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# Identification of two forebrain structures that mediate execution of memorized sequences in the pigeon

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**Helduser S, Cheng S, Güntürkün O.** Identification of two forebrain structures that mediate execution of memorized sequences in the pigeon. *J Neurophysiol* 109: 958–968, 2013. First published December 12, 2012; doi:10.1152/jn.00763.2012.—The execution of action sequences is the basis of most behavior. However, little is known about the neural foundation of visuomotor sequence execution in birds, although pigeons are a classic model animal to study sequence learning and production. Recently, we identified two structures in the pigeon brain, the nidopallium intermedium medialis pars laterale (NIML) and the nidopallium caudolaterale (NCL), that are involved in the execution of a serial reaction time task (SRTT). In the SRTT sequence execution is always cue guided. Thus the previous study could not unambiguously clarify whether NCL and NIML contribute to a memory-based execution of sequential behavior. In addition, a possibly differential role of these two structures could not be identified. Therefore, the present study was conducted to further elucidate the role of NCL and NIML in sequence execution in a task where pigeons performed a memorized four-item sequence. Transient inactivation of each NIML and NCL severely impaired sequence execution. The results confirm and extend our previous findings. NIML and NCL seem to store sequence information in parallel. However, the results support the hypothesis that NCL, in contrast to NIML, is especially required for sequence initiation.

sequence execution; tetrodotoxin; pigeon; song system

SERIAL ORDER IS FUNDAMENTAL to all forms of skilled action, from car driving in humans to singing in birds. Humans, monkeys, and pigeons can all learn to perform a serial-order task with five items and achieve similar high levels of performance (Scarf and Colombo 2008). However, pigeons were long thought to form a simpler representation of these series than primates (D’Amato and Colombo 1988; Terrace 1993). In the last years, however, the tables were turned. Recent studies applied a modified approach to test pigeons’ abilities and showed that these birds are indeed able to form a cognitive representation of sequences in a way that is qualitatively not different from humans and monkeys (Gibson et al. 2012; Scarf and Colombo 2010a, 2010b, 2011).

The question remains as to what neural substrates enable those cognitive functions. In mammals, the formation and production of sequential behavior is based on cortico-striato-thalamic loops (Eckart et al. 2009). Moreover, frontal areas have been shown to play a crucial role as well (Bailey and Mair 2007; Collins et al. 1998; Delatour and Gisquet-Verrier 2001; Gomez et al. 2002; Honda and Shibasaki 1998; Pammi et al. 2011). In a previous study we identified two areas in the pigeon brain that play a role

in the execution of a sequential visual task (Helduser and Güntürkün 2012). One of these regions is the nidopallium caudolaterale (NCL). A variety of evidence supports the idea that NCL is the avian functional equivalent to mammalian prefrontal cortex (PFC) (for a review see Güntürkün 2012). Hence, the finding that NCL is involved in sequence control is in line with this view. NCL projects to the arcopallium (Kroner and Güntürkün 1999; Zeier and Karten 1971), and the arcopallium in turn sends motor-related efferents to the brain stem (Wild et al. 1985; Fig. 1A). Via this axis, NCL is in the position to regulate body movements. The second region that was demonstrated to take part in sequence control is the nidopallium intermedium medialis pars laterale (NIML). Anatomically, this region was first outlined by Rehkemper and Zilles (1991) as area Ne9. NIML projects to the medial striatum, which in turn is connected to the dorsal thalamus (Kitt and Brauth 1982; Kroner and Güntürkün 1999; Wild 1987). Dorsal thalamic nuclei provide afferent input back to NIML (Kitt and Brauth 1982; Kroner and Güntürkün 1999; Fig. 1A). Thus NIML is part of a pallio-striato-thalamic circuit that is reminiscent of mammalian cortico-striato-thalamic loops (Reiner et al. 1998) that were shown to be crucial for sequential actions (Benecke et al. 1987; Doyon et al. 1996; Graybiel 1998; Lehericy et al. 2005).

The aim of the present study was to extend our previous findings and further elucidate the role of both NCL and NIML in sequence processing. In our earlier study we applied a serial reaction time task (SRTT). The SRTT is commonly applied to investigate sequence learning and production and proved useful to identify sequence-relevant brain areas. However, the SRTT has several disadvantages. Most importantly, sequence generation is always cue guided. Although Helduser and Güntürkün (2012) demonstrated that their animals properly anticipated the next sequential location before cue onset, the animals can in principle perform at high levels independent of sequence memory. Thus temporary lesions of NCL or NIML can never abolish correct sequential actions since cue-guided sequences are left as a default option. Therefore, our previous results—though unequivocally demonstrating that both NCL and NIML are involved in sequence execution—could not fully answer the question of in what way both structures contribute to sequential behavior. Therefore, here we designed a task based on the simultaneous chaining paradigm of Straub and colleagues (1979), in which performance is purely memory based (Fig. 1B). Our new results further support our earlier findings but also reveal partly differential roles of NCL and NIML for sequence execution.

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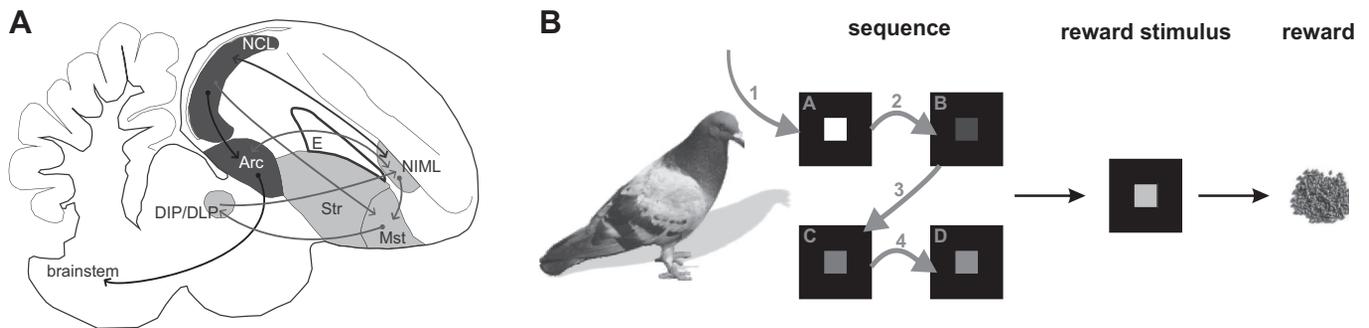


Fig. 1. Neural pathways encompassing the target areas and the experimental paradigm. *A*: connectivity of the nidopallium caudolaterale (NCL) and the nidopallium intermedium medialis pars laterale (NIML). The NCL has reciprocal connections to the arcopallium (Arc), which projects to brain stem nuclei. NIML is incorporated into a circuit that encompasses the medial striatum (Mst) and thalamic nuclei (DIP/DLP). Str, striatum; E, entopallium. *B*: schematic drawing of the experimental paradigm. Pigeons learned to peck 4 items in a fixed sequence. The different-colored items were arranged in a square on a touch screen. Gray arrows indicate the order of items ( $A \rightarrow B \rightarrow C \rightarrow D$ ). If the pigeon completed the sequence correctly, the items vanished and a single stimulus, called the reward key, appeared. A peck to the reward key resulted in reward delivery.

## METHODS

### Subjects

Eighteen naive homing pigeons (*Columba livia*) of unknown sex served as subjects in this study. The pigeons were housed in individual wire mesh cages within a colony room with a 12:12-h light-dark cycle. The animals had ad libitum access to water. During experiments the animals were maintained at 80–90% of their free-feeding weight and were fed accordingly. All experiments were in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by a national committee (North Rhine-Westphalia, Germany).

### Apparatus

The experiments were carried out in the same setup we described in a previous study (Helduser and Güntürkün 2012). The setup consisted of a custom-made operant chamber (38 cm × 38 cm × 42 cm) equipped with a touch screen (Elo 1515L, Tyco Electronics) that was mounted at the rear. A feeder provided an amount of ~0.3 g of grain as reward. The grain was delivered to a food tray that was situated centrally beneath the touch screen. A white LED illuminated the food tray during the whole intertrial interval if a food reward was gained by the animal. The box was further equipped with a set of white LEDs that functioned as houselights. Control of the setup and data collection was done by means of MATLAB (R2006b, The MathWorks) using functions of the Biopsychology Toolbox (Rose et al. 2008).

### Behavioral Paradigm

**Pretraining.** Initially an autoshaping procedure was applied to train the pigeons to peck a target stimulus on the touch screen. The stimulus was a black square (3 cm × 3 cm) with a white center (1 cm × 1 cm) that was presented centrally on the touch screen. This stimulus would later be the first item of the sequence. As during all stages of the experiment the screen background color was white. After an intertrial interval of 30 s the stimulus appeared on the screen. The appearance of the stimulus was signaled by a sound. Free reward was delivered after 5 s of stimulus presentation or upon a single peck to the stimulus. A brief sound was played as feedback signal when a peck to the stimulus occurred. The animals were transferred to a FR1 schedule when they started to respond to the stimulus. When reliable responding to the stimulus was established, sequential training began.

**Sequence training.** When the pigeons reliably pecked the stimulus, sequential responses were established stepwise. The final sequence consisted of four stimuli that were arranged in a square formation centrally on the screen. The stimuli were black squares (3 cm × 3 cm) with different-colored centers (1 cm × 1 cm). The sequence was white—

blue—green—orange. The stimuli were presented at fixed locations (white: upper left corner; blue: upper right corner; green: lower left corner; orange: lower right corner). For simplicity, the sequence items are denoted with letters A–D in the following ( $A \rightarrow B \rightarrow C \rightarrow D$ ). Hence, the pigeons performed a regular pattern of movements to peck the sequence. It is therefore possible that the pigeons learned either the sequence of colors or alternatively the sequence of spatial locations. In this way the task differs from the original task designed by Straub et al. (1979), where pigeons could only acquire a sequence of colors because stimulus locations changed from trial to trial. To provide further feedback, each of the stimuli was associated with a different sound signal that was played when the stimulus was pecked.

In the initial step of sequence training, the first two stimuli of the sequence (A and B) were presented sequentially at their respective locations. That is, first *stimulus A* was presented. A peck to it elicited its disappearance, and *stimulus B* was presented immediately. One peck to *stimulus B* resulted in delivery of a reward. When reliable pecking to both stimuli was established, the next training step was initiated. During the second training step both stimuli were presented simultaneously. To obtain a food reward the stimuli needed to be pecked in the correct order ( $A \rightarrow B$ ). To prevent a stronger association of the last stimulus of the sequence with food reward, a “reward stimulus” was introduced from this training step onwards. That is, after the sequence was completed the sequential stimuli vanished from the screen and a red stimulus was presented. One peck to the reward stimulus triggered reward delivery. The next trial started after an intertrial interval of 10 s. When performance was above 70% in two consecutive sessions the pigeon was transferred to the next stage of training. Within the subsequent training steps *stimuli C* and *D* were added to the sequence in the same manner as training steps 1 and 2. That is, after successful pecking of the already acquired part of the sequence ( $A \rightarrow B$  or  $A \rightarrow B \rightarrow C$ ), the novel stimulus (*C* or *D*) was presented alone. As soon as the novel stimulus was pecked consistently during one training session, the novel stimulus was added to the simultaneous presented stimuli (Fig. 1*B*).

In each trial, pecking a stimulus in the correct order elicited the associated sound signal and additionally the stimulus was blanked out for 250 ms. Pecking a stimulus in the incorrect order was associated with a buzzer sound. Incorrect pecks resulted in a time-out of 10 s during which the houselights were turned off and the touch screen was black. In the following trial the pigeons were required to start the sequence again at the first position (white). As long as the sequence was not violated, repeated pecks on a stimulus had no consequence. Repeated pecking did not elicit any feedback signal. If no peck occurred within 10 s, the trial was ended and the intertrial interval started. As after incorrect pecks, the pigeons had to start the sequence again at the first position (A) in the subsequent trial. Training sessions lasted until 50 rewards were obtained or maximally for 100 trials. By “trial” we refer to the period from the beginning of stimulus present-

tation until reward delivery or until the occurrence of an incorrect peck or an omission. When the pigeons reached a stable performance in the execution of the whole sequence, the animals underwent surgery for the implantation of cannulas.

**Test sessions.** After recovery from the surgery pigeons were trained again until the performance level from before surgery was reestablished. Each pigeon was then tested in four test sessions. Half an hour prior to the start of a test session the animals received an intracranial injection of either tetrodotoxin (TTX; 10 ng/ $\mu$ l, tetrodotoxin citrate, Tocris) or saline as a control condition. Injections were carried out bilaterally into the NIML or the NCL. The order of injections (“Saline—TTX—TTX—Saline” or “TTX—Saline—Saline—TTX”) was counterbalanced within the groups (NCL or NIML). Between the first and second as well as between the third and fourth test sessions lay an interval of 48 h. The second and third test sessions were separated by a period of 1 wk, during which the pigeons were regularly trained.

### Injections

To carry out TTX and saline injections, an injection cannula (C3151 8.5 mm, Plastics One) was fixed to a connector assembly (C313C, Plastics One) that was linked to a syringe (5- $\mu$ l syringe, Hamilton). The assembly was completely filled with distilled water, and the syringe was mounted to a microinjection pump (PHD 2000, Harvard Apparatus). The same was done with a second assembly. Then an air bubble of 1- $\mu$ l volume and subsequently 4  $\mu$ l of saline or TTX were drawn up. The air bubble was used to monitor the injected volume (Freund et al. 2009). The injection cannulas were inserted into the implanted cannula guides (C315G 8 mm, Plastics One), and 1  $\mu$ l of TTX or saline was injected at a rate of 0.2  $\mu$ l/min. After the amount of substance was injected the injection cannula stayed in place for a further 5 min to allow the substance to diffuse into the brain tissue.

### Surgery

The surgery has been described previously (Helduser and Güntürkün 2012). In brief, cannula guides were chronically implanted into NIML ( $n = 10$ ) or NCL ( $n = 8$ ). Stereotactic coordinates were determined with the atlas of Karten and Hodos (1967) and were as follows: NIML: AP 9.5 mm, ML 3.5 mm, DV 4.2 mm; NCL: AP 6.5 mm, ML 8 mm, DV 2.3 mm. For the anesthesia isoflurane (Forane, Abbott) was administered with an anesthetic machine (Mark 5, Medical Developments International). During surgery an analgesic (Dolorex, Intervet) was administered. The pigeons were mounted into a stereotactic device, small craniotomies were performed, and the cannula guides were inserted into the brain. The cannulas were secured to the skull with dental cement. The dental cement was anchored with six screws that were drilled into the skull. For 3 days after surgery the animals were treated with carprofen (Rimadyl, Pfizer). The pigeons were allowed to recover for at least 1 wk before training was resumed.

### Histology

For histological assessment of the injection sites the animals were killed after completion of the experiments and TTX was stained with an immunohistochemical technique. Therefore, a transcardial perfusion was performed first with warm (40°C) sodium chloride solution (0.9%) and subsequently with cold (4°C) paraformaldehyde (4% in 0.12 M phosphate buffer pH 7.4, PBS) for tissue fixation. The brain was removed and transferred to 4% paraformaldehyde with additional 30% sucrose for postfixation. After 2 h of postfixation the brain was stored overnight in a solution of 30% sucrose in PBS for cryoprotection. Then 40- $\mu$ m-thick sections of the brain were prepared with a freezing microtome. For staining of the TTX the immuno-ABC technique was applied (Freund et al. 2009). Sections were incubated with a primary antibody (mouse- $\alpha$ -TTX, 1/200 in PBS+, Hawaii Biotech) and subsequently with a biotinylated secondary antibody

(horse- $\alpha$ -mouse, 1/200 in PBS+, Vectastain, Vector, Camon). Then the sections were stained with a heavy metal-intensified 3,3'-diaminobenzidine reaction (DAB, Sigma). For a more detailed description of the histological techniques see Helduser and Güntürkün (2012). Finally, the sections were mounted and stained lightly with cresyl violet in order to visualize anatomical structures. The sections were carefully examined under a microscope, and TTX spread was reconstructed with the brain atlas of Karten and Hodos (1967). Reconstruction was done on a computer with CorelDRAW X5.

### Data Analysis

**Response times.** Response times (RTs) were defined as the latency from trial onset to the occurrence of the first peck. This calculation was applied for the first item of the sequence as well as the reward stimulus. For the second, third, and fourth sequential items RT was calculated as the latency between the last peck to the previous item and the first peck to the respective item.

For the analysis the averages of the median RTs of both test sessions in the two conditions (Saline, TTX) were calculated for each pigeon. The mean over all pigeons was taken for both the Saline and TTX conditions in both groups (NIML, NCL). Results are depicted as means  $\pm$  SE. Friedman's two-way analysis of variance (ANOVA) by ranks was applied to compare RTs of correct pecks to the four items of the sequence within each condition. Inactivation effects were assessed by means of Wilcoxon signed-rank tests. Between-group comparisons were carried out with Mann-Whitney *U*-tests for independent samples. Moreover, RTs in case of errors were analyzed separately for each type of error (see below). For statistical analysis a  $2 \times 2$  ANOVA with the between-subjects factor injection site (NCL, NIML) and the within-subject factor injection (Saline, TTX) was computed for each type of error. All statistical analyses were performed with SPSS (version 20, IBM).

**Error analysis.** We analyzed different kinds of errors to assess the effect of inactivation of the target brain regions. First, we calculated the percentage of correct pecks as a measure of overall performance. Second, we analyzed the proportion of omitted trials, i.e., trials during which at any point during the sequence no peck occurred during the stimulus presentation time (10 s), leading to the abortion of the trial. Omissions can indicate different deficits. On one hand, they can reflect an impairment of sequence recall. On the other hand, they can also be interpreted as a lack of motivation or attention. Third, we analyzed sequence-specific errors that fell into three categories: skipping errors, backwards errors, and perseveration.

Skipping errors mean that items of the sequence were left out. In our four-item paradigm it was possible to skip one, two, or three items. There were three possibilities to skip one item: pecking first *item B* as well as the transitions  $A \rightarrow C$  and  $B \rightarrow D$ . Skipping two items included pecking *C* first as well as the transition  $A \rightarrow D$ . Finally, there was one possibility to skip three items, pecking the last item (*D*) first.

Similarly, there were different ways to produce a backward error: going back one or two items within the sequence. Going back one item comprised two transitions:  $B \rightarrow A$  and  $C \rightarrow B$ . Moreover, there was one possibility to go back two items, namely,  $C \rightarrow A$ . There was no possibility to commit a backwards error including *item D*, since a peck to *item D* initiated the reward key.

Perseverations comprised the third category of sequence-specific errors. As perseveration we defined repeated pecks at one stimulus. An increase of perseverations can be interpreted as a deficit in sequence processing, with the result that the animal has difficulties in progressing to the next sequential item.

In addition, we analyzed the amount of “undirected pecks” as a measure of pecking accuracy. Pecks that were located anywhere on the touch screen outside the boundaries of the stimuli were defined as undirected pecks. If a general motor deficit was caused by TTX injections we would expect undirected pecks to increase and thus pecking accuracy to be impaired.

For the analysis, results from each error type were averaged for each pigeon over both TTX and Saline test sessions, respectively. The mean value averaged over all pigeons was then calculated. For statistical analysis values were transformed with the logit function. A  $2 \times 2$  ANOVA with the between-subjects factor injection site (NIML, NCL) and the within-subject factor injection (Saline, TTX) was computed for each type of error. All statistical analyses were performed with SPSS (version 20, IBM).

## RESULTS

### *Histological Results*

After immunohistochemical staining of TTX, brain sections were microscopically inspected to determine cannula positioning and TTX spread. From both the NIML and NCL groups two pigeons had to be excluded because of misplaced cannulas. For all other pigeons TTX spread was confirmed to lie within the targeted regions. Figure 2 depicts schematically the smallest and largest TTX spreads.

In the NIML group TTX spread lay between AP 9.0 mm and 10.75 mm. It was in all cases localized adjacent to the medial tip of the entopallium and was confined between the lamina mesopallialis and the lamina pallio-subpallialis. In no case did TTX spread beyond the lamina pallio-subpallialis, so the basal ganglia were not affected. In some cases there was a very limited spread of TTX into the lateral-most part of the entopallium and into the overlying mesopallium (Fig. 2, *left*). In the NCL group TTX spread was located in the anterior-most part of the NCL (Herold et al. 2011), in a region between AP 6.0 mm and 7.25 mm. TTX had a spherical spread that extended directly below the lateral ventricle (Fig. 2, *right*).

### *NCL and NIML Inactivation Impaired Sequence Execution*

*Decrease of correct transitions.* Inactivation severely disrupted sequence execution. Figure 3 depicts the transition probabilities between the four items of the sequence. Obviously, correct transitions were reduced, while consequently errors increased. Comparison of the percentages of correct transitions between the

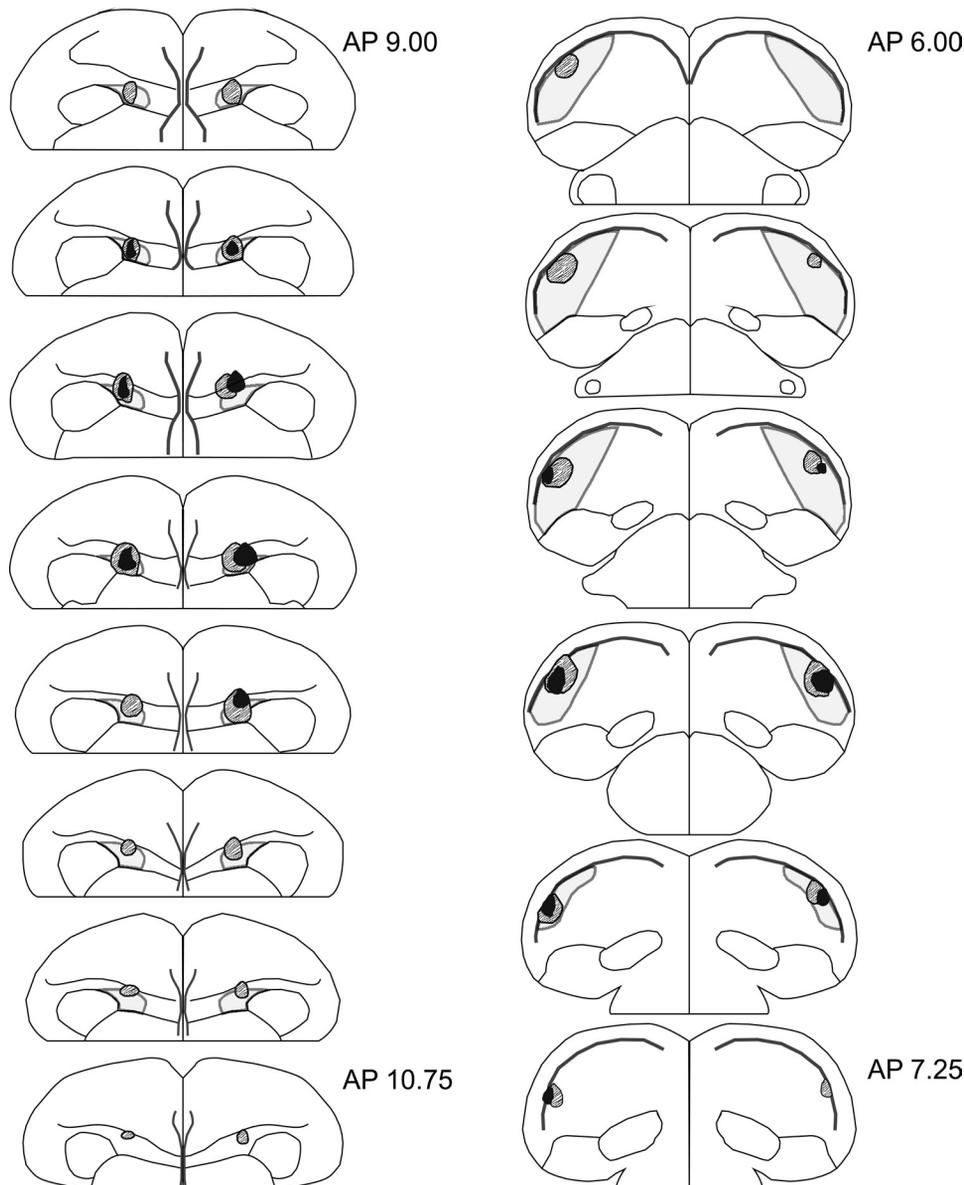


Fig. 2. Schematic frontal sections illustrate tetrodotoxin (TTX) spread. Shown is the reconstruction of the largest (shaded) and smallest (black) extent of TTX staining in pigeons of the NIML (*left*) and NCL (*right*) groups. Boundaries of NIML and NCL are depicted by gray areas. Distances between the sections are 250  $\mu\text{m}$ .

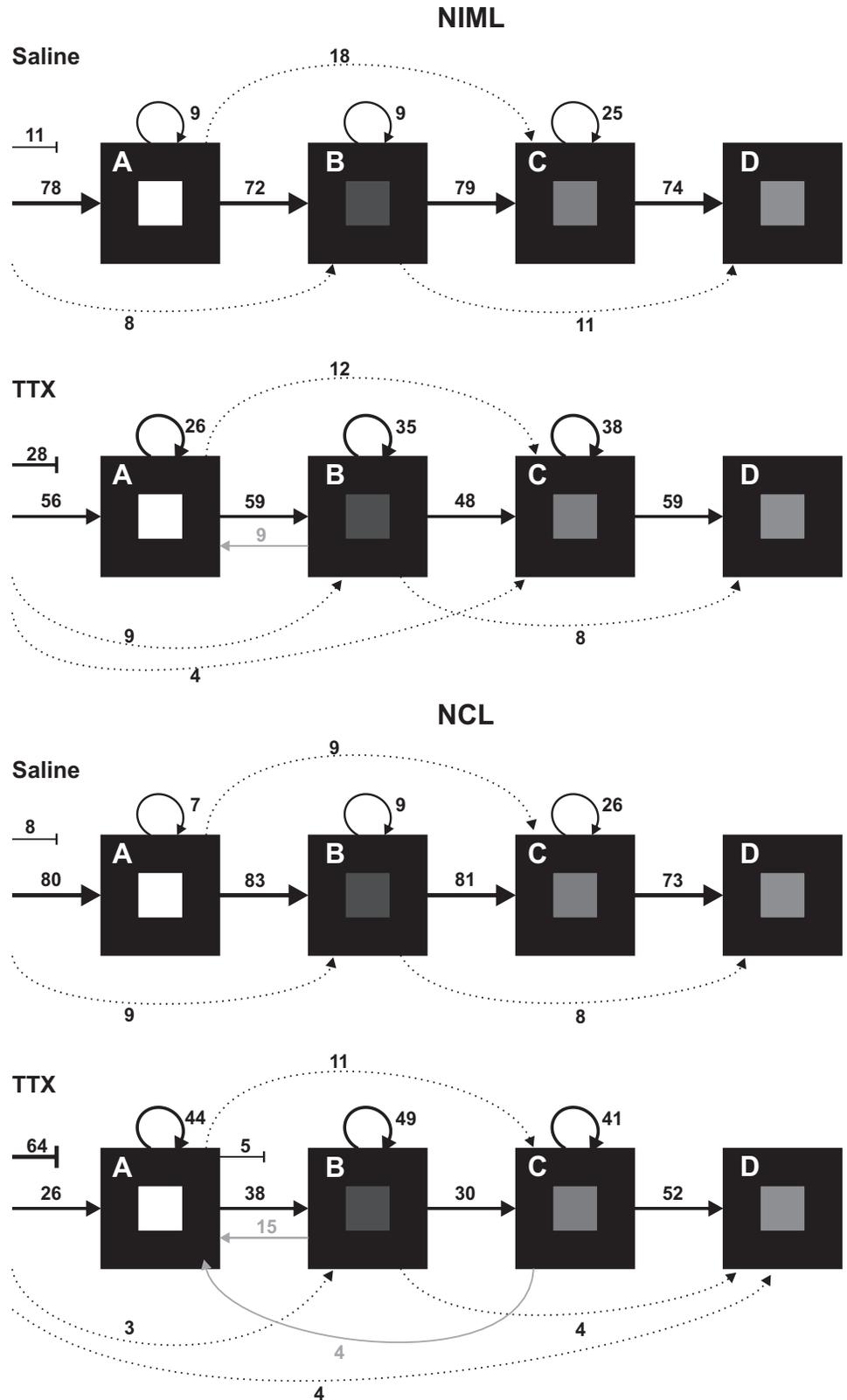


Fig. 3. Transition probabilities between items in the control condition (Saline) and after inactivation (TTX) of NIML (top) and NCL (bottom), respectively. Arrows indicate the direction of the transition for different types of errors (solid: correct pecks; dotted: skipping errors; gray: backwards errors; circular: perseveration; t-shaped marks: omission). Numbers indicate the transition probability expressed as % of pecks originating from the respective item. Transitions with probabilities <4% are not depicted. NIML and NCL inactivation decreased performance. In particular, perseveration and omissions were increased. Additionally, skipping and backwards errors were affected. The pattern of inactivation effects differed between NIML and NCL groups. See text for details.

NCL and NIML groups implies that NCL animals were most strongly impaired at sequence initiation whereas in the NIML group all transitions were equally affected. Analysis of correct transitions was done by means of a three-way repeated-measures ANOVA with factors injection (Saline, TTX), injection site

(NCL, NIML), and transition (to items A, B, C, and D). The main effect of injection was highly significant ( $F_{1,12} = 40.1, P < 0.0001$ ). Additionally, the interaction of injection and injection site was significant ( $F_{1,12} = 6.1, P = 0.03$ ). There was a trend for injection site ( $F_{1,12} = 4, P = 0.07$ ) as well as the interaction of

injection and transition ( $F_{3,36} = 2.7, P = 0.078$ ). The other factors and interactions were nonsignificant (each  $P > 0.1$ ).

**Overall performance.** In general, after inactivation of both NCL and NIML with TTX, a substantial decrease of the overall performance was apparent compared with the Saline control

condition. There was a drop of performance from  $64 \pm 4\%$  to  $13 \pm 5\%$  of correct trials (means  $\pm$  SE) in the NCL group and from  $53 \pm 6\%$  to  $30 \pm 8\%$  in the NIML group (Fig. 4A). There was a significant main effect of injection ( $F_{1,12} = 76.7, P < 0.001$ ) but no effect of injection site. The interaction of injec-

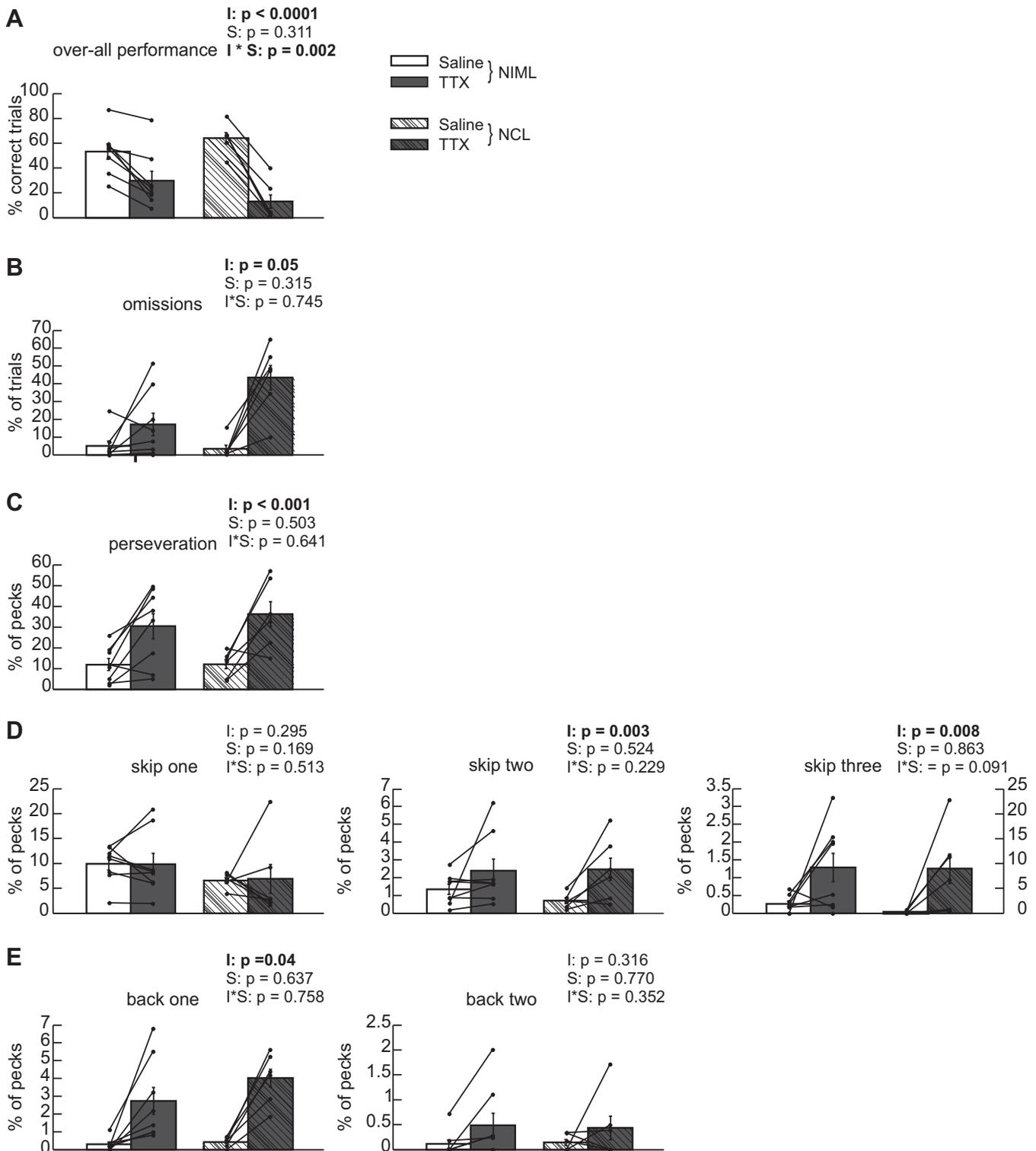


Fig. 4. Error rates of different types of errors were affected by NCL and NIML inactivation. *A*: overall performance. *B–E*: omission (*B*), perseveration (*C*), skipping (*D*), and backwards (*E*) errors. Bars show means  $\pm$  SE; lines show results from individual pigeons. *P* values computed by repeated-measures ANOVA are shown: I, within-subject factor injection (Saline, TTX); S, between-subject factor injection site (NCL, NIML); I\*S, interaction. *P* values  $< 0.05$  are marked in bold. See text for details.

tion and injection site was significant ( $F_{1,12} = 15.7$ ,  $P = 0.002$ ). Further analysis revealed that after both TTX injections into NCL as well as NIML performance decreased (each  $P < 0.01$ ). However, performance decrease was larger after NCL injections ( $P = 0.002$ ). In each case performance by far exceeded chance level. Taking into account that repetitive responses were not counted as an error, the probability of correctly performing the sequence by chance is 0.9% ( $0.25 \times 0.33 \times 0.33 \times 0.33$ ). In the following a detailed analysis of the different types of impairments is described. If not explicitly reported, error rates did not differ significantly between NCL and NIML animals ( $P > 0.05$ ).

**Omissions.** Omissions as we have defined them here comprise two qualitatively different aspects. First, an omission occurred when the pigeon did not start to peck at the beginning of a trial, i.e., the sequence was not initiated. The second possibility is aborting a trial by not proceeding to the next item of the sequence. However, the latter case occurred only scarcely (2–6%) in the both Saline and TTX conditions (cf. Fig. 3). Therefore, we did not distinguish these aspects in our analysis. In the control condition omission rates were low in both groups. NCL as well as NIML inactivation, however, caused a significant increase of omissions ( $F_{1,12} = 4.8$ ,  $P = 0.05$ ; Fig. 4B).

**Perseveration.** The amount of perseveration, i.e., repetitive pecks to a stimulus, were increased by TTX injections ( $F_{1,12} = 25.5$ ,  $P < 0.001$ ; Fig. 4C).

**Skipping errors.** There were three types of skipping errors that could occur, namely, skipping one, two, or three items of the sequence. The most common of these was the skipping of one item error (pecking *item B* first, the transitions  $A \rightarrow C$  and  $B \rightarrow D$ ). Neither NCL nor NIML inactivation affected the incidence of the skipping of one item error. The skipping of two items (pecking *item C* first and the transition  $A \rightarrow C$ ) was increased after TTX injections ( $F_{1,12} = 14$ ,  $P = 0.003$ ). Also, the skipping of three items (pecking *item D* first) was increased significantly by TTX injections ( $F_{1,12} = 10$ ,  $P = 0.008$ ; Fig. 4D).

**Backwards errors.** There were two possibilities of committing backwards errors, i.e., going back one or two items within the sequence. Only the backwards one error (transitions  $B \rightarrow A$  and  $C \rightarrow B$ ) was increased by TTX injections ( $F_{1,12} = 5.3$ ,  $P = 0.04$ ; Fig. 4E).

#### NCL and NIML Inactivation Slowed Sequential Responses

RTs were analyzed separately for correct pecks, for reward key responses, and for incorrect pecks. We first present the results for correct pecks (Fig. 5). Comparisons were adjusted for multiple testing.

**Correct pecks.** Responses to all four items of the sequence differed with respect to RT. In the TTX condition of the NIML group, RT for pecking *item A* was longer compared with all other items. This was significant between *A* and *C* ( $P = 0.012$ ) and *A* and *D* ( $P = 0.022$ ) but not for *A* and *B* ( $P = 0.071$ ). A similar pattern was obtained in the Saline condition. Here, significant differences were found for *A* and *B* ( $P = 0.04$ ) and *A* and *C* ( $P = 0.022$ ). Likewise, in the TTX condition of the NCL group the RT for *item A* was higher relative to *items B* ( $P = 0.022$ ) and *C* ( $P = 0.044$ ). Additionally, inactivation of NIML with TTX moderately slowed down responses to *items A* ( $P = 0.012$ ) and *C* ( $P = 0.036$ ) compared with the Saline condition. Injecting TTX into NCL yielded significantly slower responses for all four items compared with the Saline condition (*item A*:  $P = 0.012$ ; *items B, C, and D*:  $P = 0.028$ ), with the largest RT increase being observed at *item A*. Generally, the RT increases were larger after NCL than NIML inactivation (*A*:  $P = 0.013$ ; *C*:  $P = 0.043$ ; *D*:  $P = 0.059$ ).

**Reward key pecks.** Responding to the nonsequential reward key was affected by neither NCL nor NIML inactivation (Fig. 5). RT did not differ between NCL and NIML groups.

**Incorrect pecks.** Most errors consisted of skipping one or more items. Under some conditions, these errors were accompanied by a significant slowing down of responses. Overall, RT during a one-item skipping error was increased after TTX injection compared with control condition ( $F_{1,12} = 20.6$ ,  $P = 0.001$ ). Injection site had a significant effect ( $F_{1,12} = 9.7$ ,  $P = 0.009$ ), and there was a significant interaction of injection and injection site ( $F_{1,12} = 18.9$ ,  $P = 0.001$ ). Further analysis by means of two-tailed paired *t*-tests revealed that only NCL inactivation produced a significant RT increase ( $P = 0.003$ ; Fig. 6A). Similarly, the RTs for two-item skipping errors also were overall increased ( $F_{1,12} = 8.9$ ,  $P = 0.011$ ), showed a significant interaction ( $F_{1,12} = 7.8$ ,  $P = 0.016$ ), and evinced a significant effect for the NCL only ( $P = 0.009$ ; Fig. 6A). For the RT of three-item skipping errors there was a significant interaction of injection and injection site ( $F_{1,12} = 9.0$ ,  $P = 0.011$ ). Subsequent two-tailed paired *t*-tests again showed a significantly increased RT ( $P = 0.035$ ) after NCL inactivation only (Fig. 6A).

TTX injections also increased RT for the backwards one-item error (Fig. 6B). The main effect of injection was significant ( $F_{1,12} = 7.1$ ,  $P = 0.021$ ). There was no effect of injection site ( $F_{1,12} = 0.3$ ,  $P = 0.599$ ). The interaction reached significance ( $F_{1,12} = 6.1$ ,  $P = 0.029$ ). Further analysis revealed that only NCL inactivation resulted in RT increase ( $P = 0.007$ ), not NIML inactivation ( $P = 0.904$ ).

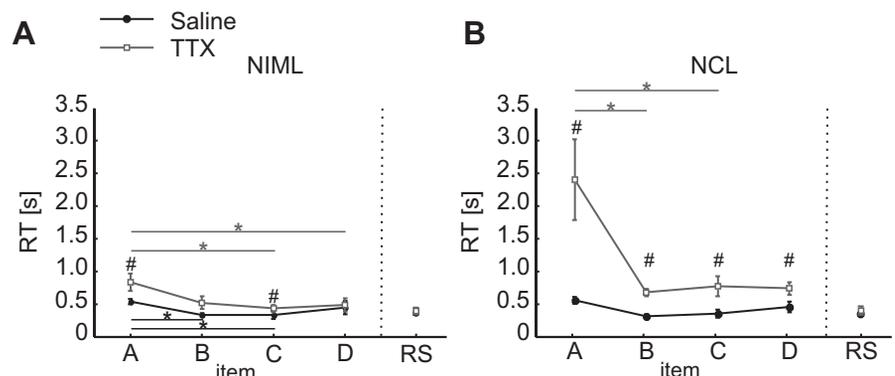


Fig. 5. Response times (RTs) for correct pecks and the reward key. RTs as a function of item for saline and TTX injections are depicted for NIML (A) and NCL (B) groups. There were RT differences between items within injection conditions. After both NIML and NCL inactivation RT increases were observed. \*Significant differences within condition; #significant differences between conditions. In contrast, there were no effects of NIML or NCL inactivation on RT for responding to the reward stimulus (RS) that was not part of the sequence. Means  $\pm$  SE are shown.

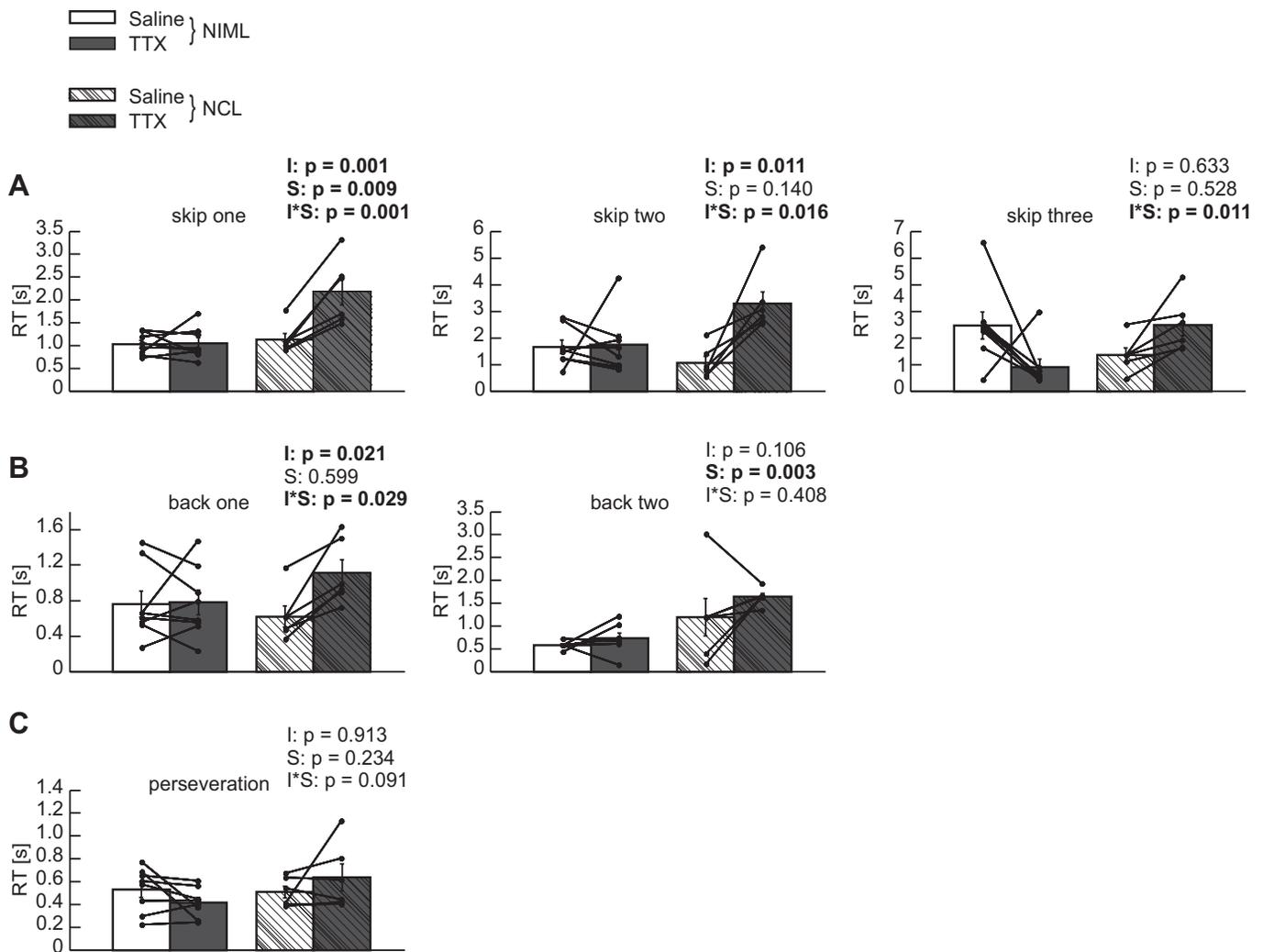


Fig. 6. RTs for incorrect pecks classified as different error types: skipping (A), backwards (B), and perseveration (C). Conventions as in Fig. 4.

In contrast to skipping and backwards errors, perseverative responses were not affected by TTX injections into NCL or NIML. There were no between-group differences, either (Fig. 6C).

#### TTX Injections Did Not Reduce Pecking Accuracy

Undirected pecks, i.e., pecks that were located outside the boundaries of the stimuli, were analyzed as a measure for pecking accuracy. Figure 7A, left and right, show typical examples of the peck distribution on the touch screen from a pigeon in the NIML group and in the NCL group, respectively. The highest density of pecks was concentrated within the area of the stimuli. Only a small proportion of pecks were located outside the boundaries of the stimuli. The peck distribution after TTX injection overlays the peck distribution after saline injection. There were no statistically significant differences (Fig. 7B).

#### DISCUSSION

We investigated the role of NIML and NCL in the execution of a memorized sequence and revealed that reversible inactivation of each of these structures severely impairs performance. Overall, the pattern of inactivation effects was similar between the NCL and NIML groups. However, increases of RT following NCL inactivation that were not seen after TTX

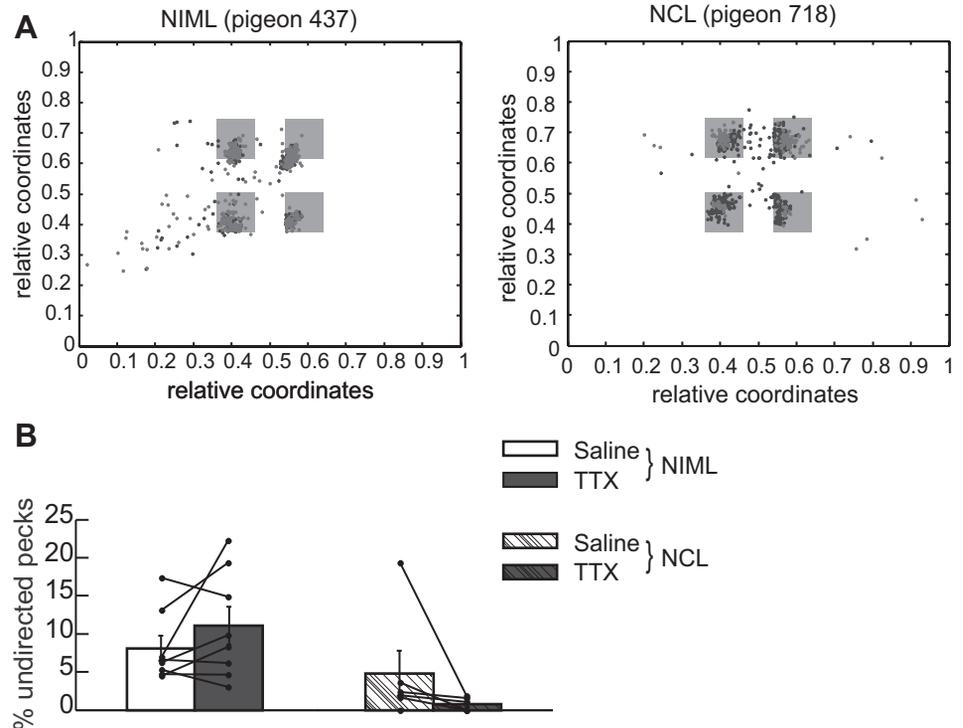
injections into NIML possibly indicate differential functions of these two structures. In the following, the different effects are discussed in detail and hypotheses for different functions of NCL and NIML are considered.

#### Contribution of NIML and NCL to Nonsequential Compounds of Behavior

We discuss our results in the light of sequence processing. However, to do so properly, we should first rule out other cognitive functions as a source for the observed impairments. Such impairments could encompass, e.g., a general motor deficit as well as attentional or working memory dysfunctions. These need to be considered especially for NCL because of its association with executive functions analogous to mammalian PFC (Diekamp et al. 2002a, 2002b; Güntürkün 1997, 2005, 2012; Kalt et al. 1999; Rose and Colombo 2005).

For two reasons, a general motor deficit is an unlikely explanation for the present data. First, although there was a general increase of RT within the sequence, RT for the final peck to the reward key was not affected. This reward peck was not part of the sequence since it was displayed without alternative sequential elements. Additionally, perseverative responses did not slow down, whereas RT to commit sequential

Fig. 7. Pecking accuracy is not affected by TTX injections. *A*: typical examples from 2 pigeons from the NIML (*left*) and NCL (*right*) groups. Black marks represent peck locations under Saline condition and gray marks after TTX injections. In both situations pecks cluster within the boundaries of the stimuli. Peck distributions after Saline and TTX injection overlap. Gray shaded areas depict the location of the stimuli. Scaling of the axes is in relative coordinates, where 1 on the *x*-axis equals 30.4 cm and 1 on the *y*-axis equals 22.8 cm. *B*: average % of undirected pecks. Bars depict means  $\pm$  SE; lines represent data from individual pigeons. There were no statistically significant effects.



errors was increased. This sequence-associated increase of RT speaks against a general motor deficit. Comparable results were observed after lesions of rat frontal cortex (Bailey and Mair 2007), where lesions increased RT especially for the first response during a sequential task without affecting response times for single responses and running speed of the rats. Second, a general motor deficit should lead to decreased peck precisions and thus to an increase of pecks that are not directed at the stimuli on the touch screen. However, this was not observed. Therefore, a general motor deficit can very likely be excluded.

Attentional processes involve the mammalian PFC (Granon et al. 2000; Muir et al. 1996). Therefore, similar deficits are reasonable for the NCL and thus could account for the observed effects. Since error patterns after NIML inactivation were comparable to the effects seen after NCL inactivation, an attention deficit could also account for NIML inactivation effects. Indeed, the increase of omissions could result from attentional impairments. Since omissions occurred almost only at trial onset it is in principle possible that the pigeons occasionally missed trial onset, although this start was saliently signaled by a clearly audible sound signal. However, other effects on error rates cannot be so easily attributed to attentional deficits since they are significantly associated with sequence information. Thus an attention deficit cannot account for skipping errors, backwards errors, or perseverations. Instead, omissions within the sequence should increase if pigeons were easily distracted from task performance, yet this was not the case.

Working memory deficits could create difficulties in remembering which items were already pecked. This explanation is especially relevant for the NCL as an avian functional counterpart of the mammalian PFC and as such associated with working memory functions (Diekamp et al. 2002b; Fuster 1973; Romo et al. 1999; Rose and Colombo 2005). In the case of a working

memory impairment perseverations should increase since pigeons could forget that they already pecked an item toward which they are just oriented. While this is possible for perseverations, it is more difficult to explain skipping or backwards errors based on a sole working memory account. Indeed, PFC lesions in monkeys also increase perseverative responses without affecting sequential responses (Collins et al. 1998).

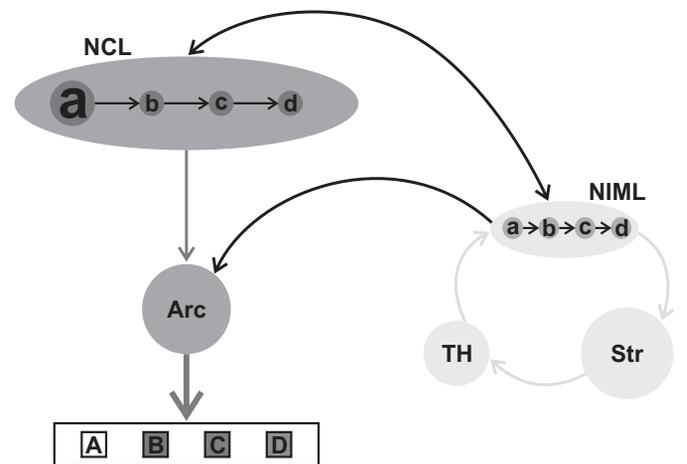


Fig. 8. Hypothetical neural system controlling sequence execution in the pigeon. The special function of NCL possibly lies in sequence initiation. Hence selection of the first sequential item is a process that relies on processes within NCL. Moreover, it is likely that NCL stores sequence information and controls transitions between items during sequence execution. A possible way the sequence could be encoded is that each item is represented by different assemblies of neurons that activate neuron groups in the arcopallium. The arcopallium then produces the according motor commands to peck the respective items. In addition, NIML contains sequence information and is capable of driving sequence execution via its projections to arcopallium. Moreover, information exchange between NCL and NIML is likely and computation of information within the NIML—striatum—thalamus—NIML loop could support sequence execution. Arc, arcopallium; Str, striatum; TH, thalamus.

Taken together, some of the observed deficits could have been induced or elevated by attentional and working memory deficits, but it is unlikely that these cognitive factors can explain the full pattern of errors. Skipping and backwards errors are especially difficult to explain without invoking a specific deficit in sequence processing.

#### *Functional Roles of NIML and NCL in Sequence Execution*

The results of the present study demonstrate that both NIML and NCL play a role in the execution of a memory-based sequence. Transient inactivation resulted in a substantial decrease of performance. Overall the pattern of error increases was very similar, and inactivation of both structures induced an increase of perseverations, skipping errors, and omissions. These results confirm and extend findings from a previous study (Helduser and Güntürkün 2012). In that study we showed that inactivation of NCL and NIML impairs the execution of a SRTT. In contrast to the present paradigm, in the SRTT behavior is cue guided. Hence, SRTT performance benefits from but does not completely rely on a memorized representation of the sequence. Since qualitatively both studies yielded equivalent results, NCL and NIML probably represent and execute sequences in both tasks in a similar way.

Taken together, our data suggest that both structures are possibly part of a functional system that controls the processing of sequential actions. We presently do not know how a sequence generation network is organized in pigeons, but the architecture of the oscine song system could provide a hypothetical framework. Song is an example of a very specialized form of sequential behavior of vocal elements (cf. Katahira et al. 2007; Okanoya 2004). Moreover, it is likely that the avian neural pathways for learning of vocalizations are derived from neural systems of a common ancestor (Feenders et al. 2008). Consequently, structures and pathways that resemble the location and connectivity of the song system are present in non-songbirds (Brenowitz 1991; Farries 2004; Feenders et al. 2008). On the basis of its hodology and location NCL is comparable to the premotor nucleus HVC (used as proper name). Likewise, NIML has a location that is similar to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) of the song system. LMAN is incorporated into a circuit (termed anterior frontal pathway, AFP) that has a structure similar to the NIML circuit (Fig. 1A).

These anatomical similarities suggest a possible functional resemblance. Indeed, HVC is crucial for the generation of learned song (Nottebohm et al. 1976) and is supposed to control the sequence of song elements (Hahnloser et al. 2002; Yu and Margoliash 1996). Our data demonstrate that NCL likewise is involved in the execution of learned sequences. Moreover, the fact that the RT for the first item of the sequence increased dramatically after NCL inactivation argues for a dominant role of NCL in sequence initiation. In further support of this notion is the high incidence of omissions that, at least in part, can be interpreted as a failure to initiate the sequence. Thus NCL can be associated with selection of the sequence at trial onset. In fact, such a function has also been attributed to PFC, the mammalian counterpart of NCL (Verwey 2001).

Inactivation of NIML resulted in impairments that partly equal those of NCL inactivation. Thus it is possible that NIML also contains sequence information. The observation that NCL inactivation severely disturbed task performance but did not

completely abolish sequence execution makes it likely that sequence information is stored or processed in parallel within NCL and NIML. The difference between NCL and NIML lies in two aspects. First, there is evidence for a pivotal role of NCL in response selection of the initial sequential item that is not evident for NIML. Second, RT increases throughout the sequence were more moderate in the NIML animals and did not extend to incorrect pecks. This suggests that NCL might have a leading role during selection and monitoring of a sequence, while sequence information is stored and/or processed in both NCL and NIML. Figure 8 summarizes our proposed model for a sequence generation network in the pigeon. The anatomical connections accord with the architecture of the oscine song system. On a functional level, however, the equivalency only partly holds true. With its function for sequence execution NCL matches HVC. Whether NCL represents sequences with the same neural mechanism as HVC yet remains an open question. NIML, on the other hand, differs obviously from LMAN. Our results show that NIML contributes to the generation of a learned sequence, while LMAN is not required for the production of learned song in adult birds (Scharff and Nottebohm 1991).

In conclusion, despite a similar anatomy, the pigeon's sequence generation network differs from the song system. The pigeon's network mediating general sequences probably has a parallel architecture, in which both NCL as well as NIML contain sequence information. Consequently, both structures may be able to mediate sequence execution. Issues that remain unclear are the nature of the sequence representations in NCL and NIML, how both systems interact during sequence execution, and how they contribute to acquisition of novel sequences.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: S.H. and O.G. conception and design of research; S.H. performed experiments; S.H. analyzed data; S.H., S.C., and O.G. interpreted results of experiments; S.H. prepared figures; S.H. and O.G. drafted manuscript; S.H., S.C., and O.G. edited and revised manuscript; S.H., S.C., and O.G. approved final version of manuscript.

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