthesia than under urethane anaesthesia. Differences could also be accounted by the small difference between the intensity of the stimulus in this and Woolley and Casseday’s 2004 study. It must also be noted that the range of BF of the units used for the calculation of bandwidth in this study is smaller than that in the Woolley and Casseday’s and, as shown in Figure 4.2, it appears that units with the larger bandwidths also tend to have a higher BF. Inhibition by tonal stimulus presentation was not quantified in this study, but was never the less encountered. This represents an important observation, as it is thought that sideband inhibition, as well as combination-sensitive facilitation or inhibition, help shape response selectivity to complex sounds within the central auditory system (but see below).

Units responding to tones and white noise present four temporal response patterns described in previous MLd studies (Coles & Aitkin, 1979; Woolley, S. M. N. & Casseday, 2004). In the
present study the sustained pattern was predominant (60%), followed by the onset pattern (20%). This is in contrast to Woolley and Casseday (2004) who found the onset pattern to dominate their sample (49%). There is no indication from their work that a neuron’s BF is correlated to a specific response pattern. Therefore, it is unlikely that the discrepancies between the two studies are due to differences in the distribution of BFs of the populations of units. Coles and Aitkin (1979) also investigated patterns of responses to tones but they pooled together responses that presented a sustained firing whether or not they were just sustained or presented an onset component (primary-like or primary-like with notch). This represented 51% of the units they recorded from. They also pooled units that had a pure onset component or were primary-like (63%). All that can be compared with their data set is that they encountered mainly onset, sustained, primary-like and primary-like with notch response patterns, as seen in the present study. They also reported units that were inhibited by tonal presentation. Patterns observed in the auditory midbrain seem to be linked to both species and anaesthesia. For instance, in the IC of the chinchilla (Nuding, et al., 1999), bat (Pollak, G. K. et al., 1978; Xie et al., 2005) or cat (Rose, J. E. et al., 1963), onset pattern was reported as the most common, but none of these studies were performed under ketamine anaesthesia. However, in the IC of the cat anaesthetized with ketamine (Aitkin et al., 1994) the sustained pattern becomes more prominent in response to both tone and noise stimuli. Similarly, Syka (2000) reported in the guinea pig under ketamine anaesthesia that more sustained patterns were observed than onset. Therefore, the interpretation of pattern predominance in anaesthetized animals is likely to depend on the type of anaesthetic used in experiments. The temporal response pattern to tone and WN was identical in 90% of the units where this similarity could be tested. Woolley and Casseday (2004) reported a similarity in patterns in only 50% of their cases. However, since many units could not be categorized in the present study, especially based on their responses to WN, the significance of this difference is unclear.
Of all the units that were classified as auditory most (81%) were found to respond to stimuli representing all categories (tones, white noise and songs). Some units, however, were found to be more selective. Five percent of the auditory units responded only to songs and 14% responded to songs plus one of the simple stimuli (e.g., a tone or white noise).

An important implication of these findings is that the choice of search stimulus may have a significant effect on the population of units represented in any study, as depicted in Figure 4.2. Thus, the proportions of MLd neuronal population assigned to a category (responding to either all or only to some stimuli) must take into account the type of search stimulus used in that particular study, and differences in search stimuli may account for differences in the proportions of units showing one or another characteristic. A second implication of these results is that the differences in response patterns to different types of stimuli could not be predicted from the unit’s frequency tuning curves; that is, frequency tuning alone cannot explain the selective response to complex vocalizations. This mirrors results obtained in guinea fowl and fish. Scheich et al. (1977), in the guinea fowl, found neurons that responded to both pure tones and conspecific vocalizations (in their case, calls) in MLd. They also found neurons that were inhibited by pure tones but excited by the calls presented. Maruska and Tricas (2009), in the torus of the damselfish obtained neuronal responses to both simple stimuli and complex conspecific sounds. They showed that these neurons had lower mean thresholds to conspecific sound stimuli than to tones (with the exception of 100Hz). In the present experiment, responses of units to songs were varied, some units responding to all songs and all elements of songs while others selectively responded to subsets of songs or to particular elements within the songs. Though this study was not designed to identify the precise features that evoked the unit’s response, some degree of feature extraction was demonstrated in MLd. This also agrees with the results of Woolley et al. (2009). Using STRFs of midbrain neuron responses to conspecific songs, four functional groups were identified according to their spectrotemporal tuning properties and how each of these groups could potentially contribute to feature extraction from the conspecific signals. The
present study did not include STRFs analysis of the recorded units, making it impossible to assign our units confidently to any particular group. However, it is clear that there are neuronal subpopulations within MLd, each extracting different features of natural sounds that potentially contribute to the discrimination of conspecific vocalisations.

The heterogeneity of responses to conspecific stimuli in the auditory midbrain- and therefore the selectivity of neurons – and a key role of inhibition in shaping this selectivity, has been described in mammals (Klug et al., 2002; Portfors, 2004; Xie, et al., 2005; Yang et al., 1992) and amphibians (Hall, J. C., 1999). Klug et al. (2002) and Xie et al.(2005) showed that the heterogeneity of responses to calls in the IC of bats is reduced or abolished when substances such as strychnine or bicuculline are applied that remove the inhibition. Neurons that responded selectively to subsets of stimuli began to respond to all stimuli following drug administration.

Several underlying mechanisms shape the selective responses of neurons to conspecific signals. Sideband inhibition as well as combination sensitivity facilitation or inhibition have been described in the bat IC (for review, see Pollak, G. D., 2010; Portfors, 2004). Sideband inhibition classically represents the inhibitory bands that surround the excitation band(s) of a neuron. This phenomenon shapes the tuning curve of each individual neuron and prevents the prediction of a neuron’s behaviour to a complex stimulus based on the excitatory responses of that neuron to simple pure tone stimuli. When bicuculline is applied, the tuning curve of neurons showing side band inhibition broadens (Hall, J. C., 1999; Klug, et al., 2002; Xie, et al., 2005; Yang, et al., 1992) loosening its selectivity to complex vocalizations (Klug, et al., 2002; Xie, et al., 2005).

The mechanism of combination sensitivity works differently. Basically, some neurons are facilitated or inhibited by combination of tones. When IC neurons in the Moustached bat were tested in response to both pure tones and bat calls, their responses to calls could not be predicted according to their response to single pure tones but could be predicted from their combination-sensitive responses to pairs of tones (Portfors, 2004). Combination sensitivity has also been reported in the mouse IC (Portfors & Sinex, 2005) but only to tone bursts, so its implication for...
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The processing of complex sounds is unclear. Similarly, combination sensitivity might also be present in the bird’s MLd, but given the paucity of studies of nuclei below the level of the telencephalon in birds, its contribution to song processing remains to be clearly established. Margoliash (1983) in the starling and Margoliash and Fortune (1992) in the zebra finch described the properties of temporal combination-sensitive (TCS) and harmonic combination sensitive (HCS) neurons in response to BOS in HVC. They distinguished several classes of TCS neurons. Some were characterized as single-syllable-TCS and responded strongly to specific syllables presented either within the song or isolated from it. The activity of the unit B109s2n2 in Figure 4.13 that fired specifically to a few notes might be similar to the single-syllable TCS neurons of HVC. Other classes of TCS neurons such as syllable pair or multiple syllable and the specific responses of these neurons need to be investigated further. This could be done by presenting sequences of syllables so as to determine the exact order that triggers the response of a unit. Margoliash and Fortune (1992) also described HCS neurons in the zebra finch HVC, excited by complex harmonic FM properties, as well as some TCS neurons exhibiting HCS properties. As none of these properties have been investigated in MLd by presentation of isolated syllables or suites of syllables, it is hard to relate these findings from HVC to the present results from MLd, although it may be that, considering the specific responses some units exhibited to songs, such combination-sensitive units are present in MLd. Never the less, it appears that the responses of some units in this study are more tightly tuned to the amplitude modulations contained within the conspecific or heterospecific songs, while other units appear to be tuned to precise spectro-temporal features within notes or syllables. This is in agreement with the disparities existing between zebra and Bengalese finch songs. Both species cover an identical frequency range and both present harmonic stacks in their songs, but diverge in some other spectro-temporal features they contain. Zebra finch songs usually contain noisy and harmonic elements and Bengalese songs contain fast repeated harmonic syllables called trills (Woolley, S. M. N. & Casseday, 2004; Zann, 1996). Many units in this study showed responses that were
‘locked’ to these temporal features, although it cannot be ruled out that they may also be tuned to the amplitude modulation of the song. Woolley and Casseday (2005) showed that 79% of the units they recorded from in the male zebra finch responded to amplitude modulated tones. This also fits the observation of Woolley et al. (2009). Among the categories of MLd neurons they described, more than half were broadband neurons whose characteristic STRF made them either good at detecting onsets and suitable for rhythm encoding (BB-F neurons) or good at encoding amplitude envelope and suitable to participate in timbre encoding. Distinct populations dedicated to encoding either spectral features (probably via combination sensitivity to different frequencies) or temporal information have also been described in fish (for review, see Bass, et al., 2005), amphibians (for review, see Bass, et al., 2005; Feng, et al., 1990; Rose, G. J. & Gooler, 2006), and in mammals (for review, see Ehret & Schreiner, 2005; Rees & Langner, 2005).

While there is a consensus on population coding of conspecific signals in the auditory midbrain, responses to heterospecific sounds and, more interestingly, selectivity to conspecific over heterospecific vocalizations, have yet to be fully characterised. In the present study, two major findings stand out. First, at the population level in MLd, neurons not only responded to both categories of songs (conspecific and heterospecific) but also responded more strongly to forward conspecific songs than to forward heterospecific songs (see chapter V for full analysis and discussion). Second, units also preferred the songs when these were played backwards.

The preference observed toward the reverse direction over the forward direction of songs in the present study is a little more surprising (Figure 4.15). Indeed, one would expect that the forward versions of conspecific and heterospecific signals would be behaviourally more relevant to the birds and therefore preferred. The preference toward reverse signals is therefore puzzling but still could be a confirmation of the results reported by Woolley and Casseday (2005). They found that in MLd, 89% of the units they recorded from were responsive to FM sweeps but most of these cells were insensitive to the direction of the sweep. Of five cells that showed a preference,
four preferred the upward direction. The zebra finch song contains more downward FM than upward FM. Therefore, the reverse songs, that are mirror images of the forward songs, contain more upward FM sweeps than downward FM sweeps and accordingly would evoke a stronger response from the units sensitive to direction. Amin et al. (2010) demonstrated that in the thalamic nucleus Ov, the STRFs of some neurons resembled those seen in MLd while some others where more like those observed in field L. But more importantly, Ov showed a great proportion of neurons sensitive to FM sweeps, a characteristic that is not seen in MLd using STRFs, and that is seen in smaller proportion in field L. Therefore, selectivity to downward sweeps could be a function not encoded in MLd but rather at the next level, in the thalamus, leading to the selectivity to forward version of songs observed in field L of female zebra finches (Hauber, et al., 2007b). However, one might argue that not both directions of FM sweeps need to be encoded to extract information of the direction of an FM sweep. Outputs from neurons showing FM sensitivity with no directional sensitivity could combine with outputs of neurons with, say, sensitivity to upward sweeps to generate sensitivity to downward sweeps further along the pathway. Further, there is no reason to assume that neurons showing sensitivity to upwards FM sweeps are excitatory in nature. It must be noted, however, that it is not clear to what extent the FM direction of the stimulus influences the bird’s behaviour. Lauay et al. (2004), showed no significant difference in a choice task in the preference for forward song over reverse song in zebra finch females, whether they had been reared with both parents or isolated from male tutors. In contrast, the ICs of all bats are known to be dominated by direction-selective neurons that favour the downward direction (Andoni et al., 2007; Casseday et al., 1997; Fuzessery & Hall, 1996; Razak & Fuzessery, 2006; Suga, 1965; Voytenko & Galazyuk, 2007) and express a range of preferences for sweep velocities that correspond to the sweep velocities in the signals they emit (Andoni, et al., 2007). This correspondence shows that a substantial portion of the IC population is tuned to respond to the features in their conspecific vocalizations. In zebra finches,
given the behavioural studies described above, it is not clear what the biological significance of the units that are specifically tuned to one FM sweep direction.

It is even more striking that there are even fewer electrophysiological studies performed in Ov than in MLD. Experiments presenting simple and complex stimulations to Ov units could provide more information on how different filters interact along the ascending auditory pathway. Never the less, the preference toward the reverse version of song stimuli in the present experiment tends to confirm the idea of distinct neuronal populations in MLD that are sensitive not just to spectral components of the stimulus but to temporal parameters as well. It is clear, however, that while filtering properties are already present in MLD, much of this information probably needs to converge at higher levels for further filtering and discrimination.

At the population level, not all songs were preferred equally (see Figure 4.19) but no single song was consistently preferred. However, single-units are able to discriminate between the varied song types by extracting intrinsic features. The heterogeneity of MLD is what makes it very different from the song system, in which stereotypy to BOS is characteristic. In contrast, MLD appears to include a large population of neurons that represent a larger range of stimulus characteristics, with units showing highly reproducible responses that indicate a higher degree of selectivity to stimulus parameters.
Chapter V

Effects of experience on MLd single-unit characteristics and responses
1 - Introduction

The previous chapter characterized the response properties of MLd units in zebra finches that had been reared in an environment where they could only hear vocalizations from their conspecifics. In these birds, most of the neurons responded to both simple and complex stimuli and, as a population, showed stronger responses toward conspecific signals. The purpose of this chapter is to describe how the tuning properties of MLd units are affected when zebra finches are raised by Bengalese finches, a closely related estrilid finch.

The role of early auditory exposure (experience) on song structure and on song preference has been demonstrated behaviourally in the zebra finch. For instance, it has been shown that male zebra finches raised in isolation, where they are deprived of a song tutor, will develop abnormal songs (Price, 1979). The temporal pattern of these abnormal songs in terms of note sequence (introductory notes, song units and bouts) is fairly well preserved, but note structures show much more variability than normal songs, either in duration and/or frequency. The role of rearing environment is also manifest in the songs of zebra finches that have been cross-fostered (Clayton, N. S., 1989; Eales, 1987; Takahasi, et al., 2006) or are hybrids between zebra and Bengalese finches (Takahasi, et al., 2006). In these birds the resulting song structure usually reflects the difference between the zebra finch and the Bengalese finch songs. The Bengalese song is longer than the zebra finch song and contains more elements and more repeated elements (Clayton, N. S., 1989) while the zebra finch song possesses more harmonics and contains “noisy” notes and call notes (Clayton, N. S., 1989; Zann, 1996). Cross-fostered zebra finches develop a song that (1) incorporates elements of the foster parents (2) contains more elements than the normal zebra finch song but fewer than the Bengalese finch song and (3) is longer than the normal zebra finch song but shorter than that of the foster male (Clayton, N. S., 1989; Eales, 1987; Takahasi, et al., 2006). Takahasi et al. (2006) also reported that, even though the cross-
fostered zebra finches learn Bengalese finch specific elements well, the element structure is actually incomplete.

The environment not only shapes song structure but also impacts on song perception and preference (Braaten & Reynolds, 1999; Clayton, N. S., 1988; Eales, 1987; Lauay, et al., 2004). The song of zebra finch males will resemble that of their tutors, be these conspecifics or heterospecifics (Eales, 1987) but, given the choice, a young cross-fostered zebra finch male will choose to learn from a conspecific tutor (Clayton, N. S., 1988; Eales, 1987). In adulthood, male and female zebra finches will prefer conspecific over heterospecific songs in a choice task whether they have been raised isolated from a male tutor or with both conspecific parents (Braaten & Reynolds, 1999; Lauay, et al., 2004). Braaten (1999) showed that females raised by both parents will prefer songs of tutored males over untutored ones, while females that were reared in an aviary with their mother and siblings (and no father) prior to the sensitive period (see chapter I, section 2.1.1) respond equally to both types of song. Females reared with both their parents show a strong preference for songs that resemble those of their father (Clayton, N. S., 1988; Miller, 1979b) and also learn to recognize the songs of their mates (Miller, 1979a).

While many behavioural studies are available which point toward a predominant role of early auditory experience and a subsequent tuning of the auditory system, very few studies have investigated the underlying mechanisms behind these observations with electrophysiological recordings of auditory tuning and responses to song. Developmental exposure to simple, synthetic sounds such as clicks and pure tones alters frequency tuning in midbrain neurons (Poon & Chen, 1992; Sanes & Constantine-Paton, 1985; Yu et al., 2007). Woolley et al. (2010) showed that early auditory experience alters the mean firing rates of neuronal responses to conspecific and heterospecific songs in cross-fostered zebra finches and that cross fostering affects the firing rate evoked by these vocalisations at a similar level. Indeed, in their study, neither the neuronal population recorded in the cross-fostered birds nor that recorded in the normally reared birds showed a bias toward the encoding of one of the song categories: conspecific and heterospecific.
songs appear to evoke similar firing rates in MLd neurons. However, in the previous chapter of the present thesis, MLd units of normally-reared zebra finches were shown to have a bias toward conspecific vocalizations (see section 3.4 in this chapter for full statistics). This chapter will provide evidence that, in zebra finches that were cross-tutored by Bengalese finches, MLd units appear to present similar characteristics to those observed in normally reared birds, but the bias toward zebra Finch vocalizations is not as pronounced.

2 – Material and Methods

The experimental procedures were carried out according to the guidelines of the Animal Ethics Committee of the University of Auckland. Six male zebra finches (>100 days old) that were raised by Bengalese finches were used in this experiment. Birds were bred in an aviary at the University of Auckland where they lived under a constant light/dark cycle of 14h/10h in the aviary were provided with food and water ad lib.

2.1 - Experimental protocol

The experimental protocol for surgery, electrophysiological recordings and analysis is the same as that of chapter IV, section 2.

2.2 - Cross-fostering

Zebra finches were cross-fostered to Bengalese finches (ZF-CF). These birds had previously been used in behavioural experiments and a full description of the rearing conditions is available in Campbell and Hauber (2009). Briefly, zebra Finch chicks (3-5 days old) were transferred into the nest of a Bengalese Finch pair in one of two outdoor aviaries in Auckland, exposed to natural photoperiod and weather conditions. The chicks were therefore reared hearing male and female Bengalese finches and tutored by a Bengalese Finch male, but were also exposed to wild birds species found commonly in suburban Auckland (Blackbird Turdus merula; House Sparrow Passer domesticus; Song Thrush Turdus philomelos; Starling Sturnus vulgaris; and Tui...
Prosthermadera novaeseelandiae). After reaching adulthood (> 100 days), the cross-fostered birds were transferred into single-sex aviaries where they were housed with fostered conspecifics. After the behavioural experiments were completed, the birds were finally transferred in an indoor aviary at the Faculty of Medical and Health Sciences. At all times, birds were held at a constant light/dark cycle of 14h/10h in the aviary and birds were provided with food and water ad lib. These birds did not hear normal conspecific vocalisations before behavioural testing (a time at which their songs were crystallised).

2.3 - General Linear Mixed Model

Each isolated single unit in this experiment, as well as those in the experiment performed in normally-reared zebra finches, was presented with 19 different stimulations each repeated 15 times, i.e. the recording session presented 285 stimuli. Also, since several units were recorded from a single animal, the data set cannot be considered to consist of independent data and is best considered in the context of pseudo-replication (for review on these issues, see Lazic, 2010; Nakagawa & Hauber, 2011). One statistical method to address these considerations is to use a Generalized Linear Mixed Model (GLMM). In order to investigate factors influencing the response of units within birds, a general linear mixed model was fitted with bird and unit within bird as random effects to allow for the clustering of units within birds, and song and direction (i.e. song played either forwards or backwards) as repeated measures with unstructured covariance. Category (reared with conspecifics or cross-tutored), song type (conspecific or heterospecific), song within type (conspecific song #1, 2 or 3; heterospecific song #1, 2 or 3), direction of song (forward or reverse) as well as the interactions of category, song type and song direction and the interaction of song with direction were included as explanatory variables. Interactions that were not significant were removed from the analysis.
3 - Results

The responses of 20 units to all stimuli were recorded in the cross-fostered zebra finch (ZF-CF) MLd (six birds, 1-6 units/birds). As seen in zebra finches raised by conspecifics (ZF-ZF), most of the units (15 out of 20) had no spontaneous activity (< 1 spikes/s). The remaining units showed low activity, < 5 spikes/s, as shown in Figure 5.1.

Latency to stimulus onset was calculated for units showing a sustained response to the WN stimulus, presented at 75 dB and values ranged from 5 to 100ms, with a mean of 55.83 ± 10.87 ms.

3.1 - Characterization of responses to all stimuli

In the previous chapter, units were classified into 4 main categories according to their evoked response to different types of stimuli. Units found in the MLd of cross-fostered birds could only be assigned to two of those categories: units not responding to WN but responding to tones and songs stimuli, and units whose responses were evoked by all stimulus types (Table 5.1). It seems probable that this limited categorization is related to the smaller number of units that were recorded from in this experiment.

**Table 5.1:** Different patterns of units when considering all 3 types of stimulation: Tones, WN and songs

<table>
<thead>
<tr>
<th></th>
<th>% units ZF-CF</th>
<th>% units ZF-ZF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Responsive only to songs</td>
<td>0</td>
<td>1.72</td>
</tr>
<tr>
<td>Responsive to tones and songs</td>
<td>25</td>
<td>12.07</td>
</tr>
<tr>
<td>Responsive to WN and songs</td>
<td>0</td>
<td>6.9</td>
</tr>
<tr>
<td>Responsive to all 3 stimuli</td>
<td>75</td>
<td>79.31</td>
</tr>
<tr>
<td>Tones &gt; WN</td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td>WN &gt; Tones</td>
<td>80</td>
<td>48</td>
</tr>
</tbody>
</table>
Figure 5.1

Distribution of spontaneous rate of units recorded in the MLd of cross-fostered birds
Five units (25%) did not respond to the white noise stimulus but did respond to both tone and song stimuli (Figure 5.2). One of these units was actually inhibited by the WN stimulation as it was by pure tones of 1, 2 and 3 kHz, but excited by a pure tone of 5 kHz and a few songs. The remaining 15 units (75%) responded to the three types of stimulations, a proportion similar to that seen in the ZF-ZF MLd. Most of these units (80%) responded more strongly to the white noise stimulation than to tones. This differs from that observed in the normal MLd, where the proportions of units responding more strongly to tones over WN and vice-versa, were equivalent.

### 3.2 - Characterisation of responses to tones and WN

Fifteen of the 20 units (75%) that were recorded from responded to the WN stimulus (Table 5.2), all (100%) responded to at least one tone and 15 (75%) responded both to the WN and at least one pure tone stimulus. Of the units that responded to at least one of the pure tone stimuli, three responded to one tone only (15%, Figure 5.3), eight responded to two tones (40%), five showed responses to three tones (25%) and four responded to four tones (20%). No unit showed evoked responses to five or six tones. Table 5.2 shows the percentages of units that responded to each tone stimulus tested: 60% responded to 1 kHz, 75% responded to 2 kHz, 65% to 3 kHz, 15% responded to 4 kHz, 30% responded to 5 kHz and 5% responded to 6 kHz.

**Table 5.2:** Percentages of units responding to each pure tone and WN stimulus

<table>
<thead>
<tr>
<th></th>
<th>WN</th>
<th>1 kHz</th>
<th>2 kHz</th>
<th>3 kHz</th>
<th>4 kHz</th>
<th>5 kHz</th>
<th>6 kHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of units ZF-CF</td>
<td>75.00</td>
<td>60.00</td>
<td>75.00</td>
<td>65.00</td>
<td>15.00</td>
<td>25.00</td>
<td>5.00</td>
</tr>
<tr>
<td>% of units ZF-ZF</td>
<td>84.48</td>
<td>67.24</td>
<td>67.24</td>
<td>48.28</td>
<td>22.41</td>
<td>8.62</td>
<td>3.45</td>
</tr>
</tbody>
</table>

These proportions are roughly the same in the MLd of both ZF-CF and ZF-ZF birds and, in both bird populations, recorded units are biased towards lower frequency tuning. In both normal reared and cross fostered birds, tones could evoke both excitation and inhibition. White noise
**Figure 5.2:**

Summary of responses of each of the 20 individual ZF-CF units to each stimulation.

The left column of the table indicates the individual units. Each row then represents the response of each unit to the 19 stimuli used in the experiment: WN, 6 pure tone stimuli, 6 conspecific songs (forward and reverse versions) and 6 heterospecific songs (forward and reverse versions).

The response of a unit to each stimulus is represented using a colour code: if the unit was inhibited by the stimulus, its response is indicated in blue; an excitatory response to a stimulus is indicated in green. Light green represents a light to moderate response; dark green represents a strong response from the unit elicited by the stimulation. No colour indicates that the unit failed to respond to the stimulation.

The preferred frequency of a unit is indicated by a crossed box.
Percentages of units responding to either 0, 1, 2, 3, 4, 5, or 6 tones
however, failed to evoke inhibition in normal reared birds, but this response was seen in one unit in the cross fostered birds.

3.2.1 - Preferred Frequency and Tuning Curve Bandwidth

The best frequency (BF) of each single-unit was derived from its response tuning curve and is characterized as the frequency that elicited the maximal response of the unit at 75dB. Of the 20 units that responded to at least one tone, seven (35%) had a BF of 1 kHz, seven (35%) a BF of 2 kHz, four (20%) a BF of 3 kHz, one (5%) responded best to 4 kHz and one (5%) had a BF of 5 kHz (Figure 5.4). As seen in the normal MLd, 1, 2 and to a lesser extent 3 kHz are the most represented units in the ZF-CF midbrain population used in this study. The tuning curve bandwidths of nine units were calculated (see Figure 4.5 in chapter IV for issues) and ranged from 1.03 kHz to 1.88 kHz. They showed a narrower range than that of the ZF-ZF units (1 to 2.84 kHz).

3.2.2 - Temporal response patterns

The four categories of temporal response patterns described in the previous chapter (onset, sustained, primary-like, primary-like with notch) were also observed in the cross-fostered bird auditory midbrain. Thirteen out of 20 units could be assigned to each of these response patterns on the basis of their PSTH at their BF (Table 5.3).

Table 5.3: Percentages of units per temporal response patterns to their preferred frequency and to WN stimulation in ZF-CF and ZF-ZF midbrains

<table>
<thead>
<tr>
<th>Preferred Frequency</th>
<th>Onset</th>
<th>Sustained</th>
<th>Primary-like</th>
<th>Primary-like with notch</th>
</tr>
</thead>
<tbody>
<tr>
<td>% units ZF-CF</td>
<td>23</td>
<td>46</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>% units ZF-ZF</td>
<td>20</td>
<td>60</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>WN</td>
<td>12.5</td>
<td>75</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>% units ZF-CF</td>
<td>18</td>
<td>59</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>% units ZF-ZF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 5.4**

**Distributions of ZFZF and ZFCF units per preferred frequency**

Black bars represent the percentages of units per preferred frequency in ZF-ZF birds. Gray bars represent the percentages of units per preferred frequency in ZF-CF birds.
Three units (23%) were classified as onset, 6 (46%) as sustained, 1 (8%) as primary and 3 (23%) as primary-like with notch. Based on their responses to WN, the temporal response pattern of eight units could be identified: one (12.5%) had an onset pattern, six (75%) had a sustained pattern and one (12.5%) had a primary-like with notch pattern. No primary-like pattern was observed in response to WN but this is probably due to the low number of units sampled. Still, it remains clear that the ZF-CF and ZF-ZF birds have similar proportions of units corresponding to each response pattern, with the sustained pattern being the most common (almost 2/3 of the observed patterns). Of the eight units for which a response pattern could be obtained from both WN and its BF, six (75%) units had an identical pattern to both white noise and BF.

3.3 - Characterization of responses to songs

3.3.1 - Characterization of pattern of responses to CON and HET songs

Cross-fostered birds were presented with the same set of conspecific and heterospecific songs, in both the forward and backward directions, as were the ZF-ZF birds. The units that were recorded from responded to both categories of songs: CON and HET. Most of the categories were determined according to the ZF-ZF unit responses to song stimulations were also observed in the ZF-CF MLd. First, units that showed strong responses to most of the components of songs were observed, whether they were CON or HET. Responses from these units were locked to the elements of the song (Figure 5.5A) and followed the temporal amplitude modulation pattern of song stimuli. However, amongst these units some responded strongly to all songs, while some showed variation in the strength of their responses, depending on the song (Figure 5.5B).

The second category of units that was formerly described contained units that responded to many, but not all, features within a song and therefore showed some degree of selectivity. Figure 5.6 shows an example of such a unit that has a very strong response toward most elements of
Figure 5.5

Examples of units responding to most elements of the song stimuli

A: Example of a unit that responses to songs were locked to the temporal amplitude modulation pattern of the stimuli, here conspecific song #1. Note how the unit responds to most if not all components within the song visible on the underlying spectrogram.

B: Example of a unit that responses were locked to the temporal amplitude modulations of the stimuli, here conspecific song #2 and heterospecific song #32. Note how the unit appeared to respond much stronger to conspecific song #2 than to heterospecific song #2.

The spectrogram under each PSTH has been shifted so as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
Figure 5.6

Example of degrees of selectivity of responses to song stimuli

This figure shows the variability in responses of a unit to two conspecific song stimuli.

Note how the unit responded to most elements of conspecific song #2 reverse (top panel) but responded much more selectively to a few elements of conspecific song #1 as indicated by the green arrows (bottom panel).

The spectrogram under each PSTH has been shifted so as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
This figure shows the variability in responses of a unit to two different song stimuli presented in the forward direction.

Note how the unit responded quite strongly to almost all features of conspecific song #2 but responded quite poorly to heterospecific song #2.

The spectrogram under each PSTH has been shifted so as to account for latency. The red arrow below each PSTH represents the beginning of sound stimulation proper (red arrow under spectrogram).
conspecific song #2 reverse (Figure 5.6A), but shows a greater selectivity when presented with conspecific song #1 (Figure 5.6B).

Units that responded preferentially to some songs or to specific features of the song were also observed. Figure 5.7 shows an example of a unit that responded strongly to conspecific song #2 but very poorly to heterospecific song #2.

A unit is shown in Figure 5.8A that shows inhibition in response to tones and white noise. The same unit’s response to song is quite different. Panel B (top) shows an increase in spontaneous activity during ‘silence periods’, while Panel B (bottom) shows a response that appears to be locked to the rhythm of the song. This underscores the complexity of the neuronal responses encountered in this study, thereby providing a challenge to standard statistical analyses.

### 3.3.2 - Response strength to songs

The amount of firing activity in response to song stimulations was calculated using the RS. The distribution of RS values (Figure 5.9) differs from that seen with ZF-ZF units. First, the values of RS fell within a narrower range (-0.28 to 17.03 spikes/s) and there was no tail towards high values of RS. Also, in the ZF-ZF auditory midbrain, other than the peak at 0, the majority of instances were confined between 0 and 5 spikes/s. The RS values of ZF-CF units also showed a peak at 0 but the shapes of the distributions were different. The frequency of values between 0 and 15 spikes/sec appears to fall less sharply in cross fostered units than in units in ZF-ZF midbrain (compare with Figure 4.14 in chapter IV). As for normal reared zebra finches, most values of RS were centred near zero, but for normal reared zebra finches the median was 2.4 while for cross fostered zebra finches it was 4.4.

RS values obtained in response to each song were quite homogenous and no category of songs - CON or HET - seemed to consistently elicit stronger responses (Figure 5.10A). The average and SEM values of RS for the different song stimuli are summarized in Table 5.4.
This figure shows a unit that was either inhibited or excited by stimulus presentation. 

Panel A: Example of inhibited responses of the unit when presented with either the WN stimulus (top panel) or a pure tone (bottom panel), here the 1 kHz frequency. The inhibition resulting from WN presentation was not observed in ZF-ZF birds.

Panel B: Example of responses of the same unit elicited by song stimuli. Note how the unit's response to conspecific song #2 (top panel) actually corresponded to “silence period” while the unit responded to most elements in heterospecific song #2 (bottom panel).

In both panels, the spectrogram of each stimulus is visible underneath the PSTH and has been shifted so as to account for latency. Red arrows mark the beginning of the sound presentation proper.
Figure 5.9

Distribution of all RS values from all 20 units to all songs

Note that most of instances are concentrated around the lowest values and that, contrary to what was seen in the “normal” MLd, no values above 17 spikes/s are observed.
**Figure 5.10**

RS values in the cross-fostered zebra finch midbrain and comparison with the “normal” zebra finch midbrain

Panel A: Mean ± SEM values of RS to each song stimulus in forward (black bars) and reverse (gray bars) directions.

Panel B: Plot of mean RS value of each individual unit for a song versus its reverse counterpart. The diagonal line represents the equality line where the response of a unit to both songs is identical.

Panel C: Mean ± SEM values of RS to forward conspecific songs and forward heterospecific songs in both the ZF-CF birds (black bars) and the ZF-ZF birds (gray bars).

Panel D: Mean ± SEM values of RS to forward songs and reverse songs in both the ZF-CF birds (black bars) and the ZF-ZF birds (gray bars).

The asterisk indicates statistical difference (p < 0.05)
Table 5.4: Mean ± SEM values of RS for each forward and reverse song stimulus

<table>
<thead>
<tr>
<th></th>
<th>CON 1</th>
<th>CON 2</th>
<th>CON 3</th>
<th>HET 1</th>
<th>HET 2</th>
<th>HET 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOR</td>
<td>4.38±1.10</td>
<td>5.53±0.93</td>
<td>5.70±1.11</td>
<td>5.63±0.93</td>
<td>5.17±1.12</td>
<td>4.56±1.01</td>
</tr>
<tr>
<td>REV</td>
<td>5.01±1.21</td>
<td>6.28±1.05</td>
<td>5.82±0.97</td>
<td>6.09±0.85</td>
<td>5.36±1.15</td>
<td>4.67±1.08</td>
</tr>
</tbody>
</table>

There were no large differences between the responses to forward CON and HET songs (5.20 ± 0.6 and 5.12 ± 0.58 respectively, Figure 5.10C). This differs from the behaviour of ZF-ZF units and suggests that early auditory experience shapes responses in the auditory midbrain in the zebra finch (Table 5.5, see below for full statistical analysis).

Table 5.5: Mean ± SEM of RS values in both the ZF-ZF and ZF-CF midbrains

<table>
<thead>
<tr>
<th></th>
<th>Forward CON</th>
<th>Forward HET</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZF-ZF all</td>
<td>5.76 ± 0.6</td>
<td>4.49 ± 0.48</td>
</tr>
<tr>
<td>ZF-ZF males only</td>
<td>5.34 ± 0.76</td>
<td>3.61 ± 0.50</td>
</tr>
<tr>
<td>ZF-CF</td>
<td>5.20 ± 0.6</td>
<td>5.12 ± 0.58</td>
</tr>
</tbody>
</table>

The response to forward and reverse song stimuli was significantly different, as shown on Figure 5.10D (Wilcoxon Signed Rank Test, p < 0.05) even though no obvious differences were seen when comparing RS values obtained from forward versus reverse presentation of a song stimulus (Figure 5.10A and Figure 5.10B). When the RS values obtained for the forward and reverse presentation of a given song stimulus in a single unit are plotted against each other, most data points fall on or near the equality line (Figure 5.10B).
3.3.3 - Temporal Sparseness Index

The Temporal Sparseness Index (TSI) was used to characterize the spike train of the evoked response to a stimulus. At the level of the neuronal population, no clear difference was visible in the response to each song stimulus (Figure 5.11A). These average and SEM values of TSI for the different song stimuli are summarized in Table 5.6.

**Table 5.6:** Mean ± SEM values of TSI for each forward and backward song stimulus

<table>
<thead>
<tr>
<th></th>
<th>CON 1</th>
<th>CON 2</th>
<th>CON 3</th>
<th>HET 1</th>
<th>HET 2</th>
<th>HET 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOR</td>
<td>0.78±0.05</td>
<td>0.74±0.05</td>
<td>0.71±0.05</td>
<td>0.71±0.05</td>
<td>0.74±0.05</td>
<td>0.77±0.04</td>
</tr>
<tr>
<td>REV</td>
<td>0.76±0.04</td>
<td>0.70±0.05</td>
<td>0.71±0.05</td>
<td>0.71±0.05</td>
<td>0.74±0.05</td>
<td>0.78±0.05</td>
</tr>
</tbody>
</table>

A Wilcoxon Signed Rank Test showed no significant difference between the forward versus reverse versions of songs stimuli (p = 34, mean ± SEM: 0.74 ± 0.02 and 0.73 ± 0.02 respectively), which differs from the findings in the ZF-ZF auditory midbrain (Figure 5.11C and D). The variation in the responses (TSI) of individual units to forward and reverse versions of a song stimulus is shown in Figure 5.11B. Table 5.7 shows the percentage of responses for each stimulus type that showed TSIs over 0.75 (i.e. units considered to be highly selective) or below 0.25 (i.e., units considered to show low selectivity). Values of exactly 1 were removed from the calculations for each stimulus, because they were more likely to represent units failing to respond to a stimulus more than an inhibitory response. For most stimulus types, at least more than half the presentations of the stimulus resulted in responses with values of TSI over 0.75, indicating a bias against sustained responses to these stimuli. However, a smaller percentage of responses had TSI’s over 0.75 in cross-fostered birds than in normal reared birds. The same was true for values of TSI below 0.25. Thus, cross fostering resulted in an increase of instances in which 0.25 < TSI < 0.75. In fact, with the exception of two song stimuli – conspecific song #2 and heterospecific song #3 – values differed by at least 10%, even reaching a decrease of 28.41
**Figure 5.11**

**TSI values in the cross-fostered zebra finch midbrain and comparison with the “normal” zebra finch midbrain**

Panel A: Mean ± SEM values of TSI to each song stimulus in forward (black bars) and reverse (gray bars) directions.

Panel B: Plot of mean TSI value of each individual unit for a song versus its reverse counterpart. The diagonal line represents the equality line where the response of a unit to both songs is identical.

Panel C: Mean ± SEM values of TSI to forward conspecific songs and forward heterospecific songs in both ZF-CF (black bars) and ZF-ZF (gray bars) birds.

Panel D: Mean ± SEM values of TSI to forward and reverse songs in both ZF-CF (black bars) and ZF-ZF (gray bars) birds.
and 35.63% in the case of heterospecific song #2 and its reverse, respectively. The observed bias against sustained responses in the ZF-CF auditory midbrain seems to be more pronounced than that seen in the normal MLd. The comparison between TSI in normal reared and cross- fostered zebra finches suggests a lower level of feature extraction in the MLd of cross fostered birds, potentially leading to lesser discrimination. These observations are in agreement with the reduced feature selectivity found with RS values (see below for full statistical analysis).

**Table 5.7:** Proportion of TSI values > 0.75 and < 0.25 for each song stimulation. Values of 1, indicating 0 spikes above threshold, were removed from the analysis. The number of units considered per stimulation was: CON1: 19; CON1r: 18; CON2: 17; CON2r: 18; CON3: 17; CON3r: 17; HET1: 17; HET1r: 17; HET2: 18; HET2r: 18; HET3: 17; HET3r: 17

<table>
<thead>
<tr>
<th></th>
<th>CON1</th>
<th>CON1r</th>
<th>CON2</th>
<th>CON2r</th>
<th>CON3</th>
<th>CON3r</th>
<th>HET1</th>
<th>HET1r</th>
<th>HET2</th>
<th>HET2r</th>
<th>HET3</th>
<th>HET3r</th>
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<tbody>
<tr>
<td>&gt; 0.75 ZFCF</td>
<td>63.16</td>
<td>55.56</td>
<td>58.82</td>
<td>44.44</td>
<td>52.94</td>
<td>52.94</td>
<td>47.06</td>
<td>47.06</td>
<td>61.11</td>
<td>72.22</td>
<td>70.59</td>
<td>70.59</td>
</tr>
<tr>
<td>&gt; 0.75 ZFZF</td>
<td>75.00</td>
<td>71.15</td>
<td>67.92</td>
<td>62.50</td>
<td>71.43</td>
<td>66.67</td>
<td>75.47</td>
<td>82.69</td>
<td>75.93</td>
<td>83.67</td>
<td>80.39</td>
<td>82.69</td>
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<tr>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>&lt; 0.25 ZFZF</td>
<td>4.17</td>
<td>3.85</td>
<td>3.77</td>
<td>1.79</td>
<td>2.04</td>
<td>5.88</td>
<td>1.89</td>
<td>1.92</td>
<td>1.85</td>
<td>4.08</td>
<td>1.96</td>
<td>1.92</td>
</tr>
</tbody>
</table>

**3.3.4 - d’ and Selectivity Index**

d’ values and the selectivity index (SI) were used to quantify the selectivity of a neuron for one stimulus versus another. As in the ZF-ZF auditory midbrain, the threshold of selectivity in the ZF-CF MLd was set at $d' > |2|$. A $d'_{A \rightarrow B} > 2$ indicated a neuron selective for stimulus A over stimulus B, while a $d'_{A \rightarrow B} < -2$ indicated a neuron selective for stimulus B over A (see Material and Methods, chapter IV). The cumulative distributions of d’ values of each song against all others for all instances (example in Figure 5.12A) suggest that, at the population level, there is
**Figure 5.12**

Selectivity of units as measured by $d'$ and Selectivity Index for pairs of song stimuli

A: cumulative distributions of $d'$ values to conspecific song #1 versus its reverse version and the other forward stimulations for each of the 20 individual ZFCF units.

Black-filled circles represents non-selective instances as determined by SI value (see Material and Methods in Chapter 4)
Unfilled circles represent selective instances as determined by SI value.
The vertical black lines delimit the non selectivity zone where $d' < |2|

B:Proportion of instances where selectivity was observed for each song stimulus.

Blue bars represent the proportion of instances where the stimuli against which the song in abscissa was plotted was the preferred stimulation (Negative preference) in both the ZF-ZF midbrain (dark blue) and ZF-CFmidbrain (light blue).
Red bars represent the proportion of instances where the song in abscissa was preferred over the other stimuli it was plotted against (Positive preference) in both the ZF-ZF midbrain (dark red) and ZF-CFmidbrain (light red).
Instances that were deemed non-selective are not represented here.
A

Cumulative percentages

Con1 vs Con1rev
Con1 vs Con2
Con1 vs Con3
Con1 vs Het1
Con1 vs Het2
Con1 vs Het3

B

% of d' values > |2|

Negative preference ZFZF
Negative preference ZFCF
Positive preference ZFZF
Positive preference ZFCF

Con1 Con2 Con3 Het1 Het2 Het3
no preference for an individual song stimulus, as most responses are found within the non-selectivity zone. Some MLd units in the ZF-CF MLd are able to discriminate between the various song stimuli, since some data points fall outside the non-selectivity interval (Figure 5.12A). Figure 5.12B shows the proportions of instances where a stimulus elicited positive or negative preference with respect to all instances (i.e. the proportion of data points that fell outside the non-selective zone depicted in the example in Figure 5.12A). Interestingly, apart for conspecific song #1, the trends of positive and negative preferences are similar between the normal and cross-fostered auditory midbrain.

At the single-unit level, only one of the 20 units recorded from had all its data points within the non-selective zone for each song when considering all possible stimulus comparisons. Consistent with the pattern observed in the “normal” MLd, units in cross-fostered birds are in their great majority able to discriminate at least one feature within the stimulus landscape.

### 3.4 - Effect of rearing environment

To fully analyse the effect of rearing environment on the response properties of MLd units, a GLMM analysis was performed. The response parameter of choice was RS because it better reflects the responses of the units to different song stimuli. While the TSI is good at detecting selectivity to certain features, its shortcoming lies in its failure to detect the strength of that response. The RS, although it provides little information regarding the temporal pattern of the evoked response, is a more direct measure of the ‘total’ response of the cell.

The GLMM analysis found that there was no indication of any difference in the effect of direction in CON or HET song differing in ZF-ZF or ZF-CF birds (3 way interaction $p = 0.35$). The 3-way interaction was therefore removed. There was neither an effect of song type (CON or HET) when the song was played forward or backward ($p = 0.97$), nor an effect of rearing (ZF-ZF or ZF-CF) when the song was played forward or backwards ($p = 0.81$). No effect of direction
(forward or backward) for the different songs ($p = 0.81$) was found. These 2 way interactions were therefore removed from the analysis.

However, there was evidence of the effect of CON or HET in ZF-ZF or ZF-CF birds ($p = 0.04$, see Table 5.8) with ZF-ZF birds having larger reductions in response to HET than to CON compared to ZF-CF birds. There was also strong evidence of an effect of the direction of the song ($p < 0.0001$), with a greater response to a song played backward than forward, and an effect of individual song within song category ($p = 0.0007$), which acknowledges the fact that all songs from one type, either CON or HET, did not seem to be treated the same way by neurons.

**Table 5.8:** Significant interactions as determined by the GLMM analysis

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual Song</td>
<td>4</td>
<td>384</td>
<td>4.95</td>
<td>0.0007</td>
</tr>
<tr>
<td>Song Direction</td>
<td>1</td>
<td>467</td>
<td>16.87</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Rearing*CON/HET</td>
<td>1</td>
<td>384</td>
<td>4.12</td>
<td>0.0429</td>
</tr>
</tbody>
</table>

4 - Discussion

The goal of this set of experiments was to assess the effect of rearing environment on the tuning properties and the evoked response of MLd units by examining these properties in cross-fostered zebra finches.

Units recorded in the MLd of cross-fostered birds showed little to no spontaneous activity and none showed a spontaneous firing rate above 5 spikes/s. The absence of units with strong discharge rates, as those seen in the normal MLd, is probably related to the smaller number of units recorded. Indeed, out of the 58 units recorded in the normally reared zebra finches, only 4 showed a spontaneous firing rate greater than 7 spikes/s.

Most of the units responded to the white noise stimulation (75%). Although this proportion of cells is slightly smaller than in the auditory midbrain of normally reared birds (84.48%), it never
the less shows that the majority of the units respond to this type of stimulation. Interestingly, in the cross-fostered birds one unit was also found that was inhibited by white noise stimulation, something that had not been observed in the normally-reared birds. Whether this is a rare occurrence that was encountered by chance or whether the occurrence of inhibition by white noise is a more common phenomenon in cross fostered birds than in normal reared birds will require more extensive studies.

In the MLd of cross-fostered birds, as in the MLd of normally reared birds, the population of neurons recorded was biased towards lower frequencies, with a large percentage of units responding to lower frequencies, especially 1 and 2 kHz. At the level of the population, 2 kHz remains the frequency that evoked responses from most cells (70%). At the level of individual units about 90% of the units had a BF of or below 3 kHz. Compared to the best frequency proportions observed in normally-reared birds, these values are roughly equivalent.

Other electrophysiological data on neuronal responses to tone stimuli in the MLd of cross-fostered birds have not been reported. This study cannot address whether the mapping of BF in MLd is equivalent in normally reared and cross fostered zebra finches since a systematic mapping of tuning properties was not performed. However, the similarities in the responses encountered in this study in the populations of MLd units from normal and cross-fostered zebra finches suggest that differences in the processing of songs observed between units of normally or cross-fostered reared zebra finches are probably not correlated with a difference in frequency tuning of MLd units. This may not be an unexpected finding, since zebra finch and Bengalese finch songs have similar power spectra, and thus frequency alone would not be expected to be a reliable feature to distinguish between these two songs. Since low-frequency feedback appears to be the most important for song maintenance in Bengalese finches (For review, see Woolley, S. M. N., 2004) and because the current study recorded primarily from lower frequency units (albeit in zebra finches), it will still have to be established whether neurons with higher BFs show similar discriminatory properties. This may be a source of discrepancy between the results.
obtained in this and (Woolley, S. M. N., et al., 2010) study, in which discrimination between CON/HET was not found, but the tuning properties of the neurons were not reported. The values of the tuning curves bandwidths ranged from 1.03 to 1.88 kHz and were thus narrower than those observed in the MLd of zebra finches reared by their conspecifics. However, many units responded to 1 kHz, and tuning bandwidth values for these units could not be calculated, potentially leading to an underestimation of the bandwidth range.

The units recorded in ZF-CF birds showed the same pattern of responses to both pure tone and white noise stimuli as those observed in birds raised by conspecifics. The sustained response pattern to stimulation was the most common amongst evoked responses, representing almost half of the observed patterns in response to pure tones and 75% of those observed in response to white noise. Onset responses and primary-like with notch responses were then seen with similar percentages. Twenty-five percent of the units in the present study did not respond to white noise, but responded to tones and songs and 75% of the units responded to all types of stimuli. Among these, 80% percent had an evoked response that was greater to white noise than to pure tones, while in the normal ZF MLd, only half of the units responding to all types of stimuli showed increased activity to white noise.

Tuning properties and response patterns to tones and WN of MLd units in the ZF-CF midbrain appeared quite similar to those in the ZF-ZF midbrain. Units in the ZF-CF were also seen responding to the various song stimuli, CON or HET, FOR or REV. In order to analyse whether there was an influence of rearing experience on song discrimination, a GLMM analysis was performed so as to control for pseudoreplication. Three main findings resulted from this analysis: (1) ZF-ZF MLd units responded more strongly to CON than to HET songs than did ZF-CF MLd units; (2) the reverse versions of songs were preferred over the forward versions in both the ZF-ZF and ZF-CF birds; and (3) there was an effect of individual songs within either the CON or HET category.
Selectivity toward the forward conspecific songs in the MLd of ZF-ZF or ZF-CF birds was tested by Woolley et al. (2010). Contrary to the results presented here, they did not find a difference in the responses to conspecific and heterospecific songs in male ZF-ZF or ZF-CF. In the present study, the differences in responses to CON or HET songs was even more pronounced in male ZF-ZF than in females ZF-ZF (Mean RS of 5.94 sp/s and 4.88 sp/s for CON and HET songs, respectively, in females and mean RS of 5.34 sp/s and 3.61 sp/s for CON and HET songs, respectively, for males). Responses to HET songs in females were about 18% lower than to CON, whereas in males, responses to HET songs were about 32% lower than to CON songs. That female zebra finches appeared to discriminate more poorly between CON and HET than males is puzzling, since one would expect this discrimination should play a major role in mate choice. However, female zebra finches will actually mate with Bengalese finch males. It would be interesting to examine the discrimination of MLd neurons in females between CON and HET songs from bird species with which female zebra finches cannot mate or from which males cannot learn. Thus far, no other study has investigated electrophysiologically the difference in response strength to conspecific signals in male and female ZF-ZF, so all possible explanations here remain speculative and highlight the need for more studies involving both sexes. Never the less, the preference toward conspecific songs vs. heterospecific songs in zebra finches raised in normal conditions or in isolation without a tutor has also been demonstrated in behavioural experiments using choice paradigms. Braaten and Reynolds (1999) showed that young zebra finches raised in isolation preferred songs of conspecifics over starling songs. Lauay et al. (2004) showed that females raised either with a male or in isolation also preferred songs of conspecific males over songs of canaries. However, zebra finches are known to incorporate Bengalese finch song features under experimental conditions; this is not the case for canary or starling songs. Thus, these experiments do not address whether the inability to incorporates canary or starling song elements is related to limitations in learning circuits or limitations in auditory processing.
Similar results were recently reported in the brainstem of the Lusitanian toadfish (Vasconcelos et al., 2011). These workers measured auditory evoked potentials (AEP) in response to some conspecific vocalizations from both males and females and also from juveniles. The heterospecific signals consisted of the mate advertising call of a sympatric fish species (meagre *A regius*) and vocalizations of the bottlenose dolphin, a potential predator of this fish. Vasconcelos and her colleagues showed that Lusitanian fish brainstem of both sexes perceived the temporal patterns, amplitude modulations and the spectral content of conspecific sounds, features that can be related to the biology of this species. Indeed, temporal patterns detection probably plays a role in mate choice, as the duration and repetition rate of the grunt signal depends on the fish size. Amplitude modulation appears to be an important feature of boatwhistles to convey information: the boatwhistles produced by males in an advertising context or by males in a territorial defence context diverge in their amplitude modulations. Finally, the spectral content, and more precisely the dominant frequency of the fish grunts, varies with the body size and therefore can serve as an indicator of mate quality. Ryan and Rand (1993) showed that female frogs also exhibited strong preference for conspecific calls over heterospecific calls using phonotaxis experiments. Even more interesting, they demonstrated that some heterospecific features can be preferred by females but only if they are integrated within a conspecific call.

While the main characteristics of tuning of units in the cross-fostered MLd appear to remain quite similar to those observed in the MLd of normally-reared birds, the processing of vocalizations differ. Cross-fostering reduces discriminatory abilities between conspecific and heterospecific vocalizations. Woolley et al. (2010) tested cross-fostered birds in their study. They found that the mean firing rate of cross-fostered bird’s neurons in response to conspecific and heterospecific songs did not differ. However, it should be remembered that in their study they did not find a significant difference for the similar experiment with ZF-ZF birds either. Also, the
ZF-ZF birds in their study showed a mean firing rate of 8.4 and 8.2 spikes/s to conspecific and heterospecific songs, respectively, while the ZF-CF birds had mean firing rates of 5.6 and 6.0, respectively, to these signals. Therefore, there appeared to be a decrease of firing rate to both types of stimuli in the midbrain of cross-fostered birds. In the present study, units from cross-fostered birds showed a mean RS of 5.20 and 5.12 spikes/s to conspecific and heterospecific songs, respectively, while units recorded in normally reared birds had mean RS values of 5.76 and 4.49 to these types of songs, respectively. It therefore appears that it is the contact with the fostering species that modulates the response towards the HET signals. Normally-reared birds, deprived of such a contact, have a reduced response to the Bengalese finch songs, while the cross-fostered birds present an equal response to the songs of both their own species and those of the cross-fostering species. This is also consistent with the observations of Lauay et al. (2004). They conducted a behavioural experiment in which zebra finch females were either reared in the presence or absence of their father and were then asked to choose between conspecific songs of varying quality: tutored male songs or untutored male songs. They showed that rearing conditions of females shape their choice toward male songs. Females that were raised with an adult male preferred songs of tutored males over those of untutored males, while females that were denied the presence of a male did not show this preference. Since the song of untutored male is abnormal (Price, 1979), the results of their experiment showed the importance of rearing environment on conspecific song choice. Based on the results of the present study, it would also be interesting, for instance, to test whether the songs of cross-fostered birds would elicit greater responses from the ZF-CF MLd units than do the CON and HET stimuli.

Finally, the GLMM analysis confirmed that there was a significant difference between the mean RS values to forward and reverse versions of songs (see chapter IV), with a preference toward the reverse songs in both the ZF-ZF and the ZF-CF birds. More interestingly, the analysis also showed that there was an effect of song within either the conspecific or heterospecific signals. This could raise a question for studies that pool many songs together and could explain the
results of Woolley et al. (2010). Pooling too many songs might eradicate the individuality of each of them and fail to identify differences. Lauay et al. (2004), showed, for instance, that normally-reared females will prefer the songs of tutored males over the songs of untutored males showing that the “quality” of a song is an important component for females performing in a mate choice paradigm.
Chapter VI

General discussion and conclusion
Despite the fact that more than half of the living bird species are passerines, and that auditory function plays a crucial role in the learning and maintenance of song, their auditory system has not been comprehensively studied. Instead, the auditory midbrain (IC) of barn owls remains the best described (both anatomically and electrophysiologically) and is commonly referred to as a model of avian auditory organisation. But extrapolating from barn owl to other species may be problematic given that their IC probably co-evolved with a series of other specialisations associated with their nocturnal hunting habits. Some of the features of the barn owl’s IC are found in other species of birds, such as the chicken (Conlee & Parks, 1986; Puelles, et al., 1994; Wang & Karten, 2010), guinea fowl (Scheich, et al., 1977), and other birds of prey (Calford, et al., 1985)

Songbirds such as the zebra finch may have evolved a unique use of their auditory system as part of their song learning processes. Songbirds use their songs in the context of courtship and territorial defence. The control of song is mediated by a series of novel forebrain circuits that rely on auditory feedback for song learning and maintenance. While selectivity to auditory inputs has been studied at the forebrain level, how this auditory input is processed at lower brainstem levels has been poorly examined, and whether the organisation of these lower circuits co-evolved with those involved in song production remains unknown. Thus, extrapolation from auditory data from the brainstem of non song-learning species may not be appropriate.

The present work was aimed at characterising the auditory midbrain of zebra finches, a major site of convergence of auditory information and where species’ vocal identification has been shown in many vertebrates.

6.1 - Anatomical considerations

The present work was able to show two MLd subdivisions using CaBPs, MLd.I and MLd.O, that appear to be quite similar to those observed in the MLd of non-songbirds (Kubke, et al., 1999; Puelles, et al., 1994; Takahashi, et al., 1987; Wagner, et al., 2003; Zeng, S, et al., 2008a). This
was quite surprising considering that the organisation of projections from the lower brainstem to MLd in songbirds differs from that seen in barn owls or chickens. In barn owls and chickens, the ‘core’ region of MLd corresponds to, and can be defined by, the terminal field of NL (Conlee & Parks, 1986; Leibler, 1975; Takahashi & Konishi, 1988a; Wang & Karten, 2010; Wild, 1995). In zebra finches, the projections of both NL and NA do not appear to follow a similar organisation to that described in chickens and barn owls, and therefore a core cannot be defined based on their terminal fields. This highlights the inaccuracies in the interpretation of data that may arise when it is assumed that data obtained from “model” species can be extrapolated to others that may (or may not) exhibit species-specific specialisations. It is now clear that segregated projections of NA and NL do not provide an accurate criterion to distinguish between the inner and outer regions of MLd. This also underscores the need to reconsider the assumptions regarding the functionality of MLd/IC subdivisions delineated with CaBPs. Given that inner and outer regions of MLd do not receive similar sets of ascending projections in different species, functional correlates between the two regions in different species need to be re-considered and not simply extrapolated from data obtained in barn owl/ chicken models.

6.2 - Functional considerations

IEGs have become a widely used tool to investigate auditory activity and selectivity in the bird brain. This study showed that the expression patterns of IEGs should be interpreted with caution. The main finding from the pilot studies included in this thesis was that levels of activity obtained from IEG expression patterns did not match those obtained when recording electrophysiologically under the same anaesthetic and stimulus conditions. For example, although it is known that the tonotopic organisation of the auditory midbrain of vertebrates is conserved, tone stimuli failed to elicit expression of IEGs in a pattern that was aligned with the known tonotopic map of the zebra finch MLd. Similarly, the presentation of noise or song stimuli did not evoke a large activation of IEGs, whereas neurons were seen to readily and
robustly respond to these stimuli in electrophysiological experiments (see chapters IV and V). Therefore, it can be questioned as to what it was about the specific neuronal populations that were shown to upregulate ZENK, that was different from those that did not. ZENK auditory-evoked activity in response to noise and songs showed that these signals seemed to be encoded by units in both MLd.I and MLd.O, and this is consistent with the electrophysiological findings. The electrophysiological experiments described in chapters IV and V revealed the complexity of analyzing responses of units to sound stimuli. While most authors have averaged data across neurons, the variability in the responses obtained in this study suggested that this would obscure the diverse response properties of MLd neurons. A second concern was associated with the difficulty of incorporating inhibitory responses in the overall analyses, given that the criterion to classify a unit as auditory required that the unit respond above threshold to at least one auditory stimulus. As a result, categorisation was based on excitatory criteria alone. It is necessary to set a criterion to accept the activity of units as auditory and this is usually a criterion related to the strength of a unit’s firing rate. This immediately discards any unit the activity of which would be inhibited by stimulus presentation, although inhibition, especially in the IC, is a phenomenon quite crucial in generating the responses of units to species-specific features of vocalisations (Feng, et al., 1990; Pollak, G. D., 2010; Portfors, 2004). For those units that strongly responded to the stimuli, single-unit recordings showed that most of the units exhibited responses to all three types of stimulus presented: tones, white-noise and songs. This study also showed that a substantial number of units showed some degree of selectivity by either not responding to one type of stimulus or by responding to a subset of either songs or elements within songs. By using zebra finches that were reared in a conspecific-only aviary (ZF-ZF) and zebra finches that were cross-fostered by Bengalese finches (ZF-CF), this thesis was able to highlight the role of rearing environment in song discrimination. While the units recorded in both categories of birds exhibited similar tuning characteristics, ZF-ZF units as a population showed a much stronger bias toward the encoding of conspecific signals than ZF-CF units and cross-fostering
was therefore shown to diminish the ability to discriminate between CON and HET signals. The GLMM analysis also raised another point to be considered, namely the effect of songs within type (CON or HET), indicating that the choice of the individual song(s) used as a stimulus may affect the results. Portfors and Sinex (2005) referring to the mammalian IC stated: “An important consideration, however, is the choice of vocalizations used as stimuli. To dissect critically how the IC encodes species specific vocalizations and determine the extent of neural selectivity and/or specializations, a full array of vocalizations should be explored as stimuli.” The present work confirms this view, but also suggests that it is essential to consider each signal separately.

Most of the studies investigating selectivity of responses to songs in the zebra finch tend to average the responses of a unit to several songs, which might attenuate or even abolish specific unit properties. For example, Woolley et al. (2010) investigated the effect of auditory experience on responses of units from normally-reared birds and cross-fostered birds and failed to demonstrate an effect of the rearing conditions on CON/HET discrimination. In their study they use a sample of 20 CON and 20 HET songs but compared the averaged response to all CON songs with the averaged response to all HET songs. This might explain why they did not detect an effect of the rearing environment on the auditory processing of songs, while the GLMM analysis in the present study did.

6.3 - Implications of the findings of this thesis and suggestions for future studies

Although some features of the zebra finch midbrain differ from those which have been previously shown in non-song learners, it still appears that some MLd characteristics are conserved across vertebrates, such as the tonotopic organisation of frequencies within the nucleus from dorsal (low) to ventral (high) or a sensitivity to species-specific features. Species-specific filtering properties in response to calls have been described in the midbrain of mammals, amphibia and fish and seem to be a primitive condition of the auditory midbrain that was also maintained in the songbird lineage. It is surprising that no studies have investigated the responses
of MLd in songbirds to calls, in particular, as calls are a shared vocalisation between males and females of species, such as the zebra finch, where only the males sings. The present work showed that the zebra finch midbrain possesses filtering properties that are species specific and that the auditory midbrain is also able to discriminate CON from HET signals. From these findings and what is known from studies of other vertebrates, it would be expected that species-specific responses to calls would be observed in MLd and probably in MLd of both sexes. However, whether songbirds can actually discriminate conspecific from heterospecific calls is difficult to predict on comparative grounds, because, so far, only one other study has explored this subject electrophysiologically in fish, where it was shown that the auditory midbrain was able to differentiate between CON and HET signals.

In this thesis it was found in normally-reared birds that there was species-specific filtering for songs in MLd with a bias toward the encoding of CON songs. In contrast to the unlearned call, song is a learned type of vocalisation for which songbirds have evolved a forebrain pathway devoted to its learning and production. It is therefore particularly interesting that discrimination between CON and HET learned signals happens at the level of the single neuron in the brainstem. Whether this discrimination in the auditory midbrain co-evolved with the emergence of the forebrain song system, which receives inputs from the central auditory pathway, or whether this discriminatory ability predates the evolution of the song system, is unknown. The forebrain song system possesses its own filtering properties and preferentially responds to and encodes the BOS, but whether these filters evolved independently of brainstem inputs, or whether, as seems more likely, the forebrain took advantages of existing functionalities in the brainstem - where we now know that CON/HET discrimination happens – is a matter of speculation.

To investigate this further, the experiments of this thesis should be repeated in species where both males and females sing and in hummingbirds and parrots. In species where both sexes sing, it can be asked whether there would be the same differences in discrimination as those observed
in zebra finches. This question is even more important when considering that in the present work the female MLd neuronal population seemed not to discriminate as well as that of the male. Could discrimination be correlated with the ability to sing and would the auditory midbrain of females that sing perform at a similar level than that of males? The second option mentioned above is to replicate the set of experiments in hummingbirds or parrots. These birds appear to have independently evolved a song system, albeit having certain differences from that of songbirds (Striedter, 2005). Would the discriminatory abilities between CON and HET observed in songbirds be also present in the brainstem of this species or would this be only seen in the forebrain?

It is not clear whether the discrimination between CON and HET songs that occurs in the midbrain is important for song learning. It is possible that the forebrain uses a separate circuit to process this discrimination in song learning. In barn owls, integration of interaural time and level differences that are used for sound localisation emerges separately in the inferior colliculus and thalamus (Perez & Pena, 2006). It may be that CON/HET discrimination occurs at more than one level of the auditory pathway.

Another important point arising from this thesis is that the bias toward the encoding of conspecific signals seen in the midbrain can be altered by experience. MLd neurons from birds that were cross-fostered with Bengalese finches were not able to show a CON/HET discrimination as strong as that found in birds reared amongst their conspecifics. It is not clear whether the effects of rearing are associated with auditory experience itself or whether it is influenced by the motor experience of singing (cross fostered zebra finches ‘learn’ and sing a Bengalese-like song). Nor is it known when during development this discrimination emerges.

The plasticity of the responses to CON and HET signals and the origin of the discrimination between these vocalisations could be investigated by testing the discrimination in MLd prior to the onset of singing. Another possibility would be to test the discrimination within MLd of adult birds whose rearing environment was modify before or after the onset of singing. Zebra finches
that have been cross-fostered with Bengalese finches until nutritional independence (35 days old) and then transferred in a cage where they had full contact with a conspecific tutor tended to learn from the conspecific male even if they still had auditory and visual contacts with the foster father (Eales, 1987). However, cross-fostered birds that where only allowed visual and auditory contacts with their conspecifics after independence learned from their foster fathers (Eales, 1987). Recordings from birds from both these rearing conditions could give great insights about the effect of motor experience, as the birds are reared hearing both CON and HET vocalizations but differ in the songs they produce.

It is also not clear how stable the discrimination between CON/HET is and whether this discrimination shows different degrees of plasticity in opened versus closed learner. Open learners continue to add either new elements to their songs or new songs to their repertoire. Experiments could be designed to examine discrimination to CON/HET components before and after those HET components are added to the bird’s own song. Similarly, neurons could be challenged with the requirement to discriminate between two different HET elements and then tested again after one of those has been incorporated into the bird’s own song. Then the bird could incorporate the second element into its BOS and neurons tested for discrimination between the two sounds again. Would the discrimination evolve as elements go from HET to CON category when being added to the BOS?

In this thesis, the response to cross-fostered songs was not tested. It remains to be seen if the cross-fostered midbrain, while losing the ability to discriminate between zebra and Bengalese finch songs, would be able to encode preferentially cross-fostered vocalisations. The same question applies to normally reared birds. Given that the cross-fostered song retains some characteristics of the zebra finch song, it could be hypothesized that the auditory midbrain of normally reared birds would respond more strongly to cross-fostered vocalisations than to Bengalese finch songs but not as strongly as it responds to the CON songs.
Finally, the organisation and/or properties of the zebra finch MLd may not be representative of that of songbirds in general. Zebra finches are non-territorial, whereas many songbird species actually are territorial and will use the song as a defence behaviour and approach and attack the intruder (for review, see Catchpole & Slater, 1995). Therefore, these birds must be able to assess whether and where an intruder is within their territory. What would the organisation and physiological properties of the auditory midbrain of these species look like? It seems appropriate to suggest that CaBPs studies, such as the one performed in this thesis and tracing experiments such as those realized by Krutzfeldt et al. (2010a) and Wild et al. (2010), should be replicated in several bird species so as to get a better understanding of the organisation of auditory projections onto MLd and the role of MLd subdivisions in relation to their behaviour. There is no reason to assume that the anatomical and electrophysiological organisation of a closed end learner such as the zebra finch is the same as that of other songbirds in which both sexes sing or that are able to learn a song each season or continuously learn their song.
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