Acoustic signalling for mate attraction in crickets: Abdominal ganglia control the timing of the calling song pattern

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Abstract

Decoding the neural basis of behaviour requires analysing how the nervous system is organised and how the temporal structure of motor patterns emerges from its activity. The stereotypical patterns of the calling song behaviour of male crickets, which consists of chirps and pulses, is an ideal model to study this question. We applied selective lesions to the abdominal nervous system of field crickets and performed long-term acoustic recordings of the songs. Specific lesions to connectives or ganglia abolish singing or reliably alter the temporal features of the chirps and pulses. Singing motor control appears to be organised in a modular and hierarchically fashion, where more posterior ganglia control the timing of the chirp pattern and structure and anterior ganglia the timing of the pulses. This modular organisation may provide the substrate for song variants underlying calling, courtship and rivalry behaviour and for the species-specific song patterns in extant crickets.

Keywords: cricket; calling song; central pattern generator; abdominal ganglia; modular organization; temporal patterns

1. Introduction

Species-specific acoustic signals for mate attraction are used in a wide range of animals like birds, frogs, fishes and insects [1-3]. These signals are crucial for an individual's mating success and play a major role in behavioural isolation and in speciation [4].

In acoustically communicating insects, anurans and fishes, signals are often composed by stereotypical pulse patterns, which are genetically determined and consistent between individuals [3, 5]. Sound production is under temporal control of the nervous system by the activity of central pattern generators (CPGs) [6-9]. The most important properties of the acoustic signals rely on their temporal features, *e.g.* rate, duration, amplitude and subdivision into pulses [10, 11]. This is well exemplified in the acoustic behaviour of the Hawaiian *Laupala* crickets [12, 13]. Here pulse rates of male songs of closely related species diverge remarkably, and in combination with female preferences, support the hypothesis that male calling songs play a causative role in the rapid speciation of this group [14, 15]. The importance of temporal cues in species recognition and in the evolution of acoustic communication systems is also demonstrated in bushcrickets [16], treefrogs [17, 18] and fishes [19, 20]. Revealing how the temporal properties of the acoustic signals emerge from the activity of the nervous system is crucial not only to understand the neural organisation of the behaviour but also to provide new notions for its genetic, molecular biological and evolutionary analysis [21].

Male crickets rhythmically rub their forewings together to produce species-specific song patterns, a calling song to attract females, a courtship song before mating and a rivalry song on encounter with other males. Here we have focused on the calling song of the two-spotted field cricket *Gryllus bimaculatus,* which is composed by 3-5 sound pulses grouped in chirps. Their acoustic signals consist of two rhythms: one slow (3-4 Hz) timing the chirp sequence

and a fast one (30 Hz) timing the pulses [22-24], however, the neural organization of these timers is not yet understood.

Initial theories assumed that the mesothoracic ganglion, which houses the forewing motoneurons [24-26], would also house the CPG for singing [27]. However, males failed to sing when the connectives behind the thoracic ganglia were cut [7, 28], and differential heating of the central nervous system (CNS) suggested that the abdominal ganglia play a crucial role in singing [29]. In line with this, recent electrophysiological recordings have shown that interneurons of the singing network span from the metathoracic ganglion complex $T3_{A1/A2}$ [30, 31] to at least the first unfused abdominal ganglion A3 [7, 8].

Altogether, this evidence points towards the importance of the abdominal ganglia in the singing behaviour of crickets. Nonetheless, it is still not clear how the singing network is organised to control the temporal patterns of chirps and pulses. In order to reveal the contribution of the abdominal ganglia for calling song generation, we performed selective lesions in the abdominal ganglia chain of male *G. bimaculatus* and subsequently followed their singing behaviour with long-term acoustic recordings.

2. Material and methods

2.1. Experimental animals

Crickets [white-eye strain of *Gryllus bimaculaus* DeGeer; autosomal recessive, *gwhite* [32], European wild-type (WT) *G. bimaculatus* and Japanese WT *G. bimaculatus*] were lab-reared in large communal terraria, until the penultimate instar, after which males were selected and kept individually in clear 17.5 x 11.5 x 13 cm containers until reaching sexual maturity. Crickets were housed at 28ºC with a 12h light:dark cycle and were provided ad-libitum with a mixture of muesli, fish food, cat food, and water. Experiments were performed from eight to eleven days post final ecdysis. The *G. bimaculaus gwhite* were larger and more robust than

our European WT colony and were more suitable to study the effect of central nervous system (CNS) lesions on singing motor activity. All experiments complied with the principles of Laboratory Animal Care [33].

2.2. Selective lesions to CNS

Male crickets were mounted ventral side up in a Plasticine block on a Peltier element (Peltron GmbH Peltier-Technik, Fürth, Germany) and cooled to 6°C. Two types of lesions were applied to the CNS (*Fig. 1A*), either a cut of the connectives between consecutive abdominal ganglia, or a mediosagittal hemisection of a particular abdominal ganglion, *i.e.* split. To expose the target ganglion and/or connective the abdominal intersegmental soft membrane was incised and the ventral cuticle was folded to one side. Exposed nervous tissue was perfused in insect saline (in mmol l^{-1}: NaCl 140; KCl 10; $CaCl_2$ 7; $NaHCO_3$ 8; $MgCl_2$ 1; N-trismethyl-2-aminoethanesulfonic acid 5; D-trehalose dehydrate 4) adjusted to pH 7.4. Fat tissue around the ganglia and connectives was removed. The split of a ganglion was applied with a blade fragment (8 x 1.5 mm; Geuder AG, Heidelberg, Germany) while connectives between two ganglia were cut with a fine pair of scissors (3 mm straight blade, Vannas Scissor, Super Fine; WPI UK, Hertfordshire, UK). After the procedure the ventral cuticle was folded back, the wound sealed by drying haemolymph and the animals recovered. Following the acoustic recordings and once the males had died, their nervous system was examined under a dissecting microscope to confirm the site of the applied lesion. Examination of the fixed tissue revealed conclusively whether a split was complete; in cases of doubt, data were discarded.

2.3. Song and video recordings

Selected males were individually kept in containers at 23-24ºC; each fitted with a standard PC microphone (Omni type; Maplin Electronics, Rotherham, UK). For two or three nights before and at least for ten nights after the lesion singing activity was recorded each night for 12 hours at a sampling rate of 48 kHz using Cool Edit 2000 software (Syntrillium Software Corporation, Phoenix, AZ, USA). Each lesioned male was video recorded (Praktica DVC 5.5 HDMI Flash Digital; Pentacon GmbH, Dresden, Germany) at least once during its lifetime. Males where placed in contact with females, to increase the probability of singing activity during the video recordings.

2.4. Data analysis

Song recordings were analysed with CED Spike2 software (CED, Cambridge, UK), using the in-built burst analysis feature, and NEUROLAB [34]. For each male, three 10 min time-windows at the beginning (1), middle (2) and end (3) of all overnight singing periods were chosen. These time-windows represent periods of stable singing activity, except where otherwise stated. The use of such temporally separated sections is sufficient to capture all the temporal variability of the calling activity produced by acoustic communicating animals [35]. From these time-windows, mean and standard deviation ($\bar{x}$±SD) of the chirp duration, the chirp period, and the interchirp interval, of the sound pulse duration and pulse period (*Fig. 1A*) were calculated. For this analysis, the following restrictions were applied: the minimum duration of pulses and the minimum interval between pulses were both set to 5 ms. Two consecutive pulses were considered to belong to a chirp if the inter-pulse interval was less or equal 50 ms. Data from single pulses were excluded from this analysis.

For a qualitative analysis of the sound patterns, the beginning of each sound pulse was plotted in sequential raster plots (symbolized by a +) for the time-windows selected (*c.f.* Fig. 1B as

an example). In the raster plot, the 1st pulse of a chirp is aligned at time zero and all preceding or subsequent pulses within +/- 500 ms are plotted to the left or right, respectively. Each pulse at time zero represents the start of a chirp, unless otherwise stated. Each row of the raster plot represents a subsequent chirp. In the normal calling song, chirps represent more than 99% of data and in the time-windows analysed individual single pulses were not included. After cutting the A3-A4 connectives or splitting the A4, single pulses occurred more frequently. Due to their long interpulse interval these pulses were plotted and quantified, in the raster plot and the corresponding cross-correlogram, as starts of chirps. Temporal progression during the overnight recording goes from the bottom of time-window 1 to the top of time-window 3. To illustrate the temporal frequency distribution of sound pulses after each lesion, a cross-correlogram is given for the three time-windows analysed. The cross-correlogram is aligned to the start of chirps and includes all pulses within +/- 500 ms, in order to show the frequency distribution of the sound pulses and the chirps. Due to the nature of the analysis, sound pulses will be evaluated more than once if they occur within the +/- 500 ms around subsequent chirps. The cross-correlogram and its inset have a bin width of 1.75 ms. The y-axis represents the normalized number of events (%) for each bin, 100% indicates that each event is represented, *e.g.* the start of the chirps; the inset is set to 10% of occurrence.

To compare the song parameters between different *G. bimaculatus* strains (European WT, Japanese WT and *gwhite* strain) statistical analysis was carried out using one-way ANOVA. The reference *gwhite* strain song parameters used for this analysis are the mean of each parameter recorded before the lesion. When appropriate, post hoc planned comparisons were performed contrasting the song parameter between strains.

Song parameters before and after lesions were compared using a two-way ANOVA with lesion and animal as between-subject main factors. The individual animals were included as a

factor in the two-way ANOVA to analyse cases where an effect of lesion could occur on just some of the animals. Unless otherwise stated, only the data of the song parameters 2-3 days before and for the first 3 days after the recovery of sound production were used. The analysis was restricted to 3 days after recovery in order to evaluate only acute effects. In locusts recovery of the flight motor pattern after deafferentation progressively occurred over a period of 1 to 2 weeks [36, 37]. When appropriate, post hoc planned comparisons were used contrasting the song parameter before and after the lesion in individual animals. Data were normally distributed and therefore the post hoc multiple comparisons, in both cases, were corrected using the Holm-Šídák test.

For statistical analysis, we used GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and Matlab (MathWorks, Inc., Natick, MA, USA).

3. Results

Long-term overnight sound recordings of the control and experimental crickets generated a data set of more than 1 million chirps. For each recording three 10-minute time-windows of stable singing activity, from the beginning, middle and end of the night [35], were analysed to provide reference data for the calling song and to scrutinize the effect of selective lesion in the abdominal ganglia chain (*Fig. 1A*). Experiments were performed on a white-eyed mutant *Gryllus bimaculatus*, *gwhite*, isolated from a wild-type lab colony [32]. Since *gwhite* males were slightly bigger, it was easier to perform the lesions of the abdominal ganglia chain.

3.1. Normal singing pattern of G. bimaculatus

In preparation to sound production, male crickets lift their front-wings and perform rhythmic opening and closing movements, with each closing movement producing a loud sound pulse

[27]. Low amplitude sound pulses also occur whenever the front-wings are lowered into resting position after singing.

As a reference, we recorded the singing activity of each *gwhite* male before any lesion was applied. During each overnight recording of a male normal calling song (*Fig. 1A*) an average of 28907±14813 chirps occurred ($\bar{x}$±SD; N=30; range 7995-59437 chirps). For the three analysed time-windows, the raster plot and corresponding cross-correlogram (*Fig. 1B*, see 2. Methods) show the high robustness of the pulse pattern constituting the chirps. The raster plot also demonstrates that the chirp period considerably varied during the three-time windows (1^{st} time window: 323±75 ms; 2^{nd}: 411±114 ms and in 3^{rd}: 294±45 ms), however, this did not alter the robustness of the pulse pattern. Within a chirp the timing of the pulses gradually becomes less precise, in *Fig. 1B* the SD for the start of the sound pulses increases from 1.1 ms for the 2^{nd} pulse, to 1.9 ms in the 4^{th} pulse.

Based on the analysis of 30 *gwhite* males (2569±1283 chirps/animal) the following reference data were obtained. Chirps have a mean duration of 124±12 ms and a mean period of 373±60 ms; the average number of sound pulses per chirp is 4.5±0.4; sound pulses have a mean duration of 17.9±2.0 ms and occur with a mean period of 30.2±2.2 ms (*Table 1*; *Video 1*). The chirp duration and the average number of sound pulses per chirp describe the chirp structure, and the chirp pattern is reflected by the chirp period.

The calling song parameters were compared between three different *G. bimaculatus* strains, European wild type (WT), Japanese WT and *gwhite* (*Table 1*). *G. bimaculatus* strains differed in the average pulse number per chirp ($F[2,47]=11.3$, $p<0.001$) and in the sound pulse period ($F[2,47]=45.5$, $p<0.001$). Pairwise comparisons revealed that European WT and *gwhite* differed in the average pulse number per chirp ($p < 0.001$) and sound pulse period ($p<0.001$). Importantly, no significant differences were found between the Japanese strains (*Table 1*), showing that the *gwhite* mutation thus does not affect singing behaviour.

3.2. General effects of the lesions

Two types of lesions were applied, either cutting both connectives between adjacent ganglia, referred to as *e.g.* A5-A6 cut, or a mediosagittal hemisection of a ganglion, referred to as *e.g.* A5 split.

The mean survival rate after lesions was 46% (N=101), ranging from 30% after cutting the connectives between $T3_{A1/A2}$ and A3 (T3-A3 cut), to 75% after splitting the A4. Survival times ranged between 10 and 48 days (median 19.5 days). The males showed no noticeable locomotor defects and could raise the front-wings in a normal way. The median recovery time of sound production after the lesion was 4.5 days, ranging from 1 day to 16 days, in an animal where both A3 and A4 were split. Recordings over three consecutive nights after the animals had recovered sound production were analysed and showed an overall reduction in calling song activity. In sham-operated animals, where the entire abdominal cavity was opened but no lesion applied, males recovered on the day of the procedure with a 12-20% reduced singing activity. On subsequent days, their singing activity was similar to the period before the procedure (*data not shown*). Previously, Jacob and Hedwig [38] showed that cutting the connectives between A6 and the terminal ganglion had no effect on the calling song pattern of the European WT *G. bimaculatus*. Therefore, this lesion was not repeated in this study.

3.3. Singing pattern after cutting the connectives between A5 and A6

After the A5-A6 cut males continued to sing (*Fig. 2*A), however singing activity was decreased by 28% in the first 3 days of recovery (before: 27887±12247 chirps/night; after: 19951±2144 chirps/night; N=5). The raster plot and the corresponding cross-correlogram (*Fig. 2B*) reveal that this lesion did not have a major effect on the pulse pattern that composed the chirps.

Comparing the behaviour before (2649±1163 chirps/animal) and after the lesion (2382±776 chirps/animal) shows a marginally non-significant change in mean chirp duration (before: 117±7 ms and after: 123±10 ms; F[1,18]=4.41, p=0.0501; *Fig. 2C* and *Table 1*). A significant increase in the chirp period from 358±59 ms to 517±108 ms (F[1,18]=15.7, p=0.001) occurred, with an increase in the interchirp interval from 263±102 ms to 402±104 ms.

The average number of pulses per chirp did not change after the lesion (F[1,18]=2.74, p=0.115; *Fig. 2C* and *Table 2*). This parameter, however became more broadly distributed as mirrored in the increase of chirps with 2-3 sound pulses from 1.9% to 9.8%, and chirps with more than 6 sound pulses from 1% to 8.1% (*Fig. S1*, Supplementary Materials). Neither the sound pulse duration (F[1,18]=2.06, p=0.168;) nor pulse period (F[1,18]=3.97, p=0.062; changed after the lesion.

3.4. Singing pattern after cutting the connectives between A4 and A5

After the A4-A5 cut, singing activity was reduced by 57%, from 35976±12377 chirps/night to 15564±3574 chirps/night (N=6) and became highly irregular (*Fig. 3A,B*). The most obvious effect, as demonstrated in the raster plot, is the loss of a stable chirp structure normally composed of 4-5 pulses per chirp. In this example, the number of sound pulses per chirp was considerably increased, up to 20 pulses per chirp occurred, and the interchirp interval became variable (*Fig. 3A*; *Video 2*). In time-window two, the chirp structure extends beyond five pulses and the interchirp interval is not clearly expressed. Additionally, the cross-correlogram shows an increasing temporal jitter of the sound pulses within the chirps in comparison to the intact animal (*cf. Fig. 1B*). Here, the SD for the start of the pulses increased from 1.2 ms, for the 2nd pulse, to 3.5 ms for the 4th pulse.

Further analysis (before: 3418±1176 and after: 2479±340 chirps/animal analysed; *Fig. 3C* and *Table 3* and *Fig. S2 and Table S1*, Supplementary Materials) showed that the chirp

duration significantly increased from 126±13 ms to 216±44 ms (F[1,21]=169, p<0.001. The chirp period also increased from 358±54 ms to 567±134 ms (F[1,21]=75.1, p<0.001) and the interchirp interval from 232±54 ms to 351±112 ms.

After the lesion the number of pulses per chirp increased from 4.5±0.5 to 6.7±1.5 (up to 71 sound pulses per chirp occurred in some animals; F[1,21]=154, p<0.0001). Once again, the distribution of sound pulses per chirp was broader after the lesion, with an increase in the percentage of 2-3 sound pulses from 1% to 16% and in the percentage of six or more pulses from 6% to 49% (*Fig. S2*). After the lesion the pulse pattern changed, the mean pulse duration decreased from 18.2±1.5 ms to 16.8±1.7 ms (F [1,21]=9.26, p=0.006) and the mean pulse period increased from 30.6±2.8 ms to 34.0±3.3 ms (F[1,21]=53.4, p<0.001; *Fig. 3C* and *Table 3*).

3.5. Singing pattern after cutting the connectives between A3 and A4

After cutting the A3-A4 connectives (*Fig. 4A*) 10 out of 17 animals survived, but only four continued to produce sound pulses. The pulses were shorter and the normal chirp structure was almost completely abolished (*Fig. 4A,B*). The production of chirps was reduced by 87%, from 21974±11090 chirps/night (range 11798 to 48108 chirps) to 3362±470 chirps/night (range 77 to 7995 chirps). Due to the drastic reduction in singing activity appropriate 10 min time-windows could not be selected, the raster plot and corresponding cross-correlogram were therefore generated for all pulses of a 12-hour recording. Note that after the lesion, single pulses with a pulse interval considerably larger than the normal pulse period (see 2. Methods) were very frequent. These were included in these diagrams and they represent the majority of pulses aligned at time zero (*Fig. 4B*).

Further analysis (before: 2521±1861 and after: 3362±5649 chirps/animal analysed; *Fig. 4C* and *Table 4* and *Fig. S3* and *Table S2*, Supplementary Materials) showed that the chirp

duration was reduced from 133±16 ms to 83±12 ms (F[1,15]=107, p<0.001; Fig. 4C and Table 4) and an extensive increase of the chirp period from 380±26 ms to 2501±1909 ms (F[1,15]=8.82, p<0.01) occurred.

A reduction in the average pulse number per chirp from 4.7±0.3 to 2.6±0.3 (F[1,15]=1131, p<0.001) occurred. Furthermore, there was an increase in the percentage of single sound pulses from 0.5±0.5% to 73±10%, and an overall reduction of the percentage of chirps with 2 or more pulses (*Fig. S3*). The pulse duration decreased from 18.9±2.4 ms to 6.2±2.4 ms (F[1,15]=331, p<0.001) and the pulse period increased from 30.6±2 ms to 42.8±3.8 ms (F[1,15]=67.4, p < 0.001).

3.6. Disconnecting the free abdominal ganglia, cutting connectives between $T3_{A1/A2}$ and A3

To test the effect of a complete removal of the abdominal ganglia chain (*Fig. 5A*), the connectives between the $T3_{A1/A2}$ and A3 were cut (T3-A3 cut) in 23 males. The procedure had a survival rate of 30% (N=7) and the recovery took from 7 to 14 days. Sound production was characterized by very low amplitude "scratchy" sounds of 1-3 ms duration (*Fig. 5B, arrowhead*) and occasional low amplitude sound pulses of 6-10 ms duration (*Fig. 5B, arrow*). None of the overnight recordings demonstrated a structured chirp pattern. Video recordings (*Video 3*) revealed that the males could raise the front-wings for several seconds as normal males do for singing, however rhythmic opening and closing movements did not occur. Low amplitude sounds were produced when the wings "quivered slightly" or when they were lowered to resting position. This behaviour observed in the video could be the basis for the sounds in the overnight audio recordings.

3.7. Contributions of different abdominal ganglia

The connective lesions clearly indicated the important role of the abdominal ganglia for singing motor pattern generation. As the lesions destroyed the flow of intersegmental activity, always more than one ganglion was disconnected from the remaining nerve cord. To gain further insight into the organisation of the singing network the functional removal of single ganglia was performed [30, 39, 40]. All interneurons in the singing network described so far, cross the ganglion midline with their main neurites or have their arborisation along the midline [8, 31]. We therefore split each ganglion from A5 to A3 along its midline, destroying any bilateral crossing neurites but leaving the connectives intact.

3.8. Splitting the A5 ganglion

After splitting the A5, singing activity was reduced by 48%, from 36389±24013 chirps/night to 19001±14542 chirps/night (N=5), and the chirp pattern was similar to the normal one (*Fig. 6A,B*). However, the cross-correlogram demonstrates a gradually increasing jitter in the timing of pulses within the chirps. The SD for the start of the 2nd pulse was 2.0 ms, whereas the start of the 4th pulse had a SD of 3.5 ms.

Detailed analysis (before: 3518±2439 and after: 2468±1751 chirps/animal analysed; *Fig. 6C*, Table *5* and *Fig. S4* and *Table S3,* Supplementary Materials) showed that the chirp duration increased from 127±16 ms to 145±19 ms ($F[1,16]=51.8$, $p< 0.001$). A marginal non-significant increase in the chirp period occurred from 360±26 ms to 386±46 ms ($F[1,16]=4.14$, $p=0.059$; *Fig. 6C* and *Table 5*), with a similar interchirp interval before and after the lesion, 259±41 and 261±27 ms, respectively.

Overall, the number of pulses per chirp increased from 4.3±0.3 to 4.8±0.4 ($F[1,16]=36.35$, $p<0.001$). The pooled data revealed a broader distribution in the number of pulses per chirp (*Fig. S4*); chirps with 2-3 pulses increased from 1.7% to 9.3% and chirps with more than 6

pulses from 2.2% to 18.5%. Neither the pulse duration (F[1,16]=0.14, p=0.714) nor the pulse period (F[1,16]=3.82, p=0.067) were affected by the lesion.

3.9. Splitting the A4 ganglion

After splitting the A4 ganglion (*Fig. 7A)*, singing activity was reduced by 83%, from 24526±13321 chirps/night to 4256±2616 chirps/night (N=5). Single pulses now were very frequent, and made a significant contribution to the pattern displayed in the raster plot and the corresponding cross–correlogram (*Fig. 7B*; *Video 4*). The sound recording and the raster plot show the robust chirp structure of normal singing was abolished and there was no clear interchirp interval. After a sound pulse a second or third pulse was generated but these were not precisely timed and the SD for the start of the 2nd pulse was 7.0 ms.

Statistical analysis (before: 2207±1199 and after: 2006±1038 chirps/animal analysed; *Fig. 7C* and Table *6* and *Fig. S5* and *Table S4*, Supplementary Materials) revealed that the mean chirp duration decreased from 117±6.6 ms to 81±16 ms (F[1,16]=144, p<0.001). The chirp period was similar before and after the lesion (F[1,16]=2.24, p=0.154; *Fig. 7C* and *Table 6*), whereas the interchirp interval increased from 265±38 ms to 345±39 ms.

A reduction in the average number of pulses per chirp from 4.4±0.4 to 2.6±0.3 occurred (F[1,16]=872, p<0.001). Additionally, the percentage of single pulses increased from 0.4±0.7% to 37±14% (*Fig. S5*). The sound pulse duration significantly decreased from 17.3±1.5 ms to 14.1±2.8 ms (F[1,16]=27.5, p<0.001) and the pulse period increased from 29.2±1.7 ms to 42.5±8.6 ms (F[1,16]=731, p<0.001).

3.10. Splitting the A3 ganglion

After this split (*Fig. 8A*), singing activity decreased by 67%, from 24903±11630 chirp/night to 8195±11091 chirps/night (N=5). The sound recording and the raster plot demonstrated that

singing was clearly structured in chirps, but the temporal precision of sound pulses within a chirp was altered (*Fig. 8A,B*). This was characterized by the broader distribution of the timing of each sound pulse within a chirp, this effect occurred already from the 2nd sound pulse onwards and was strongest for the 4th pulse, as seen in time-windows 2 and 3. The SD for the start of the pulses increased from 2.2 ms for the 2nd pulse to 4.5 ms for the 4th pulse.

Detailed analysis (before: 2741±1170 and after: 2486±829 chirps/animal analysed; *Fig. 8C* and *Table 7* and *Fig. S6* and *Table S5*, Supplementary Material) showed that the chirp duration did not change after the lesion (F[1,15]=3.04, p=0.102*)*. The chirp period significantly increased from 383±90 ms to 456±87 ms (F[1,15]=4.57, p=0.049), with an increase in the interchirp interval from 258±84 ms to 338±94 ms.

A significant reduction in the average number of pulses per chirp occurred from 4.6±0.5 to 4.0±0.3 (F[1,15]=31.0, p<0.001). The sound pulse duration did not change after the split (F[1,15]=0.79, p=0.389), however the sound pulse period significantly increased from 29.4±1.6 ms to 34.1±3.4 ms (F[1,15]=64.7, p<0.001; *Fig. 8C* and *Table 7*).

3.11. Combined splitting of the A3 and A4 ganglia

To identify and isolate the possible contribution of the A5 and A6 ganglia for singing pattern generation, a combined splitting of the A3 and A4 ganglia was performed (N=5; *Fig. 9A*). This double split caused a complete loss of structured singing activity, like cutting the $T3_{A1/A2}$-A3 connectives. After 3 days, the sound production was characterized by occasional low amplitude single sound pulses with a duration ranging from 7 to 14 ms (*Fig. 9B, arrow*). These pulses were rare and less than 50 pulses occurred per night; they were never grouped in chirps and even after 16 days the behaviour did not change. Video recordings (*Video 5*) showed that the males raised their wings and kept them risen for several seconds as in normal

singing behaviour. The lowering or the quivering of the wings generated low amplitude sound pulses.

3.12. Comparing cutting the connectives between ganglia and the split experiments

Splitting ganglia and cutting connectives have different impacts on the remaining structure of the nervous system. However, both types of experiments revealed similar effects (*Fig. 10A,B*). When compared to the normal song pattern, procedures that affected ganglia A5 and A6 most obviously caused an increase in the chirp period, the chirp duration and could increase the number of pulses per chirp. They had only small effects on the sound pulse parameters like the pulse period and pulse duration (*Fig. 10B*). When ganglion A4 was split and/or removed with the other posterior abdominal ganglia, the normal chirp structure was strongly altered by a reduction in the number of pulses per chirp, and an increase in the pulse period occurred. When only the A3 ganglion was functionally removed the chirp structure was still retained although the pulse pattern changed as the pulse period increased and became more variable. Combined splitting of ganglia A3 and A4 had the same effect as removing the whole abdominal ganglion chain (T3-A3 cut), the chirp structure and pattern was completely abolished and males could only produce occasional low amplitude sound pulses.

4. Discussion

The organisation of the pattern-generating network underlying singing is a long-standing question in cricket neurobiology and was addressed in several studies [7, 8, 27-30]. Our systematic lesions in the abdominal nerve cord reveal the specific functional importance of the different abdominal ganglia. The calling song apparently results from the activities of two-timer networks, one for chirps at 3-4 Hz and one for sound pulses at about 30 Hz as

proposed by Bentley [24]. These are housed in the abdominal ganglia chain as suggested by experiments of Pires and Hoy [29].

4.1. Methodological considerations

Before this study, the calling song of *G. bimaculatus* was documented by short recording sequences only [30, 41, 42]. Here we generated a comprehensive large-scale data set with long-term recordings, capturing more than 1 million chirps of the singing males as the basis for the behavioural analysis.

Lesion experiments are an important approach to study the organisation of neural circuits underlying behaviour and have been successfully applied in different invertebrate systems, *e.g.* crickets [27, 30], grasshoppers [39, 40, 43], leeches [44] and locusts [45, 46]. Whereas connective cuts are unambiguous, splits cannot be as precisely controlled when separating a ganglion along its midline, where interneurons of the singing network cross over or have their main arborisations [8, 31]. Inter-individual differences in neuronal network organisation can occur [47], however these were not controlled and may have contributed to slightly different effects of the procedures in individual males (*see Fig. 3C,6C,7C, open circles*).

In lesion experiments, conclusions can only be drawn from any resultant changes in behaviour. However, each experimental animal group demonstrated normal locomotor activity like running and fighting and yet very characteristic changes in singing behaviour. Therefore, any lack of singing activity was not related to general motor deficits. As functional reorganisation of the CNS may occur within 1-2 weeks after lesions [36, 37], we focussed on acute behavioural effects, within the first week after the lesion, before a major reorganisation could have occurred. Thus, in experimental males any singing activity was due to the remaining acute capabilities of the lesioned CNS.

With these considerations in mind, emphasis is laid on developing a consistent hypothesis for the functional contribution of different abdominal ganglia to the generation of chirps and sound pulses.

4.2. Evidence for the localisation of the chirp timer network

The most dramatic effect on the chirp structure occurred after cutting the connectives between A3 and A4. Crickets after this lesion generated mainly single pulses, with chirps occurring only rarely. These chirps were composed of only two to three pulses, and had a considerable extension and variation of the pulse period (*Fig. 10A,B*). This lesion therefore indicates that the normal chirp structure and pattern cannot be generated when ganglia A4, A5 and A6 are functionally removed from the CNS.

When cutting the connectives between A4 and A5 the chirp pattern and structure still occurred but both were severely altered. The change in chirp pattern reflected here by an increase in chirp period, was mainly due to changes in chirp structure, like the increase in chirp duration, the number of pulses per chirp and the pulse period. The data indicate that A4 is sufficient to generate a chirp pattern. However, when A4 was split, chirps with a normal chirp period still occurred, yet the number of pulses per chirp was reduced to 2-3. Thus, although A4 can generate the chirp pattern, posterior ganglia may contribute as well. When A4 was intact and A5 was split, a clear chirp pattern was still observed, however its structure was altered, with an increase in chirp duration and in the average number of pulses/chirp. We conclude that at least A4 and A5 house a separate chirp CPG network, which together interact in a stabilising manner during singing. Cutting the connectives between A5 and A6 had no impact on the overall chirp structure, but significantly increased the chirp period.

The lesions indicate that networks for organizing the chirp pattern, *i.e.* chirp period, and structure, *i.e.* chirp duration and number of pulses per chirp, cannot be assigned to a single

abdominal ganglion. For the correct organization of chirps with 4-5 sound pulses rather the interaction of ganglia A4 and A5 seems to be necessary, whereas the generation of the normal chirp period needs the contribution of A5 and A6 (*Fig. 10C*).

4.3. Evidence for the localisation of the pulse timer network

The abdominal ganglia also control the timing of the sound pulse pattern (*Fig. 10*). After cutting the connectives between A3 and A4, calling song activity was abolished, but animals consistently generated sound pulses of normal amplitude. These were of shorter duration, likely due to improper opening and closing wing movements. After both A3 and A4 were split, crickets would only very rarely generate low amplitude sound pulses, while the wings were quivering or lowered into resting position.

Splitting the A3 ganglion and functionally removing the A3 ascending opener-interneuron (A3-AO), which is a crucial element to generate the pulse pattern [8], only increased the pulse period on average by 4.7 ms. The effect on the pulse period was stronger when cutting the connectives between A3 and A4 or splitting A4. After these experiments, the pulse period increased 12 and 13 ms, respectively (*Fig. 10*). The contribution of A4 is also evident in fictive singing animals, where after cutting the connectives between A3 and A4 the pulse pattern became increasingly distorted [7].

Together, these data indicate that in addition to the pulse timer network in A3, ganglion A4 importantly contributes to the generation of a normal sound pulse pattern and that both might be functionally coupled (*Fig. 10C*). An additional functional contribution of A5 and A6 to the pulse pattern is indicated, since disconnecting the ganglia posterior to A4 increased the pulse period by 3.4 ms.

4.4. Interaction of chirp and pulse networks

The functions of the different abdominal ganglia seem to overlap in respect to the control of the calling song parameters (*Fig. 10C*). Two independent timing networks housed in the abdominal nerve cord control the singing activity, one for the chirp and another for the pulses [24]. The independence of the two-timer networks is suggested by the fact that the pulse pattern of subsequent chirps is not temporally coupled, but rather is restarted with each chirp. In addition, the interneurons of the singing network that mirror the pulse pattern, are not active during the interchirp interval [8]. Our data also demonstrate that the A4 ganglion has a crucial role in the neural network underlying singing. In this ganglion the two-timer networks seem to interact to combine the timing of the chirps and the timing of pulses of the calling song (*Fig. 10C*). Additionally, our video recordings indicate that courtship and rivalry singing are also impeded, when the abdominal ganglia are disconnected.

Given the central importance of the chirp and pulse structure and pattern in species recognition [11, 48, 49], the distributed organisation of distinct but functionally coupled networks along the abdominal ganglia, may ensure the temporal robustness of the singing motor system. This might be an example of degeneracy in the nervous system, where structurally different components of a network perform very similar functions [50-52].

4.5. Contribution of T2 and $T3_{A1/A2}$ to the generation of the chirp and pulse pattern

When the connectives between $T3_{A1/A2}$ and A3 were cut singing stopped, as reported before [28]. Even several days after the lesion, in front of females, these males lifted the front wings but only produced low amplitude sounds, due to quivering or lowering of the wings. Therefore, the cricket central nervous system from the brain to $T3_{A1/A2}$ does not have the ability to generate the sequential and coordinated rhythmic neural activity underlying the front-wing opener- and closer-movements, characteristic of the calling song behaviour. Instead, it requires a patterned input from the abdominal ganglia as indicated by lesioning the

connectives anterior to A3 [7, 28] and by manipulating the body temperature in singing crickets [29].

Based on lesion experiments in *G. campestris*, Huber [27] proposed that ganglion T2 houses the CPG for singing. Unfortunately, the precise localizations of the cuts in the abdominal chain were not reported, although it is noted that these males sang less frequently. Here we show that the effects on singing behaviour depend on the specific site of the lesions (*Fig. 10*). Hennig and Otto [30] showed that splitting the $T3_{A1/A2}$ ganglion complex impaired the ability of males to raise the front wings and to coordinate their movements, and concluded that the singing CPG would be housed in $T3_{A1/A2}$. However, these males had an intact abdominal chain; electromyograms of the wing muscles showed a normal chirp motor pattern, but as the wings were not raised sound production would have been impeded.

Besides housing the motoneurons that drive the wing muscles, the role of the thoracic ganglia might be more related to the preparation for singing. This also evident after disconnecting the abdominal chain as males will not sing but still lift their wings into singing position. This organisation is similar to the motor control of the pulse-song in *Drosophila*, where separate types of thoracic interneurons control either wing extension in preparation to sing, or the generation of the pulse-song [53].

4.6. Speculations on the neural organization of singing behaviour

Our results indicate that a distributed network in the abdominal chain controls the temporal features of the crickets' calling song, the sound pulses at 30 Hz and chirps at 3-4 Hz (*Fig. 10C*).

Singing activity is still maintained even when one of the abdominal ganglia is eliminated by splitting. We expect that singing-interneurons in the different abdominal ganglia project anteriorly in a parallel manner and that there is a sequential integration of the ascending

information related to the chirp pattern from the posterior ganglia (A5-A6) to the anterior one (A4). In A4, the chirp structure and pattern appear to be integrated with the pulse timer network, with the pulse pattern being further refined in the A3 ganglion, as the A3-AO interneuron activity reflects the complete final singing motor pattern [7, 8]. From the A3 and A4 ganglia, the activity of the chirp and pulse pattern might finally be carried forward to the T2 wing motoneurons (*Fig. 10C*). The distributed organization of the network is also supported by the fact that a descending T3 interneuron of the singing network [8], and a corollary discharge interneuron [54], which are rhythmically active during singing, have their axonal projections across all the abdominal ganglia. Also the activity of the descending calling song command neuron [55] may be integrated in the pattern generating networks distributed along the A3-A6 ganglia (*Fig. 10C*).

Our data indicate a form of spatial hierarchical organization from the posterior ganglia to the anterior ganglia of the abdominal nerve cord. This is functionally similar to acoustically communicating fish where the hindbrain nucleus controlling call duration projects to the nuclei setting the fundamental frequency/pulse repetition rate, which finally provide inputs to the vocal motoneurons [56-58]. Furthermore, the apparent distributed and interconnected nature of the singing network suggests that its elements are organized as modules that implement specific identifiable features of the final output [59].

4.7. Evolutionary implications

The presence of interneurons belonging to the singing CPG in the $T3_{A1/A2}$ ganglion complex [8, 31] is similar to the segmental organisation of the locust flight system [46, 60, 61] and the singing network of grasshoppers [40]. However, our lesion experiments confirmed the spatial separation of the CPG timer networks for chirps and pulses from the thoracic segments [7, 8] and extended the organization of CPG network for singing from A3 to the remaining

abdominal ganglia. This may reflect a prior evolutionary stage, where the pattern generator circuits for ventilation or locomotion might have provided the precursor networks [62]. There is evidence of some coupling between ventilation and singing in crickets [63-65], and although, the use of the same motoneurons to play different roles in different motor networks is commonly observed in insects, as in singing and flying [31, 66], the dedicated CPG networks for the two behaviours are distinct in crickets [31].

Extant cricket species exhibit a wide diversity of species-specific patterns of calling, courtship or rivalry songs [22, 67, 68]. A phylogenetic analysis of the calling song of different species of North American field cricket suggests that the song parameters have evolved separately in *Gryllus* species [69]. The described modular organization of the singing network could be suited to explain species-specific differences in song patterns and that changes in the pulse timer network can occur independently of changes in the chirp timer network. At the level of a modular neural circuit, the modulation of the connectivity and/or synaptic strength of its different components [70-72] may allow the generation of different motor patterns and that specific changes in some elements of a network occur without affecting other parameters of the same network [52, 73]. Further studies comparing the neuronal organisation underlying singing, by either lesions or electrophysiology in closely related species with different song patterns, may reveal the functional species-specific adaptations in the neural networks.

This study demonstrates how the different timescales of chirps (3-4 Hz) and pulses (30 Hz) for acoustic communication can be organized in the CNS. This problem has been explored in several systems across the animal kingdom (flies [74], grasshoppers [39], fishes [56, 57], anurans [75] and birds [76]). Detailed comparative studies may allow the identification of possible shared functional features between different species [77]. Furthermore, combining the neurophysiological data in *Gryllus* and the knowledge on the genetics of male song

production and female preference like in the *Laupala* crickets [78-80] may be fundamental to understand the genetic basis and the transcriptome profile of species-specific singing and phonotaxis behaviour.

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Competing Interests

The authors declare no competing interest for this work.

Author Contributions

P.F.J. and B.H. conceptualised and designed the experiments. P.F.J. performed the experiments and analysed the data. P.F.J. prepared the figures and drafted the manuscript. P.F.J. and B.H. revised and approved the final version of the manuscript.

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Figure Legends

Fig. 1. Calling song of *G. bimaculatus* (*gwhite*) with intact central nervous system.

(**A**) Schematic diagram of the cricket CNS (***i***), modified after Huber (1963). SOG: subesophageal ganglion, T1: prothoracic ganglion, T2: mesothoracic ganglion, $T3_{A1/A2}$: metathoracic ganglion complex, TAG: terminal abdominal ganglion. A sequence and a schematic representation of the calling song is shown (***ii***); the song parameters analysed i.e. chirp period; chirp duration, interchirp interval, sound pulse period, and sound pulse duration are indicated. Arrow in the recording sequence, represent the sound pulse at the start of a chirp, these are aligned to time zero in the raster plot and cross-correlogram. The grey area represents a +/- 500 ms time-window aligned to the 9^{th} chirp from the left (asterisk). (**B**) Raster plot and cross-correlogram of the three selected time-windows for one animal (3724 chirps). For each chirp the start of the pulses are plotted within a time-window of +/- 500 ms. The cross-correlogram and the inset, showing a higher amplitude resolution of the cross-correlogram, have a bin width of 1.75 ms. The y-axis indicates the normalized number of events for each time bin (see 2.4 Data Analysis for details). The description given here pertains to the following figures Fig. 2, Fig. 3, Fig. 4, Fig. 6, Fig. 7 and Fig.8, unless otherwise stated.

Fig. 2. Effect on sound production after cutting the connectives between A5 and A6.

(**A**) Schematic representation of the lesion (***i***) and a sequence of the song pattern after the lesion (***ii***). (**B**) Raster plot and cross-correlogram of the time-windows for one animal (3008 chirps). (**C**) Analysis of song parameters before (x-axis) and after (y-axis) the lesion (N=5), each symbol represents the mean for one individual. The line through the origin indicates where pre- and post- would have the same mean parameter value.

Fig. 3. Effect on sound production after cutting the connectives between AA and A5.

(**A**) Schematic representation of the lesion (***i***) and a sequence of the song pattern after the lesion (***ii***). (**B**) Raster plot and cross-correlogram of pulses within the time-windows for one animal (2630 chirps). (**C**) Analysis of the song parameters before (x-axis) and after (y-axis) the lesion (N=6), each

symbol represents the mean for one individual animal. Open circles represent a song parameter, in one animal, that statistically differs from the remaining values in that group, revealed by the two-way ANOVA interaction between lesion and animal factors (*cf. Table S1*, Supplementary Materials).

Fig. 4. Effect on sound production after cutting the connectives between A3 and A4. (**A**) Schematic representation of the lesion (***i***) and a sequence of the song pattern after the lesion (***ii***). Note the presence of single pulses with an inter-pulse interval greater than 50 ms, this data was included in the raster plot and in the cross-correlogram. (**B**) Raster plot and cross-correlogram of a continuous 12 h recording. Of all pulses generated 69% were single pulses (2458 chirps and 5533 singles pulses). (**C**) Analysis of song parameters before (x-axis) and after (y-axis) the lesion (N=4), each symbol represents the mean for one animal.

Fig. 5. Effect on sound production after removing the abdominal ganglion chain by cutting connectives between $T3_{A1/A2}$ and A3

(**A)** Schematic representation of the lesion. (**B)** Sequences of sounds produced before (left) and after the lesion (right), the scale bars are the same for the two sequences. The inset shows two types of signals, low amplitude sound pulses (arrow) and "scratchy" sounds of very low amplitude (arrowhead).

Fig. 6. Effect on sound production after splitting A5 along the midline.

(**A**) Schematic representation of the split (***i***) and a sequence of the song pattern after the split (***ii***). (**B**) Raster plot and cross-correlogram of the selected time-windows for one animal (3411 chirps). (**C**) Analysis of song parameters, before (x-axis) and after (y-axis) the split (N=5) with each symbol representing the mean for one individual animal. Open circles represent a song parameter that statistically differs from the remaining values in that group, revealed by the two-way ANOVA interaction between split and animal factors (*cf. Table S3*, Supplementary Materials).

Fig. 7. **Effect on sound production after splitting A4 along the midline.**

(**A**) Schematic representation of the split (***i***) and a sequence of the song pattern after the split (***ii***). Note the presence of single pulses with an inter-pulse interval greater than 50 ms, this data was included in the raster plot and in the cross-correlogram. (**B**) Raster plot and cross-correlogram of the selected time-windows for one animal, from which 34% were single pulses (2084 pulses in chirps and 1073 single pulses). (**C)** Analysis of song parameters before (x-axis) and after (y-axis) the split (N=5), with each symbol representing the mean for one individual animal. Open circles represent a song parameter that statistically differs from the remaining values in that group, revealed by the two-way ANOVA interaction between split and animal factors (*cf. Table S4*, Supplementary Materials).

Fig. 8. **Effect on sound production after splitting A3 along the midline.**

(**A**) Schematic representation of the split (***i***) and a sequence of the song pattern after the split (***ii***). (**B**). Raster plot and cross-correlogram of the selected time-windows for one animal (3371 chirps). (**C**). Analysis of song parameters before (x-axis) and after (y-axis) the split (N=5), each symbol represents the mean for one animal.

Fig. 9. **Effect on sound production after splitting A3 and A4 along their midlines.**

(**A**) Schematic representation of the splits. (**B**) Sequences of sounds produced before (left) and after (right) the splits, the scale bars are the same for two main sequences, low amplitude sound pulses are represented by an arrow.

Fig. 10. **Summary of the effects on the song parameters in the different experimental procedures and putative organization of the singing network in crickets.**

(**A**) Schematic representation of the sound production in intact (black) and in experimental animals (coloured). The diagram represents the group mean of each song parameter, chirp duration and period, average pulse number per chirp, and pulse duration and period. (**B**) Relative value for each song parameter in each group, normalized to the normal calling song pattern (Mean of parameter $_{\text{after lesion}}$ /

Mean of parameter $_{normal\ pattern}$). Stippled lines at 1, represent the value for the normal pattern; rel. Units, relative units; n.p., indicates that the specific song parameter was absent. The colour scheme is the same as in (**A**), and qualitatively represents changes in the chirp structure and pattern (blue colours) and changes pulse pattern (red colours). (**C**) Representation of the cricket CNS from T2 to the TAG, and putative neural organization underlying the singing behaviour in crickets. The stippled line on the left indicates a subset of regions along the abdominal ganglia chain where the brain command neuron for singing may project. Blue ellipse represents the putative regions for the location of the chirp timer network, and the red ellipse represents the putative regions for the location of the pulse timer network. The arrows show the direction of information flow.

Tables

Table 1. Statistical comparison of the song parameters in three *G. bimaculatus* strains, European wild-type (WT), Japanese WT and *gwhite*

Song Parameter	European WT (1)	Japanese WT (2)	*gwhite* (3)	ANOVA		Groups	Holm-Šídák Post hoc *p-value*
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	*F*	*p*		
Chirp Duration (ms)	130±24	124±21	123±10	0.66	0.521	1 *vs* 2 1 *vs* 3 2 *vs* 3	0.7176 0.5879 0.7997
Chirp Period (ms)	408±51	397±40	372±58	2.11	0.123	1 *vs* 2 1 *vs* 3 2 *vs* 3	0.6426 0.1905 0.3622
Average Pulse Number per Chirp	3.8±0.5	4.1±0.2	4.5±0.4	11.3	**<0.001**	1 *vs* 2 1 *vs* 3 2 *vs* 3	0.062 **< 0.001** 0.062
Sound Pulse Duration (ms)	18.6±3.2	18.3±1.5	18.0±2.0	0.33	0.719	1 *vs* 2 1 *vs* 3 2 *vs* 3	0.969 0.728 0.885
Sound Pulse Period (ms)	38.2±3.2	32.0±2.6	30.2±2.2	45.5	**<0.001**	1 *vs* 2 1 *vs* 3 2 *vs* 3	**<0.001** **<0.001** 0.060

(Note: data shown in bold are significant at $p<0.05$)

Table 2. Statistical analysis of the effect of cutting the connectives A5-A6 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Main Effect of Lesion	4.41	0.0501	**15.7**	**0.001**	2.74	0.115	2.06	0.168	3.97	0.062
Main Effect of Animal	**4.73**	**0.009**	1.38	0.282	**6.89**	**0.002**	**3.80**	**0.021**	**7.26**	**0.001**
Lesion by Animal Interaction	1.64	0.209	2.74	0.063	2.73	0.062	0.43	0.782	2.06	0.128

(Note: data shown in bold are significant at $p<0.05$)

Table 3. Statistical analysis of the effect of cutting the connectives A4-A5 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*
Main Effect of Lesion	**169**	**<0.001**	**75.1**	**<0.001**	**154**	**<0.001**	**9.26**	**0.006**	**53.4**	**<0.001**
Main Effect of Animal	**8.95**	**<0.001**	**10.1**	**<0.001**	**15.8**	**<0.001**	1.55	0.219	**26.4**	**<0.001**
Lesion by Animal Interaction	**6.30**	**0.001**	**3.09**	**0.003**	**9.90**	**<0.001**	**6.44**	**<0.001**	2.56	0.058

(Notes: data shown in bold are significant at $p<0.05$. Planned comparisons for the significant two-way interactions are presented in *Table S1)*

Table 4. Statistical analysis of the effect of cutting the connectives A3-A4 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*
Main Effect of Lesion	**107**	**<0.001**	**8.82**	**0.01**	**1131**	**<0.001**	**331**	**<0.001**	**67.4**	**<0.001**
Main Effect of Animal	**7.11**	**0.003**	1.85	0.182	**5.47**	**<0.001**	**5.93**	**0.007**	**3.32**	**0.049**
Lesion by Animal Interaction	1.51	0.253	1.80	0.190	**15.7**	**<0.001**	**4.09**	**0.026**	1.24	0.329

(Notes: data shown in bold are significant at $p<0.05$. Planned comparisons for the significant two-way interactions are presented in *Table S2)*

Table 5. Statistical analysis of the effect of splitting A5 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Main Effect of Lesion	**51.8**	**<0.001**	4.14	0.059	**36.4**	**<0.001**	0.14	0.714	3.82	0.067
Main Effect of Animal	**28.6**	**<0.001**	**4.24**	**0.016**	**12.0**	**<0.001**	2.35	0.098	**18.2**	**<0.001**
Lesion by Animal Interaction	**10.4**	**<0.001**	2.85	0.058	**4.2**	**0.002**	0.86	0.511	**6.13**	**0.003**

(Notes: data shown in bold are significant at $p<0.05$. Planned comparisons for the significant two-way interactions are presented in *Table S3)*

Table 6. Statistical analysis of the effect of splitting A4 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Main Effect of Lesion	**144**	**<0.001**	2.24	0.154	**872**	**<0.001**	**27.5**	**<0.001**	**731**	**<0.001**
Main Effect of Animal	**5.11**	**0.007**	0.42	0.789	**17.9**	**<0.001**	**6.11**	**0.004**	**59.3**	**<0.001**
Lesion by Animal Interaction	**9.02**	**<0.001**	0.81	0.538	**8.67**	**<0.001**	**5.46**	**0.006**	**68.8**	**<0.001**

(Notes: data shown in bold are significant at $p<0.05$. Planned comparisons for the significant two-way interactions are presented in *Table S4)*

Table 7. Statistical analysis of the effect of splitting A3 on the song parameters of *G. bimaculatus*

	Chirp Duration		Chirp Period		Average Pulse Number per Chirp		Sound Pulse Duration		Sound Pulse Period	
	F	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p* value
Main Effect of Lesion	3.04	0.102	**4.57**	**0.049**	**31.0**	**<0.001**	0.79	0.389	**64.7**	**<0.001**
Main Effect of Animal	1.38	0.288	**4.91**	**0.01**	**6.63**	**0.003**	**5.81**	**0.005**	**13.7**	**<0.001**
Lesion by Animal Interaction	**3.92**	**0.023**	0.45	0.772	2.03	0.141	0.82	0.534	3.00	0.053

(Notes: data shown in bold are significant at $p<0.05$. Planned comparisons for the significant two-way interactions are presented in *Table S5)*

Supplementary Materials

Supplementary Figures Legends

Fig. S1. Percentage of single pulses and of chirps with 2 to ≥10 pulses before (Pre; black) and after (Post; grey) cutting the connectives between A5 and A6. Data were pooled from all animals (N=5).

Fig. S2. Percentage of single pulses and of chirps with 2 to ≥15 pulses before (Pre; black) and after (Post; grey) cutting the connectives between A4 and A5. Data were pooled from all animals (N=6).

Fig. S3. Percentage of single pulses and of chirps with 2 to 10 pulses before (Pre; black) and after (Post; grey) cutting the connectives between A3 and A4. Data were pooled from all animals (N=4).

Fig. S4. Percentage single pulses and of chirps with 2 to ≥10 pulses before (Pre; black) and after (Post; grey) splitting the A5 along its midline. Data were pooled from all animals (N=5).

Fig. S5. Percentage of single pulses and of chirps with 2 up to 10 pulses before (Pre; black) and after (Post; grey) splitting the A4 along its midline. Data were pooled from all animals (N=5).

Fig. S6. Percentage of single pulses and of chirps with 2 to 10 pulses before (Pre; black) and after (Post; grey) splitting the A3 along its midline. Data were pooled from all animals (N=5).

Supplementary Tables

Table S1. Planned comparisons per animal for significant two-way interactions of cutting the connectives A4-A5 on the song parameters of *G. bimaculatus*

	Chirp Duration (ms)			Chirp Period (ms)			Average Pulse Number per Chirp			Sound Pulse Duration (ms)		
	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*P*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*P*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*
1	**151±3.1**	**224±30**	**<0.001**	**379±30**	**603±125**	**0.003**	**5.1±0.1**	**6.8±0.8**	**0.002**	**21.1±0.6**	**15.3±0.9**	**<0.001**
2	116±3.2	142±8.3	0.541	299±18	311±16	>0.999	4.0±0.1	3.9±0.6	0.814	17.3±1.6	19.2±1.8	0.417
3	**120±9.5**	**195±34**	**<0.001**	**399±48**	**685±84**	**<0.001**	**4.5±0.4**	**7.0±1.0**	**<0.001**	17.9±0.5	15.5±1.1	0.234
4	**118±7.5**	**239±4.3**	**<0.001**	**430±124**	**673±50**	**0.005**	**4.3±0.2**	**7.2±0.5**	**<0.001**	17.2±1.9	18.6±1.8	0.830
5	**126±0.1**	**221±15**	**<0.001**	**301±18**	**556±95**	**0.003**	**5.0±0.1**	**7.5±0.5**	**<0.001**	18.5±0.6	16.3±1.4	0.412
6	**125±4.6**	**273±39**	**<0.001**	**338±13**	**570±51**	**0.007**	**4.1±0.2**	**8.0±0.4**	**<0.001**	17.5±0.3	15.8±1.8	0.702

(Note: data shown in bold are significant at $p<0.05$)

Table S2. Planned comparisons per animal for significant two-way interactions of cutting the connectives A3-A4 on the song parameters of *G. bimaculatus*

	Average Pulse Number per Chirp			Sound Pulse Duration (ms)		
	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*P value*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p value*
1	**4.9±0.2**	**2.2±0.1**	**<0.001**	**16.9±3.1**	**5.4±0.6**	**<0.001**
2	**4.5±0.1**	**2.6±0.3**	**<0.001**	**21.8±0.4**	**8.6±1.8**	**<0.001**
3	**5.0±0.1**	**2.7±0.2**	**<0.001**	**20.0±0.5**	**3.8±0.3**	**<0.001**
4	**4.5±0.2**	**2.9±0.1**	**<0.001**	**16.9±1.0**	**7.1±2.4**	**<0.001**

(Note: data shown in bold are significant at $p<0.05$)

Table S3. Planned comparisons per animal for significant two-way interactions of splitting A5 on the song parameters of *G. bimaculatus*

	Chirp Duration (ms)			Average Pulse Number per Chirp			Sound Pulse Period (ms)		
	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*P value*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p value*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p value*
1	**124±6.3**	**139±3.3**	**0.048**	**4.1±01**	**4.5±0.2**	**0.046**	34.5±2.5	35.0±1.1	0.934
2	**109±3.8**	**127±4.4**	**0.028**	**4.1±0.1**	**4.6±0.1**	**0.015**	29.4±0.5	29.3±1.2	0.970
3	**151±5.6**	**168±7.1**	**0.028**	**4.7±0.1**	**5.3±0.2**	**0.007**	35.4±0.9	34.0±0.5	0.604
4	**118±8.6**	**163±7.8**	**<0.001**	**4.2±0.3**	**5.0±0.2**	**<0.001**	**30.4±0.4**	**35.3±0.4**	**<0.001**
5	134±6.4	130±6.1	0.559	4.5±0.1	4.4±0.3	0.475	32.9±1.2	33.2±1.6	0.941

(Note: data shown in bold are significant at $p<0.05$)

Table S4. Planned comparisons per animal for significant two-way interactions of splitting A4 on the song parameters of *G. bimaculatus*

	Chirp Duration (ms)			Average Pulse Number per Chirp			Sound Pulse Duration (ms)			Sound Pulse Period (ms)		
	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p*
1	**125±7.5**	**88.7±3.6**	**<0.001**	**4.9±0.3**	**3.0±0.2**	**<0.001**	15.6±1.4	16.6±2.0	0.962	**28.1±0.6**	**34.7±1.6**	**<0.001**
2	**111±2.7**	**88.8±7.1**	**0.023**	**4.2±0.1**	**2.5±0.2**	**<0.001**	17.1±0.2	13.0±1.5	0.052	**29.3±0.2**	**50.3±1.2**	**<0.001**
3	110±2.2	97.8±10	0.285	**4.0±0.1**	**2.6±0.2**	**<0.001**	19.4±0.9	17.3±1.4	0.482	**30.0±1.1**	**50.3±0.6**	**<0.001**
4	**122±6.5**	**75.7±14**	**<0.001**	**4.9±0.1**	**2.4±0.2**	**<0.001**	16.5±0.1	13.4±2.9	0.201	**27.0±1.4**	**45.2±2.1**	**<0.001**
5	**118±5.9**	**55.7±2.7**	**<0.001**	**4.2±0.1**	**2.4±0.1**	**<0.001**	**18.2±0.2**	**10.3±0.6**	**<0.001**	31.5±0.7	32.1±1.0	0.991

(Note: data shown in bold are significant at $p<0.05$)

Table S5. Planned comparisons per animal for significant two-way interactions of splitting A3 on the song parameters of *G. bimaculatus*

	Chirp Duration (ms)		
	Before $\bar{x} \pm SD$	After $\bar{x} \pm SD$	*p value*
1	132±10	114±3.9	0.177
2	118±14	120±13	0.999
3	113±1.9	131±13	0.201
4	124±10	104±6.2	0.137
5	134±1.1	120±3.7	0.452

Video Legends

Video 1. Calling song of a *gwhite* G. *bimaculatus* male, with a European WT female.

The male holds it wings well raised and generates the rhythmic calling song pattern.

Video 2. Singing behaviour of a *gwhite* male 4 days after cutting connectives between A4-A5.

The video shows a *gwhite* male and a European WT female. The male raises its wings as normal crickets but a slightly different song pattern is produced, with longer chirps and with more variable interchirp intervals.

Video 3. Behaviour of a *gwhite* male 11 days after cutting the connectives between $T3_{A1/A2}$-A3.

The video shows a *gwhite* male and a European WT female. The male responds to the aggressive female, it walks normally and finally raises its wings into singing position, however only low amplitude sounds are generated, when the wings quiver or are lowered into resting position.

Video 4. Behaviour of a *gwhite* male, 4 days after splitting A4.

The video shows a *gwhite* male and a European WT female. The male raises its wings into singing position, but does not generate coordinated opening and closing-movements of the wings. This is noticeable from time point 00:10 min, when its body shakes like during courtship behaviour. At time 01:25 min a series of low amplitude sound pulses are generated.

Video 5. Behaviour of a *gwhite* male 4 days after the combined split of A3 and A4.

The video shows a *gwhite* male and a European WT female. The male raises its wings, however only low amplitude sound pulses are produced.

Figure 1

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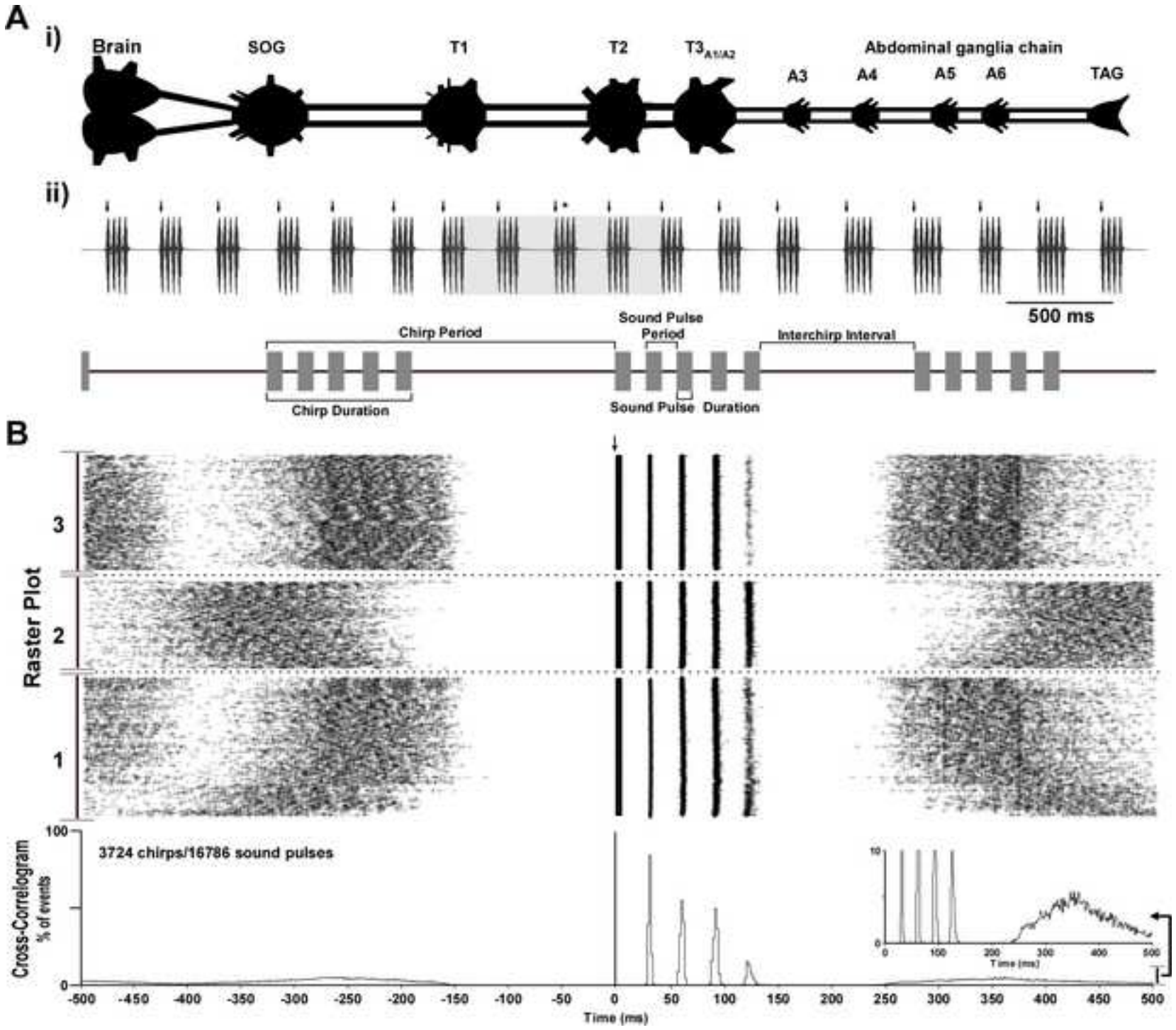

Figure 2

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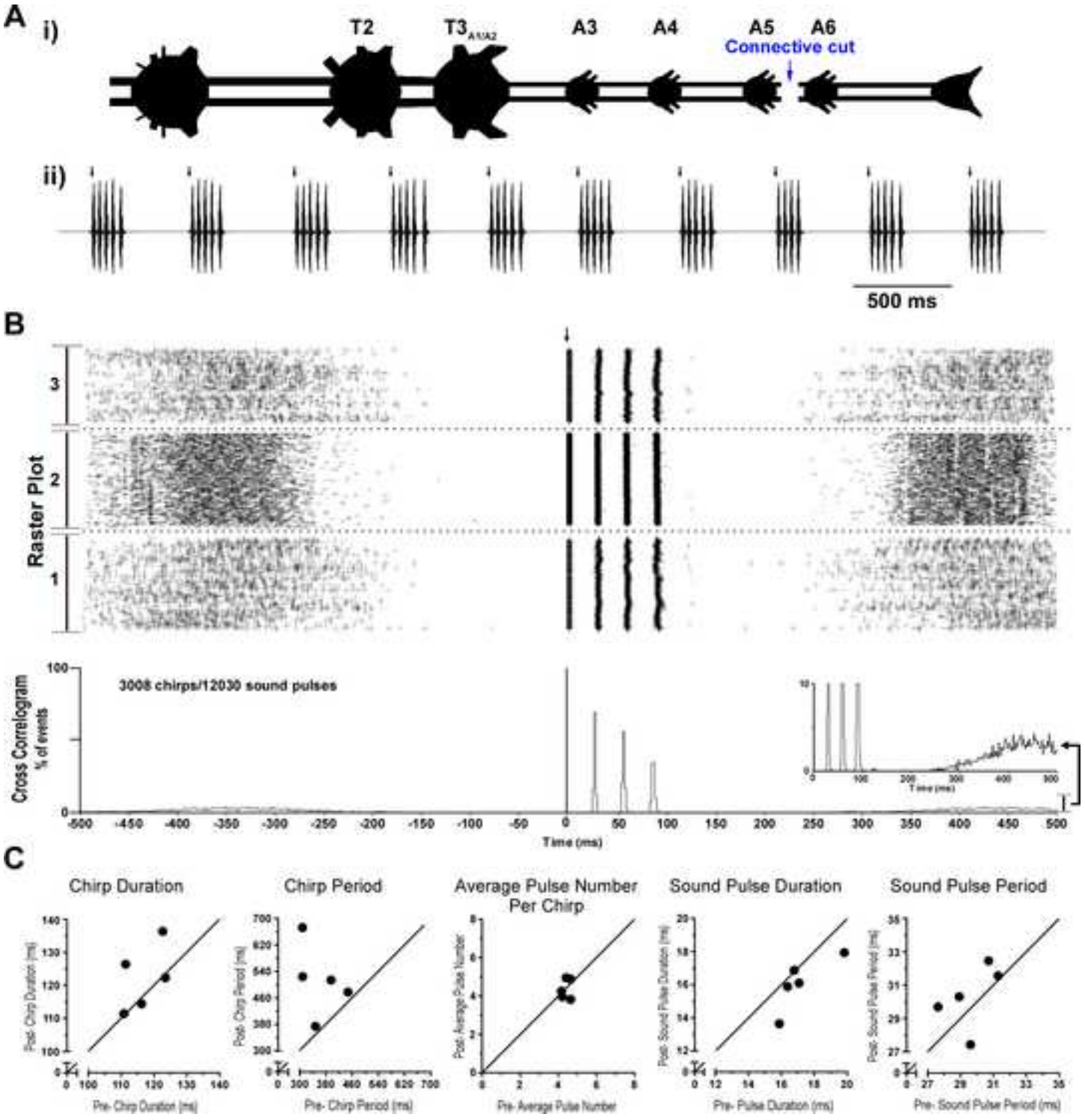

Figure 3

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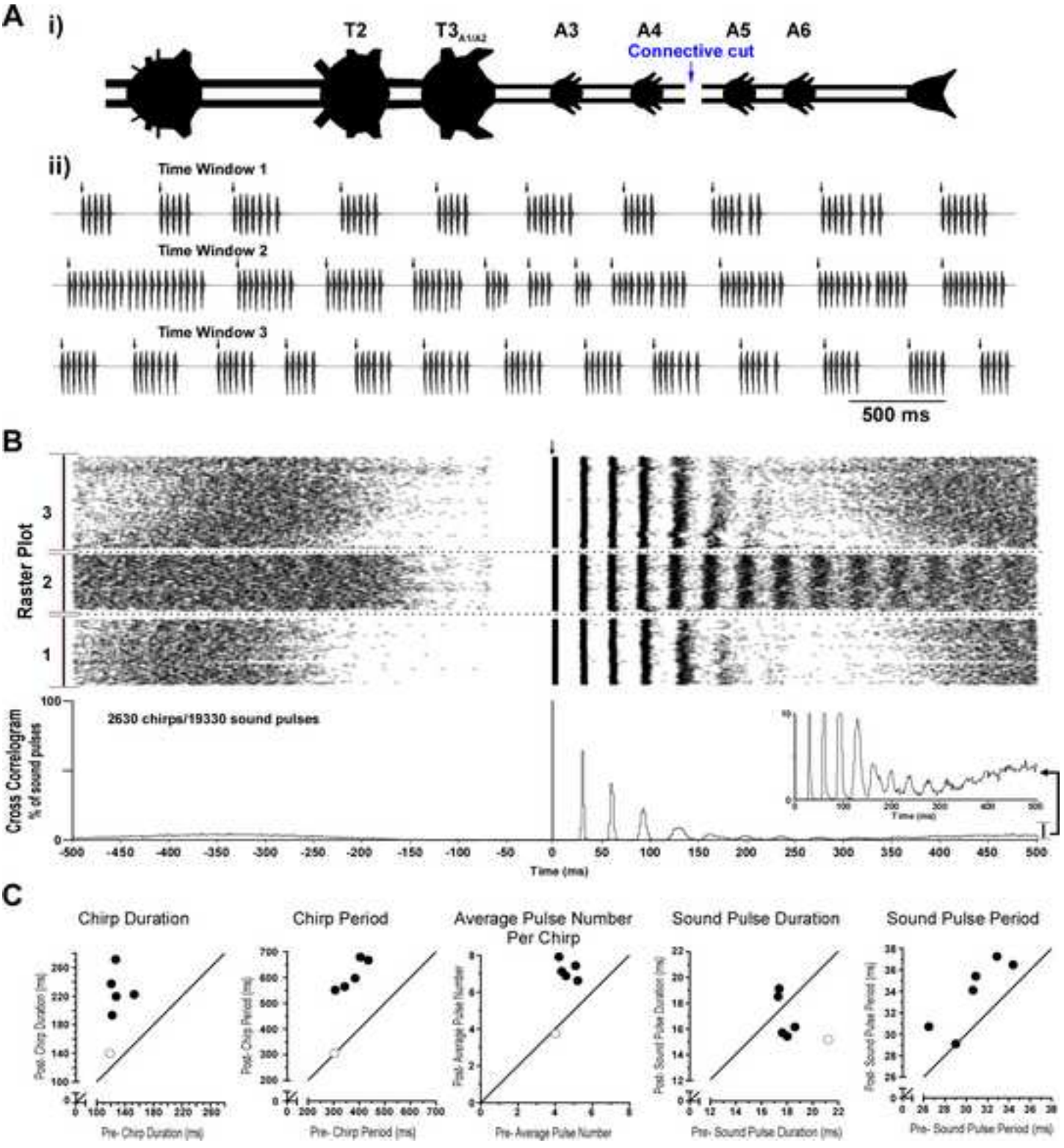

Figure 4

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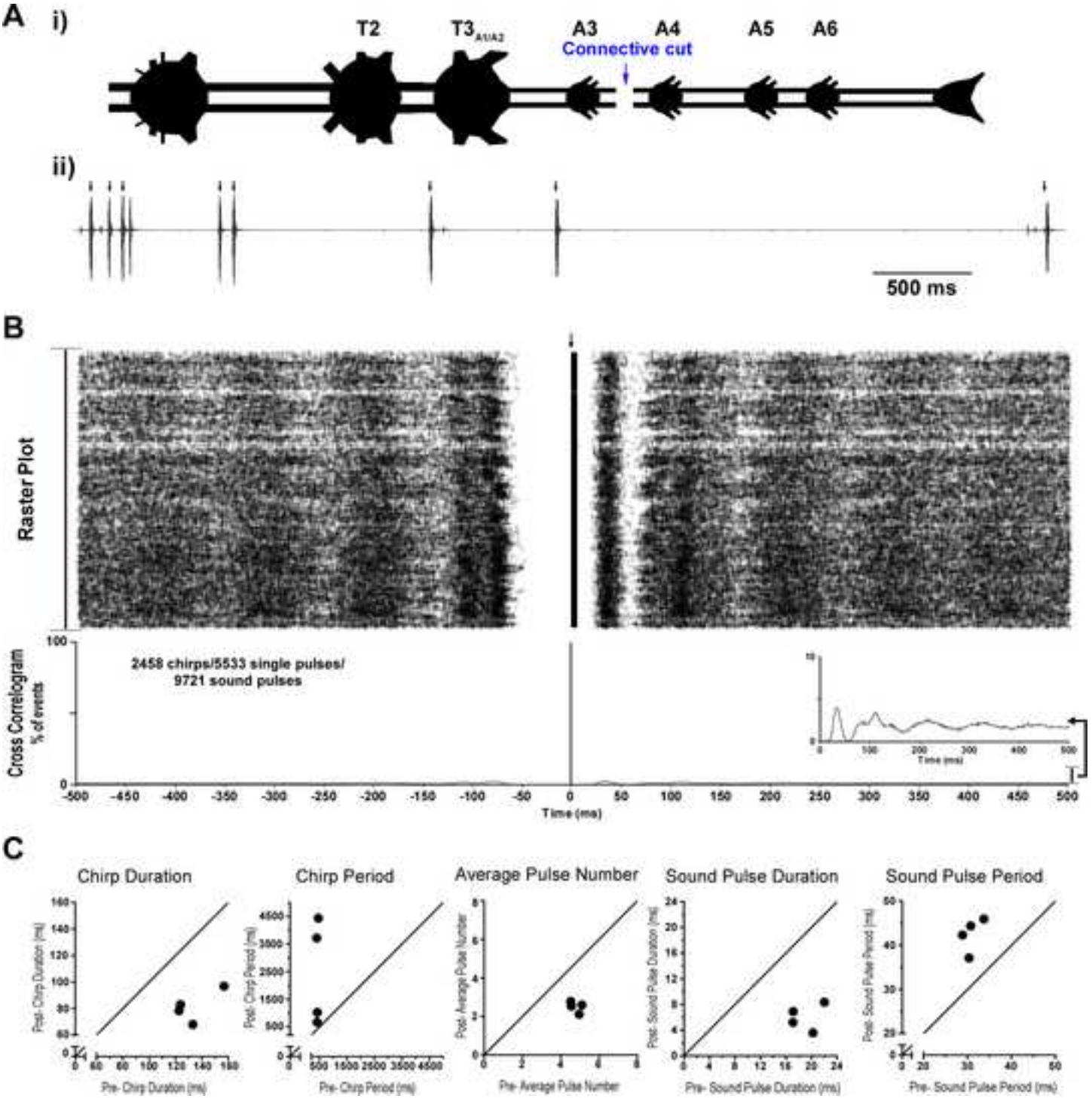

Figure 5

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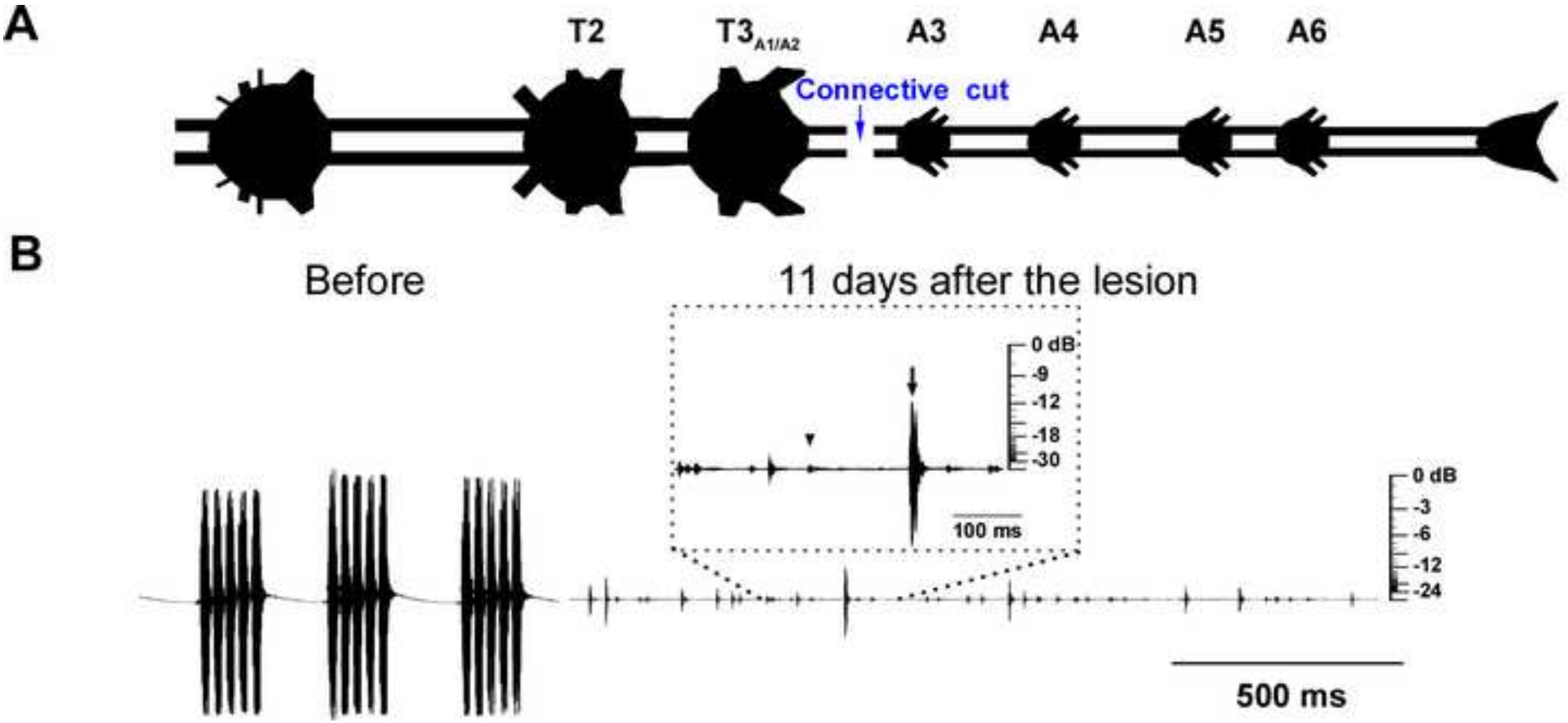

Figure 6

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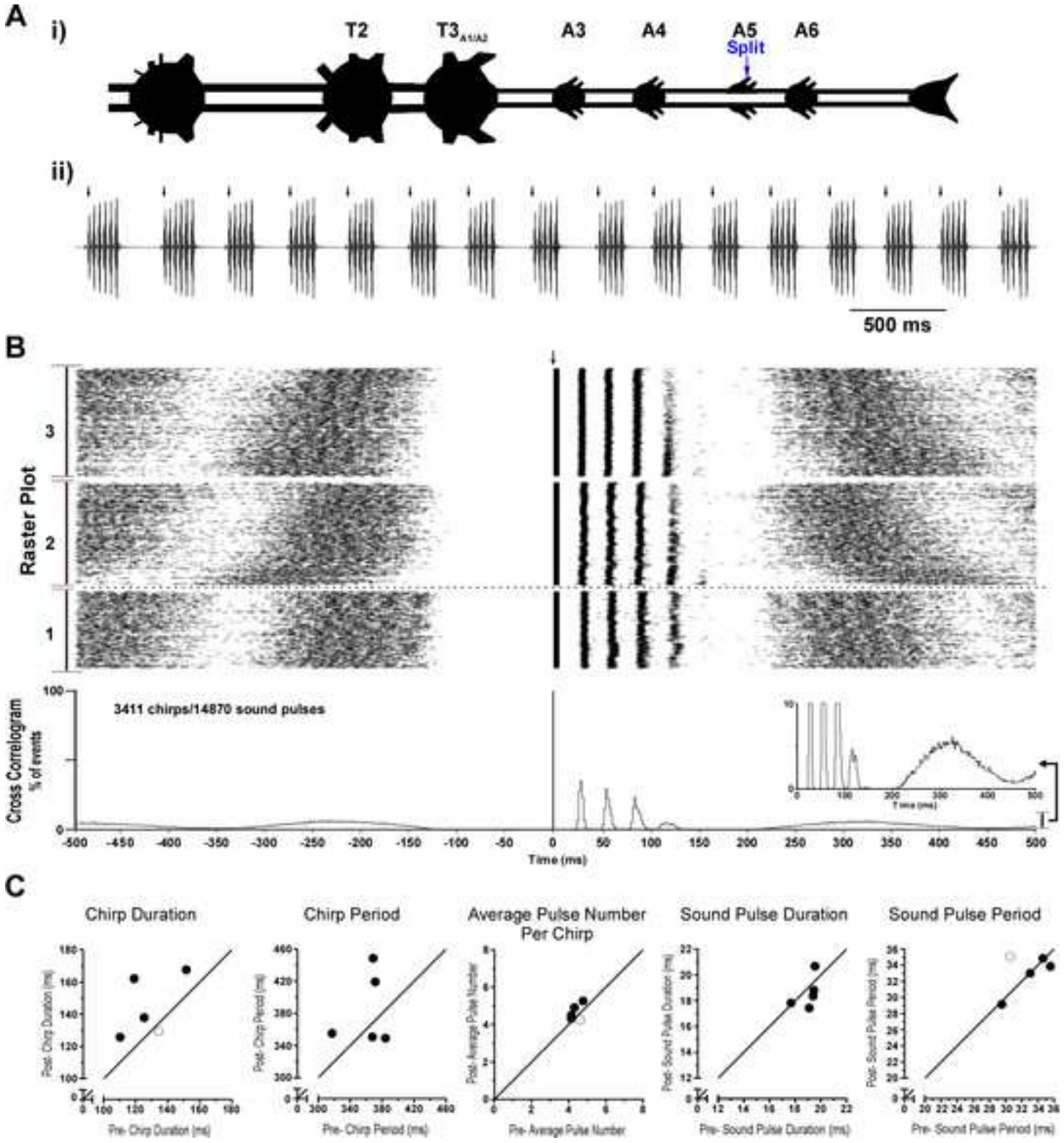

Figure 7

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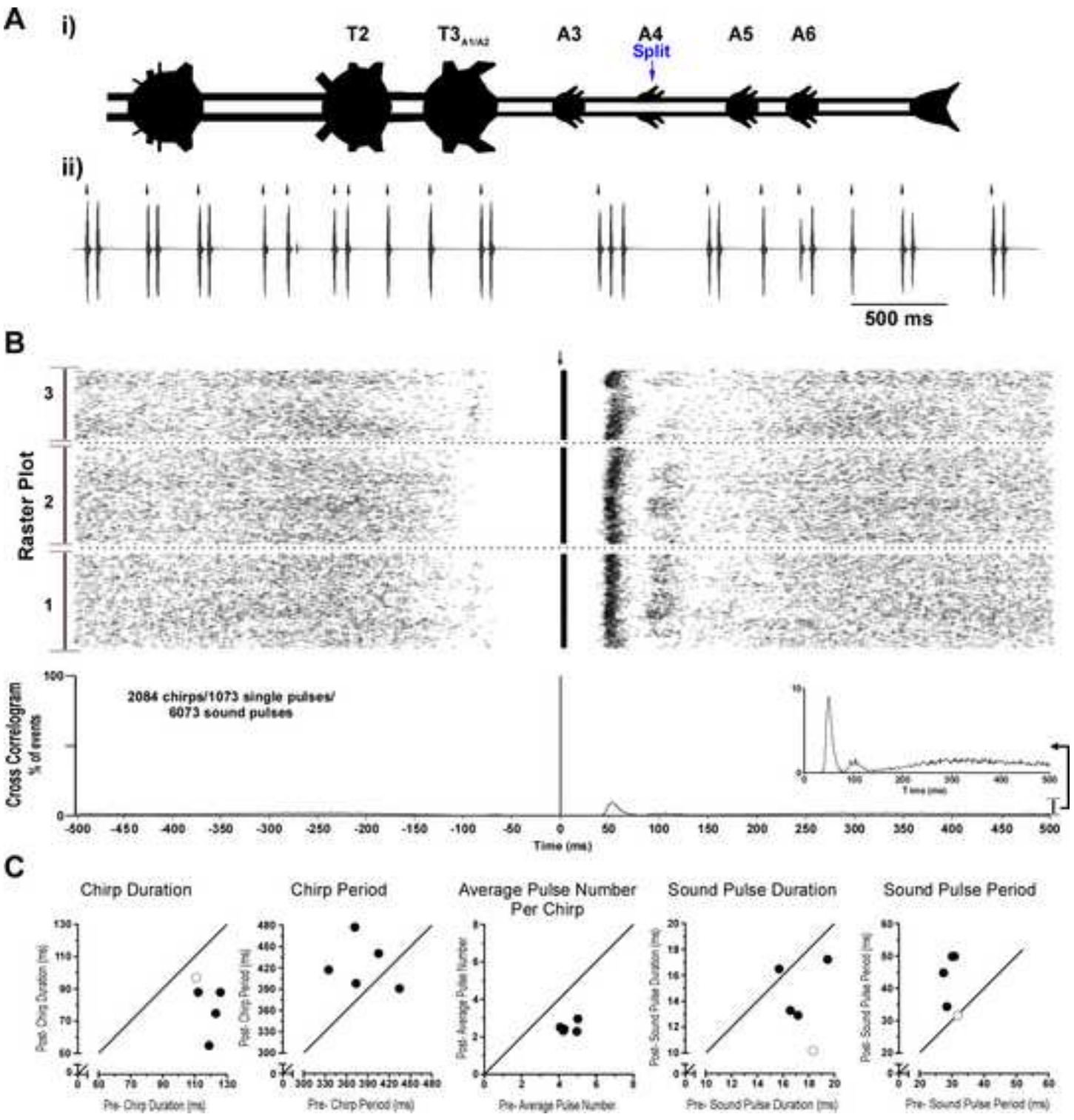

Figure 8

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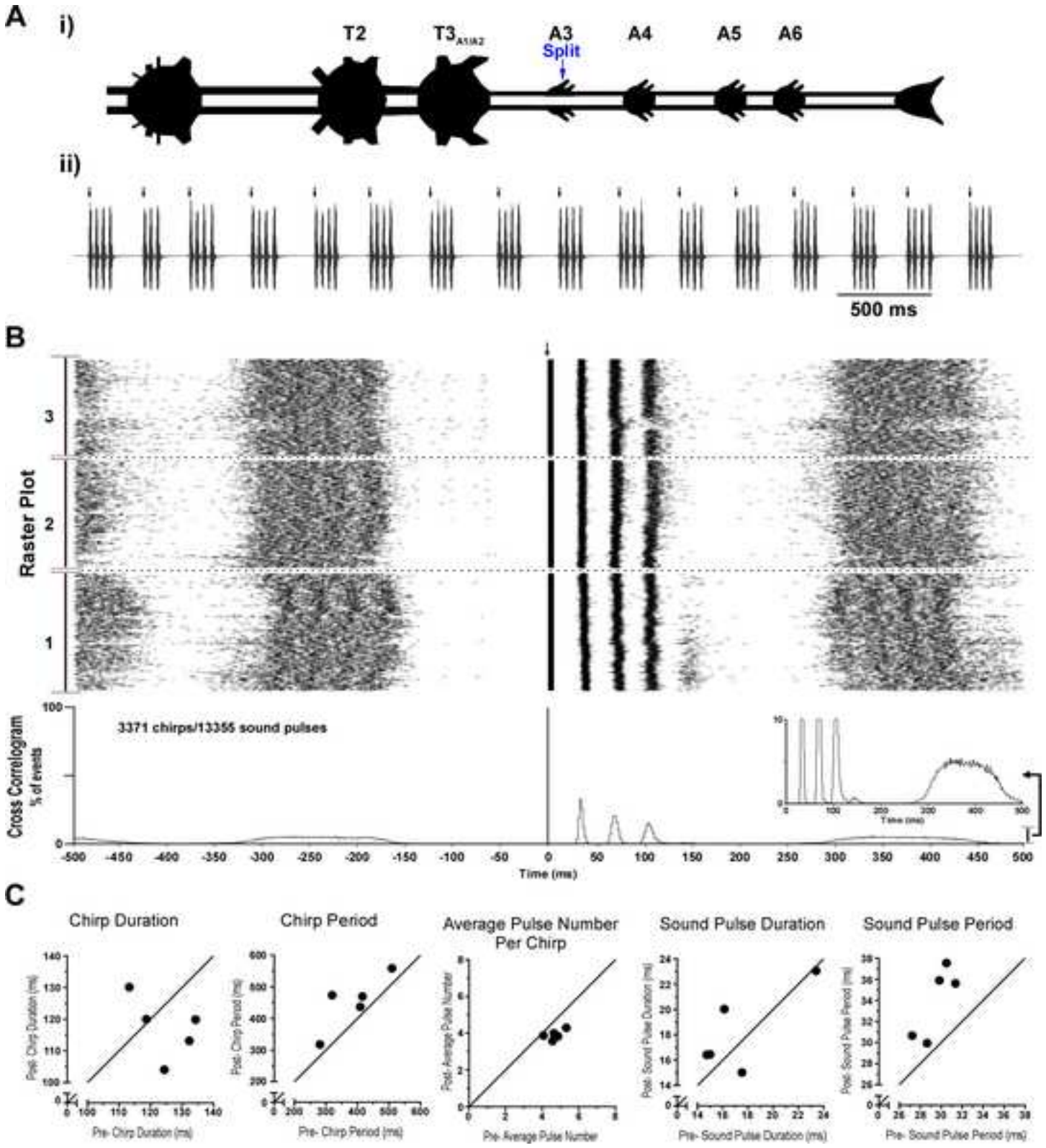

Figure 9

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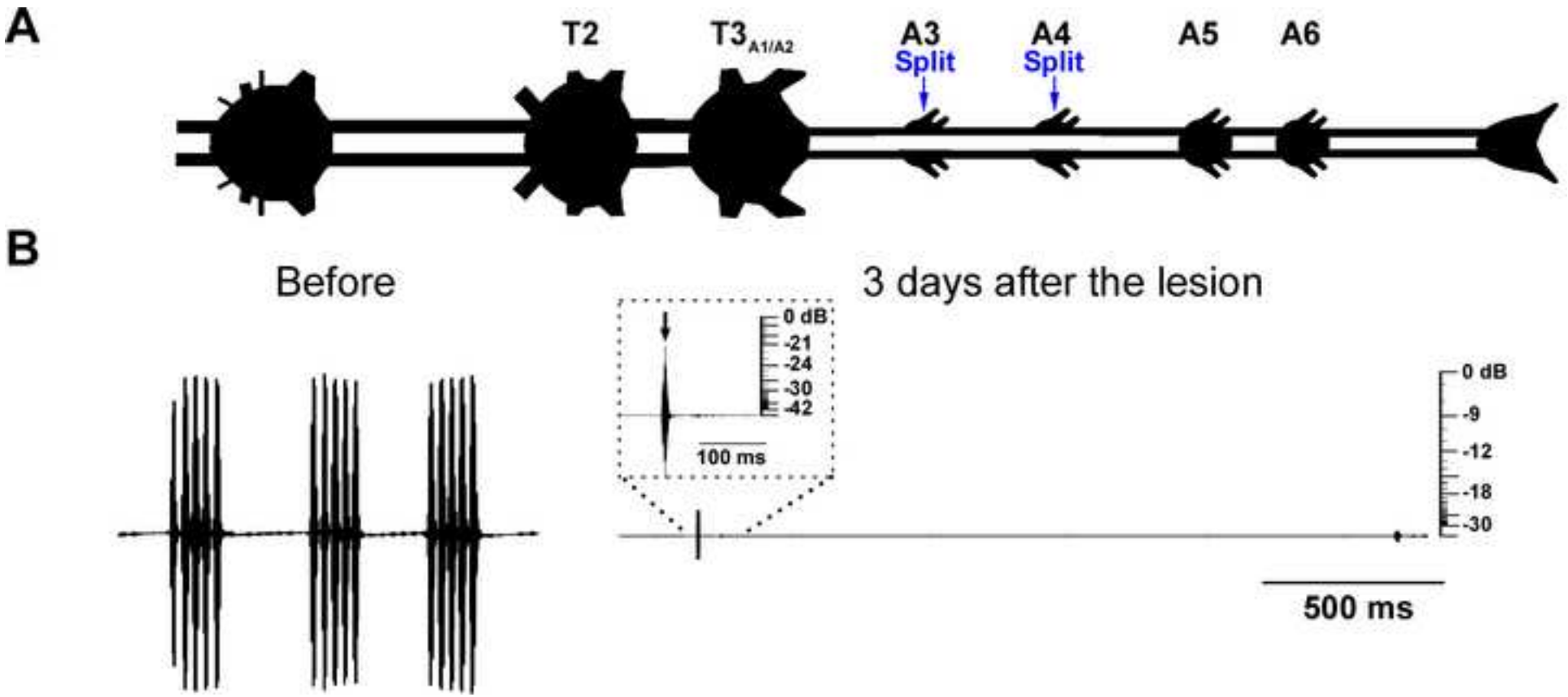

Figure 10

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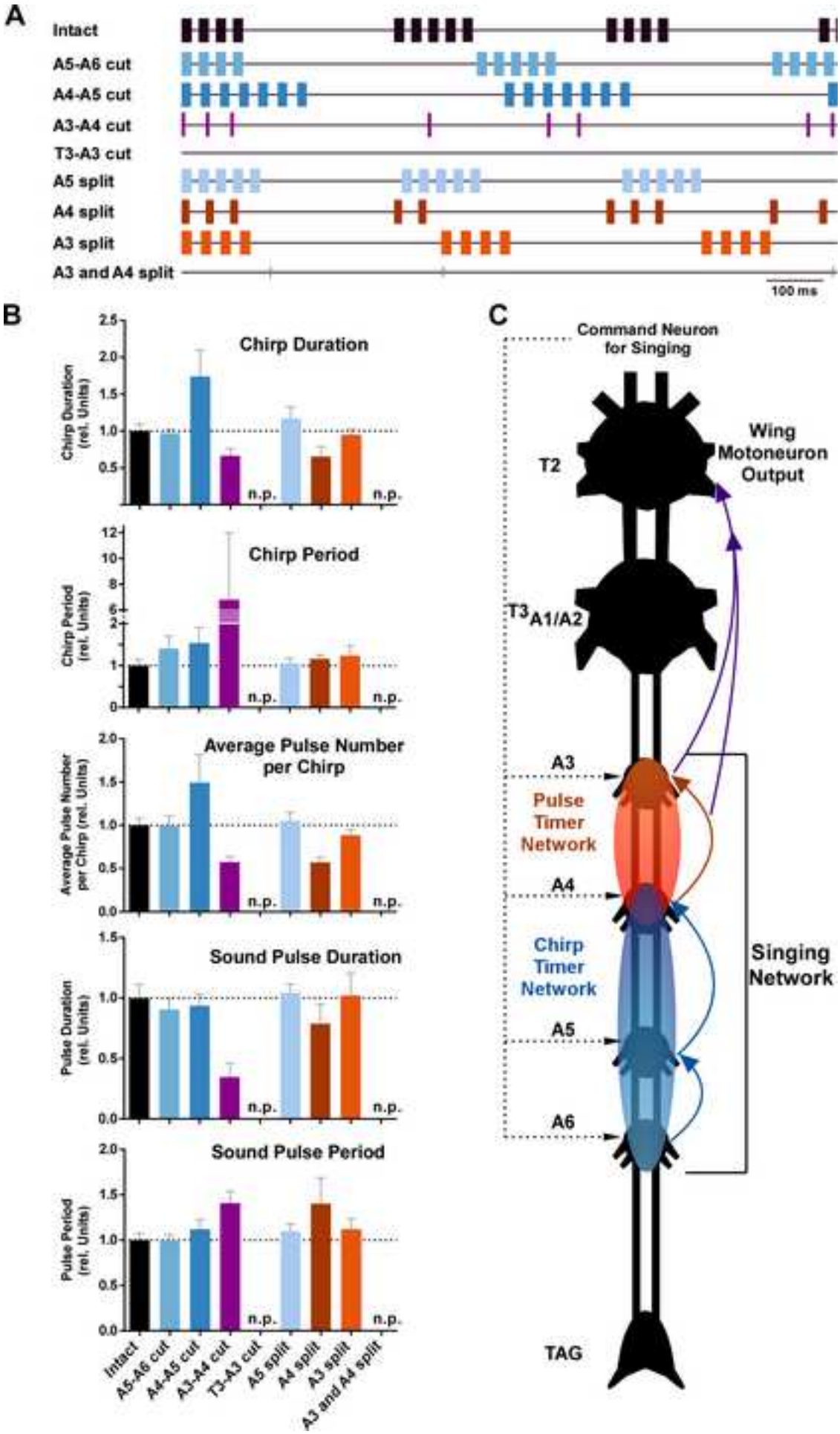

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