The role of spontaneous neural activity for song development

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Abstract

The zebra finch is a songbird that learns his song by memorizing and copying a song of an adult tutor during a critical phase for song learning. Converging evidence points to a prominent role of sleep in the vocal learning process. Spontaneous activity of neurons in the motor system during sleep — activity that is not directly linked to auditory perception or motor production — often constitutes a replay of their sensorimotor activity and depends on previous exposure to a song model. This spontaneous activity has been proposed as neural mechanism for sleep-dependent processes of vocal learning. The sensorimotor nucleus interface of the nidopallium (NIf) plays an essential role for song learning: NIf drives the spontaneous neural activity in the motor system during sleep and the production of plastic song in juveniles, and lesions of NIf in juvenile birds prevent song learning. However, a causal relation between the spontaneous activity during sleep and vocal learning has not been established yet. In this thesis we aimed at probing direct causal relationships between spontaneous activity during offline time periods (periods in which there is no exposure to song, or production thereof), specially sleep, and song development.

In song-isolated juvenile male zebra finches we bilaterally implanted stimulation electrodes into NIf. We electrically stimulated NIf and tutored the juvenile birds on a daily basis. The electrical stimulation was contingent on vocal behavior and body movement, and was sufficiently strong to drive downstream neural activity. We either stimulated birds during movement periods, no-movement periods, or both, but we never stimulated when either the bird or the tutor was vocalizing. We stimulated with a weak single-pulse stimulation (150 µA, 5 Hz), a strong single-pulse stimulation (300 µA, 10 Hz), or a strong paired-pulse stimulation (300 µA, 5 Hz, paired pulses).

The vocal learning performance, as judged by the similarity of the developing song to the tutor song, was affected by the stimulation: weak single-pulse stimulation had a facilitating effect and led to fast and precise learning of the tutor song, while strong paired-pulse stimulation did impair normal vocal learning and resulted in poor copying of the tutor song. These effects were more prominent in birds stimulated during resting periods covering sleep.

Our results indicate that alteration of neural activity in NIf and downstream areas during sleep can enhance and impair vocal learning and provide evidence for a causal relationship between spontaneous activity during sleep and vocal development.
Zusammenfassung


Wir implantierten jungen männlichen Zebrafinken, welche ohne Zugang zu Gesang aufgezogen wurden, bilateral Stimulations-Elektroden in NIf. NIf wurde elektrisch stimuliert und die jungen Vögel wurden täglich mit einem adulten singenden Vogel in Kontakt gebracht. Die Stimulation war abhängig von Bewegung und Singverhalten und stark genug um neuronale Aktivität in efferenten Regionen zu erzeugen. Wir stimulierten die Vögel entweder während sie sich bewegten, während sie sich nicht bewegten, oder beides, aber niemals wenn entweder der junge Vogel oder der adulte Vogel sangen. Die Stimulation bestand entweder aus schwachen einzelnen Pulsen (150 µA, 5 Hz), aus starken einzelnen Pulsen (300 µA, 10 Hz), oder aus starken paarweisen Pulsen (300 µA, 5 Hz, paarweise Pulse).


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Chapter 1

Introduction

There is a large body of evidence suggesting the involvement of sleep in memory consolidation and learning [Maq01, WBHS03, Shi05]. Sleep in humans has a beneficial impact on the performance increase in various tasks, e.g. visual discrimination tasks [SJH00], motor learning tasks [FHEB02], reaction time tasks [MLP+00], and spatial learning tasks [PLF+04]. The beneficial impact is ascribed to different aspects of sleep. Sometimes a nap is as good as a night [MNS03], and sometimes the intensity of distinct sleep phases, such as slow-wave sleep (SWS) and rapid eye movement (REM) sleep, is linked to the specific learning and consolidation task [SWS+00, DB10]. The interaction between exposure to a task and sleep is bi-directional: the exposure to a task affects the activity in the involved brain regions during sleep, and the activity during sleep correlates with the performance increase [HGMT04, HGM+06].

The beneficial roles of sleep for behavioral learning are usually ascribed to spontaneous neural activity during sleep, activity that is not directly linked to behavior, but often closely resembles that observed during daytime behavior: Place cells in the hippocampus of rats that were firing together during a spatial behavioral task exhibited an increased tendency to fire together during subsequent SWS [WM94]; And Spike sequences of pyramidal cells in the rat hippocampus during wheel running were ‘replayed’ at a faster timescale during SWS [NHC+99]; and this replay is coordinated between hippocampus and cortex [JW07].

To study the causal relation between sleep and behavior, sleep and neural activity during sleep have been manipulated in humans: selective disruption of distinct sleep phases resulted in no sleep-dependent performance gain in a visual discrimination task [KTR+93], and excitability enhancing transcranial direct current stimulation (tDCS) and transcranial magnetic stimulation (TMS) of the motor cortex during sleep enhanced motor learning and motor memory formation [RRK+08].

Despite numerous studies the underlying role of sleep in learning and
memory has yet to be precisely characterized. To further elucidate the role of sleep one needs to realize four goals \cite{Maq01}: First, the task-dependent and regionally specific brain activity during post-training sleep has to be characterized. Second, the causal relation between the post-training neural activity and behavior has to be demonstrated. Third, the effects of the sleep discharge patterns and the experimental effects (such as stress and circadian modification) need to be disentangled and, fourth, the effects of SWS and REM sleep should be specified.

The realization of these four goals necessitates a suitable model and the songbird is an applicable candidate. Sleep in songbirds shares many features with mammalian sleep: As in mammals, sleep is a homeostatic process in the white-crowned sparrow \cite{JVC+08} and the features of sleep structure in zebra finches are mammalian-like \cite{LSSM08}. Furthermore, the performance in an auditory perception task in European starlings benefits from sleep \cite{BNM10}. And while the mammalian neocortex and the avian pallium evolved independently, they share the extensive palliopallial connectivity that is speculated to be linked to the evolution of SWS, a sleep feature not found in reptiles \cite{Rat06}. In this introduction we will summarize the extensive knowledge on vocal learning in songbirds, specially the zebra finch, and argue why this natural process is well suited to study specific neural activity during sleep.

This thesis contributes to the realization of these four goals by testing the role of well-characterized spontaneous activity — activity that is not directly linked to auditory perception or motor production — in the song motor system during sleep on vocal development.

### 1.1 The zebra finch as model system

Only few species are capable of vocal imitation learning. Among mammals, only humans, cetaceans, pinnipeds, elephants and some bats show evidence of vocal learning \cite{JS97,DK99,PTSHW05,FSR+08}. Among birds there are three distantly related groups capable of vocal learning: parrots, hummingbirds and songbirds \cite{PMN81,JRS+00}. Vocal learning has proven to be a powerful tool to study sensorimotor learning. It is a complex natural behavior, but simple to monitor. Songbirds in particular have gained much attention lately as their song learning process shows many parallels to human speech learning, while not being equally complex. Song learning can be considered as a miniature system of human speech learning \cite{Mar70,DK99,BD02,Mar03}.

The most prominent animal model for vocal learning is the zebra finch (taeniopygia guttata). In contrast to mammals capable of vocal learning, zebra finches are simple to house in laboratory conditions and the learned vocalizations are simple. Only male zebra finches sing and their song is very peculiar: each song consists of multiple repetitions of a stereotyped song.
1.1 The zebra finch as model system

1 second

Figure 1.1 – Adult zebra finch song (sound waveform in black with corresponding spectrogram above) consists of repetitions of introductory notes followed by several renditions of the stereotyped song motif (marked by the red rectangle). By assigning letters to the individual syllables of the song motif (i for introductory notes) we can describe the song as sequence of letters: iiiiiBCDEF ABCDEF CDEF.

8 kHz
0 kHz

1 second

Figure 1.2 – Timeline of song learning. The song learning process in zebra finches is divided into two overlapping phases: In a sensory phase (15 to 60 days post hatch) juvenile zebra finches are exposed to a tutor song and acquire a memory of that song. During the sensorimotor phase (30 to 90 dph) juvenile birds gradually develop a precise imitation of the tutor song. At around 90 dph the developing song crystallizes and adult birds sing and maintain a stereotyped song for the rest of their life. (This figure is reproduced from [BD02].)

motif, itself consisting of a fixed sequence of syllables. Such a song motif lasts between half a second and a second. An example of adult zebra finch song is shown as sound waveform and spectrogram in Figure 1.1.

During song development (Figure 1.2) male offsprings imitate the song of a tutor, usually their father. In an early stage of development, the so-called sensory phase, the juvenile birds listen to the tutor and form a memory of that tutor song. During the sensorimotor phase, starting at around 30 days post hatch (dph) in zebra finches, juvenile birds vocalize and make use of auditory feedback to gradually improve the imitation of the tutor’s song. They start with a very variable song without clear structure called subsong, progress to plastic song with more structure in both the time and the frequency domain in an intermediate stage, and finally end up with a ‘crystallized’ song, usually a very precise imitation of the tutor’s song [Mar70, Mar90] (Figure 1.3). The song crystallizes at approximately 90 dph, meaning that it has reached a stable and stereotyped state. The nature of
Introduction

Figure 1.3 – Zebra finch song development. The juvenile’s song evolves gradually from babbling like subsong (42 days post hatch, topmost panel), via variable plastic song (53 dph) to stereotyped adult song (70 dph), usually a precise imitation of the tutor song (lowermost panel). The tutor song motif consists of six syllables ABCDEF (green rectangle), the imitation of the juvenile consists of five syllables, ABNEF (red rectangle): the syllables ABEF are copied and the syllables CD are replaced by a new syllable N.

this learning process is manifold: the speed of learning varies, as does the imitation quality. An adult male zebra finch typically sings more than a thousand songs per day, each consisting of multiple song motifs.

During the sensorimotor phase the juvenile birds do no longer need exposure to the tutor’s song: all they need for precise imitation learning is access to an internal representation of the tutors song [Boh90]. The absence of a song model during the sensory phase appears to postpone the sensitive phase and a model song can be acquired later in development [Eal85]. Birds that are never exposed to a tutor produce atypical song, so-called isolate song. While the juveniles depend on a tutor for learning a species-typical song, there also is a genetic component of song culture: a colony of birds founded by song-isolated individuals will establish wild-type song within a few generations through an interplay of genetic factors and social interaction [FWS09].

Auditory feedback is required for song development and maintenance of the adult song [Kon65 NN92]. Deafening of songbirds in an early stage of development obstructs regular song learning and leads to gradual song degradation in adult birds [LN00].

Our interest is the neural basis of sensorimotor vocal imitation learning. Recent advances in microtechnology made it possible not only to electrophysiologically record neurons in sleeping and anesthetized head-fixed birds, but to also to record activity from single neurons in the freely behaving an-
imial and to correlate that neural activity with behavior. These advances led to many insights into the song system \cite{NSL76, NKP82}, a network of neuronal nuclei specialized for song production, perception, and learning. In particular, neural recordings have shed light on the motor system for song production and the auditory system for song perception. However, little is known about the interaction of the sensory and the motor systems, which is at the core of sensorimotor learning.

The auditory system and the motor system are linked by the sensorimotor nucleus interface of the nidopallium (NIf). It is involved in auditory perception \cite{VBMN96} and motor production \cite{McC87}. Additionally, NIf is active during offline time periods, i.e. when no sound is perceived or produced \cite{HF07, LS11}. The working hypothesis in this thesis is that the spontaneous activity in NIf plays an important role in the sensorimotor learning process.

Here we test the role of spontaneous neural activity in NIf and downstream areas in the song motor system by perturbing the naturally occurring activity patterns during offline time periods by applying chronic electrical stimulation in NIf during song development and by analyzing the effects of this stimulation on the song learning process.

In the following sections the song system will be introduced and we will summarize what is known about zebra finch song learning and song maintenance. We will discuss sleep dependent processes and their implications for theories of song learning.

1.2 The song system

In the zebra finch brain there are several nuclei that are significantly larger in males than females \cite{NA76}. These nuclei are part of the song system, a network of neurons dedicated to vocal learning and song production \cite{NSL76, NKP82, SV90, Sch09}. The song system can be roughly divided into downstream motor pathways, an upstream auditory pathway, and several structures in which the separation into unique motor or auditory functions is not possible. We shall give an overview of the main pathways and the neural codes found in the motor systems for song production and perception, and will discuss the implications on the song learning process. The nomenclature of the different brain regions and nuclei was revised in 2004 to reflect the better understanding of the avian brain and we will use the revised nomenclature throughout this thesis \cite{RPMJ04}.

1.2.1 The neural pathways

Figure 1.4 illustrates the network dedicated to song production, perception and learning. The descending song motor pathway (SMP) consisting of
The zebra finch brain contains several neural pathways designated for song production, perception and learning: The song motor pathway (SMP) is responsible for adult song production (blue), the ascending auditory pathway for song perception (grey) and the anterior forebrain pathway (AFP) is involved in song learning and juvenile song production (red). The thalamic nucleus Uva and NIf (green) serve both motor and auditory functions. Furthermore there are nuclei responsible for respiratory control (magenta) and dopaminergic input to the song system (yellow). (This figure is reproduced from Heather Williams [website][Wil].)
HVC → RA → {DM → nXIIts} is depicted in blue and is responsible for production of stereotyped adult song.

In juvenile birds there is another important afferent input to RA that drives song, apart from HVC. The anterior forebrain pathway (AFP) HVC → area X → DLM → LMAN → {area X, RA} depicted in red is required for the production of variable juvenile songs, for developmental song learning, and for song maintenance in adult birds. The area X → DLM → LMAN → area X loop closely resembles the mammalian cortical-basal-ganglia-cortical loop [DPRS05]. There are two known inputs from the SMP to the AFP: HVC projects to area X, and just recently the projection from RA to DLM has been described [GF12]. This connection forms another loop: RA → DLM → LMAN → RA. RA is the only premotor output nucleus of the forebrain.

The ascending auditory pathway is depicted in grey. Auditory information enters the forebrain via the nucleus Ov that projects to the higher auditory areas commonly known as Field L, the avian homologue of primary auditory cortex. From Field L there are projections to secondary auditory areas CM and NCM.

The two nuclei in green, Uva and NIf, are intermingled between the motor system and the auditory pathway. Both Uva and NIf are involved in auditory processing [VBMN96, CRWM07] and motor production [McC87]. Uva is thought to be involved in patterning of the song and interhemispheric coordination [WV93, CV05]. The function of NIf is described in detail in Section 1.5. Auditory information feeds into the motor structures of the SMP and the AFP predominantly via HVC. HVC receives input from CM directly and input from CM via NIf [VBMN96, CRWM07, AK10]. Recently new feedforward and feedback connections were described between HVC ↔ Av ↔ NIf → HVC, forming a loop between auditory and motor structures [AK10].

Finally, there is substantial dopaminergic input to the song system, spe-
cially to area X: \(\{\text{SNc}^{14}, \text{VTA}^{15}\} \rightarrow \text{area X} \rightarrow \text{VF}^{16} \rightarrow \{\text{SNc, VTA}\}\), depicted in yellow [LRAB81, Bot93, GP10].

Many of the above-mentioned structures and connections have been documented early in the history of songbird research [NSL76].

### 1.2.2 The neural code of song production

In this section we summarize the current understanding of the neural code in the two distinct circuits that drive juvenile and adult song.

The neural code of adult song production in HVC and RA is well understood due to the strong correlation of neural activity and stereotyped song. The nucleus HVC, at the origin of both the AFP and the SMP, exhibits a very peculiar code during song production: the HVCRA neurons (projection neurons from HVC to RA) burst reliably at a single precise time in the song [HKF02]. Cooling of HVC slows song speed by up to 45% [LF08], an effect not found further downstream in the motor pathway. It is thought that HVC controls the timing of the song by means of a feedforward network that can control complex sequence behavior [FKH04, LJF10].

HVCRA neurons project to multiple neurons in RA and RA neurons receive input from multiple HVCRA neurons [YMSB12]. Thereby the ultraspars code in HVC drives bursting activity in RA: in adult zebra finches, individual RA neurons burst on average 12 times per song motif with activity that is very precisely time-locked to individual syllables [YM96, LF05].

The other afferent input to RA from LMAN is not needed for adult song production [BMA84]: LMAN lesions lead to a reduction in the variability in the bird’s song [KB06] and a loss of plasticity in learned vocalizations [BD00]. A reduction in variability can also be observed by changing the social context: female-directed song is more stereotyped than undirected song [SB80, HD99] and LMAN activity switches from variable firing during undirected song to very precise firing during directed song [KWD08]. Females do prefer directed song over undirected song [WD08].

In juvenile zebra finches RA activity is mainly driven by LMAN and this drive is very variable: reversible pharmacological inactivation of LMAN in juveniles can turn juvenile subsong into adult-like song [OAF05, OOG+11], while inactivation of HVC in adults leads to unstructured subsong-like song [AAF08]. Juvenile courtship song is similar to song produced during LMAN inactivation and is as stereotyped as is adult song [KD11]. And analogous to the finding that cooling of HVC slows song speed in adults [LF08], cooling of LMAN leads to slower juvenile songs: decreasing LMAN temperature leads to increased syllable durations in juveniles [AVGFT11]. LMAN lesions in juvenile zebra finches disrupt song development, stabilize the variable song development.

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14SNc: substantia nigra pars compacta.
15VTA: ventral tegmental area.
16VP: ventral pallidum.
1.2 The song system

[BMAM84], and lead to premature alteration of the HVC to RA connectivity [KM99]. Lesions of area X in juvenile zebra finches do not lead to cessation of variable song production, but impair song learning [SNN90, SN91].

Song development from juvenile to adult song is paralleled by a change in the connectivity of RA and its afferent sources LMAN and HVC. During development the drive of HVC to RA increases and HVC itself is driven by NIf [NH11]. In the following sections we will summarize evidence that the gradual replacement of RA’s primary afferent input is linked to NIf driven sleep-dependent processes.

1.2.3 Auditory properties of the SMP and the AFP

To understand sensorimotor learning we need to understand how sensory information is processed and how it influences the developing motor code. In this section we describe auditory properties in the motor systems.

The motor areas are not only active during singing but also during sleep and sedation. In these states HVC neurons of adult zebra finches respond more strongly to the playback of the bird’s own song (BOS) than to other stimuli [TD98]. HVC is the only known source of auditory input to the SMP and the AFP [VY93, RM09], and neurons in downstream areas LMAN, RA and nXIIIts respond more strongly to BOS playback than to other stimuli [WN85, DK91].

The auditory properties in the SMP depend on long sensory memories: BOS selective neurons in HVC integrate auditory input over extended time periods (80 to 350 ms), and responsiveness is weakened when the temporal features of the BOS are modified [MF92, DM00]. RA’s activity to BOS playback closely resembles RA’s motor code during song production, and the auditory activity of RA neurons is only slightly delayed in relation to motor activity by approximately 8 ms: hence, the sensory response in RA to a pattern corresponds to the production of a subsequent pattern [DM00]. Even more precise mirroring with zero delay has been found in HVC of swamp sparrows [PPNM08]. The auditory responses can thus be thought of as auditory-driven responses that also engage the song motor network in a complex manner [MS10].

The auditory activity in the SMP is rapidly modulated and suppressed by arousal [DYM98, CS03]. Different neuron types in HVC have different state-dependent auditory properties: only one of two interneuron types in HVC is selective to BOS during wakefulness, whereas all interneurons and projection neurons are BOS selective during sleep [RS03].

During development auditory selectivity in HVC switches with behavioral state: HVC is highly selective to playback of the tutor song during waking, and this selectivity shifts to BOS during sleep [NK05a]. An overall shift of selectivity from TUT to BOS during song learning develops in parallel with song [NK05a]. Even in adulthood the representation of BOS
in the SMP is plastic, and when the bird’s own song changes in adult zebra finches the selectivity to BOS changes with the song [RM07].

During song production neurons in the SMP and LMAN are not sensitive to playback of auditory stimuli, so called distorted auditory feedback (DAF) [Kon04, Leo04, KF07]. In higher auditory areas afferent to the SMP, such as Field L and CM, there are both neurons that respond to DAF and neurons that do not [KH09]. Only in Bengalese finches auditory responsiveness in HVC during song production has been documented [SB08]. This suggests that DAF responsiveness in zebra finches is gated off between CM and HVC. State-dependent auditory selectivity is likely to be gated off by Uva [AK05, CRWM07].

The state-dependent auditory response selectivity and the lack of DAF sensitivity during song production show that auditory and motor systems are gated apart during song production and indicate the involvement of sleep in song learning. There is no evidence for simple online comparison mechanisms of motor performance and auditory feedback in the SMP or the AFP. It may thus be that song-learning processes happen during offline time periods.

1.3 Song learning and induced learning

Developmental song learning is manifested on a neural level by the gradual replacement of RA’s primary drive from LMAN to HVC. To study sensorimotor learning in the zebra finch one can study the natural behavior of developmental song learning, and also induce learning in experimental conditions: induced mismatch between desired vocal output and auditory feedback leads to adaptive processes to reduce that mismatch.

Deafening induced mismatch leads to gradual degradation of adult song and the time course of that degradation is age dependent: the stability of the representation of the song increases with age [NN92, LN00]. Injuring of the tracheosyringeal nerve in adult birds leads to a short-term deficit and long-term changes of the adult song [WM92, RM07, RM09]. Perturbation of auditory feedback during singing in adult zebra finches causes their song to deteriorate slowly [LK99].

Recently a new experimental paradigm has started to further elucidate the role of the AFP in the song learning process, predominantly using Bengalese finches. By monitoring the natural variations in pitch of a targeted song element and penalizing a subset of those variations by playback of white noise, Bengalese finches rapidly shifted the pitch of their vocalizations in an adaptive fashion to avoid auditory disruption [TB07, SB09]. This DAF induced adaptive process is very precise and changes can be observed on the millisecond timescale [CTWB11]. The shift in pitch is driven by LMAN, as LMAN inactivation leads to instant regression of the pitch values closer
1.4 Learning and sleep

The gradual adaptation of song that normally occurs in pitch shift paradigms can be prevented by pharmacologically blocking the input from LMAN to RA. Interestingly, unblocking the output of the AFP after training causes an immediate transition from baseline performance to excellent performance avoiding DAF, indicating that the AFP covertly gained the ability to implement learned skill performance without contributing to skill practice [CWB12]. Thus, this instructive bias of the AFP is not just the result of random trial-and-error learning. The LMAN to RA connections are topographically organized [JSB95], and song triggered micro-stimulation in LMAN effect song features such as amplitude and fundamental frequency [KDB05]. The AFP is more than just a variability generator and can induce specific song changes.

Learned changes in the song generated by the AFP are consolidated into the motor pathway within one day (reported in zebra finches [AF09]) or within a couple of days (reported in Bengalese finches [WTCB11]), and the learned changes become AFP independent. This consolidation could happen online during singing or offline, perhaps during sleep. We will now present studies that link sleep-dependent processes to song learning.

1.4 Learning and sleep

On a behavioral level there are several findings that suggest the involvement of sleep in the song learning process. The song structure of juvenile zebra finches changes on the day following the first exposure to tutor song [TMLN01]. Furthermore, the song develops in a nonmonotonic manner: Early-morning songs following a night of sleep usually are of lower quality than the late afternoon songs. The song structure deteriorates overnight and regains structure after intense morning singing. Interestingly, no such song degradations were observed in adult zebra finches. Furthermore, and somewhat surprisingly, sleep and its associated nightly song degradations in juveniles seem to have mostly beneficial effects on song development: birds that showed stronger post-sleep degradation during development achieved a better final imitation of tutor song [DMF05].

On a neural level there is more and more evidence of offline song-learning-related activity in the SMP. In adult zebra finches it was found that ‘spontaneous’ activity of RA neurons during sleep matches their sensorimotor activity, a form of song replay [DM00]. This replay is not a unique phenomenon only observed in RA. As in RA, there is a correspondence between sensorimotor activity patterns and offline activity patterns during sleep in HVC [CRM03]. The stereotyped sequences in RA are driven from nucleus HVC: paired recordings of identified HVC_{RA} and RA neurons in sleeping adult zebra finches showed that HVC_{RA} neurons produce bursts sparsely, at a single, precise time during the RA sequence [HKF02, HKF06].
This replay of sensorimotor activity during sleep is suggested to be involved in learning and consolidation: changes in the highly stereotyped bursting patterns of RA were far more frequent after periods of sleep than after periods of waking without vocalizations [RCDM10].

These sleep patterns emerge early in development. The offline activity is not just a replay of motor activity but closely linked to auditory input: In sleeping juvenile male zebra finches there is a tutor-song-specific change in RA activity after first exposure to tutor song [SM09]. And in juvenile zebra finches the offline activity HVC could be linked to changes in behavior: the spike rate during sleep is correlated with overnight changes in song during development [CAKN07].

The spontaneous neural activity in HVC and RA is driven by the sensorimotor nucleus NIf: reversible pharmacological inactivation of NIf leads to transient abolishment of premotor-like bursting activity in HVC neurons [HF07]. Recently it was also shown that Uva input to NIf is excitatory during sleep but that Uva itself has no major role in driving sleep replay patterns, as these patterns persist during reversible inactivation of Uva [HWNN08].

The behavioral observations and the characteristics of spontaneous neural activity during sleep point to a major involvement of NIf driven spontaneous activity in song learning and maintenance.

1.5 The role of NIf

NIf has been linked to online processes such as song production, song perception, and offline processes — such as spontaneous replay of sensorimotor activity in the SMP that is hypothesised to be learning related. In this thesis we test the role of Nifs spontaneous activity on the song learning process by perturbing the naturally occurring spontaneous activity patterns with chronic electrical stimulation. To be able to assess the effects of NIf stimulation we summarise the manifold parts NIf plays in song perception, production and learning.

The nucleus NIf is the primary source of auditory input to HVC and thereby to the SMP and AFP. Anatomically, it is embedded in the auditory structures of Field L, the avian homologue of primary auditory cortex. The neurons in the Field L complex are selective to features of conspecific song [TAS+04, ND08]. Selectivity to individual songs emerges at the level of the higher auditory area CM [GM03, TAS+04]. CM is necessary for much of the auditory-evoked activity in NIf and supplies a direct source of auditory drive to HVC via its subdivision Av, that has reciprocal connections to both NIf and HVC [VBMN96, BCR+08, AK10]. Auditory responses in NIf are highly selective to BOS, and NIf is the main source of BOS selective auditory input to HVC, thereby closely linking their auditory properties [VBMN96, FM95, JM99, CS04, CRS05]. Population activity in NIf is similar
to HVC subthreshold responses and the suprathreshold selectivity to BOS is higher in HVC than in NIf [CM04]. Pharmacological manipulation of NIf eliminates most spontaneous activity and most auditory responses in HVC [CS04] and NIf lesions in adult zebra finches lead to long-term reduction of auditory activity in HVC [CRS05]. Finally, the auditory responses in NIf and HVC are state dependent: auditory responses in NIf are highly selective to the bird’s own song (BOS) during sedation and sleep, but are unselective during wakefulness [SK98, CS04].

While some argue that the nucleus NIf is the first nucleus in the downstream song motor pathway with strong excitatory input to HVC, it is not consistently included, as NIfs role in adult song production and learning is still debated. Multunit recordings in NIf showed bursting activity preceding song syllables and inactivity preceding periods of silence, hinting to a premotor role of NIf [McC87, LS11]. The premotor role of NIf in zebra finches depends on the developmental state: in juveniles pharmacological inactivation of NIf does not affect the production of subsong, but plastic song looses its song stereotypy and regresses to subsong [NH11], showing the involvement of NIf in driving plastic song. Simultaneous inactivation of NIf and LMAN in juvenile zebra finches leads to complete cessation of singing [GO11]. NIf lesions in juvenile zebra finches hinder normal song development [RGM+12], while the song of adult zebra finches following bilateral NIf lesions recovers quickly after a short period of distorted song, showing that NIf is not essential for song production in adults [CRS05]. Bengalese finches sing a complex song with a finite state syntax and NIf lesions result in the simplification of that syntax, turning the song zebra finch like [HO00, Oka04]. Thus, NIf drives plastic song in zebra finches and is involved in sequencing of the song as found in plastic juvenile zebra finch song and complex syntax song in Bengalese finches.

In zebra finches NIf is not necessary for adult song production and even auditory feedback related adaptations of song: decrystallization, a form of de-learning related to auditory mismatch induced by transecting the vocal nerve, was found even after bilateral NIf lesions [RM09]; and auditory activity in the AFP persists after NIf lesions by auditory drive from CM to HVC, and this auditory input is strong enough to change the representation of BOS in the SMP and AFP during decrystallization [BCR+08, RM09].

NIf is also involved in mediating input from the thalamic nucleus Uva to HVC. Uva projects to HVC directly and via NIf, but with separate populations of neurons [AK10]. Uva is thought to be involved in song sequencing and interhemispheric coordination [WV93, CV05] and providing feedback from the brainstem to SMP during singing [SAV04, AWS05, ARS08].

Apart from involvement in online processes for song production and song perception, NIf exhibits interesting song related spontaneous activity. As seen before, NIf drives spontaneous replay of sensorimotor activity in the SMP [HF07]. Another study points to the involvement of NIf on memory
consolidation and the involvement of NIf in learning through offline processes in the awake bird. Neural recordings of spiking activity and local field potential in NIf in adult singing zebra finches revealed an increased firing activity and coherent oscillations in the fast gamma range (90-150 Hz) for up to 30 seconds after vocal activity [LS11]. It is suggested that this activity is suited to facilitate the integration of auditory and vocal motor traces associated with vocal performance evaluation. Spontaneous motor related activity in the awake state has also been found in the hippocampus of rats and is speculated to serve similar functions as replay during sleep [CJFT11].

1.6 Vocal learning with inverse models

In this section we will give a short introduction to a model of song learning that includes a functional explanation of the spontaneous sensorimotor replay observed in the SMP. Hahnloser and Ganguli have presented a novel approach of vocal learning with inverse models. The key element is a simple Hebbian synaptic plasticity rule that in combination with random motor explorations constructs an inverse vocal model. The inverse model inverts the motor to sensory transformation by mapping sensory signals back onto the motor signals that caused them. Recurrent connections among auditory neurons form a memory of the tutor song that is recalled and written into a motor memory during an offline process, for example during sleep. This motor memory can be recalled and the bird can reproduce a copy of the tutor’s song [HG13].

The model is illustrated in Figure 1.5. It consists of an auditory brain area A and a motor brain area M. Some connections are static (filled arrowheads) and some are plastic (empty arrowheads), obeying a simple Hebbian learning rule. In a first sensory phase (Phase (1)) sequencing connections within the auditory area A are learned by a predictive Hebbian learning rule during tutor song exposure. These connections are such that the tutor memory can be replayed at any time by gating off auditory input and being initialized. In a second phase (Phase (2)) the inverse model is trained [JR92, Kaw99]. Random explorations of the motor area M produce random vocal output. The auditory area A is activated via auditory feedback and the tutor memory is gated off (inactive recurrent connections). If the auditory memory would be on, the bird would ‘hallucinate’ the tutor’s song. The map from the auditory area A to the motor area M is the inverse of the sensorimotor map, i.e. the activation of the auditory area by auditory feedback from the vocal activity driven by the motor area M. The connections from A to M are learned by a postdictive Hebbian learning rule. In a minimal form of this model, these two learned connections would already suffice to reproduce the tutor’s song. By gating off the auditory input and gating on the sequencing connections in A, the replay of the tutor memory
1.6 Vocal learning with inverse models

Figure 1.5 – Learning with inverse models. Sensorimotor learning can be modeled as a four phase process: (1) Sensory phase: auditory input (static synapses, filled arrowhead) of a tutor song leads to the formation of an auditory tutor song memory via recurrent connections among auditory neurons (plastic synapses, empty arrowhead) in the auditory area A. (2) Sensorimotor phase: random motor explorations in M lead to activation of A via auditory feedback. Neurons in A feed silently to M such that their synapses learn an inverse of the causal mapping from M to A. (3) Spontaneous replay: the auditory tutor song memory is recalled and written into a motor memory by the formation of recurrent connections among motor neurons. This happens offline, for example during sleep. (4) Song imitation: the recurrent connections in M allow the bird to sing a copy of the tutor song, whilst the inverse model is maintained by means of auditory feedback. This figure is adapted from [HG13].
in A could drive the motor area M via the inverse model and the tutor’s song would be reproduced.

However, in this minimal model there is no access to auditory feedback of a bird’s own vocalizations yet. In a third phase (Phase (3)) the auditory memory in the sequencing connections in the auditory area A is recalled and written into sequencing connections in the motor area M. This recall and replay of activity happens offline, possibly during sleep, as auditory input into A would interfere with the recall of the auditory memory in A. Finally (Phase (4)) the bird can reproduce the tutor song by means of the sequencing connections in M, and the auditory feedback leads to refinement of the inverse model while the auditory memory is gated off.

This model can explain rapid one-shot imitations of sounds, by direct activation of the motor network by an auditory memory, a phenomenon that can not be explained by reinforcement learning [FFS07, FG11] but is observed in humans and some species of birds, for example parrots and mocking birds. It can explain separated sensory and sensorimotor phases in zebra finches [Boh90] and acquisition of a tutor memory during the vocal exploration phase [Eal85]. Extracellular recordings of premotor neurons in the SMP and AFP show insensitivity to perturbations of auditory feedback during song production [Leo04, KF07, PPNM08], a phenomenon that is explained by this theory but is a puzzle from the perspective of the comparator theory used in other models [TD00, FFS07]. State-dependent gating has been observed in the auditory areas of the non-singing bird [SK98] and similarity the auditory properties in the SMP change with behavioral state [DYM98] [CS03].

There is experimental evidence of the reactivation and replay during sleep in Phase (3). Bursting patterns in RA during sleep resemble the sensorimotor patterns [DM00], and they only appear after the first exposure to a tutor [SM09] after which reactivation of memory in A and training of sequencing connections in M would take place. The spontaneous bursting patterns during sleep are driven by a sensory nucleus [HF07] and are specific to individual tutor songs, pointing to the involvement of auditory areas in this replay [SM09]: And after a night of sleep, the motor sequences are more likely to change than during periods of wakefulness [RCDM10].

1.7 The experiment

We have summarized above how song development and song maintenance in a vocal learner such as the zebra finch is manifested on a behavioural and a neural level, and how offline processes are likely to be involved in the formation of the motor code.

The aim of this thesis is to test the role of spontaneous activity in the song learning process: a causal link between song development and spon-
taneous activity, such as sleep bursts in the SMP or increased firing of NIf following vocalizations, has not been established yet. We perturbed the naturally occurring spontaneous activity patterns in NIf during song development of juvenile zebra finches with chronic electrical stimulation and studied the effects on the song learning process.

To discriminate between the effects of the state-dependent spontaneous activity in NIf, we divided the juvenile birds into different groups. A control group was not stimulated, and there were three groups with different movement-contingent NIf stimulation paradigms: one group was stimulated during all offline time periods, the other two groups either during movement (mainly during daytime) or no movement (mainly during nighttime and sleep). We never stimulated the birds during online time periods when there were vocalizations of either the juvenile bird or the adult tutor. Furthermore, we performed chronic offline stimulation with three different stimulation intensities: a weak single-pulse stimulation, a strong single-pulse stimulation, and a strong paired-pulse stimulation. We will describe the experimental design in more detail in Section 2.1 and give an overview of the different groups in Table 2.1.

If spontaneous activity plays a role in the song learning process, then there are several ways by which chronic offline stimulation could alter the naturally occurring activity patterns and influence song development:

- Block or disturb activity, negative effect.
- Induce non-natural nonsense activity, negative effect.
- Facilitate natural activity, positive effect.

We controlled that the stimulations were fully functioning, analyzed the influence of the stimulation on movement, and finally searched for offline stimulation-induced effects on the song learning process and developmental differences between the different stimulation paradigms, to elucidate the role of offline activity in NIf in song development.
Introduction
Chapter 2

Methods

In this Chapter we shall present the experimental paradigm and will describe the methods used for performing the experiment, as well as the methods used for the subsequent analysis. The song learning process is variable per se, and to test the effects of chronic state-dependent offline stimulation in NIf on song development it is important to precisely adhere to protocols and methods in order to minimize the variance that is induced by irregularities during the experiment.

2.1 Experimental design

Figure 2.1 illustrates our experimental design. Juvenile zebra finches were separated from their singing fathers at the onset of the sensory phase (15 dph) and raised in song isolation by their mother, as female zebra finches do not sing. There was social interaction: the birds vocalized and communicated with various calls, but they were never exposed to song, as only male zebra finches sing. At 35 dph, the age at which the sensorimotor period starts, the male juveniles were placed in sound-proof recording chambers, where they were kept throughout the experiment. At around 40 dph we performed a surgery on some birds and bilaterally implanted stimulation electrodes into NIf (n = 23 birds). Other birds that belonged to the control group did not undergo surgery (n = 17 birds). The birds with implants were subsequently connected to the setup via a tether cable.

To distinguish between the effects of state-dependent offline activity in NIf on the song learning process we divided the implanted juvenile zebra finches into three groups with different movement-contingent stimulation paradigms (Table 2.1). To separate periods of sleep and waking we chose for movement behavior as a proxy. Songbirds, like many small diurnal animals, frequently engage in daytime naps and frequently briefly awake throughout the night, specially when they are juvenile [M510]. We therefore did not separate the different groups by strict time of day schedule, but tried to
Figure 2.1 – Experimental design. (i) Juvenile zebra finches were isolated from their singing father (at 15 dph) and raised by their mother. (ii) Juvenile birds were implanted with stimulation electrodes in NIf at around 40 dph and placed individually in a sound proof recording setup. Movement and vocalizations were monitored and stimulations were delivered during offline time periods contingent on movement. After the stimulation was started each juvenile was exposed to a male singing tutor once per day. (iii) The chronic stimulation was restricted to the offline time periods, i.e. the stimulation was blocked whenever the juvenile bird or the tutor was vocalizing. The chronic offline stimulation was contingent on movement. We divided the stimulated birds into three groups: (a) Stimulation pulses were delivered during all offline time periods (all-stim). (r) Stimulation pulses were only delivered during no movement, primarily during sleep at night (rest-stim). (m) Stimulation pulses were only delivered during movement, primarily during daytime (move-stim).
assess the behavioral state by detecting movement.

One group was stimulated during all offline time periods (all-stim), one group only during movement (move-stim, mainly during daytime), and one group during no movement (rest-stim, mainly during nighttime and sleep). We never stimulated the birds during online time periods when there were vocalizations. We performed chronic offline stimulation with three different stimulation intensities: a weak single-pulse stimulation (150 uA / 5 Hz), a strong single-pulse stimulation (300 uA / 10 Hz) and a strong paired-pulse stimulation (300 uA / 5 Hz). The stimulation consisted of biphasic current pulses that were timed according to a Poisson process. The control group was not stimulated. The morning after the stimulation was turned on, or at a corresponding age of the control birds, we started tutoring the juveniles in a controlled manner. Every morning they were exposed to an adult male tutor that would sing to them for approximately 90 minutes per day. The tutor song was the only exposure to song except for their own song.

The vocalizations inside the recording chamber were monitored and recorded. After the offline stimulation phase we tested the effect of the NIf stimulation on ongoing song production by song-triggered stimulation, and removed the tether cable. At the end of the experiment we sacrificed the birds and extracted the brain for histological analysis. The recorded vocalizations were analyzed and we searched for stimulation-induced effects on song development.

### Table 2.1 – Birdcount by stimulation paradigm. The total number of birds was \( n = 40 \).

<table>
<thead>
<tr>
<th></th>
<th>move-stim</th>
<th>rest-stim</th>
<th>all-stim</th>
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<tr>
<td>Weak single-pulse</td>
<td>2</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Strong single-pulse</td>
<td>2</td>
<td>2</td>
<td>-</td>
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<tr>
<td>Paired-pulse</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>

2.2 Surgery and implantation

For the chronic stimulation we bilaterally implanted juvenile zebra finches with bipolar stimulation electrodes in NIf at around 40 dph. Birds were anesthetized and fixed in the stereotactic apparatus at a head angle of 65° degrees. The electrodes were positioned according to stereotactic coordinates (see schematic in Figure 2.2(a)). The reference point of the coordinate system is known as ‘Lambda’, located where the blood vessels between cerebellum and the two forebrain hemispheres meet. In juvenile birds this point of reference is not always unambiguously visible, introducing a certain
Methods

(a) Electrode positioning

(b) Stimulation response bird b11r16

**Figure 2.2** – Electrode implantation and stimulation response: (a) The stimulation electrode was positioned in NIf according to stereotactic coordinates and fixed to the skull with dental cement. We inserted a recording electrode in HVC to record stimulation induced multi-unit activity. HVC is the primary downstream target of NIf. (b) Overlay of multiple extracellular traces recorded in HVC aligned to stimulation pulses in NIf (biphasic current pulses of 0.2 ms pulse width, 150 µA current intensity). About 2 ms after the stimulation pulse there is a burst of activity in HVC that lasts for approximately 10 ms. The strong stimulation response in HVC to NIf stimulation proves the proper positioning of the stimulation electrode.

level of uncertainty about the coordinates. We will discuss the effects of this uncertainty in Section 3.1.1. We bilaterally opened craniotomies of 2.0–3.5 mm AP and 1.5–1.9 mm ML from Lambda. The handmade stimulation electrodes, each consisting of two parallel stainless steel wires separated 1.0 mm and de-insulated tips (approx. 250 µm), were inserted rostro-caudally at an angle of 65° from the horizontal plane and parallel to the midline. The electrodes were positioned at 3.2/2.2 mm AP (anterior and posterior steel wire), 1.7 mm ML and inserted to a depth of 2.7 mm DV (anterior steel wire), where they were attached to the skull with help of insect pins and dental cement.

After the electrodes were implanted we tested their stimulation effectiveness by recording the stimulation response to NIf stimulation in HVC, the primary downstream target of NIf (Figure 2.2). We placed an extracellular recording electrode in HVC and stimulated NIf with biphasic current pulses of 0.2 ms pulse width. The current intensity was varied to determine current threshold at which the stimulation response in HVC became apparent. For all birds included in our analysis the stimulation worked properly during the surgery and we recorded stimulation responses in HVC at currents that were lower than the current used during the subsequent chronic offline stimulation phase.

We did not record from nuclei other than HVC to minimize the overall surgery time. After the first couple of surgeries we found that the stimu-
2.3 Tutoring song-isolate juvenile zebra finches

2.3 Tutoring song-isolate juvenile zebra finches

Juvenile zebra finches were raised in song isolation by their mothers and were never exposed to any male song, neither adult song nor the early subsong of their siblings. At 35 dph, the onset of the sensorimotor learning phase, we placed the juveniles individually into sound proof recording chambers. We delayed the first exposure to a song model to after implantation of the electrodes, or to a comparable age for the control (non-stimulated) birds. We tutored the juvenile birds by exposing them to an adult male tutor. These tutors had been tutored themselves by button-press triggered song playback. An example of song development in one of the tutors is shown in Figure 14. The tutors did all achieve a precise imitation of the same song template and their songs did not differ much: the mean similarity of the six tutors was at 80% (see Section 2.4). We therefore did not expect to see differences in song development due to different complexities in song models. Note that we chose to use live tutors instead of tutoring with song playback, as the overall percentage of birds that learn from playback alone is quite low (own observation and [DPK+12]) and social interaction is known to be important for zebra finches and helps to reduce the strain of the experiment. In previous experiments we have found that the learning success with button-press triggered playback significantly depends on the motivation of the juvenile bird: the number of tutor songs was restricted, and the shorter the time window within which the playback songs were consumed, the better the final imitation of the tutor song.

Every morning we inserted a tutor into the recording chamber with the juvenile bird for about 90 minutes. We only analyzed juvenile vocalizations when they were in isolation because song separation in different birds is hard, especially when the juvenile’s song starts to resemble the tutor’s song. The number of tutor songs per tutoring session was variable, usually hundreds of song motifs. It is documented that songbirds can copy a model song with very limited exposure [PMN92]. In zebra finches 40 playbacks of the...
song motif per day, lasting a total of 30 seconds, can be sufficient for fairly complete imitation [TLMN99]. The exposure time in our experiment should thus suffice for good imitation of the tutor song. Across days, each bird was exposed to only one tutor.

2.4 Song recordings

We recorded the vocalizations of the experimental birds throughout development. The microphone signal was band-pass filtered between 100 Hz and 10 kHz, and was digitized at a sampling rate of 32 kHz with 16-bit precision. Because one hour of continuous recording results in 230 MB of data we decided to minimize the recording of undesired signals, whilst maximizing the percentage of recorded songs. We thus developed a method to identify zebra finch vocalizations.

To separate bird vocalizations from undesired signals such as wing flaps we made use of the harmonic structure of zebra finch song (harmonic sounds are characterized by signal intensity at every integer multiple of the fundamental frequency). On 16-ms sound windows we calculated the spectral density \( \Phi(\omega) \). We defined the harmonic power at a certain frequency as 

\[
 h(\omega) = \prod_{i=1}^{N} \Phi(i \cdot \omega),
\]

which is the product of spectral densities at the frequency \( \omega \) and multiples thereof (usually we chose \( N = 7 \)). Finally, we defined a harmonic level

\[
 H = \frac{\max_{\omega} h(\omega)}{\min_{\omega' < \omega} h(\omega')},
\]

which is higher for sounds with a harmonic structure than for broadband sounds of the same overall intensity. We calculated \( H(t) \) as a function of time \( t \) discretized to 4 ms steps. Figure 2.3 illustrates the superior discrimination of zebra finch vocalizations provided by the harmonic level function \( H(t) \) in comparison to sound amplitude alone, i.e. root-mean-square (RMS) of the sound waveform in identical 16 ms windows.

We streamed the recorded sounds to a file on a hard disk whenever \( H(t) \) was above a given threshold for more than 50 % of the time within 0.6 s time windows. This criterion for song recording was evaluated every 4 ms. Each recorded file included one additional second of data both before reaching the recording criterion and thereafter. On a typical day we recorded about two hours of data, separated into 1,500 files. Typically, bouts of vocalizations triggered the recording, but recordings were not triggered by individual calls or noise such as wing flaps and similar. We only recorded cage noise when it was intermingled with vocalizations (see Figure 2.3).
2.4 Song recordings

Figure 2.3 – Song recording. We used the harmonic function $H(t)$ (red line, lower panel) to separate bird vocalizations from ambient noise. The signal consists of a song with two motifs, followed by wing flap noise and a long harmonic call. The values of the harmonic function are higher for the bird’s vocalizations than for noise, whereas the RMS power (white line, lower panel) is more variable and higher for noise than most song syllables. This figure is printed from a MATLAB program called filebrowser developed by Andreas Kotowicz, a PhD-student at the Institute of Neuroinformatics.

(a)  
(b)

Figure 2.4 – Motion detection. We used webcams with infrared filters to observe the movement of the juvenile birds. (a) The webcam captured images of the birds in the plexiglass cage at a frame rate of 10 Hz. (b) Motion was detected by calculating differences between successive frames. The main difference between frames stems from the movement of the tether cable that amplifies head movement.
2.5 Motion detection

One of our goals was to test the roles of brain activity during sleep and waking periods for song learning. In our experiments it was impossible to distinguish sleeping from waking states using traditional methods of electroencephalography (EEG) recordings [TB88, JVC+08] because electrical stimulation leads to large stimulation artifacts that prevented us from classifying EEG signals into different sleep and waking states. We therefore used movement behavior as a simple proxy for assessing wake/sleep state: a sleeping bird barely moves, whereas a waking bird typically exhibits continuous movement behavior.

Movements of the juvenile birds were observed with a webcam. In addition to the bird lamp that operates on a regular day-night cycle, we illuminated the cage with an infrared light emitting diode (LED). The webcams were equipped with an infrared filter and, thereby, infrared image acquisition was independent of the bird lamp. The images covered the entire area of the plexiglass cage. We acquired images at a frame rate of 10 Hz and calculated difference images between subsequent frames (Figure 2.4). We divided each image into a grid of 10 by 10 segments and calculated in each segment the intensity change defined as the mean absolute pixel difference. We defined the motion value at a given time as the maximum intensity change over all segments. We chose small segments to minimize dependence of the measured motion value on the bird’s location (close to the camera or far away).

We encountered two difficulties with motion detection. First, the cameras did not always operate stably over the entire course of the experiment (there were occasional system crashes). Second, the webcams and microphones were not always connected to the same computer (we communicated motion values via the local network using a user datagram protocol (UDP) connection). Whenever either the image acquisition software crashed or there was a network problem, the motion values were no longer transmitted and the electrical stimulation was no longer influenced by movement. Our software was such that the experimenter received a warning email whenever there were no incoming motion values, to make it possible to quickly restore the normal system operation. In rare cases of network problems, when the warning email could not be sent, we restored normal operation during one of the frequent checks of experiment progress. Overall, problems with the motion detection were rare events: Only in the case of three birds we encountered problems with motion detection. For two of the birds the stimulation was not contingent on movement and a third bird was stimulated during all offline time periods instead of offline no-movement periods for two days.

We divided the bird’s behavior into movement and no-movement periods by thresholding motion values. The motion values changed at a rate of 10 Hz,
2.5 Motion detection

Figure 2.5 – Movement behavior. We monitored the fraction of time the juvenile birds were considered to be moving throughout development and illustrate the values for three birds with different movement-contingent stimulation (color coded, averaged per hour; [a] rest-stim, [b] move-stim, and [c] all-stim). Black crosses mark the time points at which the tether cable was attached and removed, respectively. Black circles mark the time points at which the stimulation was switched on and off. The birds were predominantly moving during daytime and resting during the night, were more active in the morning than in the afternoon, and there was less movement in the beginning of the night than in the early hours in the morning. Whenever the tether cable was attached the movement values were higher.
much faster than the behavioral state changes we are interested in. Awake birds often hopped around with short intermittent pauses of less than two seconds duration. We thus extracted the onset of non-movement periods by motion values that remained subthreshold for a duration of two seconds. The only two parameters used to distinguish movement from no-movement periods were the motion threshold and the duration of this motion detection window. We used this division into movement and no-movement periods in our various stimulation paradigms.

For each bird and each hour we calculated the fraction of time in which there was movement. These fractions are shown for three birds that were subjected to different stimulation paradigms in Figure 2.5. The fraction of movement appeared much larger when the birds were connected to the setup with a tether cable than in untethered birds (see Figure 2.4(b)). We did not record and analyze movement of control (unstimulated) birds, because these were not implanted with stimulation electrodes and were not tethered; which is why their movement could not be compared to that of stimulated birds.

2.6 Electrical stimulation

Electrical stimulation in NIf was restricted to offline time periods. We delivered Poisson stimulus trains, i.e. trains in which inter-stimulus intervals were exponential random variables that were independent and identically distributed. Delivered stimuli were logged continuously to disk. 11 birds were stimulated at a mean rate of 5 Hz, 4 birds were stimulated at a mean rate of 10 Hz, and 8 birds were stimulated at a rate of 5 Hz with paired-pulse stimulations (see Table 2.1).

Whenever a harmonic vocalization of either the juvenile bird or its tutor was detected, stimulation was blocked and resumed one second after the end of the harmonic vocalization (Figures 2.6 and 2.7). Harmonic vocalizations were defined as times at which the harmonic function $H(t)$ exceeded a given threshold (Equation 2.1 in Section 2.4).

In Figure 2.7 we show exemplary histograms of stimulus times relative to song onset for songs that lasted at least one second. As can be seen, the stimulation rate drops to zero roughly at song onset — note there was a small latency of less than 10 ms between harmonic sound detection and stimulus blocking, explaining why a few stimuli were delivered after harmonic sound onset (Figures 2.7(a) and (c)). In this Figure, the minimum blocking period was two seconds, corresponding to the minimum song duration plus 1 s blocking after song offset. Two seconds after song onset, the stimulation density increased gradually. We observed a gradual increase in stimulation density before song onset and the gradual decrease in the stimulation density after song offset (Figure 2.7): birds sing bouts in which multiple songs follow each other with short latency, and thus there were songs before song onsets
2.7 Quantification of song development

For each bird we assessed the development and quality of its tutor song imitation. We used an established method to calculate song similarities: Ofer Tchernichovski and colleagues have developed a measure of song similarity [TCH+00] specialized for zebra finch song (also, they are frequently revising the measure). This similarity measure is an estimate of the proportion of sounds in two vocalizations that correspond with each other. Correspondence is established based on features such as pitch, frequency modulation, amplitude modulation, Wiener entropy, spectral continuity, and syllable duration. The measure has been widely used in the songbird community and is implemented in a software called Sound Analysis Pro. We used the standard parameter settings provided with the software.

We compared the developing song and tutor song on the level of song motifs. In the next section we develop a method for song motif selection that allows us to select the most representative song motifs from developing variable songs.

We illustrate our song analysis methods on one of our experimental birds named k5r16 (Figure 2.8). This bird was first exposed to tutor song at the age of 47 dph. At 50 dph the syllable sequence consisted of repetitions of a single syllable, revealing that the juvenile copied the tutor song using a serial repetition strategy. Between 50 dph and 60 dph the song motif emerged and continued to develop on the syllable level thereafter.
Methods

Figure 2.7 – Stimulation histograms aligned on song onsets and song offsets. Stimulation pulses were blocked by vocalizations and restarted one second after the vocalization offset. For two birds ([a] and [b]: p4r16, 5 Hz stimulation; [c] and [d]: b11r16, 10 Hz stimulation) we plot the histograms of delivered stimulation pulses aligned to song onset (left column) and song offset (right column). For each bird we selected all songs for one day of singing with minimum duration of one second and inter song interval before and after the song of one second. For each histogram we plot five examples of included songs. Stimulations were blocked 10 milliseconds after the song onset, corresponding to the response-delay of the recording-system. For a minimum of two seconds all stimulations were blocked, one second of minimum song duration and one additional second after song offset.
2.7 Quantification of song development

Figure 2.8 – Song development. The juvenile bird k5r16 was first exposed to the tutor (lowermost spectrogram) at the age of 47 dph and gradually copied the tutor song. The final song motif was present from 60 dph on after which the song continued to develop on the syllable level. The adult song at 95 dph (iACDE) was very similar to the tutor song (iACDE). The white scale bar in the topmost spectrogram denotes 0.1 seconds and the spectrograms include frequencies from 0 to 8 kHz.
2.7.1 Clustering of syllable archives

In a first step of our analysis we created archives with all syllables, calls, and noises that were recorded during the experiment. All files were recorded at 32 kHz and for each file we calculated (i) the RMS values on sliding windows of 16 ms duration and non-overlap of 1/16 ms, and (ii) the spectrogram with a frequency resolution of 62.5 Hz and a time resolution of 4 ms. From these recordings we extracted sound chunks defined by RMS threshold crossings, referred to as (archive) ‘elements’. On a typical day such an archive consisted of approximately 30,000 elements. From the onset of each element we defined cluster windows by considering spectrograms containing 30 time bins (120 ms duration) and 128 frequency bins (frequencies up to 8 kHz). Such cluster windows constitute fingerprints of elements. The size of the cluster window was chosen in a tradeoff between including most parts of long syllables and not combining several short syllables into the same window: sometimes two introductory notes were combined in the same cluster window, but this did not affect the clustering as there were plenty of elements for each interval between the introductory notes.

We clustered the cluster windows using the Euclidean distance. For computational reasons we reduced the dimensionality of the cluster windows from $30 \times 128 = 3,840$ to 100 using principal component analysis (PCA). The 100 largest principal components covered most of the variance in the data (usually more than 99%). Figure 2.9 illustrates 16 consecutive elements of such an archive, with the original cluster window in the top panel and the projections onto the 100 principal components in the bottom panel.

To improve automated clustering we reversed the timeline of song development: we used a recursive procedure to sort the elements in archive of day $i - 1$ by using day $i$ as reference. On the last experimental day the

Figure 2.9 – Syllable archives. We created archives of syllables, calls and noises, and defined a spectrogram of 120 ms duration aligned to the onset of the sounds as cluster-window (upper panel, 16 consecutive archive elements). To relate the archive elements to each other we projected these windows onto the 100 largest principal components (lower panel) and used the Euclidean distance as distance measure. These projections maintain the major part of the sound structure.
2.7 Quantification of song development

Figure 2.10 – Syllable development. The syllables of the final song motif emerge at different points in time and develop individually. At 56 dph for example syllable S3 is very similar to the final version, while syllable S4 only appears at 58 dph. The syllables are tracked back in time by an iterative and automated procedure.

Bird’s song was typically stereotyped and consisted of a small set of distinct syllables. In the archive of this last day we manually assigned each syllable type to a different cluster and added clusters for introductory notes, calls and noise. We iteratively sorted archive of day $i - 1$ based on nearest neighbor distance to elements in archive $i$: an element of archive $i - 1$ is assigned to cluster $j$ if the nearest neighbor of this element in archive $i$ lies in cluster $j$. Using this iterative procedure we tracked each syllable back in time, from the stereotyped song backwards through song development. We illustrate this process for bird k5r16 in Figure 2.10. The song of this bird consists of four syllables (see Figure 2.8) that we tracked from 95 dph back to day 48. The various syllables emerge at different time points during song development and change gradually towards their final version.

A difficulty in creating archives was the choice of sound RMS threshold to extract elements from the raw sound files: that threshold should be low enough to detect low intensity syllables, but high enough to separate different syllables into separate elements. Because we calculated the RMS values on sliding windows of 16 ms duration, this separation was especially difficult in cases in which inter-syllable intervals were short. In Figure 2.8 we show a song spectrogram and corresponding sound RMS curve to illustrate that tradeoff. Our preference was to choose a low threshold that resulted in occasional elements consisting of two concatenated syllables. We designed a procedure to automatically solve such concatenation problems: In the archive of the final day we sorted all the elements in a cluster by duration and defined an expected duration of corresponding syllables in the previous archive archive by the mode of the distribution plus two times the distance
between the mode and the 95% quantile. Elements that were assigned to a cluster by similarity whose duration was much longer than expected, were split into two new elements, the second of which was again clustered according to the procedure described above. Up to 15% of elements were split in this way, depending on the bird.

2.7.2 Syllable development and overnight deterioration

We used the syllable archives to analyze the development of individual syllables by calculating the mean Euclidean distance of each element to its 20 nearest neighbors within the same cluster. The distance to the 20 nearest neighbors decreases with increasing syllable stereotypy, shown for Syllable S3 in Figure 2.11. For the illustration we grouped the syllables in a particular cluster into groups of 100 by time of occurrence and calculated the median of the mean distance to the 20 nearest neighbors per group. The medians were normalized to the final median at the end of development. For the syllable S3 in bird k5r16 the median distance started to decrease at 54 dph, one week after the first tutor exposure, and it reached a stable value at 70 dph. Between 54 dph and 62 dph the median distance decreased rapidly, but not monotonically: it decreased during daytime singing and increased overnight. This effect is known as post sleep deterioration: it has previously been reported that syllable structure deteriorates overnight and stabilizes during daytime singing, especially during early song development [DMF+05]. Note that the distance decrease during the day is not due to a reduction in sound amplitude, because the sound amplitude actually increased during the day, which would lead to larger distances (ceteris paribus). At 70 dph the median distance stabilized and the overnight deterioration effect gradually disappeared.

On a typical day a bird sings about 3,000 repetitions of a given syllable. We assigned a goodness value to each of these repetitions to discriminate variable form stereotyped renditions. We sorted all renditions of a given syllable per day by their mean distance to the 20 nearest neighbors in an ascending order. We defined the ranking level $g$ as the index $n$ of a syllable rendition in that sorted array divided by the total number of renditions $N$, $g = n/N (1 \leq n \leq N)$. The syllable renditions shown in Figure 2.10 are the ones with the lowest (i.e. best) ranking level.

2.7.3 Selection procedure for song similarity calculation

For the calculation of song similarity we analyzed syllable transitions to determine the most representative syllable sequences. To this end we paired all syllables with an inter-syllable interval shorter than 50 ms and counted the number of transitions from syllable type $i$ to syllable type $j$. From these counts we extracted the syllable transition probabilities shown in Equation
Figure 2.11 – The stereotypy of syllables increases with age and the distance between renditions of a syllable decreases over the course of song development. Furthermore, this decay is non-monotonic: the distances decrease during the day and increase overnight. For each day we grouped the renditions of syllable $S_3$ by time into groups of 100 elements and calculated the median value of the mean nearest neighbor distance per group (blue lines, linear fit in red). The distances are normalized to the final value at the end of development.
for bird k5r16 at 90 dph.

\[
T^{90} = \begin{pmatrix}
    0.25 & 0.6 & 0 & 0 & 0 \\
    0 & 0 & 0.99 & 0 & 0 \\
    0 & 0 & 0 & 0.99 & 0 \\
    0 & 0 & 0 & 0 & 0.98 \\
    0.58 & 0 & 0 & 0 & 0
\end{pmatrix}
\] (2.2)

\( T_{i,j}^{n} \) is the probability that on day \( n \) syllable type \( i \) is followed by syllable type \( j \). Introductory notes are of type 1 and the syllables in the song motif are of type 2 and onwards, e.g. \( T_{1,1}^{90} \) is the probability that the introductory note is repeated, and \( T_{1,2}^{90} \) denotes the probability that there is a transition from an introductory note to the first motif syllable S1. Normally, the song at 90 dph consists of a stereotyped syllable sequence and the transition probabilities \( T_{i,i+1}^{90} (i = \{2, 3, 4\}) \) are very close to one.

\[ 1 - \sum_{j=1}^{5} T_{i,j}^{90} \] is the probability that the bird stops singing after syllable \( i \). In k5r16 the sum of the columns of \( T^{90} \) is close to one for all syllables but S4, which is the typical final syllable of a motif (syllable S4 is followed by an introductory note in 58% of the cases and by a song ending in 42% of the cases).

At 60 dph bird k5r16 sang all syllables that were present in its final song motif at 90 dph (see Figure 2.10). The preferred syllable transitions at 60 dph corresponded to the transitions at 90 dph, but the transition matrix at 60 dph was less sparse than at 90 dph.

\[
T^{60} = \begin{pmatrix}
    0.28 & 0.37 & 0.08 & 0.01 & 0 \\
    0.01 & 0.01 & 0.90 & 0.03 & 0 \\
    0 & 0.01 & 0 & 0.96 & 0 \\
    0.18 & 0.01 & 0.01 & 0 & 0.57 \\
    0.12 & 0.02 & 0.02 & 0.02 & 0
\end{pmatrix}
\] (2.3)

We defined songs as consecutive syllables with inter-syllable intervals shorter than 50 ms. We calculated a song ranking level \( s \) for each song by combining song syllable and transition goodness: \( s \) was defined as the mean syllable ranking level \( g \) per song divided by the mean transition likelihood between the song syllables. The most representative songs were defined as the songs with the best song ranking level \( s \) and a duration close to the mode of the song duration distribution.

In Figure 2.12(a) we plot the duration of songs against their ranking at 90 dph. Most songs are 0.75 seconds long and correspond to the duration of the song motif. We selected the ten song motifs with the best (i.e. lowest) and worst (i.e. highest) ranking levels and a duration within 0.05 seconds of the mode of the song duration distribution. For crystallized song the differences between highest and lowest ranked songs are subtle (Figures 2.12(b) and (c)).
2.7 Quantification of song development

Figure 2.12 – At 90 dph the song has crystallized and the differences between renditions of the song motif are subtle. The duration of the song motifs is stereotyped and the typical song motif lasts 0.75 seconds. The song ranking level depends mostly on the syllable ranking, as the syllable transition likelihood is equal for all complete song motifs. Spectrograms of the 10 most stereotyped song motifs (red dots in (a)). Spectrograms of the 10 least stereotyped song motifs (green dots in (a)). The white bar in the top spectrogram denotes 0.1 s and the spectrograms include frequencies from 0 to 8 kHz.
Figure 2.13 – At 60 dph the bird sings plastic song and the song motif starts to emerge. While this song motif is clearly visible in some vocalizations, it is not present in others. Our method of selecting the most representative songs allows us to extract the repetitive song motif from variable plastic song. (a) At 60 dph both song duration and ranking level are variable. (b) Spectrograms of the 10 lowest ranked songs (red dots in (a)). A repetitive song motif becomes apparent. (c) Spectrograms of the 10 highest ranked songs (green dots in (a)), consisting of variable plastic song. The white bar in the top spectrogram denotes 0.1 s and the spectrograms include frequencies from 0 to 8 kHz.
2.7 Quantification of song development

The same song selection criterion was used for the late plastic song phase. At 60 dph the vocalizations were not stereotyped yet: syllable S4 had just emerged (see Figure 2.10), the syllable structure was variable, and the syllable transitions were not close to unique yet. The distribution of song durations is multimodal, with one mode corresponding to songs containing S4 and the other mode to songs not containing S4 (Figure 2.10). In Figure 2.13(b) and (c) we show the ten lowest and highest ranked songs. The benefits of our song selection method are apparent: while the highest ranked songs are already very similar to the final crystallized song copy at 90 dph, the songs with low rankings are very variable versions of plastic song. Our method allows to select the emerging song motif from a pool of variable songs. All spectrograms shown in Figure 2.8 were selected with the here presented method.

2.7.4 Calculation of song similarity

For each day during song development we extracted the 10 most representative songs corresponding to the emerging song motif. For the comparison we extracted 10 tutor song motifs with the same method (Figure 2.14(a)). In Figure 2.8 we show spectrograms of selected songs from different days throughout development.

For every day we calculated the similarities between the 10 selected sequences and 10 tutor song motifs with Sound Analysis Pro, resulting in 100 similarity values (Figures 2.14(b) and (c)). The similarity values are very variable during the early plastic song phase and the mean similarity increases gradually from 50% at the beginning of tutoring to 80% on the last day of tutoring. The song similarity continues to increase after the tutoring phase. The song gradually crystallizes and the variability of song and similarity values decreases, as can be seen by the reduced span of the blue bars (Figure 2.15).

2.7.5 Distribution of syllable durations

During the subsong phase the syllable-duration distribution is broad and lacks any peaks. As soon as different syllables emerge, the syllable-duration distribution changes from a broad into a multimodal distribution. By plotting the distribution for each day of development we can assess the transition from subsong to plastic song and the age at which the song starts to crystallize. Syllable duration distributions can provide us with insights into the developmental state that can not be gained by the similarity to the tutor song, as juveniles can sing a perfect adult-like song during the plastic song phase long before the song has crystallized [KD11].

In Figure 2.16 we plot the syllable-duration distributions for k5r16. A first peak in the distribution emerges at 50 dph at a duration of 100 ms. The
Figure 2.14 – The variability of the similarity between the tutor song and the developing juvenile song decreases with age. (a) 10 tutor song motifs extracted for the calculation of song similarity. (b) The similarity values between the tutor song motifs and the juvenile song motifs at 60 dph (see Figure 2.13(b)) range from 56% to 98%. (c) All similarity values between the tutor song motifs and the juvenile song motifs at 90 dph (see Figure 2.12(b)) are above 75%.
Figure 2.15 – The similarity of the juvenile’s song to the tutor song increases with age. The black bar marks days with tutor song exposure and the red bar days with offline stimulation. The black squares denote the mean similarity $\mu$ and the blue bars span two standard deviations ($\mu \pm \sigma$). The development of these similarity values is consistent with the development illustrated by the spectrograms in Figure 2.8. As the song matures the stereotypy of the song motif increases and the standard deviation of the similarity values decreases.

Figure 2.16 – The distribution of syllable durations (color coded) changes with song development from a broad unimodal into a multimodal distribution with peaks for each syllable type. The distribution develops up to 73 dph, thereafter there are only minor changes.
juvenile bird’s song at 50 dph consisted of repetitions of similar syllables of similar durations (Figure 2.8). These syllable primitives subsequently develop into the different syllables present at later stages of development. The syllable-duration distribution changes from a broad distribution before 50 dph to a narrow unimodal distribution at 50 dph and then changes gradually into a multimodal and stable distribution at around 73 dph. The peak corresponding to syllable S4 (see Figure 2.10) is the last peak to appear. S4 is first observed at around 60 dph and develops into the longest syllable of the song (> 200 ms). Hence, two weeks after the first appearance of Syllable S4 the syllable durations stabilize.

2.8 Histology

At the end of the experiment we sacrificed the birds and extracted the brains for histological inspection (Figure 2.17). We made sagittal sections of 100 μm thickness. We verified that there was no implantation-induced tissue damages in addition to the traces of the stimulation electrodes. We were able to confirm the location of the electrode tips. The stimulation electrodes were implanted parallel to the midline and usually both tips of the electrodes were visible in the same sagittal section. In rare cases we had to inspect neighboring slices to localize both electrode tips. To delimit the location of NIf we injected a fluorescent tracer into HVC of the bird b11r16 and sacrificed the animal three days later. The bright field micrograph reveals the position of the stimulation electrode tips and the fluorescence micrograph reveals the location of NIf. The two electrode tips were in (posterior electrode tip) or close (anterior electrode tip) to NIf.
Figure 2.17 – Histology b11r16: (a) A bright field micrograph and (b) a fluorescence micrograph of a sagittal brain section. We labeled HVC and HVC projecting NIf neurons by injecting a fluorescent tracer in HVC that was taken up by HVC neurons and the axons of the neurons projecting to HVC. The fluorescent tracer travelled up these axons and labeled the cell bodies in NIf. White arrowheads mark the positions of the stimulation electrode tips. The stimulation electrode was implanted in (posterior electrode tip) or in close vicinity (anterior electrode tip) to NIf.
Chapter 3

Results

3.1 Chronic NIf stimulation

Electrical stimulation had been used in the past in songbirds to perturb ongoing neural activity. Stimulation had been applied to nuclei in the SMP and AFP during song production to study their motor functions. Stimulation in HVC had effects mostly on the song motif level, whereas stimulation in RA had effects mainly on the syllable level [VMK94]. Unilateral stimulation in LMAN during song led to real-time modulation of the ongoing song pattern [KDB05], including changes in song amplitude or pitch. Electrical stimulation in premotor nuclei that are within the recurrently connected song system, but not in output structures such as nXIIIts, can disrupt the ongoing song motor sequence [AWS05].

Electrical stimulation in HVC led to short-latency readjustment of the activity in the contralateral HVC, revealing mechanisms of interhemispheric coordination [VSM05]. In the singing bird unilateral HVC stimulation led to observation of an interesting property of song control: the behavioral effectiveness of the stimulation rapidly switches between hemispheres during song [WHKH08].

Studies were not only performed in the motor pathway but also in the auditory pathway: manipulating the singing-related activity of feedback-sensitive thalamic neurons by electrical stimulation subsequently triggered vocal plasticity [LM10].

1stimulation protocol: 7 biphasic pulses at 400 Hz, 0.4 ms per phase, currents from 30-70 µA.
2stimulation protocol: 10-220 monophasic pulses at 400 Hz, alternating phase, 0.4 ms per pulse, currents from 10-100 µA.
3stimulation protocol: 5 biphasic pulses at 400 Hz, 0.4 ms per phase, currents from 15-60 µA.
4stimulation protocol: single biphasic pulse, 0.2 ms per phase, currents from 100-1000 µA.
5stimulation protocol: 60 biphasic pulses at 300 Hz, 0.3 ms per phase, currents from 17-40 µA.
In our experiments, we restricted stimulation to offline time periods and made it contingent on movement. The percentage of time in which stimulation was ongoing ranged from 30% to 70%. The number of stimulation pulses per day for a single bird ranged from 130,000 to 600,000 (86,400 seconds per day, 5 or 10 Hz stimulation frequency, 30% to 70% of unblocked time periods). Chronic electrical stimulation could cause brain tissue damage; therefore, we have to assess the extent to which simulation caused lesions in and around NIf. In the following Section we show the effects of NIf stimulation on downstream targets, affirm that stimulation is effective over long time period (i.e., does not diminish over the time course of an experiment), and show that chronic NIf stimulation does not lead to tissue damage.

3.1.1 NIf stimulation responses in HVC and RA

NIf stimulation did not induce any obvious immediate changes in behavior; therefore, we were not able to assess effectiveness of simulation by simply observing birds. We assessed the effect of a single current pulse in NIf in terms of induced changes in activity in downstream nuclei HVC and RA, and the strength of NIf stimulus effects as a function of the location of the stimulation electrodes: the goal was to mimic variations of electrode position in our experiment.

In acute experiments in head-fixed anesthetized adult male zebra finches, we placed extracellular recording electrodes in HVC and RA and a pair of stimulation electrodes in NIf. The stimulation electrodes were identical to the ones used for chronic implantation: the stripped tips of the stimulation electrodes are large (approximately 250 µm long) and the distance between the tips was approximately 1 mm.

We found that stimulation responses in HVC and RA did not critically depend on the position of stimulation electrodes; stimulation responses were qualitatively similar over a wide range of positions (Figure 3.1). In HVC, NIf stimulation led to an activity burst of 10 ms duration, with a response onset 2 ms after stimulation onset. In RA, NIf stimulation led to a similar response with onset latency of about 7 ms. The spatial range over which we found reliable downstream activation by NIf stimulation was wide (>500 µm).

These experiments were performed on adult male zebra finches. While the connections from NIf to HVC are already established in juvenile birds singing subsongs, the functional connection from HVC to RA develops only throughout the process of song learning. We thus assume that the strength of NIf stimulation on RA activity develops in parallel with the ontogeny of song.

6These auxiliary experiments were part of a semester project conducted by Balthasar Bänninger at the Institute of Neuroinformatics.
3.1 Chronic NIf stimulation

Figure 3.1 – Stimulation responses in HVC and RA. Stimulation in NIf elicits a strong stimulation response in the song motor pathway, in both HVC and RA. The effects of a stimulation pulse are qualitatively similar over a wide range of stimulation electrode positions: stimulation at (a) 2750 µm DV and (b) 3250 µm DV both lead to a burst of activity in HVC at a latency of 2 ms and a burst of activity in RA at a latency of 7 ms.
In all juvenile birds that were implanted with stimulation electrodes we recorded NIf stimulation responses in HVC during the implantation surgery ($n = 23$ juveniles). In all birds we verified that the current threshold for stimulation responses in HVC was lower than the currents applied during chronic stimulation (see Table 3.1).

We do not assume in our experiments that the stimulation effects in HVC, RA, and elsewhere are caused by stimulation restricted entirely to NIf; rather, it is more plausible to assume that stimulation also affected auditory regions surrounding NIf.

### 3.1.2 Stimulation responses at the end of the experiment

Over the course of a chronic stimulation experiment we delivered hundreds of thousands of current pulses per day. We considered the possibility that NIf stimulation could lose its effectiveness on HVC and RA over the course of an experiment. Therefore, at the end of the experiment we performed another surgery on two of our experimental birds to record NIf stimulation responses in HVC, approximately 50 days after the implantation surgery. These two birds were stimulated during offline movement periods with 10 Hz 300 $\mu$A pulse trains during three weeks, resulting in a total of approximately 6,000,000 stimulation pulses that were delivered over the course of the experiment. In both birds we observed HVC responses above a single-pulse current threshold of 40 $\mu$A, well below the 300 $\mu$A that birds were exposed to during the experiment (Figure 3.2).

These findings confirmed that the effects of chronic offline stimulation were stable over time and that even millions of pulses did not reduce the effectiveness of NIf stimulation on activity in downstream HVC.

### 3.1.3 HVC activity in between stimulation pulses

Stimulations in NIf did not only lead to an initial burst of activity in HVC, but also led to changes in spontaneous activity over an extended period of time. While a strong stimulation pulse leads to a strong immediate activation of HVC it leads to an extended period of inactivity following the initial activation, a weak stimulation pulse leads to a weak immediate activation of HVC and increased activity in between stimulation pulses (Figure 3.3). In the case of the birds recorded at the end of song development shown above, weak stimulations led to more spontaneous activity in HVC in between stimulation pulses compared to spontaneous baseline activity and strong stimulation to a prolonged period of inactivity.

### 3.1.4 No stimulation induced tissue damage

Over the course of the experiment we regularly verified the correct flow of stimulus current by measuring the induced voltage drop over a 10 kΩ
3.1 Chronic NIf stimulation

Figure 3.2 – Sustained HVC activation by NIf stimulation. The stimulation response in HVC to NIf stimulation is not reduced after the chronic offline stimulation phase and the intensity of the evoked activity directly following the stimulation pulse is positively correlated with the stimulation intensity. For the two birds b11r16 (left column) and b12r16 (right column) we show spontaneous activity recorded in HVC ((a) and (b)), the stimulation response in HVC to NIf stimulation at a current intensity of 50 $\mu$A ((c) and (d)), the stimulation response at a current intensity of 100 $\mu$A ((e) and (f)), and the stimulation response at a current intensity of 300 $\mu$A ((g) and (h)).
Figure 3.3 – Activity in between stimulation pulses. The amount of spontaneous activity in HVC in between stimulation pulses is negatively correlated with the stimulation intensity. For two birds ((a) b11r16; (b) b12r16) we calculated the average spontaneous activity (RMS energy) between stimulation pulses for different stimulation intensities (upper panels, more than 50 stimulation pulses per current intensity, stimulation artifacts were removed, current intensity in $\mu$A). From these averaged RMS curves we subtracted the mean RMS of baseline activity without stimulations and plotted the cumulated sum over time in the lower panels, starting after the primary stimulation response (at 30 ms after the stimulation pulse): strong stimulation abolishes spontaneous activity (negative slope, less activity than baseline), whereas weak stimulation leads to an increase of spontaneous activity (positive slope, more activity than baseline).
3.2 Motion contingent stimulation

The offline stimulation in our experiment was contingent on movement (see Section 2.1 and Table 2.1): one group was stimulated during all offline time periods (all-stim), one group during offline movement periods (move-stim), and one group during offline no-movement periods (rest-stim). The offline stimulation was designed to alter the naturally occurring state-dependent activity patterns such as sleep bursts in the SMP [HF07] or increased firing in NIf after vocalizations [LS11]. To assign differences in song development to the desired effects of our stimulation, we had to ensure that the offline stimulation did not alter other features of behavior that indirectly affected the song learning process in some sort of feedback loop: Birds that were stimulated during the movement periods could have tried to avoid the stimulations by not moving anymore. Or birds that were stimulated during no-movement periods could have moved more and physical fatigue could have led to impaired learning. In this section we describe the mutual relationship between the stimulation protocol and movement.

3.2.1 Daily stimulation density in all 3 bird groups

We compared stimulation density between birds subjected to different stimulation paradigms. All stimuli were logged for each bird, allowing us to measure the fraction of time in which stimulation was active or blocked, respectively. For each hour we calculated the ratio between the total number of stimuli delivered and the theoretical limit, and calculated the stimulation density as the median ratio per hour over the entire experiment (Figure 3.4). During daytime, when the birds were animated and moving a lot, the stimulation density was equally high for the move-stim and the all-stim birds (red and black), whereas the stimulation density was almost zero for rest-stim birds (green). During nighttime, when birds were resting, the stimulation density was similarly high for rest-stim and all-stim birds (green and black), whereas stimulation density was low for move-stim birds (red). In move-stim and all-stim birds, the daily stimulation densities were opposite to the known trend of song behavior: the frequency of vocalizations decreased throughout
Figure 3.4 – During the night birds with all-stim (black) and rest-stim (green) were stimulated intensively, whereas birds with move-stim (red) received little stimulation. During the day the stimulation was intensive for the all-stim and move-stim birds, whereas the birds with rest-stim received little stimulation. The increase of the stimulation density (fraction of time with active stimulation) during the daytime for the all-stim and move-stim birds reflects the decreasing vocalization frequency. Each bird is plotted with single dots and the mean density per movement paradigm as line.
the day, whereas the stimulation densities increased from about 30% in the morning to about 70% in the late afternoon.

On average across days and birds, move-stim birds were stimulated 35 ± 5% ($\mu \pm \sigma$) of the time (range 30% to 43%, $n=6$ birds), rest-stim birds were stimulated 43 ± 4% of the time (range 38% to 48%, $n=11$ birds), and all-stim birds were stimulated 72 ± 5% of the time (range 63% to 76%, $n=6$ birds).

### 3.2.2 Stimulation and movement in all 3 bird groups

We analyzed the movement behaviors of birds subjected to offline stimulation. For each bird and each hour we calculated the movement density as the ratio of time with movement (Figure 2.5). For each bird we then computed the median hourly movement density, providing us with a daily movement pattern per bird. We used the median instead of the mean, because the median is robust against short time periods in which there were problems with motion detection (e.g. in the morning of day 59 for bird p8r17, Figure 2.5(e)).

The median movement density was in the range 80–100% during daytime and around 10% during nighttime (in the case of some birds, movement density was high even during nighttime). We compared hourly movement densities among birds that were subjected to different stimulation paradigms, Figure 3.5. Move-stim and all-stim birds moved equal amounts during daytime (Wilcoxon rank sum test, median density 95% in $n=6$ move-stim birds, median density 93% in $n=6$ all-stim birds). Both move-stim and all-stim birds moved more during the day than did rest-stim birds: the movement density of rest-stim birds was lower during a total of 8 hours of daytime (see blue asterisks in Figure 3.5).

The amount of rest changes over the course of the night: the movement density increased from the early to the late night hours (Figure 3.6). There was a positive correlation between time of night and movement density for 21 out of 23 birds. Interestingly, there were again differences between bird groups: move-stim birds showed the strongest increase in movement density across the night, whereas rest-stim birds showed the weakest increase.

In summary, we observed subtle differences in movement patterns between the various bird groups. We do not believe that these differences affected song learning abilities. Moreover, both during the day and at night, birds adapted their behavior to increase the number of received NIf stimuli, not to decrease that number. Thus, birds did not show stimulus-aversive behaviors and did not engage in stimulus-avoidance strategies, suggesting that stimuli were perhaps addictive but were not perceived to be noxious.

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7 Wilcoxon rank sum test, median density 91% in $n=11$ rest-stim birds, $p < 0.05$.
8 $p < 0.001$, binomial test.
9 Median increase in movement density per hour: 0.026 in $n=6$ move-stim birds, 0.012 in $n=6$ all-stim birds, and 0.006 in $n=11$ rest-stim birds.
Figure 3.5 – The movement density is high during daytime and low during nighttime (dots denote median movement density over all days with stimulation for each bird, line denotes the mean movement density per movement paradigm). During daytime the movement density is higher for birds with stimulation during periods of movement (all-stim and move-stim) than birds without stimulation during periods of movement (rest-stim): the blue asterisks mark hours where these differences are significant (Wilcoxon rank sum test, $p < 0.05$).
3.2 Motion contingent stimulation

Figure 3.6 – The movement density increased with the duration of the night. For 21 out of 23 birds there was a positive correlation between movement density and duration of sleep. Each dot denotes the median movement density for a bird, each line the mean values per paradigm.
3.3 Offline stimulation and song development

We analyzed the song development of 40 juvenile zebra finches (see Table 2.1). 23 birds were stimulated with different frequencies and currents and 17 were not stimulated (control group). Birds were divided into the following 4 groups:

- Control group: 17 birds
- Weak single-pulse group: 5 Hz single pulses of 150 µA, 7-14 days of stimulation, 11 birds (4 all offline (non-vocal) time periods, 5 offline movement periods, 2 offline no-movement periods)
- Strong single-pulse group: 10 Hz single pulses of 300 µA, 20-24 days of stimulation, 4 birds (2 offline movement periods, 2 offline no-movement periods)
- Paired-pulse group: 5 Hz paired pulses of 300 µA, 14-24 days of stimulation, 8 birds (2 all offline time periods, 2 offline movement periods, 4 offline no-movement periods)

We describe and quantify the song learning process in four parts. First, we show the results for the control group to establish a baseline for comparison with stimulated birds. In a second part, we show that weak single-pulse birds learn fast and precisely, even slightly better than controls. In a third part we show that strong single pulses do not impair song learning. In the fourth part we show that paired pulses impair song learning.

3.3.1 Song development in control birds

Song learning in the 17 control birds was very variable: both the similarity to tutor song and the speed of learning varied among individual birds. The mean similarity of control birds’ songs gradually increased from below 50% at the onset of tutoring to about 60% after two weeks of tutor exposure (Figure 3.7). The variability of song similarity remained large thereafter.

In Figure 3.8 we show the song development of two control birds, examples of a ‘good’ and a ‘bad’ learner. The ‘good’ learner (r15y2) was first exposed to a tutor at 37 dph. Only 11 days later, at 48 dph, he copied all 5 syllables of the tutor song motif in the correct order. The song of that bird continued to develop on the syllable level until it reached a similarity of about 80% two weeks after first exposure to the tutor. The mean similarity to the tutor song did not change after 55 dph, but the variance of similarity decreased. The ‘bad’ learner (g20r15) showed a completely different song.

\[^{10}\] The indicated stimulation frequencies correspond to mean rates of Poissonian stimulus trains unrestricted by vocalizations and movement.
Figure 3.7 – Similarity development for 17 control birds as a function of days after first exposure to the tutor. The mean similarity of all control birds (blue line) rises from below 50% to about 60% after two weeks of tutoring.
development: he was first exposed to tutor song at 38 dph, at 50 dph the song transformed into plastic song with little resemblance to tutor song, and at 70 dph the achieved song similarity was near 40%. Imitation was poor even though this juvenile was tutored in more than 20 sessions and the tutor was singing between 100 and 500 song motifs per session.

### 3.3.2 Weak single pulses are associated with fast and precise learning

The weak single-pulse group was stimulated with 150 $\mu$A single current pulses delivered randomly at a mean rate of 5 Hz and for a period of 7-14 days. There were 4 all-stim birds, 5 move-stim birds, and 2 rest-stim birds, yielding a total of 11 birds. In all birds, NIf stimulation during the implantation surgery led to reliable HVC activation. Note that 5 Hz stimulation corresponds to a mean inter-stimulus interval of 200 ms, which is shorter than the tutor song motif.

On the 14th day of tutoring the stimulation was stopped in all birds and only 3 of 11 stimulated birds produced an imitation of tutor song with similarity below the median similarity of the control group (Figure 3.9(c)). 8 birds produced a song with a tutor song similarity of 68% or higher, such precise imitation was achieved by only 2 of 17 control birds. Stimulated birds were not only better than control birds at imitating tutor song, they also learned so faster: on the 14th day of tutoring the song similarities were higher in stimulated than in non-stimulated birds ($p = 0.048$, Wilcoxon rank-sum test). We did not observe differences in song development between rest-stim, move-stim, and all-stim birds.

Surprisingly, weak single-pulse NIf stimulation (150 $\mu$A at 5 Hz) seemed to facilitate song learning and led to fast and precise imitation of the tutor song. As examples we show data from a move-stim and a rest-stim bird. The latter bird was a bad learner with a tutor song similarity below the median similarity of the control group and even for this bird there was no indication that the stimulation had detrimental effects. Both birds were stimulated for an extended period of time and learned the better part of their song motif during the stimulation phase. After the stimulation was stopped the song continued to evolve and gradually crystallized.

**p10r16 – Weak single-pulse rest-stim bird**

The bird p10r16 was stimulated for 14 days during no-movement periods. In these 14 days the bird acquired a large portion of tutor song.

The bird was implanted with stimulation electrodes at 39 dph and connected to the setup with a tether cable at 41 dph, after which stimulation started. During the implantation surgery we observed a response to NIf stimulation in HVC at low current intensities starting from 50 $\mu$A. The
3.3 Offline stimulation and song development

Figure 3.8 – Development of the song and the similarity to the tutor song for two different control birds, a ‘good’ learner r15y2 and a ‘bad’ learner g20r15. (a) and (b) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (c) and (d) The diagrams show the similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation. While r15y2 acquires a precise imitation within two weeks after the first exposure to the tutor, g20r15 does not copy the tutor song.
Figure 3.9 – Weak single-pulse stimulation leads to fast and precise learning. One week after tutoring onset the stimulated birds reach a median similarity (red lines) to the tutor song of 65%, two weeks after tutoring onset 73%. The stimulated birds were better imitators than the control birds (14th day of tutoring, \( p = 0.048 \), Wilcoxon rank-sum test). Each asterisk marks an individual bird. The colors of the asterisks color code the stimulation paradigm: blue corresponds to control, red corresponds to move-stim, green to rest-stim, and black to all-stim. The similarity values are shown (a) on the 7th day of tutoring, (b) on the 10th day of tutoring, and (c) on the 14th day of tutoring. On the 10th day of tutoring the stimulation was active for 7 out of 11 stimulated birds and only these birds are shown in the box plot. On the 14th day of tutoring the stimulation was stopped for all stimulated birds.
3.3 Offline stimulation and song development

Figure 3.10 – Song development p10r16 (weak single-pulse rest-stim): (a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
stimulation current of 150 $\mu$A was high enough to elicit a strong stimulation response in HVC (Figure 3.12(a)). The histology showed that the stimulation electrodes were placed properly (Figures 3.11(c) and (f)). On the morning of day 42 we started the tutoring sessions. On the last day with active stimulation, at 55 dph, the bird had learned a good imitation of the tutor song and the song similarity to the tutor song was close to 90% (Figure 3.10). The similarity rapidly increased starting from 51 dph, the final similarity value was reached at 56 dph. The juvenile bird properly copied two syllables from the tutor and merged another two tutor syllables into a single one. p10r16 was a fast learner and acquired his final song on the 15th day of tutor exposure. The song barely changed thereafter.

The distributions of syllable duration revealed major transitions during song development (Figure 3.11(a)): there was a major shift from 48 to 49 dph at which the peaks corresponding to the different syllables gradually appeared. Three distinct peaks were present from 56 dph on and the durations only slightly changed thereafter.

In accordance with the development of similarity there was a major change in syllable stereotypy that occurred between 52 and 57 dph (the days with strongest change were 54 and 55 dph), the last two days with offline stimulation (Figure 3.11(b)). The syllable stereotypy evolved monotonically and we did not observe post sleep deterioration effects.

The bird p10r16 was predominantly stimulated at nighttime. During some nights there was an increase of movement in the early morning hours that blocked the stimulations, which led to a drop of mean stimulation frequency from 5 Hz to an average of 3 Hz per hour (Figures 3.11(c) and (d)).

We tested the effects of a single stimulation pulse on song production: we detected a specific song syllable and triggered a stimulation pulse at a random delay after detection. This stimulation had no effect on song production at the current intensity that was used for chronic stimulation (Figure 3.12(b)). When we increased the current intensity to 300 $\mu$A and thereafter to 400 $\mu$A, clear effects of stimulation on song became apparent (Figures 3.12(c) and (d)).

In summary, the bird p10r16 was a fast learner that acquired a precise copy of the tutor song within two weeks while being stimulated.
3.3 Offline stimulation and song development

Figure 3.11 – Summary experiment p10r16: (a) Development of syllable duration distribution by age. (b) Syllable distance development by age. (c) Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. (d) Color coded mean stimulation frequency per hour for each day of the stimulation period. (e) and (f) Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.12 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current of 100 µA. (b), (c), and (d) Song triggered stimulation p10r16. The top panels show exemplary spectrograms of the bird's song motif. Each line underneath the spectrogram corresponds to the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. The effect of song triggered stimulation starts to be apparent at a current intensity 300 µA and is clearly visible at a current intensity of 400 µA.
p4r16 – Weak single-pulse move-stim bird

The bird p4r16 was stimulated for 13 days during offline movement periods. On the 14th day of tutoring this bird was one of the three stimulated birds with a similarity to the tutor song below the median similarity of the control group (see lowermost red asterisk in Figure 3.9(c)). The tutor song similarity developed in a non-monotonic manner, but there is no indication that the song development was hindered by the stimulation. The song motif emerged during the phase with active offline stimulation.

At 40 dph the bird was implanted with stimulation electrodes and the tether cable was attached on the afternoon of day 43. Thereupon the stimulation was switched on and we started tutoring the bird at 44 dph. On the afternoon of 56 dph the stimulation was switched off. The song motif of the juvenile bird was apparent at 52 dph and at 62 dph the song motif crystallized (Figure 3.13(a)). The similarity to tutor song did not develop monotonically but reached a minimum of 45% at 55 dph, after which it gradually increased to about 70% (Figure 3.13(b)). Although the similarity between the juveniles song and the tutor song was low at the end of the stimulation phase, there were no indications of impaired song development caused by stimulation.

The distribution of syllable duration evolved gradually from 48 dph to 62 dph, the age at which the song motif crystallized (Figure 3.14(a)). The syllable stereotypy showed gradual development similar to that of syllable duration, with the steepest increase up to 62 dph (Figure 3.14(b)). Stimulation was most intense during the afternoon when the bird was active without vocalizing. Also, there were a few stimulations during the early morning hours in which the bird moved, still in the dark (Figures 3.14(c) and (d)).

The stimulation electrodes were positioned properly in both hemispheres (Figures 3.14(e) and (f)); we recorded responses in HVC to NIf stimulation at a current intensity as low as 50 µA, well below the intensity of 150 µA that was chronically applied during the experiment (Figure 3.15(a)). When we stimulated in NIf during song production we found effect that depended on the stimulation current: at 150 µA there was no effect (Figure 3.15(b)), at 300 µA there were occasional stoppings, and at 400 µA there were frequent stoppings (Figures 3.15(c) and (d)).

The crystallized song motif of p4r16 was present at 62 dph, 18 days after first tutor exposure. However, the song motif emerged already during the offline stimulation phase and gradually developed towards a good copy of tutor song. The similarity to tutor song was low at the end of the stimulation phase, but closer inspection of song development did not indicate detrimental effects of the offline stimulation on song development.
Figure 3.13 – Song development p4r16 (weak single-pulse move-stim): (a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
3.3 Offline stimulation and song development

Figure 3.14 – Summary experiment p4r16: (a) Development of syllable duration distribution by age. (b) Syllable distance development by age. (c) Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. (d) Color coded mean stimulation frequency per hour for each day of the stimulation period. (e) and (f) Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.15 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current of 100 µA. (b), (c), and (d) Song triggered stimulation p4r16. The top panels show exemplary spectrograms of the birds song motif. Each line underneath the spectrogram corresponds the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. The effect of song triggered stimulation starts to be apparent at a current intensity of 300 µA and is clearly visible at an intensity of 400 µA.
Summary of weak single-pulse stimulation

11 birds were stimulated for a period of 7 to 14 days at a frequency of 5 Hz and a current of 150 µA. The stimulated birds copied the tutor song faster and more precisely than control birds. We did not observe differences between rest-stim and move-stim groups, possibly because of the small number of birds per group and the high inter-individual variability. In all birds we observed a stimulation response in HVC to NIf stimulation, well below the current that was applied during chronic offline stimulation: 150 µA pulses evoked a strong response in HVC. However, these pulses were not strong enough to perturb the ongoing motor program: song triggered NIf stimulation at 150 µA did not affect song production in all 5 birds with song-triggered stimulation. We saw the first song stoppings at 300 µA and reliable stopping at 400 µA.

Offline NIf stimulation with a current intensity of 150 µA at a frequency of 5 Hz did not affect on the ongoing motor program and seemed to have a facilitating effect on song learning that led to fast and precise imitation of the tutor song.

3.3.3 Strong single-pulse stimulation — no effect

For a second group of four birds we increased the stimulation current to 300 µA and changed the mean rate of unrestricted stimulation to 10 Hz (2 move-stim and 2 rest-stim birds). In comparison to the previous experiment, we quadrupled the stimulation intensity (we doubled both the stimulation current and the rate of pulses). Furthermore we extended the stimulation phase to a minimum of 20 days.

By doubling the stimulation frequency we reduced the time between individual pulses and we reduced the likelihood that there was normal neural activity in NIf at any given time during stimulation.

To our surprise, the stimulated birds showed normal song learning. Their learning was not as fast and precise as the weak single-stim group, but on the population level 3 out of 4 birds produced a higher similarity to tutor song than the similarity of the control birds after 20 days of tutoring (Figure 3.16). We show the song development for two stimulated birds that ended up with a similarity to the tutor song of more than 70% at the end of the stimulation phase. One bird was a move-stim, the other a rest-stim bird. For both birds there was no indication that the stimulation had a negative effect on song development.

b12r16 – Strong single-pulse move-stim bird

The bird b12r16 was stimulated for 20 days during offline movement periods at a current of 300 µA and a frequency of 10 Hz. Despite and during this
Figure 3.16 – Similarity development strong single-pulse stimulation: The song similarity to the tutor song of birds with single-pulse stimulation (move-stim in red and rest-stim in green) was similar to the control group (mean similarity in blue). At the end of the stimulation period (marked by rectangles) 3 out of 4 stimulated birds did sing a song with higher tutor song similarity than the mean similarity of the control birds.
Figure 3.17 – Song development b12r16 (strong single-pulse move-stim): (a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
very intensive stimulation the bird developed a song that contained several tutor syllables.

We implanted stimulation electrodes at 39 dph. We connected the bird to the setup with a tether cable at 45 dph and started the stimulation on the afternoon of day 46. We started the tutoring sessions at 47 dph. At 54 dph, only one week after the first tutoring session, the song motif of the juvenile bird closely resembled the tutor song motif (Figure 3.17(a)). The song continued to develop and at 66 dph, on the last day of stimulation, the similarity was above 70% (Figure 3.17(b)). Thus, the song motif developed during the stimulation phase.

The syllable durations evolved gradually until 63 dph after which durations were stable and the song continued to develop only on the syllable level (Figure 3.18(a)). The apparent change in syllable durations around 75 dph is due to a very subtle song change: two syllables that were separated after 75 dph by our analysis method could not be separated on earlier days and they were linked together in a single syllable. This did not affect our analysis. The syllable durations reached a stable state before the stimulation phase was over.

The syllable stereotypy was very low during development (Figure 3.18(b)). There were large daily changes, with an overall trend of increasing stereotypy during the day and a decrease of stereotypy overnight. The magnitudes of overnight deterioration and daily variance in stereotypy decreased with age, and almost vanished at approximately 72 dph.

The bird b12r16 was moving a lot throughout the day and was therefore intensely stimulated between vocalizations (Figures 3.18(c) and (d)). The average stimulation frequency during the day was around 5 Hz. Additionally, the bird was stimulated during movement at late hours of the night.

During surgery, stimulation was highly effective. Starting from a minimum current of 60 µA we observed a stimulation response in HVC (Figure 3.19(a)), in accordance with proper positioning of the stimulation electrodes (Figures 3.18(e) and (f)). At the end of the experiment, on day 83, we recorded again the stimulation response in HVC for the same single 300 µA pulses in NIf: approximately 50 days after the implantation surgery the stimulation response was very comparable to what we found during the implantation surgery (see Figure 3.2, right column).

Although HVC response to 300 µA NIf stimulation was strong both at the beginning and at the end of the experiment, we did not find effects of NIf single pulses on produced song amplitudes, even when song-triggered NIf stimuli were delivered at 500 µA (Figures 3.19(b) and (c)). However, when we stimulated with a paired-pulse paradigm, i.e. two biphasic pulses separated by 4 ms, there was a drastic effect of immediate song truncations (Figure 3.19(d)).
### 3.3 Offline stimulation and song development

**Figure 3.18** – Summary experiment b12r16: 

- **(a)** Development of syllable duration distribution by age. 
- **(c)** Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. 
- **(d)** Color coded mean stimulation frequency per hour for each day of the stimulation period. 
- **(e)** and **(f)**: Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.19 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current intensity of 100 µA. (b), (c), and (d): Song triggered stimulation b12r16. The top panels show exemplary spectrograms of the birds song motif. Each line underneath the spectrogram corresponds the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. There was no effect of song triggered stimulation with single pulses, even at a current intensity of 500 µA. Stimulation by two consecutive pulses with 4 ms delay led to constant stopping of the ongoing song.
3.3 Offline stimulation and song development

k4r16 – Strong single-pulse rest-stim bird

The bird k4r16 was stimulated for 23 days during offline no-movement periods with 300 µA single pulse trains of 10 Hz mean rate. Although the bird was not a fast learner and did not copy the tutor song within two weeks, the bird succeeded in copying three syllables of tutor song after three weeks into the stimulation. The bird merged the other two remaining tutor syllables into one new syllable. The largest increase in similarity to tutor song, the crystalization of the juveniles song motif, and the largest increase in syllable stereotypy all happened within the stimulation phase.

We implanted the stimulation electrodes at 42 dph. On day 46 the tether cable was attached and stimulation started. The tutoring sessions started at 47 dph. At the end of the stimulation phase, at 69 dph, the juveniles song reached a similarity to tutor song of more than 70% (Figure 3.20). The bird k4r16 was a slow learner: The song motif started to emerge only at 60 dph, 14 days after the first tutor exposure. From 60 dph to 67 dph the similarity to tutor song increased from 50% to 70%.

The syllable duration distribution exhibited the first peak at 56 dph (Figure 3.21(a)); it crystallized at around 77 dph, 10 days after the final song motif was present in the juveniles song. The syllable stereotypy showed the daily increases and nightly decreases between 64 dph and 69 dph, the days on which the song motif emerged (Figure 3.21(b)).

Stimulation density was high with an average above 7 Hz during the night when there was little movement and an average of 2 Hz in the late afternoon when the bird was inactive (Figures 3.21(c) and (d)).

Song-triggered stimulation on 78 dph with 300 µA single pulses did not perturb song production. However, stimulation with paired pulses separated by 4 ms led to occasional stoppings (Figure 3.22).
Figure 3.20 – Song development k4r16 (strong single-pulse rest-stim): (a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
3.3 Offline stimulation and song development

Figure 3.21 – Summary experiment k4r16: (a) Development of syllable duration distribution by age. (b) Syllable distance development by age. (c) Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. (d) Color coded mean stimulation frequency per hour for each day of the stimulation period. (e) and (f) Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.22 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current of 150 µA. (b) and (c) Song triggered stimulation k4r16. The top panels show exemplary spectrograms of the birds song motif. Each line underneath the spectrogram corresponds the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. The effect of song triggered stimulation is only apparent for the paired-pulse stimulation paradigm.
Summary of strong single-pulse stimulation

We stimulated a total of four birds during at least 20 days with 300 $\mu$A single pulse trains of 10 Hz mean rate (two move-stim and two rest-stim birds). They received each about 6,000,000 pulses.

We found no evidence that stimulation impaired the song learning process, despite the high intensity of stimuli: all birds were able to copy a significant part of the tutor song during the stimulation phase. After approximately three weeks of tutoring, at the end of the stimulation phase, three of four birds had developed a song with tutor song similarity higher than the mean similarity of equally tutored control (non-stimulated) birds.

3.3.4 Paired pulses hinder song learning

The paired-pulse groups were stimulated with two 300 $\mu$A biphasic pulses separated by 4 ms and delivered randomly at 5 Hz for a period of 14-24 days. There were 8 birds in total: 4 rest-stim birds, 2 move-stim birds, and 2 all-stim birds. By design, the total number of pulses received by these birds was comparable to the 10 Hz single-pulse birds.

Two weeks after first tutor exposure 6 of 8 birds produced a song with tutor song similarity below the mean similarity of equally tutored control birds (Figure 3.23). This ratio did not change after the stimulation was switched off: paired-pulse birds did not copy the tutor song as well as did either control birds or single-pulse birds.

In contrast to single pulses, paired pulses had non-negligible effects on song production. We illustrate poor song copying in one move-stim bird and one rest-stim bird.

g12r17 – Strong paired-pulse rest-stim bird

The bird g12r17 was stimulated for 24 days during offline no-movement periods with 300 $\mu$A paired biphasic pulses of 5 Hz mean rate. We implanted the stimulation electrodes at 45 dph, the tether cable was attached on the afternoon of day 46. The bird was stimulated from 46 dph to 70 dph and tutoring started at 47 dph.

Two weeks after tutoring onset, at 60 dph, syllables of the final song motif started to appear. A song motif emerged at 64 dph and did not change much until the end of the experiment at 83 dph, even though stimulation was switched off at 70 dph (Figure 3.24(a)). The similarity of the bird’s song to the tutor song stabilized near 50% and the final song motif was reached before stimulation was switched off at 70 dph (Figure 3.24(b)). Only one syllable was properly copied, but the other elements of the pupils song were not present in the tutor song.

The syllable duration distribution shows two glitches at 67 dph and 72 dph, which was caused by our method of splitting syllables but not by a
Figure 3.23 – Similarity development – 300 µA and 5 Hz paired-pulse stimulation: (a) Birds with strong paired-pulse stimulation were worse imitators of the tutor song than control birds (full-stim in black, move-stim in red, rest-stim in green and mean of the control group in blue). After two weeks of tutoring (black rectangle), 6 of 8 stimulated birds produced a song with tutor song similarity below the mean similarity of equally tutored control birds. This ratio did not change, once the stimulation was switched off (small squares). (b) Box plot of the similarities comparing the control birds to the paired-pulse stimulated birds on the 14th day of tutoring. The paired-pulse stimulated animals did sing poor copies of the tutor song and the similarity values were lower than the control group values.
3.3 Offline stimulation and song development

Figure 3.24 – Song development g12r17 (strong paired-pulse rest-stim):  
(a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. 
(b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
Results

significant syllable development (Figure 3.25(a)). The syllable stereotypy evolved slowly with few changes between 60 and 80 dph (Figure 3.25(b)). Overall, there was very little development of the song after 64 dph. The bird did not move much during the night but was stimulated with an average of 4 paired pulses per second during the night (Figures 3.25(c) and (d)).

During the implantation surgery we recorded a strong HVC response to NIf stimulation above a threshold of 25 µA (Figure 3.26(a)). Song triggered paired-pulse stimulation at 70 dph led to transient effects on song and song stoppings (Figure 3.26(b)).
3.3 Offline stimulation and song development

Figure 3.25 – Summary experiment g12r17: [(a)] Development of syllable duration distribution by age. [(b)] Syllable distance development by age. [(c)] Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. [(d)] Color coded mean stimulation frequency per hour for each day of the stimulation period. [(e)] and [(f)] Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.26 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current intensity of 100 µA. (b) Song triggered stimulation g12r17: the top panel shows an exemplary spectrogram of the bird’s song motif. Each line underneath the spectrogram corresponds the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. The stimulation led to transient effects and stop-pings.
b7r17 – Strong paired-pulse move-stim bird

The bird b7r17 was stimulated for 17 days during offline movement periods with a 300 μA paired pulses delivered at mean rate 5 Hz. At the end of the stimulation period the bird produced plastic songs with little resemblance to the tutor song. Only one syllable of the crystallized song motif was a precise copy of a tutor syllable.

We implanted stimulation electrodes at 45 dph and we attached the tether cable and turned on stimulation on the afternoon of day 46. The tutoring sessions started at 47 dph. The first syllables appeared after 10 days of tutoring at around 56 dph (Figure 3.27(a)). On the day we turned the stimulation off, at 63 dph, the similarity of the juvenile’s song to the tutor song was below 50%. The song continued to develop thereafter, but at 67 dph the similarity to tutor song stabilized near 55% (Figure 3.27(b)).

The syllable durations distribution developed gradually and reached a stable shape at 65 dph (Figure 3.28(a)). The syllable stereotypy did not decrease over the course of development, but the daily fluctuations reduced with increasing age (Figure 3.28(b)). The stimulation was most dense in the afternoons when the bird was moving but not vocalizing (Figures 3.28(c) and (d)).

The stimulation electrodes were implanted at the correct target location (Figures 3.28(e) and (f)) and during the implantation surgery we recorded a strong HVC response to NIf stimulation above a threshold current of 60 μA (Figure 3.29(a)). Song-triggered stimulation did affect the ongoing song production: paired pulses induced stoppings in most cases (Figure 3.29(b)).
Figure 3.27 – Song development b7r17 (strong paired-pulse move-stim): (a) The spectrograms illustrate the song motif at different stages of development with the tutor song motif in the lowermost panel for comparison. The white scale bar in the topmost spectrogram denotes 0.1 seconds. The spectrograms include frequencies from 0 to 8 kHz. (b) The similarity of the birds own song to the tutor song as a function of age. The black squares denote the mean similarity $\mu$ and the blue line spans two standard deviations from $\mu - \sigma$ to $\mu + \sigma$. The black bar marks days with tutor song exposure and the red bar days with electrical stimulation.
3.3 Offline stimulation and song development

Figure 3.28 – Summary experiment b7r17. (a) Development of syllable duration distribution by age. (b) Syllable distance development by age. (c) Color coded average percentage of movement periods per hour for each day. The crosses denote the point in time at which the cable was attached and removed, the circles the point in time where the stimulation was switched on and off. (d) Color coded mean stimulation frequency per hour for each day of the stimulation period. (e) and (f) Histology showing the stimulation electrode positioning for both hemispheres.
Figure 3.29 – (a) Stimulation response in HVC to NIf stimulation during implantation surgery at a current intensity of 100 μA. (b) Song triggered stimulation b7r17: the top panel shows an exemplary spectrogram of the bird’s song motif. Each line underneath the spectrogram corresponds the RMS energy of a single song motif. The time of stimulation is indicated by the green dotted line. The stimulation leads to stoppings in most cases.
3.3 Offline stimulation and song development

Figure 3.30 – Box plot of the similarity to the tutor song after three weeks of tutoring. The weak stimulation led to fast and precise imitation, the strong single-pulse stimulation to normal song copying and the strong paired-pulse stimulation to below average imitation. The movement-contingent stimulation paradigms are color coded: all-stim birds are plotted in black, rest-stim birds in green and the move-stim birds in red.

Summary of strong paired-pulse stimulation

The two paired-pulse birds (300 µA/5 Hz, paired-pulse stimulation) shown were less successful in copying the tutor song than were either control birds and single-pulse birds. In both birds, song-triggered stimulation was highly effective and led to transient effects and stoppings.

3.3.5 Offline stimulation affects song learning

We have shown song development of the control birds and the birds belonging to three different stimulation paradigms: a weak single-pulse group, a strong single-pulse group, and a paired-pulse group.

Weak single-pulse birds (150 µA, 5 Hz) learned fast and precisely, strong single-pulse birds (300 µA, 10 Hz) learned less well, and paired-pulse birds (300 µA, 5 Hz, paired pulses) were poor learners. When evaluating song similarity after three weeks of tutoring we found large differences in tutor song similarity (Figure 3.30 and Table 3.1). The median similarity of the control group was 63%. 9 of 11 birds in the weak single-pulse group learned a song with higher similarity than 63%. The median similarity of weak single-pulse birds was 82.5%, higher than that of control birds (p = 0.058, Wilcoxon
rank-sum test\textsuperscript{11}. The strong single-pulse group achieved a similarity of 71%, comparable to that of the control group ($p = 0.14$, Wilcoxon rank-sum test). Paired-pulse birds achieved a median similarity of 54%, comparable to that of control birds ($p = 0.21$, Wilcoxon rank-sum test), and smaller than that of weak single-pulse stim birds ($p = 0.016$, Wilcoxon rank-sum test).

For this analysis we combined birds in different movement paradigms (all-stim, rest-stim, and move-stim birds). The number of birds per group and paradigm was not large enough to unambiguously discriminate between the effects of move-stim and rest-stim. However, song development of move-stim birds was comparable across stimulation groups: in move-stim birds we did not see either facilitating effects of weak single-pulse stimulation or impaired learning under paired-pulse stimulation. Such absence of effect could be explained by large inter-individual variability, or perhaps stimulation during movement phases has no impact on song imitation learning. If we only consider birds that were stimulated during rest (all-stim and rest-stim) then the difference between the weak single-pulse birds and the paired-pulse birds is highly significant ($p = 0.003$, Wilcoxon rank-sum test). This is an indication that stimulation during rest (and sleep) is responsible for the observed differences, but not the stimulation during movement periods.

\textsuperscript{11}The bird p20r16 was only recorded for 20 days after tutoring onset. If we include his tutor song similarity value of 68% at the end of song recording into the analysis, then the p-value equals $p = 0.043$. 
Table 3.1 – Similarity after three weeks of tutoring, stimulation response threshold, and stimulation duration for all birds and stimulation paradigms: (1) 150 µA single-pulse stimulation, (2) 300 µA single-pulse stimulation, (3) 300 µA paired-pulse stimulation; (a) all-stim bird, (m) move-stim bird, (r) rest-stim bird, and (c) control bird.
Chapter 4

Discussion

We explored effects of offline stimulation in NIf on vocal learning for different stimulation intensities and different movement contingent stimulation paradigms. The stimulations were either weak single pulses (150 $\mu$A, 5 Hz), strong single pulses (300 $\mu$A, 10 Hz), or strong paired pulses (300 $\mu$A, 5 Hz, paired pulse with 4 ms interval between pulses), and the juvenile birds were either stimulated during periods of rest (periods containing sleep), during periods of movement, or during all offline periods. We found differences in song development between the groups with different stimulation intensities: weak single-pulse stimulation led to fast and precise learning, and paired-pulse stimulation had a non-significant detrimental effect on song development.

4.1 Tutoring in experimental conditions leads to large variability of song learning performance

The zebra finch is a social animal and the main purpose of vocalizing is communication. Juvenile zebra finches learn their song best in a colony of birds, their natural environment. There exist various ways of training a juvenile zebra finch to sing in experimental conditions, but the juvenile birds tutor song imitation quality is negatively correlated to the extent of experimental control \cite{DPK+12}: one-to-one live tutoring is the best method to get a fairly complete imitation, birds with self-elicited tutor song playback show high inter-individual variability, and passive playback results in a poor imitation of the model song. Furthermore the imitation is better when the playbacks are not all delivered in the morning, but separated into two sessions per day or delivered in the evening before the lights are switched off.

In previous experiments we tutored juvenile birds with button-press triggered tutor song playback and found that the imitation quality depended on motivation to elicit song playbacks: The more eager the juvenile birds
pressed the button and the faster they consumed the limited number of playbacks, the better the final imitation. The total number of elicited playbacks did not correlate with imitation quality.

In the present study we used limited exposure (about 90 minutes per day) to a live tutor to train the juvenile birds. Nevertheless, the variability of the song learning performance was large. We observed differences in song development between birds that belonged to different movement-contingent stimulation paradigms and that were stimulated with different intensities, but we also observed differences within groups of identical experimental conditions. Even in the control group, the group with the least experimental stress, the inter-individual variability was considerable (see Figure 3.7).

The large baseline variability complicated the interpretation of the collected data: some effects might be hidden away and could only be traced with an experimental paradigm accompanied by a smaller baseline variability, as it is not feasible to excessively increase the number of experiments per condition. A more complex social setting during the experiment, for example by constant company of a female bird, might reduce the variability in the process of song learning and the additional experimental cost of separating vocalizations of non-isolated birds could be balanced by a reduction of the number of experiments.

In a recent study song-isolated juvenile zebra finches were exposed to live tutors for 2 hours per day for 5 consecutive days, beginning at 43-53 dph, and raised until adulthood in isolation [RGM+12]. The final tutor similarity of the control group in adulthood (>90 dph) was around 75%, comparable to the 70% we observed for the control group after 4 weeks of tutor exposure (at around 70 dph). However, from the published results we cannot assess the observed variability: for some experiments the similarity is stated for each control bird individually, for others they only state the mean similarity per group.

4.2 Representative song motifs can be extracted from variable plastic song

Additional to the differences between individual birds, there were large intraday differences for individual birds, specially during the plastic song phase [DMF+05]. During courtship behavior, juvenile birds are capable of producing song with much more mature properties, although they mostly sing immature song while isolated [KD11]. Therefore, not each produced song represents what the juvenile bird has learned to produce by that time.

We developed a method to select the most representative song motifs for each day of singing. We combined a measure on the syllable level (syllable stereotypy) and a measure on the motif level (syllable transition likelihood) to rank all syllable sequences and extract song motifs corresponding to the
4.3 The stimulation effects seemed to be restricted to the neural processes of the song system

most likely syllable transition consisting of the most typical syllable renditions. This method allowed us to assess song development by analyzing a small number of song motifs, and variable practice song did not blur the assessment of song development (see Section 2.7.3).

4.3 The stimulation effects seemed to be restricted to the neural processes of the song system

Despite the large variability between the individual birds we found that the three groups of birds with different stimulation intensities differed in the speed and precision of learning to imitate the tutor song. Birds with weak single-pulse stimulation in NIf learned faster and more precisely than did control birds. Birds with strong single pulse stimulation learned comparably to control birds, and birds with strong paired-pulse stimulation ended up with significantly lower similarity values compared to birds with weak single-pulse stimulation.

We ascribe these differences to the modulation of spontaneous neural activity and networks of the SMP. As judged by movement patterns, there are no indications that the offline stimulation led to unwanted effects such as sleep deprivation or changes in daily activity. We observed only small differences in movement patterns: birds with rest-stim moved less than birds with move-stim and all-stim. Hence, birds seemed to have adapted their movement to increase received stimulation and there was no modulation of movement behavior to avoid stimulation pulses. Similarly, we found no differences in vocalization activity between the birds with different stimulation paradigms. Therefore, offline stimulation seems to only mildly interfere with normal natural behavior.

4.4 NIf stimulation might affect the neural activity involved in offline learning in multiple ways

We pooled the experimental birds by movement contingency of stimulation and by the stimulation paradigm, and we only observed significant differences between the different stimulation paradigms (see Section 3.3.5).

We did not record the effect of NIf stimulation on spontaneous neural activity in song system during development, but only recorded the stimulation response in HVC to NIf stimulation with single pulses during the implantation surgery, and for two birds at the end of the experiment at 90 dph, after more than three weeks with chronic offline stimulation. In these birds we found that on a short timescale (< 25 ms) the stimulation intensity correlated with the amount of evoked activity in the SMP. But in between stimulation pulses the correlation between stimulation intensity and amount
of spontaneous activity was negative: strong stimulation pulses led to long periods of inactivity in HVC, whereas weak pulses led to an increase of spontaneous activity.

Several studies in humans have shown that excitability modulating transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) of the motor cortex M1 before, during or after learning a motor skill task, can affect the performance and offline performance gains. An increase of slow wave activity (SWA) during sleep can be triggered by a learning task and this increase correlates with improved performance of the task after sleep. SWA can also be induced by TMS: stimulation during sleep led (< 1 Hz) to a deepening of sleep and an increase in EEG slow wave activity, which is thought to play a role in brain restoration and memory consolidation. In rats, intracortical electrical stimulation with single current pulses (0.2–4.0 Hz) during sleep leads to slow waves similar to the naturally occurring slow waves in the local field potential. It is therefore conceivable that activity-enhancing stimulation can lead to performance improvement in a motor learning task.

Stimulation with single (0.5 ms duration) and paired pulses (biphasic pulses of 0.6 ms duration, 10 ms ISI) have been used in a couple of experiments in rats to study the effect of disrupting sharp-wave ripple events in the hippocampus on a spatial learning task: ripple events are characteristic for SWS and have been proposed to constitute a neural mechanism for sleep-dependent memory consolidation, and the disruption of these events leads to the impairment of a hippocampus dependent spatial learning task.

In birds that were stimulated with strong stimulation pulses we did not observe the fast and precise learning of the group with weak single pulse stimulation. However, although the number of stimulation pulses in the strong single-pulse group and strong paired-pulse group was comparable (10 Hz single pulse and 5 Hz paired pulse), we only observed impaired song learning in the paired pulse stimulation group. This is likely due to different effects of single pulse stimulation and paired pulse stimulation on network activity and on synaptic properties. In support of this idea, we have found that paired-pulse stimulation in NIf during song production led to song stoppings whereas single pulses did not affect song production.

There is an inherent difference between single spikes and bursts in the SMP. During song production, projection neurons in HVC fire one burst per song motif and neurons in RA fire multiple bursts per song motif; and bursting activity in the SMP is a characteristic of sensorimotor replay during sleep and depends on prior exposure to a tutor’s song. Furthermore, the effects of repeated stimulation with paired pulses (4 ms interval between pulses) might not be temporally confined. Repeated

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1Reviewed in [RRK+08].
4.5 Stimulation-induced effects on vocal learning could be due to modulation of slow wave sleep

Stimulation with high frequency stimuli can lead to long-term potentiation (LTP), as has been shown in the hippocampus [BL73, BGM73, BC93]. Some types of LTP are associated with the NMDA (N-methyl-D-aspartate) receptor complex, although it is not a unitary phenomenon [MB04]. NMDA receptors are also involved in learning processes in the SMP of the zebra finch. The formation of a tutor song memory is linked to NMDA receptor activation in song related regions: injection of a NMDA receptor antagonist into the forebrain in (or near) LMAN or into HVC, the primary afferent target of NIf, prior to tutoring of juvenile zebra finches led to impaired vocal learning [BNN96, RGM12], possibly due to a blockage of tutor song memory formation. It is possible that repeated stimulation with paired pulses leads to synaptic changes in the song motor system that interfere with the tutor song memory formation and natural vocal learning.

Not all birds with strong paired-pulse stimulation showed impaired song learning. Repeated stimulation could have led to habituation and adaptation [TS66, RAB09], which would have affected the effect of the stimulation. As we might not only have stimulated neurons in NIf but also axons of UVAHVC neurons, minor differences between the location of the electrode tips and distance between them could have led to different adaptation patterns. However, we did not observe large differences between placements of the stimulation electrodes. Song triggered stimulation showed that birds with stimulation-induced effects on song production were also more likely to show impaired song learning.

4.5 Stimulation-induced effects on vocal learning could be due to modulation of slow wave sleep

By excluding move-stim birds from our analysis and only considering birds that were stimulated during rest (both all-stim and rest-stim birds) the relation between stimulation paradigm and effect on song development is striking (black and green asterisks in Figure 3.30): the difference between the facilitating effect of weak single-pulse stimulation and the detrimental effect of strong paired-pulse stimulation was large. Thus, stimulation induced effects on song development could be ascribed to processes during rest.

The amount of movement periods increased throughout the night and therefore rest-stim was most intense during the first hours of the night. In zebra finches the amount of slow wave sleep (SWS) decreases during the night, whereas the amount of REM sleep increases; head movements are infrequent during SWS and more frequent during REM sleep [LSSM08]. In our experimental setup head movements are well detected due to the attached cable. Thus, because movement is infrequent during SWS, both rest-stim and all-stim birds were stimulated intensively during SWS.

Although the number of move-stim birds per group was too small to
significantly assess the effects of the stimulation, we speculate that the effects of move-stim were not identical to the effects of rest-stim and all-stim. The move-stim birds in the weak single pulse group did not belong to the best learners and the move-stim birds in the paired-pulse group did not belong to the worst learners. In the current experimental setup the stimulation started only one second after the last harmonic sound, but there were many songs of both the tutor and the juvenile bird that were not immediately followed by stimulations because the birds continued to sing and call. It might thus be that spontaneous activity in NIf directly following vocalizations [LS11] was not perturbed extensively enough.

4.6 What could be done?

Primarily a more complex social setting might lead to less variable learning behavior and simplify the future study of the neural basis of vocal learning.

While sleep phases have been studied previously in the zebra finch, and their sleep structure has been shown to be mammalian-like [LSSM08], the observed sleep phases remain to be described with respect to the spontaneous sensorimotor replay observed in the SMP [DM00], both in juvenile and adult birds. This relation should be established by combining recordings of EEG and spontaneous spiking activity in the SMP during sleep [HKF06], and it would be interesting to describe the effect of different stimulation paradigms on spontaneous activity and the different sleep phases.

In adult zebra finches one could study the effect of offline stimulation on learning in a pitch-shift paradigm [SB09]: penalizing a range of natural variation in pitch of a syllable with loud noise playback leads to adaptive changes of the targeted syllable and to modulation of the firing activity directly following vocalizations [LS11] or spontaneous activity during sleep [DM00]. Possibly, electrical stimulation after playback (offline) could disrupt the ability of birds to adapt their behavior to escape the noise.

It is known that NIf and HVC are involved in memory formation of the tutor song because perturbations of HVC activity during tutor song exposure blocks the acquisition of a tutor song memory and leads to bad song imitation [RGM+12]. If activity in NIf in juvenile birds directly after the exposure to a tutor song is involved in memory formation, one could test this by perturbing spontaneous activity directly following the last syllable of the tutor song motif. Although this perturbation could possibly coincide with subsequent song motifs in a song bout, the first song motif of each song bout would be unperturbed.

Similarly to spindle-contingent electrical stimulation of the hippocampus in rats [GBW+09, ESW10], one could stimulate in NIf with paired pulses triggered by bursting activity recorded in RA [DM00]. These experiments would be very demanding because they require stability of single- or multi-
unit recordings in RA over and extended period of time. While this might not be suited for perturbation of spontaneous activity during developmental learning, it could be combined with a pitch-shift paradigm, where effects can be observed overnight or within a couple of days.

4.7 Summary

In the Introduction, we have presented four points that Maquet proposed to be essential for understanding the role of spontaneous activity on learning [Maq01]: first, the spontaneous activity has to be precisely characterized; second, the causal relation between spontaneous activity and learning needs to be established; third, the experimental side-effects (such as sleep deprivation) and effects of activity modulation need to be disentangled; and fourth, the roles of the different offline phases, such as SWS and REM sleep, need to be specified. This thesis contributes to the understanding of the role of sleep in vocal learning. The here presented experiments establish a causal relation between spontaneous activity and vocal learning with a method that has little side-effects. The songbird is a suitable model system for further studies on the role of spontaneous activity in learning and memory consolidation and is likely to contribute to a better understanding of both avian and mammalian sensorimotor learning and sleep.
Appendix A

A LabVIEW Songbird Recording System

In this following Appendix I want to present the recorder, a LabVIEW songbird recording and controlling environment. The development of the recorder was a major project and I spent many hours over a period of three years on programming, refining and adapting the code to the ongoing experiments in the lab. The current version of the recorder is running on all computers in the songbird lab used for behavioral experiments and electrophysiological recordings (15 computers and 34 setups, used by more than ten doctoral and post-doctoral students, at present).

LabVIEW is a dataflow programming language and the graphical arrangement and design of the code has a significant impact on readability, usability and maintainability. Graphical programming languages have the advantage that they are structured in a top-down manner, and LabVIEW code is almost self-explanatory if one retains certain rules and conventions. I highly recommend The LabVIEW Style Book by Peter A. Blume [Blu07]: it is useful for beginners and advanced programmers and will be a major help in understanding the recorder project, as I adopted many of the concepts presented in this book. Therefore, this appendix is not intended to be a meticulous documentation but rather a conceptual overview of the recorder project.

A.1 Motivation – analysis and control in songbird experiments

The experiments in the field of vocal learning in songbirds have changed significantly over the last decade with an increasing demand for automated interaction with short latencies. The experimental software usually has two components: recording of behavioral data, such as vocalizations and neural activity; and a real-time controlling component, such as vocalization- and
movement-dependent electrical stimulation for the experiments presented in this thesis.

When I started working in this lab there were (at least) three different software solutions programmed in different languages for different experiments: one using MATLAB, one using MATLAB and SIMULINK, and one using LabVIEW. With every new setup that was added, there was another individual software solution, and small changes of the experimental paradigm did usually not only result in changes of the settings or parameters, but often necessitated adaptation of the software itself. This increasing individualization led to many problems, such as the diminishing compatibility between the different setups, dependencies on specific hardware configurations, and a prerequisite of good programming skills of each experimentalist. To remedy these problems I programmed a one-for-all solution that meets the following requirements:

- Performance and reliability: the recorder is a reliable recording and control solution with a short response time (10 ms) that is economical with computing resources.

- Flexibility: the recorder can be configured for a wide range of different experiments and hardware configurations.

- Scalability: the recorder can manage several setups at the same time, as many experiments are streamlined and done in parallel. We have several PCs that manage and record experiments in 6 setups in parallel.

- Maintainability and expandability: the recorder is simple to adapt and expand without having to modify the code at the core.

### A.2 Concepts

The best way to get acquainted with LabVIEW is the extensive collection of exemplary virtual instruments (VIs) included in their software distribution. For almost every problem there are toy examples that can be combined as building blocks for a larger project. Additionally there are a few concepts documented online with well-arranged tutorials that I used in the current implementation of the recorder. National Instruments, the producer of LabVIEW, is one of the major manufacturers of data acquisition (DAQ) hardware and the integration of the DAQ hardware in LabVIEW is neatly described in a tutorial on DAQmx. The recorder has recording and controlling functionality and there is a need for synchronized input and output.
output with short latency described in a tutorial for synchronization\textsuperscript{2}. The flexibility of the recorder is based on an implementation using object-oriented programming (OOP) and different experiments have custom written objects for their special purposes\textsuperscript{3}. Finally, to configure an individual recorder instance I made use of the LabVIEW statechart module\textsuperscript{4}. Additionally, there are many online forums with examples and helpful members.

### A.3 Separation of high- and low-priority tasks

The time-critical controlling tasks of the recorder are separated from low-priority tasks, such as recording and user interaction.

#### A.3.1 High-priority recorder elements

The controlling of the experiment consists of the following time-critical sequence: signal acquisition, data analysis, and signal generation. This sequence runs at a rate of 250 Hz: every 4 ms a buffer of data is acquired, analyzed and the calculated output buffer is generated on the hardware device. The response time of the current implementation is 10 ms.

Programmatically this sequence is separated into a data acquisition and generation instance, where the signal is read from and committed to the hardware in a synchronized manner, and a data analysis instance, where a new output buffer is determined based on the input buffer. These two high priority loops only depend on themselves. Data is passed between them via queues that serve as data transport tool and as triggering mechanism: data that enqueued triggers the execution of the code at the receiving end.

#### The data acquisition and generation loop

Every 4 ms a buffer containing 128 data samples is read from and written to the DAQ hardware, resulting in a sampling rate of 32 kHz. The read and write tasks are synchronized by sharing a common sample clock.

Buffered output can be tricky on non-real-time targets when the buffer sizes are small and the time interval between the calculation and the generation of the output buffer is short. Every 4 milliseconds a new buffer needs to be written to the DAQ device and there is no guarantee that this new buffer is calculated in time: many processes run in parallel with the recorder and it is likely that there are delays every once in a while. This is solved

\begin{itemize}
  \item \textsuperscript{2}http://zone.ni.com/devzone/cda/tut/p/id/3615 – Tutorial on M Series Synchronization with LabVIEW and NI-DAQmx.
  \item \textsuperscript{3}http://zone.ni.com/devzone/cda/tut/p/id/5786 – LabVIEW Object-Oriented Programming.
  \item \textsuperscript{4}http://zone.ni.com/devzone/cda/tut/p/id/7425 – LabVIEW Statechart Module Tutorial.
\end{itemize}
by allowing regeneration of the output buffer: the device will reproduce the 
last buffer and skip the next one whenever the newly calculated output 
buffer is not written to the device in time. This ensures the stability of 
the data acquisition and generation, but might lead to undesired output. 
However, these events can be tracked and are rare under normal conditions: 
in the current implementation of the recorder the ratio of delayed (and thus 
skipped) buffers is in the order of one per million, corresponding to about 
one delayed buffer per hour.

The data analysis loop – T-D-A-E

250 times per second the recorder needs to calculate a new output buffer 
depending on the input buffer. This is done in a sequence of steps that are 
best illustrated by using an example. For the offline stimulation experiment 
presented in this thesis we had to deliver stimulation pulses contingent on 
vocalizations of the juvenile bird: only when there were no vocalizations of 
the juvenile bird or the tutor we would deliver stimulation pulses. Vocal-
izations were detected by thresholding the harmonic level \( H(t) \) (see Section 
2.4). In a first transformation step \( T \) the spectral density is calculated 
on the input buffer containing a signal acquired from the microphone. On 
this transformation \( T \) the harmonic level is calculated, and we call these 
functions (like \( H(t) \)) with scalar output ‘detectors’ \( D \). These scalar values 
are used to decide about the output by instances called ‘activators’ \( A \): if 
the values of the harmonic level is in the desired range the ‘effectors’ \( E \) are 
triggered, and they commit the desired output buffer, in our case a bi-phasic 
pulse, back to the data acquisition and generation loop.

Each iteration of the data analysis loop consists of transformations, detec-
tors, activators and effectors: \( T-D-A-E \). All transformations, detectors, 
activators and effectors are implemented with OOP and their inputs and 
outputs are clearly defined: they are individually configurable and can be 
assembled freely for each individual experiment. New classes can be added 
with minimal effort and knowledge. Thereby the recorder is simple to adapt 
to many new experimental paradigms.

A.3.2 Low priority recorder elements

There are several components to the recorder that are decoupled from the 
time critical signal acquisition, analysis, and generation. These components 
have a limited interaction with the time-critical loops to ensure that the 
time-critical loops are not delayed by unnecessary overhead.

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http://www.ni.com/white-paper/3874/en – Analog Output Regeneration in NI-
DAQmx.
A.3 Separation of high- and low-priority tasks

File saving

The activators include the logic to determine the output and trigger saving when there is data of interest that should be saved to disk. The user can specify how much data before and after a saving trigger should be additionally included in such a data file: a circular buffer holds as many pre-trigger buffers as requested and the recorder streams the data in the circular buffer, and the data acquired during and after the saving trigger to disk until the desired number of post-trigger buffers is reached. Each data file is accompanied by a text file, containing meta-data such as the exact timestamp, the length of the file, or information on detector levels, effector triggers and similar. These meta files can subsequently be parsed by the data analysis software and data of interest can specifically be loaded and analyzed.

Graphical user interface

The graphical user interface (GUI) displays some information about the ongoing experiment (Figure A.1): it indicates saving, displays the spectrogram of the sounds inside the recording box and allows for minor interactions such as changing the thresholds and triggering saving.

Plugins

While the recorder GUI is minimalistic and does not have many possibilities to interact, there are many different plugins in the recorder, specific for certain experiments and applications, that can interact with the time-critical components. For example, there is an oscilloscope plugin (Figure A.2) which allows to monitor the electrode signal. Or there is a plugin for sound playback, similar to a media-player, and one can automate the playback of the last files that were recorded. These plugins interact but do not interfere with the core of the recorder. There is a clear hierarchy between the core input-analysis-output loop and all peripherals.

The data stream for the oscilloscope for example is provided by the data acquisition loop: each newly acquired buffer is also made available for plugins and replaced as soon as another buffer of data is available. There is no control if this buffer has been accessed by a plugin before it is replaced. It is possible that the oscilloscope plugin occasionally misses the reading of a buffer content, but this only happens on rare occasions where computing resources are scarce. By this construction, the optimal mode of operation is ensured.

To playback sounds an effector commits a sound waveform in buffers of 128 samples to the data acquisition and generation loop. The playback plugin loads a file containing the data to be played back, which can be time consuming, creates a reference to it and only passes this reference on to the effector. The time consuming steps are processed by the plugin and the
Figure A.1 – The recorder GUI displays information on the ongoing experiment: Four experiments are managed by a single recorder instance. (1) Saving is active in the last of the four setups (light green indicator). (2) Control to select setup and experiment (currently setup ISO18, bird r20y2). (3) Indicator of delayed buffers that were skipped. (4) Control to select activators and detectors for the given setup. (5) The panel shows the detector level in white and the threshold for this level in red. (6) Spectrogram of the sound in setup ISO18.
A.4 Memory management

LabVIEW handles many of the memory management details that the programmer must handle in a text-based programming language. It takes some time to understand how LabVIEW handles variables and where variables are copied. Specially in larger projects with many sub-VIs one has to be very careful how this data is stored, accessed and passed between different instances of the project. In the recorder large variables are accessed and manipulated in several VIs at an overall loop-rate of 250 Hz. I was very careful to streamline the time-critical loops to achieve fast and reliable operation: if LabVIEW created a copy of a large array once per loop iteration, this would have severe implications on performance.

LabVIEW has a property that is annoying and reasonable at the same time: single arrays can only be stored in contiguous memory and fragmented memory will therefore limit the size of any single array. Thus, it is not advisable to create arrays that are larger than 10 megabytes. While this calls for attention in the handling of large arrays, it also ensures high performance.

Here a few additional tips that I found useful to improve the performance of the recorder:

Figure A.2 – Recorder plugin: The oscilloscope plugin is one of many custom-made extensions to the recorder. It can be used to display one or many recording channels at the same time. This is particularly useful when recordings are made with tetrodes or electrode arrays.

passing of a reference to the recorder core does not interfere with the timing of the high-priority loops.

The flexibility and expandability of the recorder reduces the need for additional instruments.
• Local and global variables should be avoided, as these are always accompanied by additional copies of variables. If global accessibility is necessary one should use functional global.

• If large arrays have to be accessed in several VIs, they should be packed in queues. Dequeueing and enqueueing operations are fast and no additional copy of the array is created.

• Complicated data structures should be avoided. A cluster of two \( n \)-element arrays is better than a \( n \)-element array of a two element cluster. If complicated data structures are accessed, the in place element structure should be used. The simpler the data structure, the less likely LabVIEW will create unnecessary copies.

• There is a useful toolkit to profile the performance and memory usage that helps to find the most likely candidates for improvements.

A.5 Summary

The recorder has proven to be a powerful tool and is widely used in our lab. It runs reliably on multiple experimental setups, recording and controlling various experiments. While there were no major changes to the core of the recorder for the last two years, there are multiple new activators and plugins that have been added. The current implementation meets the requirements listed in Section A.1 and I hope the recorder will continue to contribute to experiments and findings for many years to come.

6https://decibel.ni.com/content/docs/DOC-2143 – Basic Functional Global Variable Example.
Appendix B

Documentation of Analysis Scripts

The analysis of all the recorded sound files, movement-log files and stimulation-trigger-log files was done in MATLAB with several scripts and functions. The most important functions are mentioned here, without going into unnecessary detail. The functions and scripts are listed in the order of usage.

f_importI_tutoring_josh.m

As explained in Section 2.7.1 we created archives of syllables, calls and noises based on RMS threshold crossings (using fb.plugin.RMS_500_7K). Additional to the sounds we included meta-information such as the time points of the stimulation pulses and the social state of the bird (isolated or in company of a tutor). The only parameter we needed to specify was the RMS threshold for the sound detection.

\[
\text{Flats} = \text{func_recluster_and_split}(..., \\
\quad \text{Flats}, ..., \\
\quad \text{indices_recluster}, ..., \\
\quad \text{index_reference_bird}, ..., \\
\quad \text{index_reference_tutor})
\]

Once all archives (stored in Flats) were imported, we clustered all syllables during song development by type. To cluster the archive of day \(i\), we used the archive of day \(i + 1\) for the undirected isolate times, and an additional tutor archive for the tutoring sessions. Examples of the sorted syllables are shown in Figure 2.10.

\[
\text{[Flat,songs]} = \text{get_best_song_candidates}(...)
\]

\footnote{All functions and scripts are included in the songbird group’s MATLAB code repository. Calling \texttt{InitVariables}(-1) adds all functions and scripts to the search path.}
From the sorted archives we extracted 10 songs per day to calculate the similarity to the tutor song. For the song selection we had to specify the duration of the song and the maximal duration of inter-syllable-intervals. We show examples of these songs for each bird used to illustrate song development (see Figures 3.8(a) and (b), 3.10(a), 3.13(a), 3.17(a), 3.20(a), 3.24(a), and 3.27(a)).

SAPsim = SAP_do(birdname)

The similarity of the extracted songs to the tutor songs were then calculated with Sound Analysis Pro using the standard settings. The calculated similarities are shown in Figures 3.8(c) and (d), 3.10(b), 3.13(b), 3.17(b), 3.20(b), 3.24(b), and 3.27(b).

summary_of_experiments(birds)

For all stimulated birds we have log-files with stimulation-time-points and movement-values. These log-files are parsed by functions within the summary_of_experiments script and we illustrate the movement behavior and stimulation density in Figures 3.11, 3.14, 3.18, 3.21, 3.25, and 3.28. This data was then used for the group statistics.
Bibliography


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