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Research Report

Ultrastructural and electrophysiological analysis of Area X in the untutored and deafened Bengalese finch in relation to normally reared birds

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ABSTRACT

Birdsong learning bears many similarities to human speech acquisition. Although the anterior forebrain pathway (AFP) is believed to be involved in birdsong learning, the underlying neural mechanisms are unclear. We produced two types of abnormal song learning: young birds untutored from adult "song tutors", or birds deafened by bilateral cochlear removal before the onset of sensory learning. We then studied how ultrastructure and electrophysiological activity changed in an AFP nucleus, Area X, among these birds at adulthood. Our results showed that, although the size of Area X did not change significantly, the numbers of synapses per unit area and compound synapses and the percent of concave synapses increased significantly in the untutored or deafened birds. The percent of perforated synapses or axo-spinous synapses decreased compared to the normally reared birds, suggesting a decreased efficiency of synaptic transmission in the untutored or deafened birds. We then identified several types of spontaneously firing cells in Area X. Cells with fast and slow firing rates did not show significant electrophysiological differences among the groups, but cells with moderate firing rates, most likely DLM-projecting neurons, fired at significantly lower rates in the untutored and deafened birds. In addition, cells firing irregularly were only found in the deafened birds. Thus, the decreased or irregular electrophysiological activity in the untutored or deafened birds, together with the corresponding ultrastructural findings, could be implicated in the abnormal song production in these two types of birds.

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1. Introduction

Songbird vocalizations are among the most complex animal behaviors. Birdsong learning bears striking similarities to certain aspects of human speech acquisition (Catchpole and Slater, 2008). Like humans, young birds must hear and memorize adult tutor songs or "templates" during a sensitive period, the first stage of sensory learning. In the second stage, a sensorimotor learning period, young birds gradually refine their initially variable and noisy vocalizations until they match the template memory of the tutor songs (Marler, 1970). The second stage ends with song "crystallization", a process wherein the learned songs are highly stereotyped (Konishi, 1965; Marler, 1970). Songbirds provide us an advantageous model for studying the neural mechanisms of vocal learning (Williams, 2004).

Birdsong is controlled by a well-described set of interconnected song control nuclei (Fortune and Margoliash, 1995; Nottebohm et al., 1976, 1982; Vicario, 1991). They consist of two main pathways: (1) the motor pathway, which starts from the high vocal center (HVC) to the robust nucleus of the archopallium (RA) and finally to the tracheosyringeal portion of the hypogloss nucleus; and (2) the anterior forebrain pathway (AFP), which starts from the HVC and projects through Area X, the nucleus dorsolateralis anterior, the pars medialis (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN), in turn to RA. The motor pathway must be intact, as any lesion of this pathway will prevent normal song production. In contrast, bilateral lesions of Area X or LMAN disrupt normal song development in juvenile birds but do not affect the production of stereotyped songs by adult birds (Bottjer et al.,1984; Scharff and Nottebohm, 1991; Sohrabji et al., 1990).

There are five physiologically and anatomically distinct cell types in Area X, including spiny neurons (SN), long-lasting afterhyperpolarization neurons (LA), low-threshold spike neurons (LTS), fast-spiking neurons (FS) and aspiny fast-spiking neurons (AF). Of them, one type (AF) is similar to neurons in the mammalian globus pallidus, and the other four types are very similar to their counterparts in the mammalian striatum (Farries and Perkel, 2002). The pallidum-like cell type morphologically corresponds to the projection neurons of Area X, which possess spiny dendrites and contain γ-aminobutyric acid (GABA) and are thus inhibitory neurons (Farries and Perkel, 2002; Grisham and Arnold, 1994). In contrast, LA have a large cell body with thin aspiny dendrites and can be labeled with antibodies to choline acetyltransferase (ChAT) (Farries and Perkel, 2002; Zuschratter and Scheich, 1990). LTS are interneurons with thin aspiny dendrites and contain somatostatin, neuropeptide Y, and nitric oxide synthase (Anderson and Reiner, 1990; Bottjer,1997; Figueredo-Cardenas et al., 1996; Kawaguchi, 1993). All types of neurons can fire spontaneously except SN and FS.

It has been shown that deafening adult songbirds decreases the amplitude of spontaneous synaptic activity, alters spontaneous action potential activity in HVC_X neurons, and selectively decreases their dendritic spine sizes and stability (Tschida and Mooney, 2012). These changes precede and predict subsequent birdsong degradation. AFP neurons also display song motor activity during adult singing, which is closely locked to the acoustic features of the bird's song (Hessler and Doupe, 1999; Brainard and Doupe, 2000). In addition, an intact AFP nucleus is crucial for adult song plasticity following interruption of auditory feedback (Brainard and Doupe, 2000; Tschida and Mooney, 2012). Therefore, Area X located neural circuits might also be required for normal song production by adults. While it is established that AFP is involved in normal song learning and adult song plasticity (Bottjer et al., 1984; Brainard and Doupe, 2000; Hessler and Doupe, 1999; Scharff and Nottebohm, 1991; Sohrabji et al., 1990; Tschida and Mooney, 2012; Williams and Mehta, 1999), it is not thoroughly clear how AFP is involved in song learning and plasticity.

To address this issue, we first produced two types of abnormal song learning: young birds were either untutored from adult songs, or deafened by bilateral cochlear removal before the onset of sensory learning (at post-hatching day 20). This resulted in seriously degenerated songs for the untutored birds and an incapability to produce any audible songs, bearing similarities to a human deaf mute (Peng et al.,2012), for the deafened birds. Nevertheless, the sizes of all song nuclei remained unchanged in spite of significant changes in singing. We thus further studied how ultrastructure and electrophysiological activity changed in an AFP nucleus, Area X, to investigate its potential roles in song learning and plasticity.

2. Results

2.1. *Area X nucleus volumes*

The mean volumes of Area X in the untutored ($0.54 \pm 0.028\ mm^3$) and deafened males ($0.56 \pm 0.036\ mm^3$) were slightly smaller than those of the normally reared birds ($0.57 \pm 0.039\ mm^3$), but there were no significant differences among these values ($n=5$ for each group, $F_{(2,15)}=38.19$, $p=0.58$).

2.2. *Ultra-structural changes of Area X*

We measured the following ultra-structural parameters of Area X in the normally reared, untutored and deafened birds:

2.2.1. *Synapse density, the length of the PSD and perforated synapses*

The number of synapses per unit area ($100\ \mu m^2$) increased significantly in the untutored ($p<0.05$) or deafened ($p<0.001$) birds, compared to the normally reared ones (Fig. 1A–D). The untutored and deafened birds did not show significant changes in the length of the PSD ($p>0.05$) (Fig. 1A–C and E). There was a significantly decreased percent of perforated synapses in the untutored or deafened birds compared to the normal birds (Fig. 1A, B and F).

2.2.2. *Synapse curvature and compound synapse*

The percent of concave synapses increased significantly in the untutored and deafened birds, compared to the normally reared birds ($p<0.05$) (Fig. 2A and D). Neither the untutored nor deafened birds showed any significant changes in the percent of convex synapses (Fig. 1G) or straight synapses (Fig. 2E) compared with the normally reared birds ($p>0.05$), although they showed a trend toward a decrease or an increase, respectively.

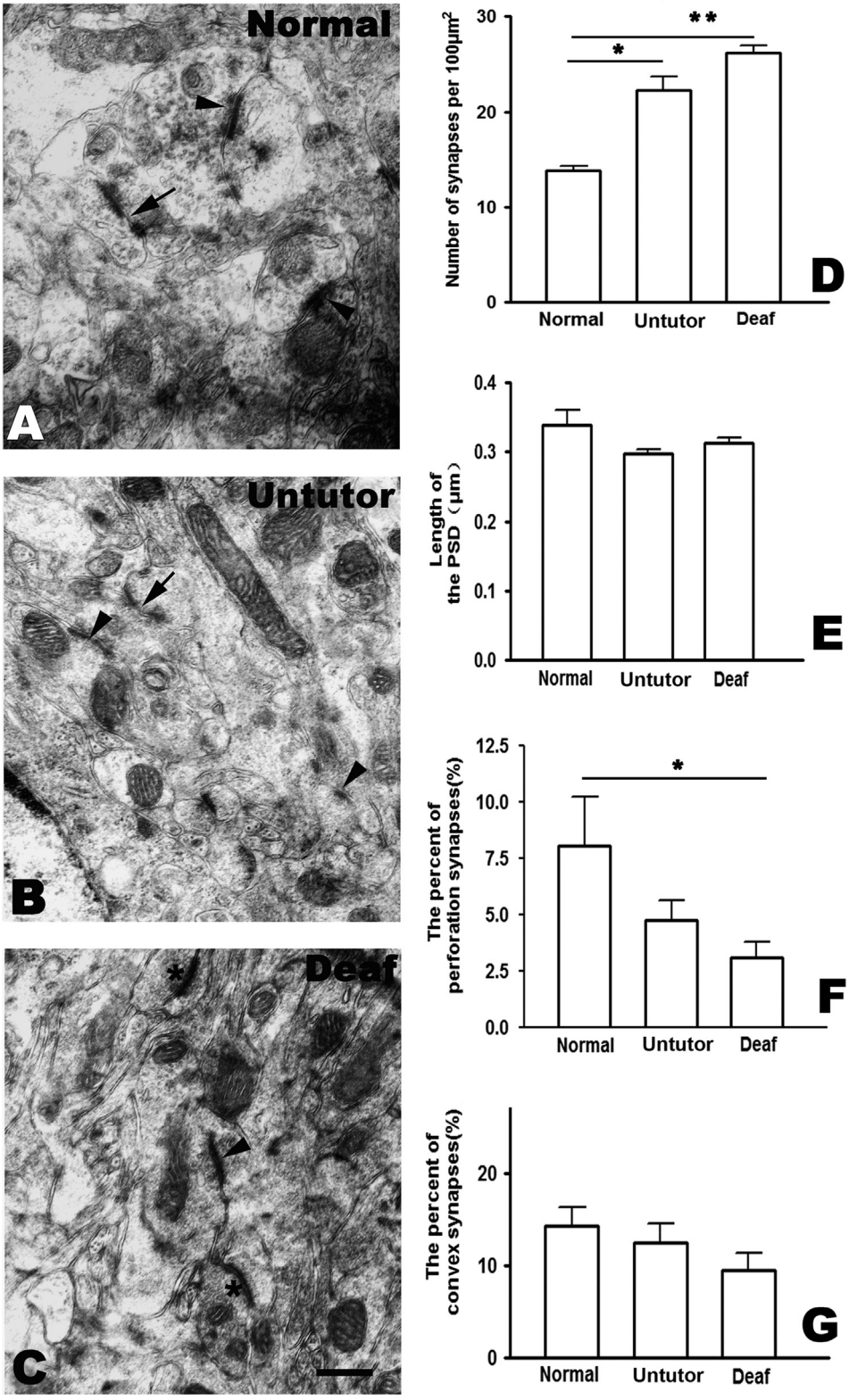

Fig. 1 – Comparison of Area X ultrastructure among normally reared (normal, (A)), untutored (untutor, (B)) and deafened (deaf, (C)) Bengalese finches. Some straight synapses are indicated by arrowheads and concave synapses by asterisks. The arrows in (A) and (B) indicate perforated synapses. (D)–(G): Comparison of synapse density (D), the length of the postsynaptic membrane density (PSD) (E), the percent of perforated synapses (F), and the percent of convex synapses (G) among normally reared (normal), untutored (untutor) and deafened (deaf) Bengalese finches. Scale bar in (C)=1 μm for (A), (B) and (C).

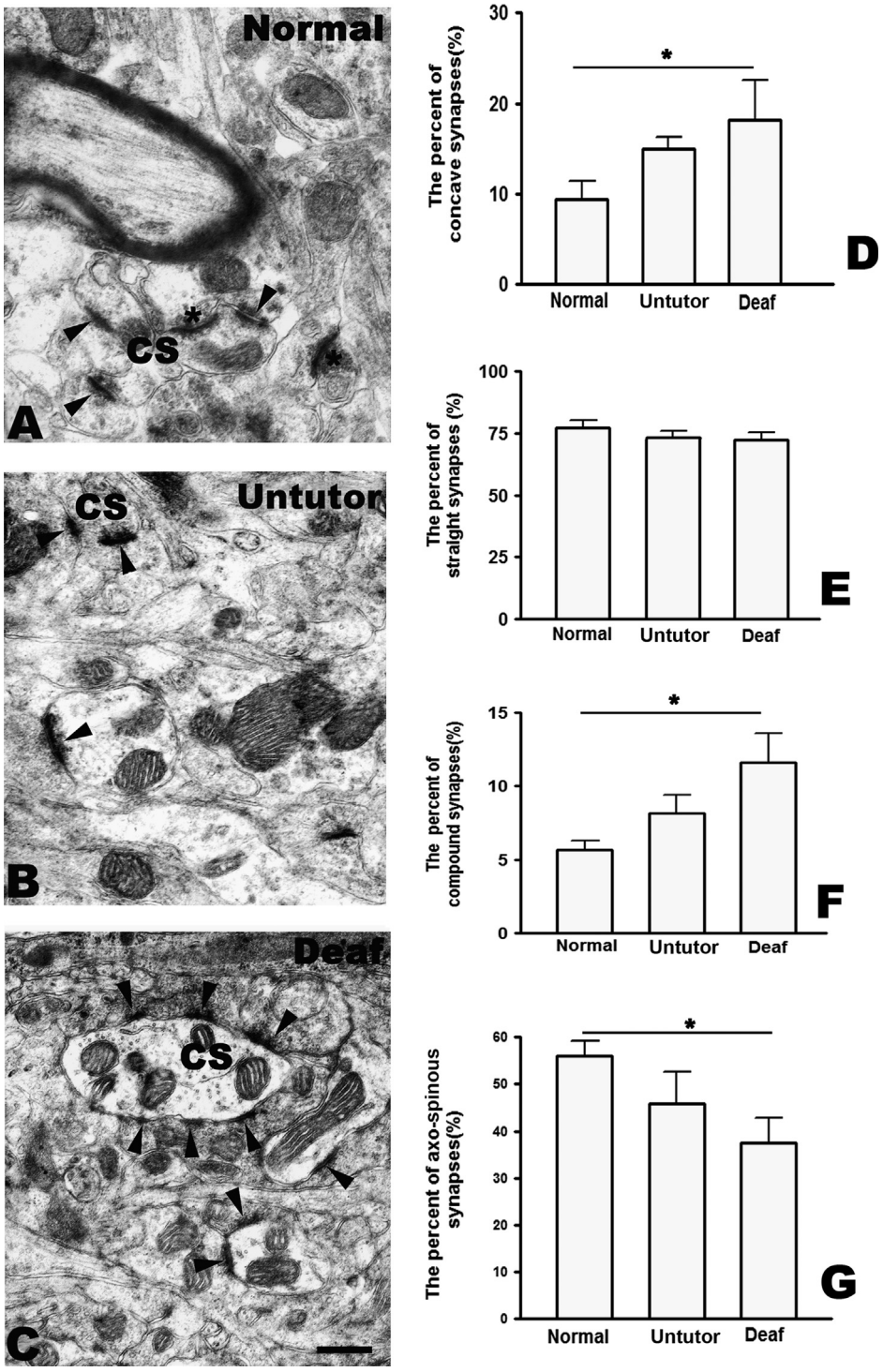

Fig. 2 – (A)–(C): Comparison of Area X ultrastructure (compound synapses, CS) among normally reared (normal, (A)), untutored (untutor, (B)) and deafened (deaf, (C)) Bengalese finches. Some synapses in (A), (B) and (C) are indicated by arrow heads (most of them are straight synapses), and concave synapses in (A) are marked by asterisks. (D)–(G): Comparison of the percent of concave (D) and straight (E) synapses and the percent of compound (F) and axo-dendritic synapses (G) among normally reared (normal), untutored (untutor) and deafened (deaf) Bengalese finches. Scale bar in (C)=1 μm for (A), (B) and (C).

Compared with the normal birds, the untutored and deafened birds had an increased percent of compound synapses (Fig. 2A–C and F). However, the difference was only significant when the normally reared birds were compared to the deafened birds ($p<0.05$), but not when they were compared to the untutored ones ($p>0.05$) (Fig. 2F).

Table 1 – Distribution of spontaneously firing types in Area X across different studied groups

Groups	FSF and MSF	SSF	ISFUA	ISFEA	IISF
Normal	$N=12$	$N=6$	$N=12$	$N=8$	/
	$n=112$	$n=9$	$n=223$	$n=16$	
$n=360$ total	31.11%	2.50%	61.94%	4.45%	/
Isolate	$N=5$	$N=4$	$N=5$	/	/
	$n=40$	$n=13$	$n=64$		
$n=117$ total	34.19%	11.11%	54.70%	/	/
Deaf	$N=5$	$N=4$	/	/	$N=5$
	$n=68$	$n=10$			$n=31$
$n=109$ total	62.38%	9.17%	/	/	28.44%

N: The number of birds recorded in each of the studied groups (normal, isolate and deaf).
n: The number of recordings in each of the studied groups.
FSF, MSF and SSF: Fast, moderate or slow spontaneous firing, respectively. ISFUA and ISFEA: Intermittently spontaneous firing with unequal or equal amplitudes. IISF: Irregularly intermittent spontaneous firing.

Table 2 – Some electrophysiological measurements for FSF, MSF and SSF (means ± SD).

	Groups	Burst firing rate/s^{-1}	Burst width/ms	Spikes per burst	Spike firing rate/s^{-1}	ISI	CV
FSF	Normal ($N=12$, $n=85$)	11.71 ± 1.19	13.32 ± 2.63	2.98 ± 0.38	89.20 ± 11.10	22.09 ± 5.14	6.60 ± 3.75 [a]
	Untutored ($N=5$, $n=15$)	8.52 ± 2.79	11.78 ± 3.98	2.75 ± 0.48	83.34 ± 9.14	19.24 ± 3.05	1.85 ± 0.99
	Deaf ($N=5$, $n=62$)	10.42 ± 2.87	12.92 ± 2.64	2.91 ± 0.56	81.29 ± 8.97	16.77 ± 1.11	2.67 ± 1.15
MSF	Normal ($N=8$, $n=27$)	1.35 ± 0.75[a]	6.30 ± 2.01	1.58 ± 0.48	48.10 ± 5.14 [a]	45.62 ± 9.35 [a]	11.48 ± 6.84 [a]
	Untutored ($N=4$, $n=25$)	0.65 ± 0.31	5.81 ± 1.82	1.45 ± 0.59	34.62 ± 4.63	30.54 ± 6.92	22.42 ± 6.47
	Deaf ($N=3$, $n=6$)	0.39 ± 0.15	5.01 ± 1.71	2.09 ± 0.73	31.56 ± 5.99	38.03 ± 9.25	4.89 ± 2.87
SSF	Normal ($N=8$, $n=9$)	-	-	-	5.15 ± 0.95	478.98 ± 221.28 [a]	9.53 ± 1.42 [a]
	Untutored ($N=4$, $n=13$)	-	-	-	4.27 ± 0.76	107.00 ± 30.93	27.78 ± 9.83
	Deaf ($N=3$, $n=10$)	-	-	-	4.86 ± 0.89	320.30 ± 139.44	24.35 ± 8.74

N: The number of birds recorded in each of the studied groups (normal, untutored and deaf).
n: The number of recordings in each of the studied groups. Superscript "a" indicates significant differences between the normally reared birds and the untutored or deafened birds.
FSF, MSF and SSF: fast, moderate and slow spontaneous firing, respectively.

2.2.3. *Percents of the types of synapses*

We also compared the percents of the axo-dendritic and axo-spinous synapses among the three groups. There was a decrease in the percent of axo-dendritic (axo-spinous) synapses in the untutored or deafened birds compared to the normally reared birds. However, only the percent of axo-spinous synapses in the normally reared birds was significantly higher than in the deafened birds (Fig. 2G), suggesting that fewer spines might appear in the dendrites of the deafened birds.

2.3. *Electrophysiology of Area X neurons*

FSF, MSF and SSF could be found in all the studied groups (normal, untutor and deaf), whereas ISFEA was only found in the normally reared and untutored birds, and IISF only in the deafened birds. The distribution of the above spontaneously firing types in Area X was shown in Table 1.

2.3.1. *FSF, MSF and SSF*

Several measurements for FSF, MSF and SSF were listed in Table 2, including firing rates, burst width, spikes per burst, spike firing rate, ISI and CV. FSF fired at the highest spontaneous rates in all the studied groups (normal, untutored and deaf) (Fig. 3A1–A3; Table 2). The measured parameters did not show any significant differences between the normally reared birds and the untutored or deafened birds except CV (Table 2). As the waveforms did not keep uniform, MSF were not regarded to be single units. All the examined measurements showed significant differences between the normally reared birds and the untutored or deafened birds except burst width and spikes per burst (Fig. 3B1–B3; Table 2). SSF were clearly distinguished from the others by their spontaneous firing at the lowest rates and by the absence of bursts in the waveform (Fig. 4A1–A3). There were significant differences in ISI and CV between the normally reared birds and the untutored or deafened birds (Table 2).

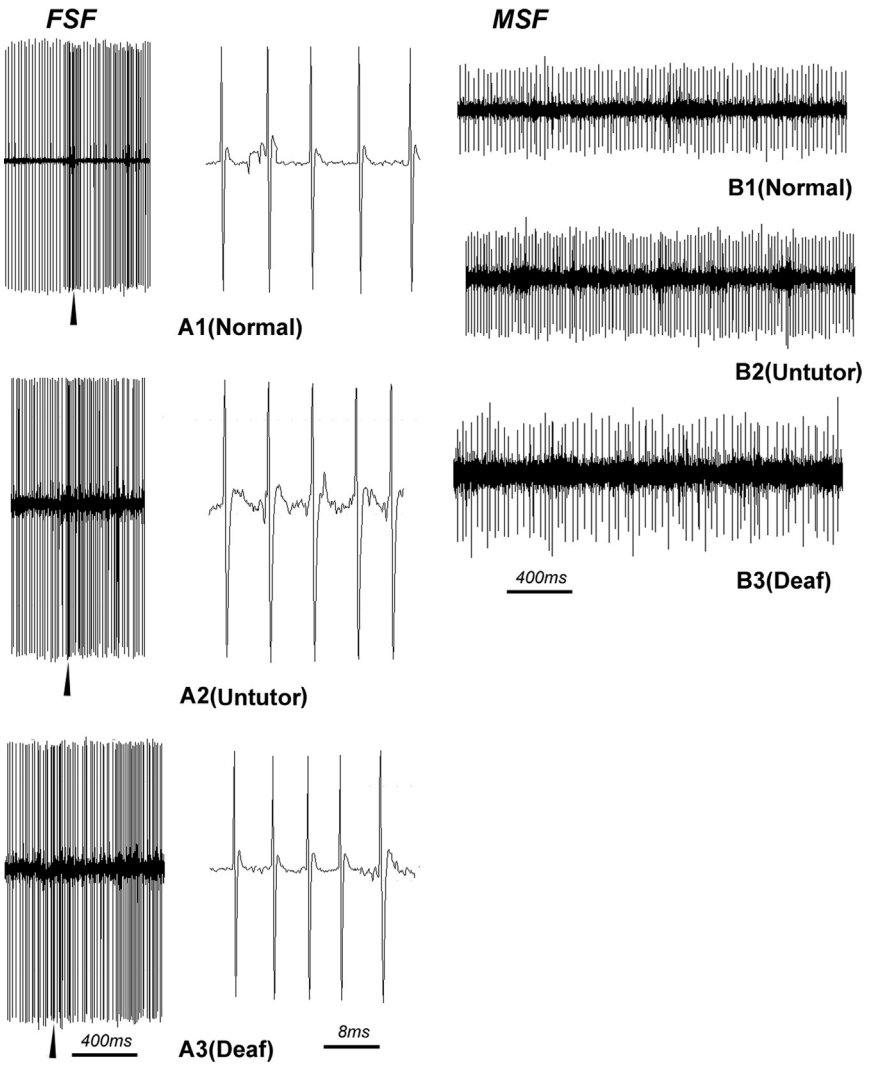

Fig. 3 – Representative waveforms of the spontaneous activity of Area X neurons in normally reared (normal), untutored (untutor) and deafened (deaf) Bengalese finches. (A1)–(A3): Fast spontaneous firing (FSF). (B1)–(B3): Moderate spontaneous firing (MSF).

2.3.2. *ISFUA, ISFEA and IISF*

ISFUA characteristically fired in bursts interspersed with silent intervals over 300 ms (ranging from 310 to 420 ms) (Fig. 4A1 and A2). The average number of spikes per cluster was 37.41 ± 9.28 ($n=223$), and 22.68 ± 6.45 ($n=64$) in the normally reared and untutored birds, respectively. They showed significant differences between the two groups ($t=18.56$, $p<0.001$). In each ISFUA, individual spikes varied largely in magnitudes (thus not regarded to be single units), forming spindle waveforms (Fig. 4A1 and A2). In addition, the average intervals were significantly decreased in the untutored birds (0.51 ± 0.045 s, $n=64$), compared to normally reared birds (0.67 ± 0.079 s, $n=223$; $t=28.98$, $p<0.001$). Like ISFUA, ISFEA also consisted of clusters of electrical activity. However, ISFEA fired more regularly with almost equal silent intervals in each of the studied birds, with the silent intervals ranging from 375 to 425 ms (398.34 ± 25.82 ms). IISF were distinguished by their irregular intermittent firing intervals (Fig. 4C), with ISIs ranging from 3 to 400 ms. The average firing rate was $18.53 \pm 7.97\ s^{-1}$ (ranging from 12 to $25\ s^{-1}$, $n=31$). There were only a few bursts in the waveform (the burst rate was only $0.75 \pm 0.30\ s^{-1}$, $n=31$).

3. Discussion

3.1. *Implications of ultrastructural variations for song learning*

To our knowledge, this is the first quantitative electron microscopy study on Area X. We showed that, compared to normally raised birds, the number of synapses per unit area, the number of compound synapses, and the percent of concave synapses increased significantly in untutored or deafened birds, while the percent of the perforated synapses and axo-spinous synapses decreased.

Our results showing an increase in the number of synaptic contacts per unit area in untutored or deafened birds are consistent with some previous reports. Using Golgi-Cox

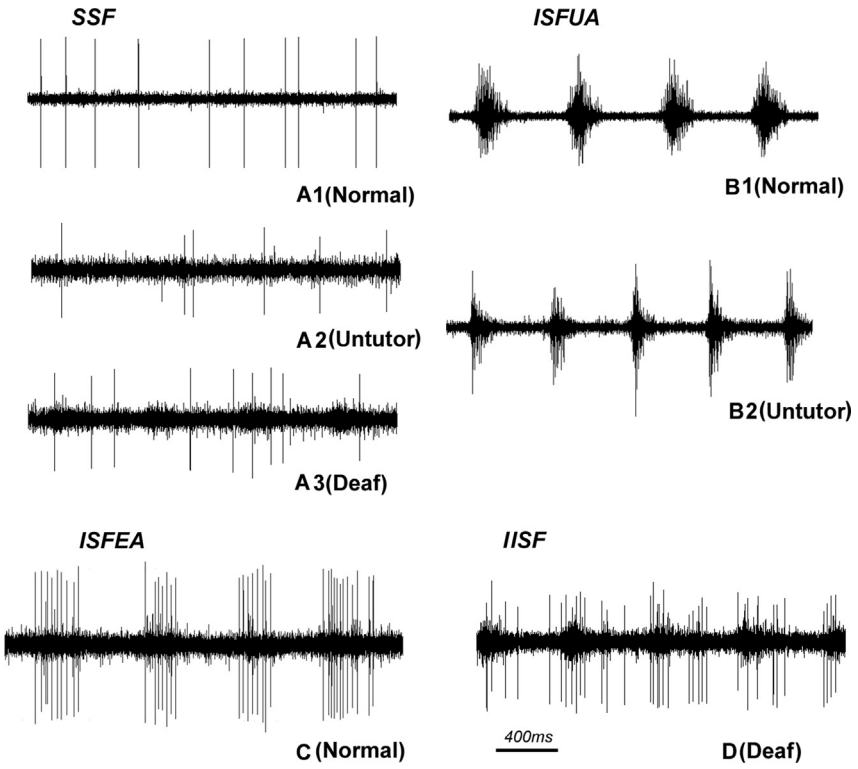

Fig. 4 – Representative waveforms of the spontaneous activity of Area X neurons in normally reared (normal), untutored (untutor) and deafened (deaf) Bengalese finches. (A1)–(A3): Slow spontaneous firing (SSF); (B1) and (B2): Intermittently spontaneous firing with unequal amplitudes (ISFUA); (C): Intermittently spontaneous firing with equal amplitudes (ISFEA); (D); Irregularly spontaneous firing (IISF). ISFUA is regarded to be produced by a single cell whose spike amplitude varied systematically. However, it might be also caused by a population of cells that became coactive at regular intervals.

staining, Wallhäusser-Franke et al. (1995) have shown that spine frequencies in LMAN of song-deprived male zebra finches increase 41%, compared with those of social, song-experienced males. Another study reported that the total number of RA synapses tripled between post-hatching day 28 and 56 in the zebra finch but decreased significantly between post-hatching day 56 and adulthood. In addition, monocular deprivation starting at hatching results in a 33% increase of synaptic density in the nucleus rotundus, a thalamic visual relay station of the tectofugal pathway in birds (Nixdorf and Bischof, 1987). These data agree with a synapse selection hypothesis, which suggests that the pruning of synapses is necessary for the learning of motor programs, including birdsong. The process of synapse selection consists of two stages, a genetically controlled stage for establishing a possible network of neural connections, and an activity-dependent stage for eliminating inappropriate synapses (Changeux and Danchin, 1976). The process of synapse selection might be disrupted in untutored and deafened birds, resulting in an increase in the number of synapses. However, it is necessary to point out that some learning events appear to induce a proliferative process. Spatial learning in a complex environment has been shown to induce an increase of dendritic spines on basal dendrites of CA1 pyramidal cells in the hippocampus of rats (Moser et al., 1994)or cortical and striatal neurons of rats (Comery and Shah, 1995), suggesting that a causal relationship between the number of synapses and learning needs to be studied in more detail.

The PSD and other synaptic features including perforation and curvature are generally regarded to be correlated to the efficiency of synaptic transmission (Calverley and Jones, 1990; Elizabeth, 2000). A decrease of perforated synapses is seen in aged rats with spatial memory deficits (Geinismann, 1986). In the perforated synapse, there is an enlargement of the contact area between the neurotransmitter release site and the postsynaptic density (PSD) (Calverley and Jones, 1990; Carlin and Siekevitz, 1983). This is thought to facilitate the recycling of synaptic vesicles and facilitate the movement of metabolites from the PSD to non-PSD regions (Vicario, 1991), thus enhancing the efficiency of synaptic transmission (Calverley and Jones, 1990; Carlin and Siekevitz, 1983; Geinismann, 1986). Based on these data, our data showing a significantly decreased percent of perforated synapses suggests a lower level of activity in the synapses of these birds than in the synapses of normal birds. This is partly confirmed by our subsequent electrophysiological study showing less electrophysiological activity in the untutored or deafened birds, including spontaneous firing spikes or bursts.

There are more mitochondria in convex synapses than in straight or concave synapses in the occipital cortex of rats, suggesting higher levels of activity in convex synapses (Dyson and Jones, 1980). In the cochlear nuclei of normal-hearing cats, PSDs typically bulge into the presynaptic end-bulb, forming convex synapses, but convex synapses are less apparent in deafened cats (Ryugo et al., 1997). In addition,

convex synapses have been reported to be predominant in the occipital cortex of mature rats compared to straight or concave synapses (Markus and Petit,1989).These reports are largely in accordance with our study showing that normal birds had fewer concave synapses than untutored or deafened ones. Further, there was a decreased number of concave synapses and an increased number of straight synapses in untutored and deafened birds in contrast to normal birds, although these results did not reach significance.

In addition, it has been reported that long-term potentiation in the CA1 hippocampus could promote the formation of multiple spine synapses between a single axon terminal and a dendrite (Toni et al., 1998, 1999). The present study showed that untutored and deafened birds had an increased percent of compound synapses in comparison with normal birds. It is difficult to explain this result with reference to the previous reports. The reason for such an increase may result from the aberrant formation of synapses in the deafened or untutored birds due to abnormal auditory inputs.

The intrinsic structure of the PSD matrix is affected by the level of activity in synapses (Bailey and Chen, 1983; Desmond and Levy, 1986; Greenough and West, 1978). Cats with greater levels of spike activity in the endbulbs of Held have smaller active zones than those with low spontaneous discharge rates (Ryugo et al., 1997). In addition, an increase in PSD size has been reported in the cochlear nuclei of deaf cats (Ryugo et al.,1997) or mice (Lee and Cahill, 2003). In contrast, a decrease in PSD size can be caused by excessive auditory stimulation (Ryugo et al., 1997). Our previous reports have shown that, compared with normal-hearing birds, untutored or deafened birds have longer PSDs in the HVC and RA. This is consistent with reports in the cat or mouse (Ryugo et al.,1997; Lee and Cahill, 2003). Although the present study did not find significant differences in the length of PSDs among the three groups, the length of the PSD did decrease in the untutored or deafened birds.

3.2. *Comparison of electrophysiological data with previous reports and their implications for song learning*

To our knowledge, no study has reported in detail on the spontaneous electrophysiological properties of Area X in vivo in untutored, deafened or normally reared birds. Nevertheless, some studies have reported the intrinsic physiological properties of Area X and its adjacent region, the medial striatum (MSt), in brain slices of the zebra finch (Lee and Cahill, 2003; Farries and Perkel, 2000), and the evoked neural activity following song playback or song production (Solis and Doupe, 1999; Hessler and Doupe, 1999). In comparing our data with the electrophysiological activities recorded in Area X neurons in vitro, one must be cautious about critical differences in the recording temperature and techniques that will affect firing rates. In addition, neurons in brain slices are largely cut off from synaptic input, while neurons in vivo are generally subjected to barrages of synaptic input. This will also affect firing rates or patterns.

Based on the differences in firing rates and patterns, several types of spontaneous activities were identified in Area X in the present study, and some measurements for these types of neural activities were compared among the studied three groups (normal, untutor and deaf). Although these classifications were arbitrary, the criteria were distinct and the classifications were reliable. Due to the limits of the present study, we could not determine whether the distinguished types of spontaneous activities were constituted in Area X. However, some intrinsic properties of constituting neurons could be reflected from our quantitative analysis of some electrophysiological parameters, including firing rates, ISI and CV.

As mentioned above, some previous reports have indicated that Area X contains all the four cell types found in the mammalian striatum, as well as a fifth mammalian pallidum-like cell type (Lee and Cahill, 2003; Kawaguchi, 1993; Kubota and Kawaguchi, 2000). Of the five cell types, SN is the most common type and is remarkably similar to the principal mammalian striatum cell type, the medium spiny neuron (MSN) (Lee and Cahill, 2003; Kawaguchi, 1993; Kubota and Kawaguchi, 2000). Meanwhile, FS neurons correspond to the mammalian striatum fast-spiking class in electrophysiological and morphological characteristics (Kawaguchi, 1993; Kubota and Kawaguchi, 2000). As both types of Area X neuron did not fire spontaneously, our recorded spontaneous firings could not be produced by these two neuron types.

All the other neuron types can fire spontaneously. LA neurons fire at the lowest rates (0.7 ± 0.2 Hz) (Lee and Cahill, 2003), whereas LTS neurons fire quickly with plateau-like spikes (the sustained firing reaches the same amplitude of the beginning spikes) (Lee and Cahill, 2003; Kawaguchi, 1993; Kubota and Kawaguchi, 2000). In the present study, the low spontaneously firing neurons had the same ranges of those reported for mammalian LA, and the firing pattern of ISFEA identified only in the normally reared birds was similar to mammalian LTS. However, there are no corresponding reports of mammalian neurons with the firing patterns of ISFUA identified in the normally reared or untutored birds. In addition, although the firing pattern of ISFUA was assumed to be produced by a single cell whose spike amplitude varied systematically, it might be also caused by a population of cells that became coactive at regular intervals. This issue could be resolved using a whole-cell recording technique.

The fifth mammalian pallidum-like cell type (AF), which is regarded to be most like the DLM-projecting neurons in Area X (Luo and Perkel, 1999), has the propensity to fire spontaneously at a relatively high rate (18.4 ± 11.6 Hz) and could sustain firing beyond 100 Hz after delivery of depolarizing pulses during spontaneous firing (Lee and Cahill, 2003). Considering these spontaneous firing rates, FSF and MSF in our study most probably corresponded to the AF cell type. If this was true, there were two types of DLM-projecting neurons. Perhaps one subtype is a mature and the other is an immature subclass. It is also possible that there are two types of DLM-projecting neurons with different physiological properties. These need to be further studied in future. According to our study, the spontaneous firing rates of spikes or bursts for both FSF and MSF were significantly higher in the normally reared birds than in the untutored or deafened birds. Area X basal ganglia circuits can affect the cortex through the thalamus by generating post-inhibitory rebound potentials or by gating excitatory drive. Thus, it is possible that the decrease of the spontaneous firing rates in DLM-projecting neurons could be correlated to the changes in song behavior: compared with the normally reared

birds, no audible songs were detected in the deafened males, while less complex but more variable songs were evident in the untutored birds (Peng et al.,2012).

4. Experimental procedures

4.1. Animals

Bengalese finches (*Lonchura striata*) used in this study were raised in the breeding colony at Beijing Normal University (Beijing, China) or bought in a local market. The birds were kept in a cage (50 × 62 × 38 cm) under a 14/10 h light/dark cycle at 20–30 °C. Each cage was equipped with nest boxes and perching sites and contained 5–8 birds. The birds were provided with fresh water and seeds at all times, and green vegetables and cuttlebones were occasionally provided.

At post-hatching day 20 (PD 20), birds were divided into experimental groups. In the isolation or song-deprived group, adult males were removed when the young hatched (leaving 4–6 hatchling birds and their mother in a cage). These birds were housed in a single room isolated from con-specific songs and were allowed to keep acoustic and visual contact with each other. In the cochlear removal group, the hatchling birds were surgically deafened by bilateral cochlear removal at PD 20, following the procedure described by Konishi (1965). Briefly, the birds were anesthetized with intramuscular injections of 25 mg/kg Nembutal. Through the oval window, both cochleae were excised using a fine tungsten wire hook; the cochleae were then examined microscopically for the presence of lagenae in order to confirm complete removal. After the skin incision was sealed with cyanoacrylic glue, the birds were returned to their parents and raised until adulthood (PD > 90). Thirty control birds (16 males) were raised by their parents until adulthood and kept acoustic and visual contact with other conspecific birds in the same room. These birds produced normal songs.

4.2. Electron microscopy

Experiments were conducted on 13 adult male Bengalese finches (~120 days after hatching), including four normally reared, five untutored and four deafened birds. The birds were deeply anesthetized with a lethal dose of urethane and perfused with 0.9% NaCl for 1 to 2 min, followed by cold (4 °C) phosphate buffer (0.1 M, pH = 7.4) containing 4% paraformaldehyde and 1% glutaraldehyde for 20–25 min. The reproductive glands were inspected to determine sex (only the males were included in this study). Well-developed testes were found in each male bird, indicating that they were mature. The brains were dissected out and stored in the same fixative overnight at 4 °C. On the following day, sagittal sections of the bilateral hemispheres were cut with a vibratome at a thickness of 100 μm. These brain sections were collected in phosphate buffer. All the sections containing Area X were dissected out with a sharp blade. They were then postfixed in 1% osmium tetroxide for at least 1.5 h at 4 °C. They were immersed in propylene oxide and distilled water, dehydrated in a graded series of ethyl alcohols, and finally embedded flat in Epon 812. Ultrathin sections (around 75 nm) were cut with an LKB ultramicrotome. They were mounted onto 200-mesh grids with formvar film and counterstained with double oxygen uranium and lead citrate. They were then observed in an electron microscope.

For each of the studied birds, either side (left or right) of the brain was used (at least five blocks could be obtained). At least three blocks were chosen to cut for ultrathin sections. For each block, there were at least 5–6 copper grids on which at least five sections could be mounted. Photos were taken along the diagonal of the trapezoid section in order to be random. For each bird, at least 30 digitalized microphotos were taken.

4.2.1. Measurement of ultra-structural parameters

To quantitatively assess the number of synapses in Area X, micrographs intended for counting were randomly chosen within Area X, excluding its borders. For each of the studied birds, 30–50 micrographs were taken at a magnification of 7000 ×. The total area for each micrograph was around 3.0×10^5 μm^2, resulting in a magnification of 20,000 × after photographic enlargement. The micrographs were coded and analyzed by a person who did not know the treatment condition of the birds. Synapses were distinguished by some characterized features, including the presence of a postsynaptic membrane density (PSD), a clearly visible synaptic cleft separating pre- and postsynaptic elements, and synaptic vesicles adjacent to the presynaptic membrane.

4.2.1.1. *Synapse density*. The number of synaptic contacts per unit area (100 μm^2) was measured by counting the number of synapses in each image. To calculate the number of synaptic contacts per unit volume, we used the stereological formula of DeHoff and Rhines (1961): $Na = Nv/d$, where Nv represents the number of synapses per unit volume, Na is the mean number of synapses per unit area, and d is the mean length of profiles of synapses (PSDs). This formula provides accurate and consistent results on truly representative samples (Colonnier and Beaulieu, 1985).

4.2.1.2. *Length of the PSD*. The PSD was distinguished as an asymmetric thickening of the postsynaptic membrane. It often occurs with the accumulation of presynaptic vesicles (Cant and Morest,1979; Fekete and Rouiller, 1984). PSD length was measured using Image Pro Analysis Soft (Media Cybernetica, L.P., Silver Spring, MD) and SPOT (Enhance 2e; Diagnostic Instruments). For each of the studied birds, we examined over 100 PSDs (100–120) whose entire length was located within the micrograph frame.

4.2.1.3. *Synapse curvature, the number of perforated or compound synapses and synapse types*. There are at least three types of synapses, classified according to curve type: straight, convex (the postsynapse extending forwards to the presynaptic side) and concave (the postsynapse extending backwards from the presynaptic side) synapses. The percentage of each type of synaptic curvature was counted in all the examined synapses. In addition, the incidence of synapses with perforated PSDs (postsynaptic membrane density is interrupted, ≤100 μm) or compound synapses (the synapses are formed by one presynapse and two or more

postsynapses) was also counted. We also counted the numbers of axo-dendritic and axo-spinous synapses and compared their differences among the three groups.

4.2.2. *Analysis of data for ultra-structural parameters*

For each of the mean values (means ± SD, dependent variable) described above, one-way analysis of variance (ANOVA) (SPSS for Windows 16.0) was performed to analyze the differences among the three groups of birds (normally reared, $n=4$; untutored, $n=5$, and deafened, $n=4$). All data subjected to ANOVAs were normally distributed (one sample Kolmogorov–Smirnov test). Post hoc tests (Duncan's multiple-range test) were used to determine which individual groups differed from each other. Significance was set at $p<0.05$.

4.3. *Electrophysiological recording*

Experiments were conducted on 22 adult male Bengalese finches (~120 days after hatching), including 12 normally reared, 5 untutored and 5 deafened birds. The birds were deprived of food and water for 1 h before electrophysiological recording. They were anesthetized with 20% urethane in 20–30 μl aliquots at 30 min intervals (60–100 μl total administered into the pectoral muscle). They were then fixed in a stereotaxic device (45° head angle) by ear bars. The scalp was dissected along the midline, and Area X's location was determined using stereotaxic coordinates at approximately 1.5–2.2 mm lateral to the midline and 4.0–4.5 mm anterior to the bifurcation of the Y sinus. A small craniotomy (~500 μm wide) was made over Area X, and the dura was slit open with a fine insect pin. Etched tungsten electrodes (1.5–2.5 MΩ) coated with solder glass were lowered into Area X at an approximate depth of 3.0 mm. Then they were progressed forward at 1–5 μm at a time, controlled by a hydraulic micromanipulator (Narishige, Japan, MO-302). For spontaneous potential recordings, spike signals were amplified using a SWF-1W microelectrode amplifier (Chengdu Instruments), filtered (300-Hz high-pass filter, 3-kHz low-pass filter), and digitized with 16-bit precision at a sampling rate of 20 kHz. These spike signals were stored in blocks of 100–300 on a Dell PC running Biology Signals Gather System software V2.0j (Chengdu Instruments). Each recording for the ongoing activity of Area X neurons lasted for 0.5–1 min before moving to the next recording site. For most birds, both sides of the brain were recorded.

At the end of each experiment, electrolytic lesions were made with 50 μA current at selected locations for reconstructing recording sites. The birds were then perfused and fixed as described above. After the brains were placed in 30% sucrose solution until they sank to the bottom, they were cut sagittally. Alternate sections (40 μm) were mounted onto gelatin-coated slides and stained with 0.1% cresyl violet. Only recordings in Area X confirmed by histological verification were included in the present study.

To obtain Area X volume, all the images of Area X were captured by SPOT (Enhance 2e; Diagnostic Instruments). For each of the three groups (normally-reared, untutored and deafened birds), five birds were chosen to measure the size of Area X. After the border was outlined, the sizes of Area X were obtained using Image-Pro Plus 5.2 (Media Cybernetics, L. P., Silver Spring, Maryland). The Area X volumes could be obtained by summing the areas and then multiplying by 40 (section thickness) and two (sampling interval).

4.3.1. *Measurement of electrophysiological parameters*

Spontaneous firing rate: The average number of spikes per second.

Coefficient of variation (CV) of the instantaneous firing rate (SD/mean): The instantaneous firing rate R(t) was used to demonstrate the activity at time t of a neuron. It was a continuous function defined by the inverse of the closest inter-spike interval, as described by Hahnloser et al. (2006).

$$R(t) = 1/t_{i+1} - t_i, \quad \text{for } t_i < t \le t_{i+1}$$

where t_i is the time of the ith spike.

Burst firing rate: We identified a group of at least two spikes as a burst if the instantaneous firing rate continuously exceeded 100 Hz. The firing rate was defined as the number of bursts per unit time.

Spikes in bursts: The average number of spikes per burst.

Burst width: The duration of the burst.

4.3.2. *Analysis of data for electrophysiological parameters*

Once the electrode was lowered to Area X, large action potentials of single or multiple units could be differentiated from background neural activity. Recordings were judged to be single units on the basis of inspection of the uniformity of waveforms, and the presence of a refractory period in the distribution of interspike intervals (ISIs). We obtained a total of 360, 117 and 109 stable recordings of spontaneous activity of Area X neurons (single or multiple units) in 12 normally reared, 5 untutored and 5 deafened birds, respectively. According to the different firing rates, the recorded electrophysiological activities (not including intermittently spontaneous firing) could be clearly classified into three types, i.e., fast, moderate or slow spontaneous firing (FSF, MSF and SSF, respectively).

Both FSF and MSF were characterized by their propensity to fire spontaneously at high rates with ISI less than 50 ms, and usually just 20–30 ms. FSF fired at relatively high spontaneous rates ($>70\ s^{-1}$, ranging from 71 to 110 s^{-1}), with many bursts in the waveform. In contrast, MSF fired at relatively low spontaneous rates (around 40 s^{-1}, ranging from 35 to 50 s^{-1}) with few or no bursts in the waveform. SSF were distinguished from the above types by their spontaneous firing at very low rates ($<8\ s^{-1}$, ranged from 0.5 to 8 s^{-1}) and by the absence of bursts in the waveform. As the spontaneous firing rates did not overlap, and differed largely among the above three firing types, they were not difficult to be distinguished out. The three types of spontaneous activities appeared in all the studied groups (normal, untutor and deaf). Some measurements (firing rates, burst width, spikes per burst, spike firing rate, ISI and CV) for these types were further analyzed, and compared among the studied three groups.

As to intermittent firing, three types could be distinguished out on the basis of different firing patterns. They included intermittently spontaneous firing with unequal (ISFUA) or equal (ISFEA) amplitudes (the heights of the extracellularly recorded spike waveforms), and irregularly intermittent spontaneous firing (IISF). It is needed to point

out that the firing pattern of ISFUA could be regarded to be produced by a single cell whose spike amplitude varied systematically. However, it might be also caused by a population of cells that became coactive at regular intervals.

Analysis of variance (ANOVA) was used to compare the averages (dependent variables) among the three experimental groups (independent variables) using SPSS for Windows 16.0 (Chicago, IL), as described above. The Kolmogorov-Smirnov test was used to detect the distributions of the examined electrophysiological parameters.

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