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In vivo MR imaging of the seasonal volumetric and functional plasticity of song control nuclei in relation to song output in a female songbird

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In temperate zone songbird species, seasonal plasticity in the morphological and functional state of brain regions involved in song production occurs in association with seasonal changes in song output. Following $MnCl_2$-injections in HVC (used as proper name) of female starlings, in vivo tract-tracing by Manganese Enhanced-Magnetic Resonance Imaging (ME-MRI) provided repeated measures of the volume of two HVC targets, the nucleus robustus arcopallii (RA) and area X, along with measures of the activity of the caudal motor pathway and rostral basal-ganglia pathway that control singing. Mn^{2+}-labeling (volume labeled and signal intensity) of both nuclei was dramatically reduced in July (post-breeding season) when birds did not sing, compared to March (breeding season) when birds produced song. Seasonal changes in telencephalon volume did not exceed 4% and were not significant but were surprisingly correlated with individual measures of song rate and song bout length. Although individual song rates were variable in March, all $MnCl_2$-injections led to a reliable labeling of area X and RA. In July, delineation of area X was only possible in two birds and RA could be delineated in 50% of the population; its volume had decreased by 46% as compared to March. The birds in which RA could be delineated in July had in March a higher activity of the HVC to area X projection as reflected by the total amount of Mn^{2+} accumulated in area X, which suggests unexpected relationships between the two types of HVC projection neurons.

Keywords: Seasonal plasticity; Female; Starling; Songbird; Song control nuclei; RA; Area X; Telencephalon; Magnetic resonance imaging; Manganese; ME-MRI

Introduction

The analysis of singing behavior and of the network of brain nuclei that controls this behavior in songbirds is a commonly used model to approach the relationship between structure and activity of brain circuits and the behavior they encode. A songbird learns to produce socially relevant songs during maturation, and some species can learn new song types throughout adult life (Brenowitz and Beecher, 2005). In addition, during adulthood song output varies over the course of a year under influence of endocrine and environmental factors, and is most prominent during the breeding season in males. Both song learning and seasonal plasticity of song are paralleled by morphological changes of the song control nuclei. The seasonal changes have an amplitude which is among the largest ever described in the brain of warm-blooded vertebrates (Tramontin and Brenowitz, 2000), but the interactions between neuronal activity, endocrinology and behavior that lead to this plasticity, as well the functional relevance of this phenomenon are subject of ongoing debate (Brenowitz, 2004; Ball et al., 2004).

The relationship between individual variations in morphology and activity of the song control nuclei and the different parameters of song production would currently deserve additional investigations (DeVoogd, 2004). Correlations between morphological features of the song system and aspects of the singing behavior have been identified in several comparative studies in a variety songbird species (DeVoogd et al., 1993; Székely et al., 1996; Garamszegi and Eens, 2004; Brenowitz, 1997; MacDougall-Shackleton and Ball, 1999), but are less clear when comparing individual variations within a same species (e.g. compare: MacDougall-Shackleton et al., 1998; Ward et al., 1998; Airey and DeVoogd, 2000; but see Garamszegi and Eens, 2004). Recent studies in male European starlings, *Sturnus vulgaris*, indicate that the relation between brain and behavior could be experience-dependent (Sartor and Ball, 2005) and even change between

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seasons as a function on the social context in which song is produced (Heimovics and Riters, 2005). Therefore, it would be desirable to relate behavioral activity not only to the structure of the song control nuclei at one given time but most importantly to their changes in time. When using a classical histological approach, this type of studies either requires very large numbers of animals to overcome the influence of individual variation in the population or cannot simply be done because the brain can only be studied at one given time point. Thanks to the development of in vivo Manganese Enhanced-Magnetic Resonance Imaging (ME-MRI), a technique that can provide repeated measures of the volume of the song control nuclei as well as functional information on this circuitry (Van der Linden et al., 2002; Van Meir et al., 2004), the behavior of one individual can be related to its own changes in neuronal substrate. This approach was used here to explore whether individual differences in seasonal changes of the song control system are related to variation in song output or to variations in intrinsic properties of specific nuclei in this circuit.

To further analyze relationships between song production and size and function of the song control nuclei we used here captive female European starlings, *Sturnus vulgaris*, kept in semi-natural conditions as a large flock in an outside aviary. Female starlings, contrary to females of other songbird species such as zebra finches or canaries, are fully capable of producing elaborate songs (Hausberger and Black, 1991; Pavlova et al., 2005) and variations of song output between individuals and between seasons is relatively large (Henry and Hausberger, 2001) allowing for meaningful studies of correlations between their song behavior and selected neuronal parameters.

We repeatedly measured the volumes of telencephalon, cerebellum, the robust nucleus of the Arcopallium (RA) and area X. The last two structures are song control nuclei which are respectively required for song production in adulthood and song learning in juveniles (Nottebohm et al., 1976; Simpson and Vicario, 1990; Yu and Margoliash, 1996; Scharff and Nottebohm, 1991). Additionally, Manganese labeling of RA and area X provided functional information on the connections between HVC and these two song control nuclei. Brain measures obtained during the singing season (March) or their changes from March to July, when birds do not sing, were related to individual measures of song behavior obtained in March.

Materials and methods

Animal housing and behavioral observations

All observations were performed on a group of female starlings that were kept in the same conditions and were studied during three consecutive years preceding the repeated MRI measurements reported here in the context of a long term study of song behavior in female starlings. A detailed report of these behavioral observations will be published elsewhere. Birds were housed in a large outdoor aviary (12.10 × 8.10 × 2.12 m) provided with 20 nest boxes and a few branches. Food and water were available ad libitum. All birds were individually marked with a numbered metal ring and color bands. All females were at least 4 years old in 2003.

The singing activity of female starlings shows a clear annual cycle, with highest song output from November to the beginning of April (Henry and Hausberger, 2001), and an almost complete absence of song during the period of molt (Feare, 1984). We recorded the spontaneous singing activity of the female starlings during two periods representing different female song activity, namely at the end of February–beginning March (high song output) and at the end of June during molt (low song output). The frequency of spontaneous singing activity of all female starlings was observed and quantified every day between February 24 and March 9, 2003 (14 observation days), and between June 23 and June 27, 2003 (5 observation days). After each observation period, all females were brought into the laboratory for brain imaging (March: n = 23 birds; June: n = 22 birds).

On each day, observations lasted 1 h and were always made between 0930 and 1300 h from a permanent hide situated 3 m away from the aviary. The presence or absence of singing activity of each female during the preceding 60 s was recorded every minute by an electronic timer (Casio) using a fixed interval sampling method. This yielded for each observation day a score between 0 and 60. This score was converted to the percent of time that each female spent singing (Eens et al., 1993; Riters et al., 2005). This provided an accurate estimate of the song rate. For statistical analyses, the mean song rate over all days during each observation period (March or June) was used for each female.

Song recordings and analyses

Additionally, the songs of 11 females from the same group were recorded daily between February 11 and March 13, 2003 to obtain a measure of song complexity (see below). For song recording, we used clip microphones, Philips SBC ME 600, attached inside the nest boxes and connected via a long cable to a Recording Minidisk Sony MZ-R700 located in the hideout with the observer. Each time a bird sung near a nest box, the observer recorded this song. When birds are perched near a nest box, they often sing multiple songs such that sufficient material can be recorded to obtain accurate measures of song bout length and repertoire size (see Pavlova et al., 2005).

Sound was analyzed using Avisoft SASlab Pro software. Classification of the song into phrase types was made by visual examination of the sonograms obtained for all recorded songs and following the song terminology employed by Eens (1997).

Both male and female starlings produce most of their songs in song bouts. A song bout is defined as a period of at least 5 s of song, containing silence no longer than 1.5 s (Eens et al., 1991a,b; Eens, 1997). A song bout is composed of complex sequences of discrete units—"phrases" (see Fig. 1). Phrases are often repeated two or more times before the next phrase type is introduced. The number of different phrase types that one bird produces is defined as its repertoire size. As another measure of song complexity, we also calculated the average song bout length of each female.

We recorded between 16 and 39 (mean ± SD = 27 ± 8.2) song bouts for each female, which has been shown to be sufficient to determine the entire repertoire size in females of this species (Eens, 1997; Pavlova et al., 2005). Repertoire size was determined by counting novel phrase types as they appeared throughout the song bouts. By plotting the cumulative number of new phrase types against the total number of analyzed phrases (Adret-Hausberger and Jenkins, 1988; Eens et al., 1991a,b; Chaiken et al., 1993; Mountjoy and Lemon, 1995; Eens, 1997), we could verify that the complete repertoire size of each female was obtained (Pavlova et al., 2005).

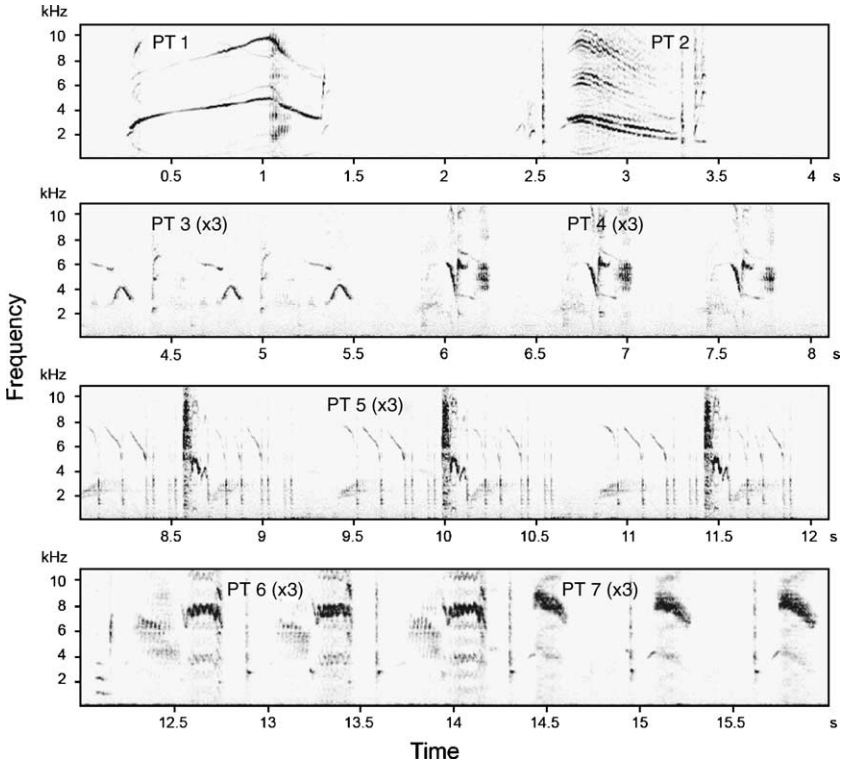

Fig. 1. Sonogram of a complete song bout of a female starling. This song bout lasted for 16 s and contains 7 phrase types (PT) and 17 phrases.

Manganese injections and imaging procedures

The brain of all subjects was scanned for the first time between March 10 and 21 (n = 23). Three months later, between July 6 and July 17, the birds were scanned for the second time (n = 22).

Twenty-four hours before each MR measurement birds received a 70-nl injection in the right HVC (previously called High Vocal Center, now used as proper name, see Reiner et al., 2004) of a 100-mM iso-osmotic $MnCl_2$ solution which was infused at a rate of 1 nl/s. HVC (Fig. 2), is an important sensorimotor region that serves as a relay center within the vocal network connecting the brain areas involved in hearing, song production and vocal learning. Two separate pathways emerge from HVC. A caudal pathway projects to RA, and plays a critical role in song production (motor pathway) (Nottebohm et al., 1976; Simpson and Vicario, 1990; Yu and Margoliash, 1996). A rostral pathway projects to a region in the basal ganglia, area X, which is important for song acquisition during ontogeny and presumably song stability in adults (Cortical-basal ganglia circuit) (Hessler and Doupe, 1999; Jarvis et al., 1998; Liu and Nottbohm, 2005; Scharff and Nottebohm, 1991). Mn^{2+}-ions are paramagnetic contrast agents that are transported along axons (Pautler et al., 1998). As previously reported (Van der Linden et al., 2002; Van Meir et al., 2004), the accumulation of manganese within RA and area X after anterograde axonal transport from HVC allows outlining the boundaries of these two nuclei.

For these injections, birds were first anesthetized with an intramuscular injection of 5 ml/kg of a mixture containing 0.33 ml xylazine (Rompun: 20 mg/ml), 2.10 ml ketamine (Ketalar: 50 mg/ml) and 4.33 ml saline solution. Anesthesia was maintained by administration of one fifth of the initial dose every 30 min through a catheter positioned in the chest muscle. The head of the animal was fixed in a stereotaxic device with the beak positioned 45 degrees below the horizontal plane of the ear bars. An incision was made in the skin and the skull was opened at the appropriate coordinates. The V-shape formed by the vena cerebralis dorsocaudalis and two branches of the sinus transversus at the border of the two telencephalic lobes was taken as zero point for positioning the injection needle. From this point, the injection coordinates were +3.0 mm lateral to the right, 0.0 mm rostro-caudal and −0.7 mm deep. The skull was closed with dental cement (Dentalbiolux, Int., Brussels, Belgium), the skin was sutured and the bird was allowed to recover. In July, 3 out of 22 birds could not be reinjected into the right HVC because scar tissue had grown over the injection site above HVC. For this period, data about volumes of song control nuclei as determined by the Mn^{2+} accumulation are thus available from 19 birds that completely recovered from the first $MnCl_2$-injection in March. 3D MRI measurements were obtained however from all 22 birds.

Twenty-four hours later birds were anesthetized again as described above and their head was immobilized in a non-magnetic Teflon™ stereotaxic head holder combined with a circular receive-only surface coil (diameter 24 mm) and a transmit head coil (Helmoltz: diameter 45 mm). The stereotaxic headholder consisted of a beak holder and ear plugs which positioned the head of all starlings in an identical position relative to the transmit, receive and gradient coil, this was done to minimize geometrical distortions and inhomogeneities in signal distribution over the head (similar setup as shown in Van Meir et al., 2005).

Imaging was carried out on a 7-T horizontal bore MR microscope (SMIS, Surrey Medical Imaging Systems, MRRS, Guildford, UK), provided with 8 cm aperture gradient coils (Magnex Scientific Ltd., Oxfordshire, UK) with a maximum gradient strength of 0.40 T/m.

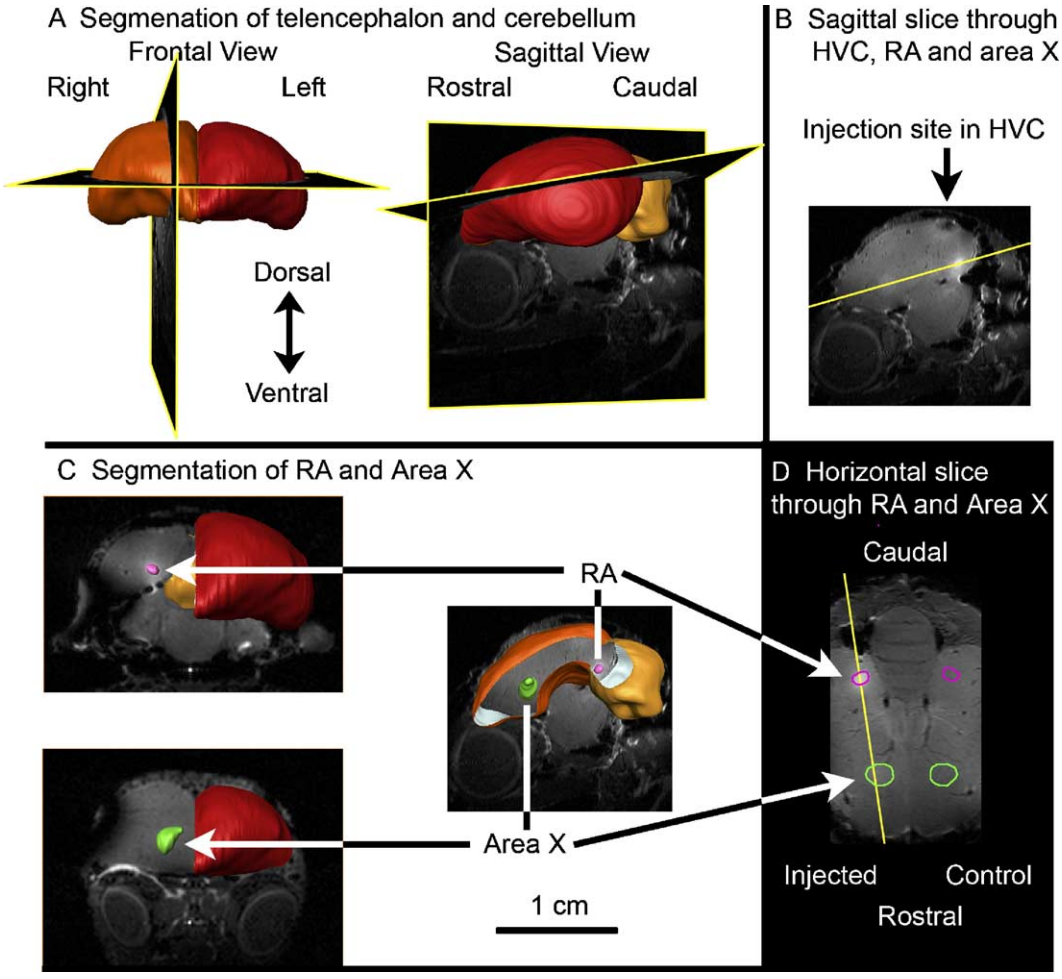

Fig. 2. Illustration of data processing. (A) The left and right telencephalon (red and orange) and the cerebellum (yellow) are presented as 3D reconstructions in frontal (left) and sagittal view (right). The brain is sliced through the song control nuclei in sagittal (B) and horizontal direction (D). (B) On the sagittal slice, the injection spot in HVC is indicated and Mn^{2+} labeling is clearly visible in the two main HVC targets, RA and area X. (C) 3D volume reconstructions of both RA and area X are presented on frontal (left), and sagittal (right) views. (D) The horizontal slice illustrates how RA (pink) and area X (green) were delineated as well as the control regions in the left brain hemisphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

One set of gradient echo (GE) scout images containing 3 sagittal, 3 horizontal and 3 coronal slices and one set of 20 coronal GE images were first acquired to determine the position of the brain (field of view [FOV] = 40 mm, echo time [TE] = 5 ms, repetition time [TR] = 500 ms, acquisition and reconstruction matrix = 128 × 128). Multiple scout images were obtained in order to position a 3D scan as standard as possible in all individuals and during the multiple measurements within each animal. The orientation of the coronal slices were taken to conform the canary brain atlas of Stokes et al. (1974; see also Van der Linden et al., 1998).

To visualize the Mn^{2+} labeling of the tracts emerging and nuclei receiving projections from HVC, a T1-weighted high resolution spin echo 3D dataset of the brain was obtained for each bird (spectral width = 25 kHz, TE/TR = 18/300 ms; FOV = 25 × 25 × 25 mm^3; 1 average; acquisition matrix = 128 × 128 × 256; image reconstruction matrix = 256 × 256 × 256).

During the whole experiment (3h), the anesthetic was infused in the chest muscle at a constant rate of 0.15 ml/h. The body was wrapped in a jacket and laid on a warm water pad connected to an adjustable heating pump (Neslab Instruments, ex111, Newington, CT, USA). Temperature was continuously monitored with a cloacal probe (SA-instruments, Inc., New York, USA) and maintained at 41.5 ± 0.5°C. Respiration rate and expired CO_2 concentration were monitored with a capnometer (Capstar-100, CWI Inc., Diss Norfolk, UK) to keep the starlings in optimal and stable physiological conditions during the experiment.

In July, the birds were kept anesthetized after the imaging procedure described above that visualized the connections of the right HVC. They were then injected with $MnCl_2$ in the contralateral left HVC following the same protocol as described above. This was done to evaluate possible effects of the injection in HVC in March on the second measurement in July for the right hemisphere. Twenty-four hours later again a T1-weighted 3D image was acquired. Experimental procedures were in agreement with the Belgian laws on “Protection and Welfare of Animals” and approved by the Ethical Committee of the University of Antwerp.

Data processing

The volumes of the song control nuclei RA and area X in the right (injected) hemisphere, the volume of the cerebellum and

volumes of the left and right telencephalon were measured in the 3D-images collected in March and in the first set of images collected in July by manually segmenting these structures on the T1-weighted 3D dataset using Amira software version 3.1 (Mercury Computers Systems, San Diego, CA, USA) (Fig. 2).

The mean signal intensity (SI) within RA and area X was calculated as the percent difference relative to a control region of the exact the same size located at the same level as each respective nucleus in the left (contralateral) hemisphere (Fig. 2D). The total amount of Mn^{2+} that was transported to RA and area X was calculated by multiplying volume of each nucleus by its respective SI. The second set of 3D-images collected in July was used only to calculate the volumes of area X and RA in the left hemisphere.

Statistical analysis

Changes in behavioral or morphological measures between March and July were analyzed by paired or unpaired *t* tests or two way analyses of variance (ANOVA; SPSS, Inc., Chicago, IL, USA) with repeated measures as appropriate. Relationships between individual differences in song features and brain measures were analyzed with the Pearson's product moment correlation coefficient. Differences or correlations were considered significant for $P < 0.05$. All data in the text and figures are represented by their mean ± standard deviation (SD).

Results

Seasonal changes

Singing behavior

In March, females spent 18 ± 12% (mean ± SD) of their time singing, with average female singing rates ranging from 5 to 48%. In contrast, in July, none of the females were observed singing during the 5 days observation period. Such a difference in singing occurrence is obviously significant by a chi-square test (χ^2 = 41.172, $P < 0.0001$).

In March, we also observed large individual differences in song bout length and repertoire sizes among the 11 females. Average song bout lengths varied between 12 and 25 s (mean ± SD = 18 ± 3 s), while repertoire size varied between 11 and 23 phrase types (mean ± SD = 19 ± 4).

Telencephalon and cerebellum volume

No difference was found between the left and right telencephalon volume, and no change in these measures took place between March and July ($March_{right}$: 506.59 ± 22.65 mm^3; $July_{right}$: 503.26 ± 27.34 mm^3; $March_{left}$: 504.04 ± 26.15 mm^3; $July_{left}$: 505.38 ± 31.71 mm^3; two-way repeated measures ANOVA with brain side and season as repeated factors: side: $F_{1,21} = 0.025$, $P = 0.877$; season $F_{1,21} = 0.148$; $P = 0.705$; interaction: $F_{1,21} = 2.440$; $P = 0.133$). Because there was no left–right difference, we used the average volume of both sides during subsequent analyses. Similarly the volume of the cerebellum did not vary between March and July (March: 153.72 ± 11.44 mm^3; July: 152.49 ± 11.97 mm^3; paired *t* test: $t_{21} = 0.713$; $P = 0.484$).

Song control nuclei

Injection of manganese in HVC reliably labeled after 24 h the two nuclei that are the main targets of HVC-projections, RA and area X, thus allowing the calculation of their volume (see Figs. 2 and 3). This transport and accumulation of manganese was, however, dramatically affected by the season. In March, both RA and area X could be readily detected and delineated in all birds (23 of 23 injected birds), whereas in July RA could only be delineated in 10 out of 19 injected birds and only 2 birds showed a detectable area X (see general decrease of labeling in Fig. 3: in July RA is very faint and area X barely visible).

The decrease in SI measured for RA was significant with an unpaired *t* test comparing all RA measurements obtained in March ($n = 23$) with the RA measures obtained in July for the 10 subjects in which RA could be delineated ($t_{31} = 2.2618$; $P = 0.0309$) (Fig. 4). However, when a paired *t* test was used to compare the repeated SI measurements of RA in the subset of 10 birds that had been measured twice, the statistical significance of this difference

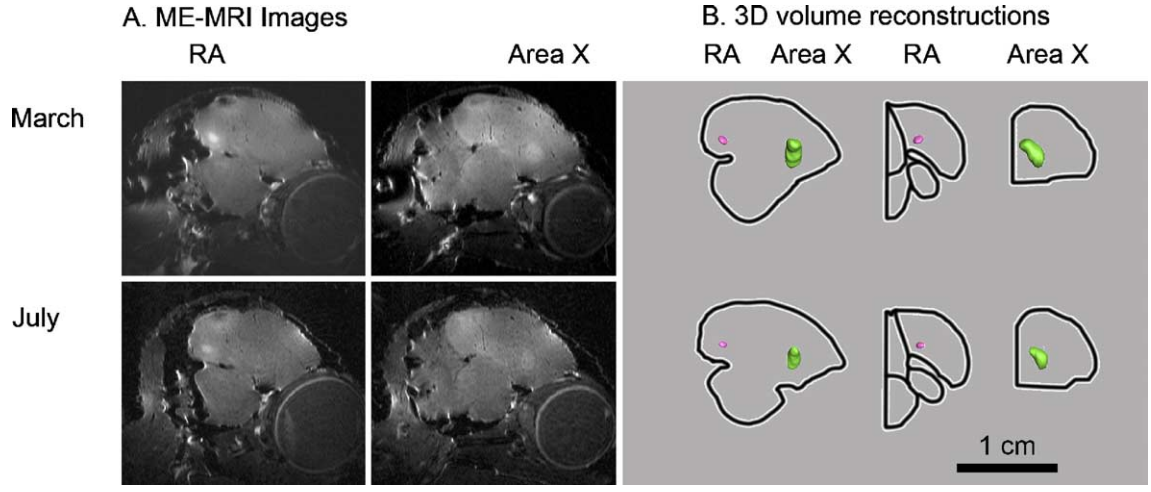

Fig. 3. Seasonal changes in manganese-dependent SI and volumes of RA and area X. (A) Illustration of changes in labeling of RA (left) and area X (right), between March (Top) and July (Bottom) in a bird where both RA and area X could be visualized in both seasons. (B) 3D volume reconstructions of the song control nuclei RA (pink) and area X (green) in sagittal (left) frontal (middle and right) direction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

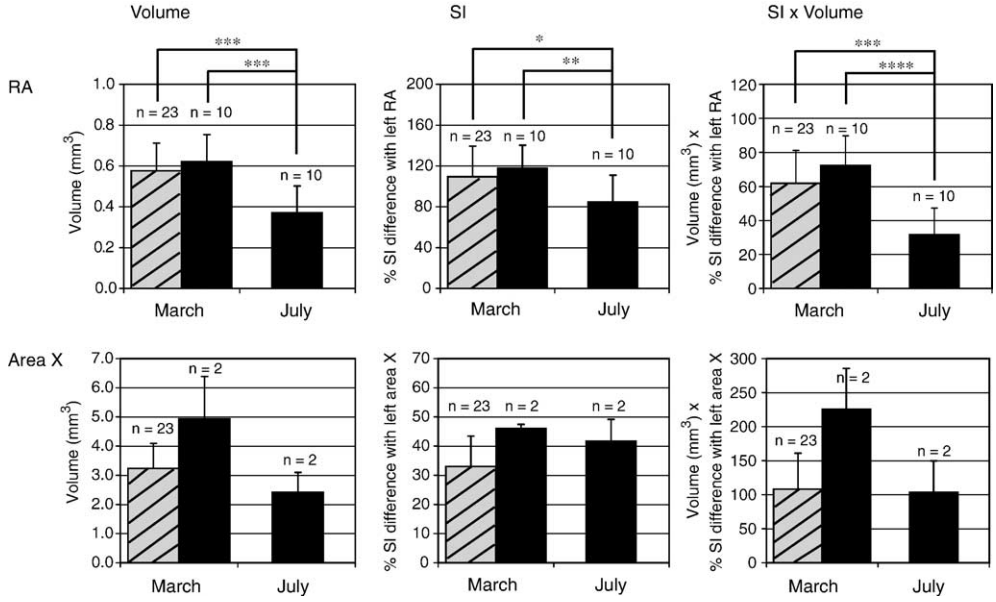

Fig. 4. Quantitative analysis of seasonal changes. Seasonal changes in volume, SI and SI × volume (from left to right) in RA (top) and area X (bottom). Dashed gray bar indicates the group means with SD from all birds measured in March. The black bars represent the means and SD of values for birds in which the song control nuclei could be delineated both in March and July. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

increased approximately 10-fold ($t_9 = 3.9582$; $P = 0.0033$) despite the decrease in the number of measures.

The decrease in signal intensity was associated with major changes in the volume that was highlighted by the manganese accumulation. The RA volume decreased by about 46% between March and July. This decrease was significant (see Fig. 4) and again, the difference in volume between March and July was slightly more significant despite the major decrease in the degrees of freedom, when comparing by a paired t test the 10 birds that could be visualized both in March and July ($t_9 = 5.5231$; $P = 0.00037$) than when comparing by an unpaired test all 23 birds imaged in March with the 10 birds that revealed a detectable RA in July ($t_{31} = 3.8989$; $P = 0.00048$).

As could be expected from the significant decrease in both volume and SI, the total amount of Mn^{2+} transported to RA, as estimated by the product of the volume by SI, decreased significantly regardless of whether paired (paired t test: $t_9 = 8.3521$; $P = 0.00002$) or unpaired measurements (unpaired t test: $t_{31} = 4.3678$; $P = 0.00013$) were used. Again the paired test led to a higher significance than the unpaired approach.

Similarly, the area X detected in two birds in July was approximately 50% of the original volume measured in March (3.90 mm^3 and 5.96 mm^3 in March versus 1.94 mm^3 and 2.90 mm^3 in July, respectively; average value for 23 birds in March: 3.24 ± 0.86 mm^3; Figs. 3B, 4). The SI measured in the area X of the two birds in which this nucleus could be identified in July did not obviously differ from the SI measured in March and correlatively no major seasonal change in the total amount of Mn^{2+} transported to area X could be identified (Fig. 4).

To test whether this decrease in the volumes labeled by manganese could be due to a lesion at the injection site, we also injected $MnCl_2$ in the left (previously uninjected) brain side of all birds that were injected in the right HVC in July (Fig. 5A). Following this second injection of $MnCl_2$, RA could be delineated in 11 out of 19 birds, including 7 of the birds in which RA had also been visualized in the right hemisphere. Thus in 3 birds RA was seen in the right hemisphere only, in 4 birds it was seen in the left hemisphere only and in 7 birds RA was seen on both sides. In the latter 7 birds, RA volumes in the two hemispheres did not differ significantly (RA_{right}: 0.35 ± 0.16 mm^3; RA_{left}: 0.37 ± 0.06 mm^3; a paired t test between left and right brain-side: $t_6 = -0.424$; $P = 0.687$). Area X was detected in two subjects out of 19 on the left brain side. These two birds were the same as those in which this nucleus could be detected on the right side and area X had similar volumes on both sides (1.94 mm^3 and 2.90 mm^3 versus 2.40 mm^3 and 2.83 mm^3 in the left and right hemisphere respectively). Detectability of RA and area X in July was thus similar in the two brain hemispheres independent of whether they had been previously injected or not, suggesting the volumetric measurements were not affected by the repeated injections.

Finally, we evaluated whether there were differences in March between the group of females in which RA could be delineated both in March and July ($n = 10$: only injections in right hemisphere) and the group where RA could be visualized only in March ($n = 9$) (Fig. 5B). Unpaired t tests did not reveal any significant difference between the two groups of females neither for RA volume ($t_{17} = 1.689$, $P = 0.110$), nor for SI in RA ($t_{17} = 0.325$; $P = 0.749$) nor for the total amount of Mn^{2+} transported to RA ($t_{17} = 1.735$, $P = 0.101$). However, there was a difference between these two groups based on RA detectability in July when comparing the volumes of area X: birds in which RA was detected in July had in March an area X that was almost significantly larger ($t_{17} = 2.007$, $P = 0.053$). No difference between these groups was detected for SI in area X ($t_{17} = -0.698$, $P = 0.495$), but the total amount of manganese that was transported to area X in March was significantly higher in the group of birds where RA could be delineated in July ($t_{17} = 2.247$, $P = 0.038$).

Correlations between individual variations in MRI measures and song behavior in March

The Pearson's correlation was then used to examine whether the three measures of singing behavior collected in March (song bout

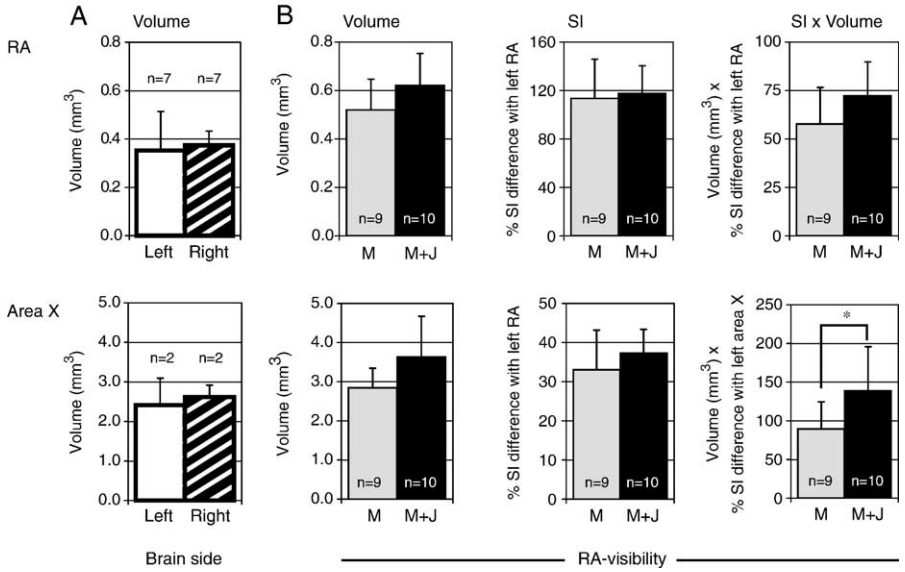

Fig. 5. Validation of repeated measures. (A) Comparison between the volumes of RA (top) and area X (bottom) between left (white) and right (dashed) hemisphere in July. (B) Comparison between the group of 10 birds where RA could be repeatedly delineated (in March and July: Black) and the group of 9 birds where RA could be delineated only in March (Gray). *$P < 0.05$.

length, repertoire size and song rate) were in any way related to the brain measures obtained by MRI in manganese-injected birds.

In the 11 females that had their songs tape-recorded in March, the three measures of singing that had been collected were not correlated with each other although song rate tended to co-vary with song bout length (Pearson's correlation: song rate versus song bout length: $r = 0.538$, $P = 0.087$; repertoire size versus song bout length: $r = 0.486$, $P = 0.130$; song rate versus repertoire size: $r = 0.136$, $P = 0.691$).

No significant correlation was detected between the song measures (obtained in 11 or 23 birds) and the different brain measures (See Table 1). The manganese signal intensities in RA and area X were, however, positively correlated ($r = 0.684$; $P < 0.001$). The volumes of RA and area X were not significantly correlated

Table 1
Correlations between brain and behavior in March

		Song			Volume				SI		SI × volume	
		SBL	RS	SR	Tel	Cb	RA	Area X	RA	Area X	RA	Area X
Song	SBL (n = 11)	–	0.486	0.538	−0.102	0.261	0.359	0.313	−0.229	−0.040	0.022	0.145
			0.130	0.087	0.766	0.438	0.278	0.348	0.498	0.908	0.950	0.671
	Rep size (n = 11)			0.136	0.054	−0.057	−0.157	0.028	0.180	−0.146	0.126	−0.138
				0.691	0.874	0.868	0.645	0.934	0.597	0.669	0.711	0.686
	Song rate (n = 23)				−0.264	0.054	0.112	0.057	0.031	−0.031	0.108	−0.001
					0.224	0.807	0.612	0.796	0.888	0.889	0.623	0.997
Volume	Tel (n = 23)					0.137	0.347	**0.437**	−0.082	0.022	0.234	0.333
						0.534	0.104	**0.037**	0.709	0.922	0.282	0.120
	Cb (n = 23)						−0.135	0.112	0.129	0.322	−0.028	0.282
							0.540	0.610	0.559	0.134	0.900	0.192
	RA (n = 23)							0.399	−0.320	−0.090	**0.498**	0.230
								0.059	0.136	0.683	**0.016**	0.291
	Area X (n = 23)								0.013	0.162	0.405	**0.741**
									0.953	0.460	0.055	**<0.001**
SI	RA (n = 23)									**0.684**	**0.649**	**0.494**
										<0.001	**0.001**	**0.017**
	Area X (n = 23)										**0.517**	**0.770**
											0.011	**<0.001**
SI × Volume	RA (n = 23)											**0.656**
												0.001
	Area X (n = 23)											–

Pearson's correlation coefficients (r) between measures of song behavior, different brain measures, and manganese accumulation in RA and area X reflected by the volume outlined by manganese, the manganese signal intensity (SI), and the total amount of manganese accumulated (SI × volume). Significant correlations are indicated in bold.

Abbreviations: SBL = song bout length; rep. size = repertoire size; Tel = telencephalon; Cb = cerebellum; RA = nucleus robustus arcopallii. In each case, the table provides the correlation coefficient r (1st line) and the associated probability (2nd line).

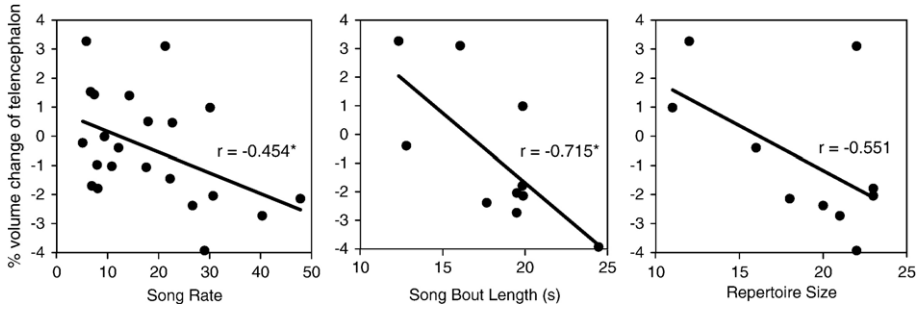

Fig. 6. Correlation between the seasonal changes in telencephalon volume and the three measures of song output. From left to right: Song rate, song bout length and repertoire size. Significant correlations are indicated with $*P < 0.05$.

(although there was a trend: $r = 0.399$ $P = 0.059$). The volumes defined by $MnCl_2$ were also not correlated with the signal intensities in these nuclei (RA: $r = -0.320$, $P = 0.136$; area X: $r = 0.162$, $P = 0.460$). The volume of area X was significantly correlated with the telencephalon volume ($r = 0.437$, $P = 0.037$) but this correlation was not significant for RA ($r = 0.347$, $P = 0.104$). The total amounts of Mn^{2+} transported to RA and to area X were also significantly correlated ($r = 0.656$; $P < 0.001$) and these total amounts also frequently correlated positively with the corresponding volume or SI measures as could be expected (see detail in Table 1).

Relationship between seasonal changes in telencephalon and cerebellum volume and song output

Repeated measures of the telencephalon and cerebellum volumes were obtained in 22 birds. Since birds completely stopped singing in July the song output in March could be considered equal to the change in song output from March to July. We found a significant negative correlation between the song rate in March and the percent change in telencephalon size between March and July ($r = -0.454$, $P = 0.034$). Given that there was no significant overall change in telencephalon volume between March and July, this correlation means that in the birds that sang a lot in March, the brain tended to shrink from March towards July, whereas in the low rate singers the size of the telencephalon tended to increase during the same period.

In the subset of 10 subjects in which the song bout length and repertoire size were calculated there was also a significant correlation between changes in telencephalon size between March and July and song bout length ($r = -0.715$, $P = 0.020$) and a trend to significance for repertoire size ($r = -0.551$, $P = 0.099$) (Fig. 6).

None of the three song measures were correlated with the seasonal change in cerebellum size (all $P > 0.203$; data not shown).

Relationship between singing behavior, season and manganese-dependent RA measures

Seasonal changes in volume, SI and total amount of Mn^{2+} transported (SI × volume) could only be assessed in RA in a subset of birds where this nucleus could be repeatedly delineated. To test whether the differences in March song rates were related to seasonal changes in these measures, we separated the 10 subjects in which RA was repeatedly visualized into Low rate Singers and High rate Singers ($n = 5$ in each group) and separately plotted volumes, SI and SI × volume in these two subsets of birds (see Fig. 7). A two-way ANOVA with season as repeated factor and groups (High versus Low singers) as independent factor confirmed a significant decrease of the RA volume between March and July ($F_{1,8} = 28.646$; $P = 0.001$) but identified no difference between groups ($F_{1,8} = 0.163$; $P = 0.697$) and no interaction between season and groups ($F_{1,8} = 0.452$; $P = 0.521$). The manganese-dependent SI within RA also changed significantly with seasons ($F_{1,8} = 13.972$; $P = 0.006$) and interestingly was, in addition, significantly different between the two groups (higher in High than in Low singers; $F_{1,8} = 5.650$; $P = 0.045$), but no interaction between season and groups was present ($F_{1,8} < 0.001$; $P = 0.985$). The group difference was nearly significant for SI × volume ($F_{1,8} = 4.947$; $P = 0.057$). SI × volume was also significantly higher in March than in July ($F_{1,8} = 72.083$; $P < 0.001$) and again no interaction was found between season and groups ($F_{1,8} = 1.300$; $P = 0.287$).

Thus, in this subgroup of 10 animals, SI in RA was significantly higher in birds with high song rate, despite the lack of significant correlation between SI and song rate in March when all animals were included (Table 1).

To further evaluate the relation between song rate and RA visualization, we then compared the SI measurements in March

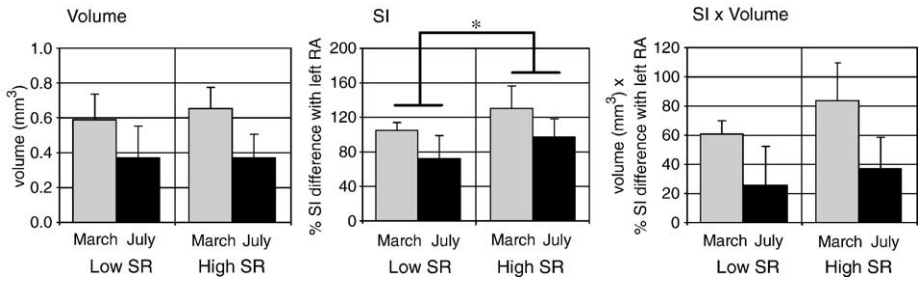

Fig. 7. Effect of song rate in March on repeated measures of RA. Seasonal changes in RA measures (volume, SI and SI × volume) in a group of birds where RA could be delineated both in March (Gray bars) and July (Black bars). The birds were additionally divided into a group with individuals singing at low song rate ($n = 5$) and a group with individuals singing at high song rate ($n = 5$). $*P < 0.05$.

between the two groups of females defined by a differential RA-visualization in July, adding this as a second factor to the analysis besides the factor indicating whether a bird had a high or a low song rate in March. The top panel of Fig. 8A illustrates the distribution on individual average song rates measured in March, i.e. just before the first set of in vivo imaging data were collected. Arrows in this figure indicate the subjects in which RA could be delineated with ME-MRI in July. Based on these behavioral data, birds were subdivided in two subgroups (Low versus High song rate [SR]; see vertical bar in Fig. 8A) and further divided based on whether RA was detected in March only or in March and July. The fact that manganese accumulation was sufficient to detect RA in July did not obviously relate with the average occurrence frequency of singing in March and this observation could be statistically confirmed through the analysis of song rates by a two-way ANOVA with song rate (High versus Low singers) and the RA-visualization (visible versus not-visible in July) as independent factors (Fig. 8A, bottom panel; Table 2). This analysis detected as expected a significant effect of the song rate factor ($F_{1,15} = 40.141$; $P < 0.001$) but there was no effect of the factor RA-visualization ($F_{1,15} = 1.887$; $P = 0.190$) and importantly no interaction between the two factors ($F_{1,15} = 0.505$; $P = 0.488$).

The same analysis was then carried out for all measures of RA and area X that had been collected in March (volume, SI and SI × volume) (Fig. 8B and Table 2).

In RA, there was no significant effect of the factors RA-visualization or song rate on the volume, SI or SI × volume (all $F_{1,15} < 3.480$, $P > 0.082$; see detail in Table 2). However, we found an interaction between song rate and RA-visualization for the SI in RA ($F_{1,15} = 5.030$; $P = 0.040$): depending on which RA-visualization-group the birds belonged to, high SI in RA was associated with low or high song rate. In birds with a low song rate, SI measured in March in RA was higher in the group where RA could be delineated in March only than in the group where RA could be delineated in both March and July and an opposite pattern was seen in the group of females with high song rate. Post hoc pairwise comparisons however did not detect any significant difference between the four individual groups (Newman–Keuls: all $P > 0.117$).

In area X, as shown before (Fig. 5), the factor RA-visualization (in July) had an effect that was almost significant for the volume of area X ($F_{1,15} = 4.060$; $P = 0.062$) and significant for the amount of manganese (SI × volume) that was accumulated in area X in March ($F_{1,15} = 4.994$, $P = 0.041$) but had no significant effect on the density of the manganese labeling, SI ($F_{1,15} = 0.456$; $P = 0.510$). For all these measures in area X, there was no additional

Table 2
The effect of song rate and RA-visibility in July on measurements in March

	Song rate March		RA visibility July		Interaction	
	$F(1,15)$	P	$F(1,15)$	P	F(1,15)	P
RA						
Volume	1.197	0.291	2.496	0.135	0.002	0.965
SI	0.005	0.944	0.231	0.638	5.030	**0.040**
SI * volume	1.192	0.292	3.671	0.075	3.480	0.082
Area X						
Volume	2.292	0.151	4.060	0.062	0.020	0.890
SI	0.793	0.387	0.456	0.510	0.553	0.469
SI * volume	0.244	0.628	4.994	**0.041**	1.134	0.304

Two-way ANOVAs analyzing the effect of song rate (Low vs. High song rate) and RA-visibility (RA visible in March and July or in March only) on measures of volume, SI and SI × volume obtained in March for RA and area X. Song rate and RA-visibility are independent factors. P values indicated in bold are considered significant ($P < 0.05$).

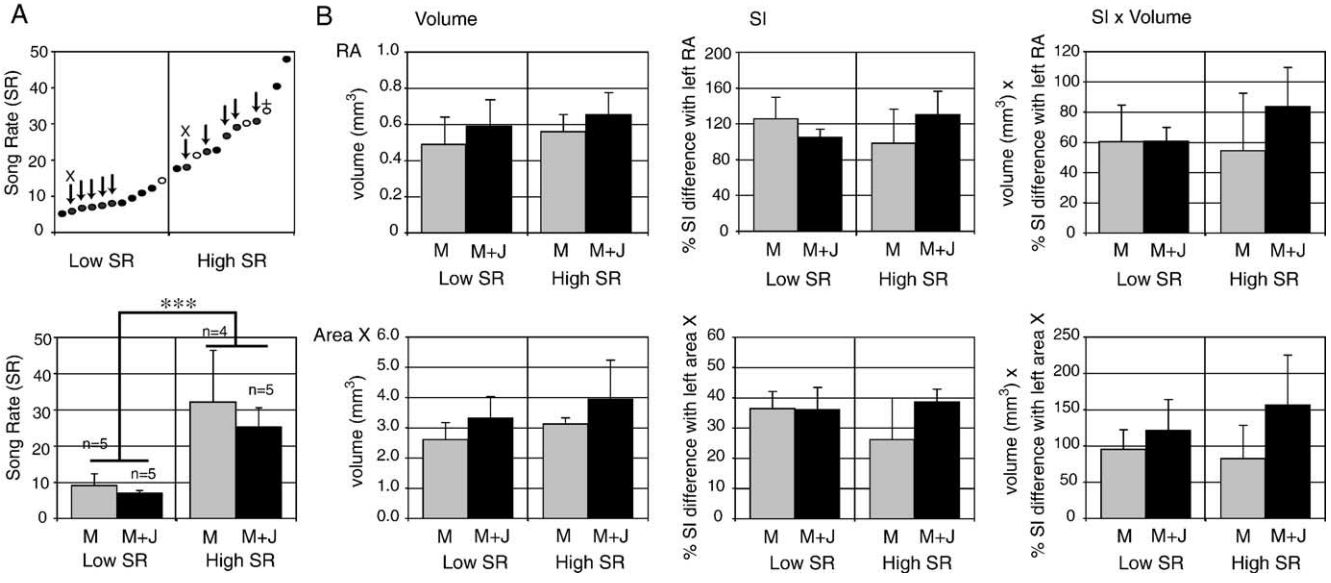

Fig. 8. The effect of song rate in March and RA-visibility in July on measurements in March. (A) Top: the individual song rates measured in 2003 ranked in increasing order. Arrows indicate the individuals from which RA was visible by ME-MRI in July. The two individuals from which area X could be delineated in July are indicated with 'X'. Open dots are the subjects that were only injected in March. One bird that died between the first and second measurement is indicated with +. Bottom: Comparisons of the mean song rates for the subgroups formed by song rate (SR: Low SR versus High SR) and RA-visualization (Gray bars = visible in March only; Black bar = visible in March and July). (B) Comparisons of the mean song control nuclei measures obtained in March for the subgroups based on song rate (SR) observed in March (Low SR versus High SR) and RA-visualization (Gray bars: visible in March only; Black bars: visible in March and July) *$P < 0.05$, ***$P < 0.001$.

significant effect of song rate and no interaction between the factors song rate and RA visualization (all $F < 2.292$; $P > 0.151$; see Table 2 for detail).

Discussion

We visualized by repeated ME-MRI tract-tracing the seasonal changes in volume of two song control nuclei and the activity of their respective afferent pathways emerging from HVC in individual female starlings following manganese injection in HVC. From March to July there was a significant decrease of area X and RA volumes and a major decrease in the activity of the rostral basal–ganglia pathway and caudal motor pathway projecting to these nuclei as determined by the density of manganese labeling. This regression of the song system occurred together with a complete suppression of singing activity. Although none of the song control nuclei volumes measured in March or July were related to any of the song parameters obtained in March, song rate and song bout length were correlated with the change in telencephalon size.

Volumetric measures

Two studies previously compared free ranging male and female songbirds and found that the relative seasonal change of the song control nuclei was similar or larger in females than in males (red-winged blackbirds, *Agelaius phoeniceus*, Kirn et al., 1989; dark-eyed juncos, *Junco hyemalis*, Deviche and Gulledge, 2000). The seasonal volumetric changes found here in female starlings where RA and area X could be repeatedly visualized also have a similar amplitude as those observed in free ranging males (Riters et al., 2002). The in vivo volumetric measurements presented here thus agree with previous histological studies.

Interestingly, the seasonal changes in RA volumes were more significant when comparing repeated measures on the same subjects than independent measures on a larger group of birds despite the fact that fewer cases were available in the former than in the latter case. This illustrates the power of in vivo imaging, which allows repeated measures that can assess variations in time without the confounding factor of individual differences. In addition, in vivo imaging can assess dynamic relationships between changes in brain features and in behavior.

Although seasonal changes in telencephalon volume did not exceed 4% and were not significant, they were surprisingly correlated with individual measures of song rate and song bout length (Fig. 6). This volume tended to increase from March to July in females with a low song output or short song bout length but decreased in birds with a high song output or song bout length. These brain changes appear counterintuitive, but it must be noted that in female dark-eyed juncos, who never sing, telencephalon width increases after the breeding season (Deviche and Gulledge, 2000), whereas in female red-winged blackbirds, who do sing during the breeding season, brain weight decreases after the breeding season (Kirn et al., 1989). The present findings based for the first time on repeated measures in the same subjects suggest a relationship between the amount of singing activity and telencephalon size. This relation is anatomically specific since no correlation was detected between cerebellum changes and singing activity. The correlation does not seem to reflect a larger transient development in March of the telencephalon in high rate singers (that would lead to a bigger decrease after the breeding season) because in March, the telencephalon volume was not correlated with singing rate. It is however possible that these correlations are obscured by stable individual differences and only become apparent when rate of changes in individual measures are considered, which can only be done by in vivo MRI. Whether this relationship between changes in the telencephalon and in singing activity reflects direct causal links relating brain to behavior or vice versa remains to be elucidated.

Manganese accumulation in RA and area X

Mn^{2+} uptake in cells depends on the activity of voltage-gated calcium channels (Aoki et al., 2002; Duong et al., 2000; Lin and Koretsky, 1997) and Mn^{2+}-ions have the capacity to move along neuronal pathways (Pautler et al., 1998; Saleem et al., 2002; Van der Linden et al., 2002; Watanabe et al., 2001). Both properties lead to the ability to perform activity-dependent tract-tracing (Pautler and Koretsky, 2002). Hence, measurements of SI in RA and area X should reflect the activity of the two pathways emerging from two separate neuronal populations within HVC (Van der Linden et al., 2004).

Because area X-projection neurons in HVC are considered to be stable in number throughout adult life (Alvarez-Buylla and Kirn, 1997), the decrease in SI within area X could be due to a change in activity of these neurons presumably present in similar numbers at both seasons. In a longitudinal ME-MRI study of female starlings, we previously reported that treatment with testosterone dramatically increases the manganese dependent SI in area X (Van Meir et al., 2004). From March to July, female starlings experience a dramatic drop in estrogen (and possibly androgen) levels (Dawson, 1983), which should affect the physiology of HVC to area X projecting neurons that express both androgen and estrogen receptors (Bernard et al., 1999; Johnson and Bottjer, 1995; Sohrabji et al., 1989). This change in activity should be quite dramatic since manganese uptake visualized area X in only two out of 19 birds in July, but in all birds in March.

In contrast, the SI changes in RA could have two causes. First, as discussed above for area X, the activity-dependent Mn^{2+}-uptake through voltage-gated calcium channels in HVC neurons projecting to RA could be reduced following seasonal changes in steroid levels. Second, the decrease in number of RA-projecting neurons which is known to occur for this neuron-type between the breeding and non-breeding season (Alvarez-Buylla and Kirn, 1997) could also contribute to a decreased Mn^{2+} transport. Interestingly, although two mechanisms are available to explain decreased manganese transport to RA, this decrease was less prominent than in area X. In HVC, RA-projecting neurons express androgen but not estrogen receptors whereas area X-projecting neurons express both types of receptors. Potentially, the more important decrease in manganese transport to area X could thus relate to the modulation by estrogen of area X-projection neurons.

In one subgroup of females, the boundaries of RA could still be delineated in July and hence volume determination was still possible, whereas in the other birds the clear localization of this nucleus became impossible. This could indicate a differential contribution, in the two mechanisms described above. One subgroup could for example have experienced a smaller decrease in amount of RA-projecting neurons within HVC.

The different pattern of seasonal change for RA (nucleus visible or not in July) was interestingly related to the total amount of Mn^{2+}

accumulated in area X in March: females that retained a detectable RA in July were accumulating more manganese in area X in March than females in which RA became undetectable (Fig. 5). The seasonal plasticity in morphology of the caudal motor pathway (amount of RA-projecting cells in HVC) might therefore depend on the morphology (volume of area X and amount of X-projecting cells in HVC) of the rostral basal–ganglia pathway. The causal links involved remain however unclear.

In the group retaining a detectable RA in July, a higher manganese SI was detected in RA, both in March and July, in females that were singing at high rates compared to low rate singers (Fig. 7). Additionally, a higher Mn^{2+}-accumulation was observed in March in the area X of females that retained a detectable RA in July (Figs. 5B and 8). Together these observations indicate that the survival or activity of the RA-projecting neurons in July might be affected not only by the specific activity of this pathway (as reflected by song output measured in March) but also by the activity or density of the rostral forebrain pathway projecting to area X. This functional interaction between both types of HVC projection neurons is also supported by the significant interaction (Fig. 8 and Table 2) between singing rate (associated with activity in the HVC to RA projection; Fig. 7) and RA detectability in July (associated with accumulation of manganese in area X; Fig. 5) in the control of the manganese SI observed in RA in March.

Available evidence for regulatory interactions between stable X-projecting and replaceable RA-projecting neurons (Holzenberger et al., 1997; Scharff et al., 2000), and the effect of singing activity on survival of RA-projecting neurons (Li et al., 2000) and HVC-size (Sartor and Ball, 2005) indicates that both mechanisms described above could be at work. However, as shown here, discriminating experience-dependent from stable individual differences in neuronal plasticity requires repeated measures of brain and behavior.

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