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Research report

Neural substrates for serial reaction time tasks in pigeons

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ABSTRACT

Most behavior is composed of action sequences. Pigeons were often used as a model to study sequence learning and execution. Yet, virtually nothing is known about the neural structures underlying sequential behavior in pigeons. We therefore applied a serial reaction time task (SRTT) that is commonly used to investigate sequential behavior. During task performance either the nidopallium caudolaterale (NCL) or the nidopallium intermedium medialis pars laterale (NIMl) was transiently inactivated with tetrodotoxin (TTX). Since prefrontal structures play a role in sequence acquisition and performance in mammals and since the NCL is functionally analogous to the prefrontal cortex, NCL was chosen a possibly critical structure of our study. The NIMl is equivalent by hodology and topology to the song nucleus LMAN. Since LMAN plays a key role in song learning and since song consists of learned vocalizatory sequences, we hypothesized that NIMl could also be a candidate for sequence performance in a non-songbird. Moreover, TTX injections into the entopallium were performed as a control. Indeed, inactivation of both the NCL and the NIMl resulted in an increase of sequence specific errors. Hence, we could identify components of neural systems in the pigeon that underlie sequence execution.

1. Introduction

The majority of our daily behavior consists of overlearned action sequences. Corticostriatal circuits are crucial for sequential behavior and especially its acquisition [1]. Imaging studies have found striatal and motor cortical activations during sequence learning and performance [2–6]. Moreover, fronto-parietal networks show learning and performance related activation [7].

Pigeons are a classic bird model for sequence learning and reach similarly high levels as monkeys and humans [8]. The neural foundations of sequential behavior in pigeons, however, are practically unknown. Yet, parallels to the mammalian neural structures involved in sequential behavior indicate candidate areas in the pigeon brain. In addition, the song system of oscines could be an excellent source of information for the neural fundaments of sequence acquisition and performance in birds. Therefore, we focused on two brain areas that comprise similarities to mammalian circuits and have parallels to the oscine song system.

In human and other mammals there is ample evidence that prefrontal areas play a role for learning as well as execution of sequences [7,9–14]. The pigeon's nidopallium caudolaterale (NCL) is densely innervated by dopaminergic fibers of the vetral tegmental area and the substantia nigra [15]. Moreover, the NCL is reciprocally connected to processing areas of all sensory modalities and projects to major motoric nuclei, e.g. the arcopallium [16,17]. In addition, the NCL has been shown to be involved in a number of higher level processes that are commonly associated with mammalian prefrontal cortex (PFC) like working memory and behavioral flexibility [18–20]. Since the anatomical and functional characteristics of the NCL highly resemble the mammalian PFC it has been proposed that the NCL is the avian equivalent to the PFC [21,22]. Therefore, we hypothesize that the NCL plays like the PFC a role in sequential behavior. The extent of NCL was recently redefined by Herold et al. [23].

In addition, if one considers bird song as a form of sequential behavior, the neural system for song production of oscine birds could hint at further candidate areas for sequences in the pigeon. The song system is composed of two interconnected circuits (Fig. 1B). On the one hand, there is a posterior pathway that is composed of two pallial nuclei [24]. On the other hand, there is a basal-ganglia loop called the anterior forebrain pathway (AFP [25,26]). The magnocellular nucleus of the anterior nidopallium

Abbreviations: AFP, anterior forebrain pathway; DI, difference index; DIP, nucleus dorsointermedius posterior; DLM, dorsolateral anterior thalamic nuecleus; DMA, nucleus dorsomedialis anterior thalami; DMP, nucleus dorsomedialis posterior thalami; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MSt, medial striatum; NCL, nicopallium caudolaterale; NIMl, nidopallium intermedium medialis pars laterale; PFC, prefrontal cortex; RA, robust nucleus of the arcopallium; RT, reaction time; SRTT, serial reaction time task; TTX, tetrodotoxin.

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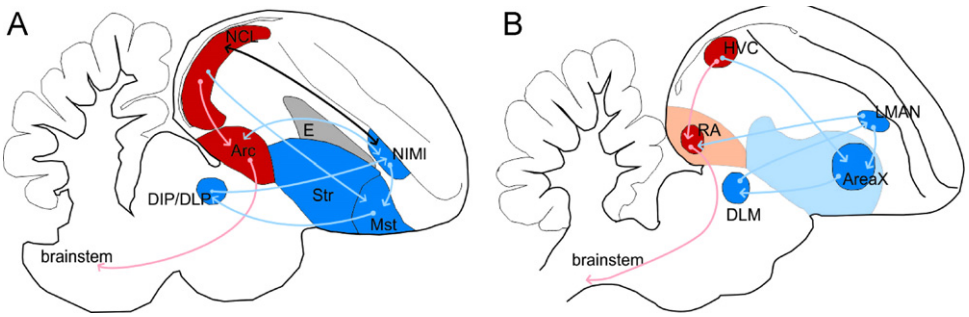

Fig. 1. Comparison of the connectivity of several brain areas in the pigeon to the song system. In the pigeon brain (A) pathways have been identified that highly resemble the structure of the song system (B, zebra finch). The song system comprises two subsystems. On the one hand a motor pathway (red) for production of learned song and the AFP (blue) that is associated with song learning. See text for a detailed description. Arc: arcopallium; DIP: nucleus dorsointermedius posterior; DMP: nucleus dorsomedialis posterior; DLP: nucleus dosolateralis posterior; E: entopallium; Mst: medial striatum; NIMI: nidopallium intermedium medialis pars laterale; RA: robust nucleus of the arcopallium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(LMAN) has a central part in the AFP; it connects the AFP to the posterior pathway [27].

Comparative studies suggest that the song system is a specialization of an ancient neural system and that song system-like motor pathways are present in non-songbirds [28–30]. In the pigeon the nidopallium intermedium medialis pars laterale (NIMl) has been identified as a region that resembles LMAN in respect to localization and connectivity [17]. It was first outlined anatomically by Rehkämper and Zilles [31] (area Ne9). Based on the similarities to LMAN we chose NIMl as a second area that could be involved in a neural system for sequential behavior in the pigeon

Hence, we tested the hypothesis that both NCL and NIMl are involved in the execution of sequential behavior. We applied the serial reaction time task (SRTT) that is commonly used to investigate sequential behavior [32,33].

2. Methods

2.1. Subjects

In this study 28 adult homing pigeons (*Columba livia*) of unknown sex were used. The animals were housed in individual wire mesh cages within a colony-room with a light dark cycle of 12 h. During the experiments the pigeons were maintained at 90–80% of their free-feeding weight and were fed accordingly with mixed grain. Water was supplied ad libitum. All experiments were in accordance with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by a national committee (North Rhine-Westphalia, Germany).

2.2. Apparatus

The experiments were carried out in a custom made operant chamber (38 cm × 38 cm × 42 cm) that was equipped with a touch screen (Elo 1515L, Tyco Electronics). The touch screen was mounted at the rear of the operant chamber; at this side the chamber had an opening so that the entire area of the screen was accessible to the pigeons. A feeder was situated centrally beneath the screen. The feeder was adjusted to provide a total amount of 10–15 g of grain within 50 reinforcements. Moreover, the operant chamber had two rows of white LEDs that served as houselights and a further LED that illuminated the feeder tray when reinforcement was delivered. Controlling the set-up and recording the data was done in Matlab (R2006b, TheMathWorks) applying functions of the Biopsychology Toolbox [34].

2.3. SRT-task

2.3.1. General procedure

A 5-choice-serial reaction time task was designed that required the pigeons to peck five target areas in a fixed sequence (Fig. 2). The target areas on the touch screen, termed keys, were represented by black squares (2 cm × 2 cm) that were arranged in a semicircle (9.5 cm diameter) on a white background. The keys were spaced at steps of 45° with the centers of the keys lying on the virtual line of the circle. This arrangement ensured that the pigeons could reach all keys with little and equal effort. The pigeons were trained to peck the key that was indicated by the appearance of a small white dot of 3 mm diameter at its center. The white dot will be referred to as the cue in the following. The keys were cued in a fixed sequential order (3-5-1-4-2-5-1-4-3-5-1-4-. . .). Key number 4 is a branching point within the sequence; it is followed either by key 2 or key 3. Overall, the probability of transition 4 → 2 is 1/3 and of transition 4 → 3 2/3. Both key 2 and key 3 are followed by key 5. All other keys are always preceded by the same key within the sequence. The sequence was constantly repeated and the pigeons were rewarded on a fixed ratio schedule of 11 (FR11). Thus, a given trial could start at any position within the sequence and all keys were rewarded with the same likelihood. This prevented the development of a pecking bias for a special key.

2.3.2. Training

Initially the pigeons were trained in an autoshaping-procedure to respond to the cue. Therefore, only one key including the cue was presented centrally on the touch screen. Free reward was delivered after 5 s cue presentation or after one peck to the cue. The pigeons were transferred to a FR1 schedule when they began to respond to the cue. From the FR1 condition onwards pecks located at any position outside the key resulted in a period of 3 s lights off. Once the subjects acquired a stable performance, all five keys were presented as described above. In each trial a key was cued randomly. Pecking a non-cued key terminated the trial and resulted in 3 s lights off. In the further training steps the sequence was applied. The number of pecks within a trial was increased to FR2, FR5, FR8 and finally FR11 when a stable performance was reached in the previous condition. Note, that in this respect the FR schedules indicate the number of subsequent cues that had to be pecked to gain a reward and not number of pecks to a single key. The cue stayed visible maximally for 10 s at the first position in a given trial and 2 s at all following positions or until a peck occurred. In case that the pigeon did not peck within this timeframe the trial was terminated with the disappearance of the keys. A correct peck elicited a response-to-cue interval of 250 ms after which the cue appeared at the next sequential position or the trial was terminated with a reward if the required number of correct pecks was reached. Wrong pecks as described above resulted in the termination of the trial and 3 s lights off. Moreover, as an additional feedback signal correct pecks elicited a click sound and wrong pecks a buzzer sound. There was an inter-trial interval of 10 s between each trial. A training session lasted until 50 rewards were earned or after 1 h.

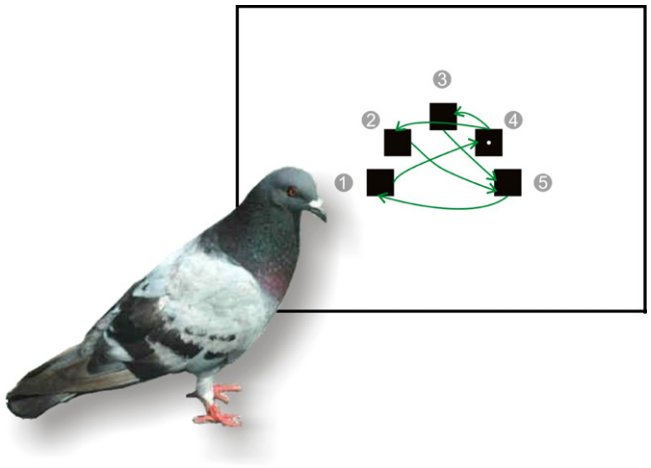

Fig. 2. Schematic overview of the experimental paradigm. Five pecking keys (black squares) were arranged in a semi-circle on a touch screen. A cue (white dot) indicated the target key the pigeon had to peck. The keys were cued in a fixed sequence (3-5-1-4-2-5-1-4; depicted by the arrows).

2.3.3. Test sessions and injections

As during training sessions, one trial of a test session consisted of 11 pecks. However, a wrong peck did neither elicit a period of lights off nor did it terminate the trial. Only the buzzer sound was played as feedback signal and the next sequential position was indicated. A trial was rewarded depending on the last peck in the trial. Moreover, the maximal number of rewards was doubled to 100. These changes in the procedure were introduced in order to be able to collect enough data during one session. In addition, not terminating a trial after a wrong peck allowed the pigeons to peck more freely, hence providing a better possibility to explore the processing of the motor sequence.

Since the SRTT can be performed without any knowledge about the occurrence of a sequence, a sequence violation was implemented in test sessions as a means to control for learning of the sequence. Sequence violation means that on sequential positions 5, 6 and 7 within a trial, keys were cued that were not in accordance with the sequence; for instance 3-5-1-4-5-2-3-4-3-. . . instead of 3-5-1-4-2-5-1-4-3-. . .. Sequence violation occurred randomly in one third of test session trials. If the pigeons had indeed learned the sequence, an increase of the reaction time in responding to the violated positions can be expected [35].

Half an hour prior to the start of a test session either 1 μl Saline or 1 μl tetrodotoxin (TTX, 10 ng/μl, tetrodotoxin citrate, Tocris) were bilaterally injected into the nidopallium intermedium medialis pars lateralis, the entopallium or the nidopallium caudolaterale (see Section 2.4). Note that entopallium injections were performed as a control; the entopallium is situated adjacent to NIMl and spread of TTX from NIMl injections into this visual area could not entirely be excluded.

For the injections a matching cannula (C315I 8.5 mm, Plastics One) was connected to a connector assembly (C313C, Plastics One) that was linked to a syringe (5 μl syringe, Hamilton Company). The assembly was completely filled with distilled water and the syringe was mounted to a microinjection pump (PHD 2000, Harvard Apparatus). A second assembly was prepared in the same way. Then 1 μl of air and subsequently up to 4 μl of Saline or TTX were drawn up with each injection cannula. The positions of the air bubble were marked on the tubing of the assemblies. Then the cannulae were inserted into the implanted cannula guides (see Section 2.4) and the injections were performed at an injection rate of 0.2 μl/min. The position of the air bubbles was used to control that the desired volume of substance was injected [36].

Each pigeon was tested in two test blocks with each block comprising one session with Saline and one session with TTX injection. The order of injections (Saline–TTX or TTX–Saline) was counterbalanced. The second session took place 48 h after the first one in order to avoid accumulating effects of injections.

2.4. Surgery

When the pigeons had acquired a stable performance during training sessions they were chronically implanted with intracranial cannula guides (C315G 8 mm, Plastics One) and dummy cannulae (C315DC 8.5 mm, Plastics One). Guides were implanted into the NIMl ($n = 10$), the NCL ($n = 9$) or the entopallium ($n = 9$). Coordinates according to the stereotactic atlas of Karten and Hodos [37] 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; entopallium: AP: 10.5 mm, ML: 6 mm, DV: 4.0 mm. For surgery the pigeons were deeply anesthetized with isoflurane (Forene®, Abbot) by means of a Komesaroff closed circuit anesthetic machine (MARK 5, Medical Developments International). The pigeons were mounted into a stereotactic device. The plumage on the scalp was cut. A topical analgetic (Xylocain®, Astra Zeneca GmbH) was administered to the scalp. Then the scalp was cut open at the midline to expose the scull. Small craniotomies were made, the *dura mater* was transected and the cannula guide was lowered into the brain. Up to six screws were fixed to the scull. The opening of the scull surrounding the cannula guide was covered with Vaseline and the cannula guide was embedded in dental cement. Dental cement was distributed over the scull covering the screws in order to secure the cannula guides to the scull. After the dental cement was hardened the scalp was sutured. The wound was treated with Xylocain® (Astra Zeneca GmbH) and an antibiotic powder (Tyrosur®, Engelhard Arzneimittel). In addition, an analgetic (Dolorex®, Intervet) was administered.

2.5. Histology

Half an hour before the beginning of the perfusion, 1 μl TTX was injected into the brain. Additionally, 200 units of sodium heparin were administered. The animals were deeply anesthetized with equithesin (0.5 ml/100 g body weight). A transcardial perfusion was performed. First, the animals were perfused with 40 °C warm sodium chloride (0.9%) and subsequently with 1 L of ice-cold 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4, PBS). The brain was removed and transferred to 4% paraformaldehyde with additional 30% sucrose for post-fixation. After 2 h of post-fixation the brain was stored overnight in a solution of 30% sucrose in PBS for cryoprotection. Afterwards, the brain was embedded in gelatin (15% gelatin + 30% sucrose in PBS). The gelatin block was immersed over night in 4% paraformaldehyde with 30% sucrose and in 30% sucrose in PBS, respectively. Frontal sections (40 μm thickness) were prepared using a freezing microtome.

An immuno-ABC-technique was applied in order to stain the TTX within the brain sections. In brief, sections were incubated in a primary antibody (mouse-α-TTX, 1/200 in PBS+; Hawaii Biotech). In a second step, the sections were incubated in a biotinylated secondary antibody (horse-α-mouse, 1/200 in PBS+; Vectastain, Vector, Camon). Finally, the sections were incubated in an avidin–biotin–peroxidase solution (Vectastain ABC-Elite kit, Vector, Camon) and a heavy metal intensified 3′,3-diaminobenzidine reaction (DAB; Sigma) was performed. For a detailed description of the immuno-ABC-technique see Ref. [36].

The sections were mounted and stained lightly with cresyl violet in order to visualize anatomical structures. The sections were examined under a microscope and TTX spread was reconstructed using the brain atlas from Karten and Hodos [37]. Reconstruction was done on a computer using CorelDRAW® X4.

2.6. Data analysis

2.6.1. Errors

In order to assess the effect of the inactivation of the different brain structures seven types of errors were defined and the occurrence thereof was compared between control conditions (Saline) and inactivation sessions (TTX). The first category of errors comprises three types of sequence specific errors, namely "perseveration" (a repeated peck to a given key), "skipping" (omitting of one position of the sequence), and "backward moving" (jumping one item backwards in the sequence). An increase of error rates in this category indicates deficits in sequence processing. For the analysis transitions from key 1 to 2, 2 to 1, 3 to 1 and 4 to 5 were considered as skipping errors; transitions from key 1 to 5, 4 to 1 and 5 to 3 were regarded as backwards errors. The second category of errors reflects the general sequence performance of the animals. It includes "trial omissions" and "trial abortions". Trial omissions and trial abortions possibly result from deficits of sequence initiation and sequence continuation. Trial omissions means that no peck occurred within 10 s after beginning of a trial and trial abortions that no peck occurred within 2 s after the first peck of a trial (see Section 2.3.2). The third category comprises two types of errors which possibly reflect mere visuomotor deficits and were termed "pecks on-the-way" and "undirected" pecks. An error en passant was defined as a peck that is executed when the pigeon pecks a key it passes while moving to the next cued key. Transitions from key 2 to 4, 3 to 4 and 5 to 2 were defined as errors en passant. A peck was evaluated as undirected if it occurred anywhere outside the pecking keys on the screen. Both these errors could depend on a perceptual deficit leading to impaired discrimination of the cue and poor coordination of pecks due to imprecise vision. We expected that especially entopallial injection to increase errors of this category. Errors were expressed as percentage of all pecks of trials during a test session. Wilcoxon signed rank tests were calculated for each type of error to test for differences between Saline and TTX condition. For the analysis the data from both test blocks was averaged. To compare error rates between injection groups Kruskal–Wallis one-way analysis of variance and post hoc multiple comparisons were applied in both conditions (Saline, TTX).

In addition, for comparison of the relative changes of error rates a difference index DI = (Saline − TTX)/(Saline + TTX), with Saline = error rate after Saline injection and TTX = error rate after TTX injection, was calculated for each type of error. The comparison between the groups (NIMl, entopallium, NCL) was conducted by means of Kruskal–Wallis one-way analysis of variance. All statistical analyses were performed with the statistic toolbox of Matlab (R2006b, TheMathWorks).

2.6.2. Reaction times

The reaction time (RT) was defined as the latency between the onset of the cue and the detection of a peck. RTs averaged over all pigeons of each group were calculated for the first pecks of trials, correct pecks and incorrect pecks (regardless of the type of error).

For the comparison between the normal sequence and the sequence violation RTs of correct pecks were averaged for peck 2–11 of a trial over each test session and mean values over all pigeons were calculated. (Note that the first peck was excluded from this analysis because of significantly longer RT and high variability.) Comparisons of RTs were done using Friedman analysis of variance and Wilcoxon singed rank tests.

3. Results

3.1. Histology

After thorough examination of the brain sections, 3 pigeons from the NIMl group and one pigeon from each the entopallium and the NCL group had to be excluded from the analysis due to misplaced cannulae. In the remaining pigeons the TTX spread was confined to the desired regions. The maximal TTX spread in the NIMl group reached from AP 9 mm to 10.5 mm and was located lateral and superior the entopallium in the area that was shown by Kroner and Gunturkun [17] to have a similar connectivity as the oscine LMAN. In most cases small parts of the lateral most entopallium showed traces of TTX as well (Fig. 3A). However, it was impossible to exclude that the TTX-injections did not have affected the

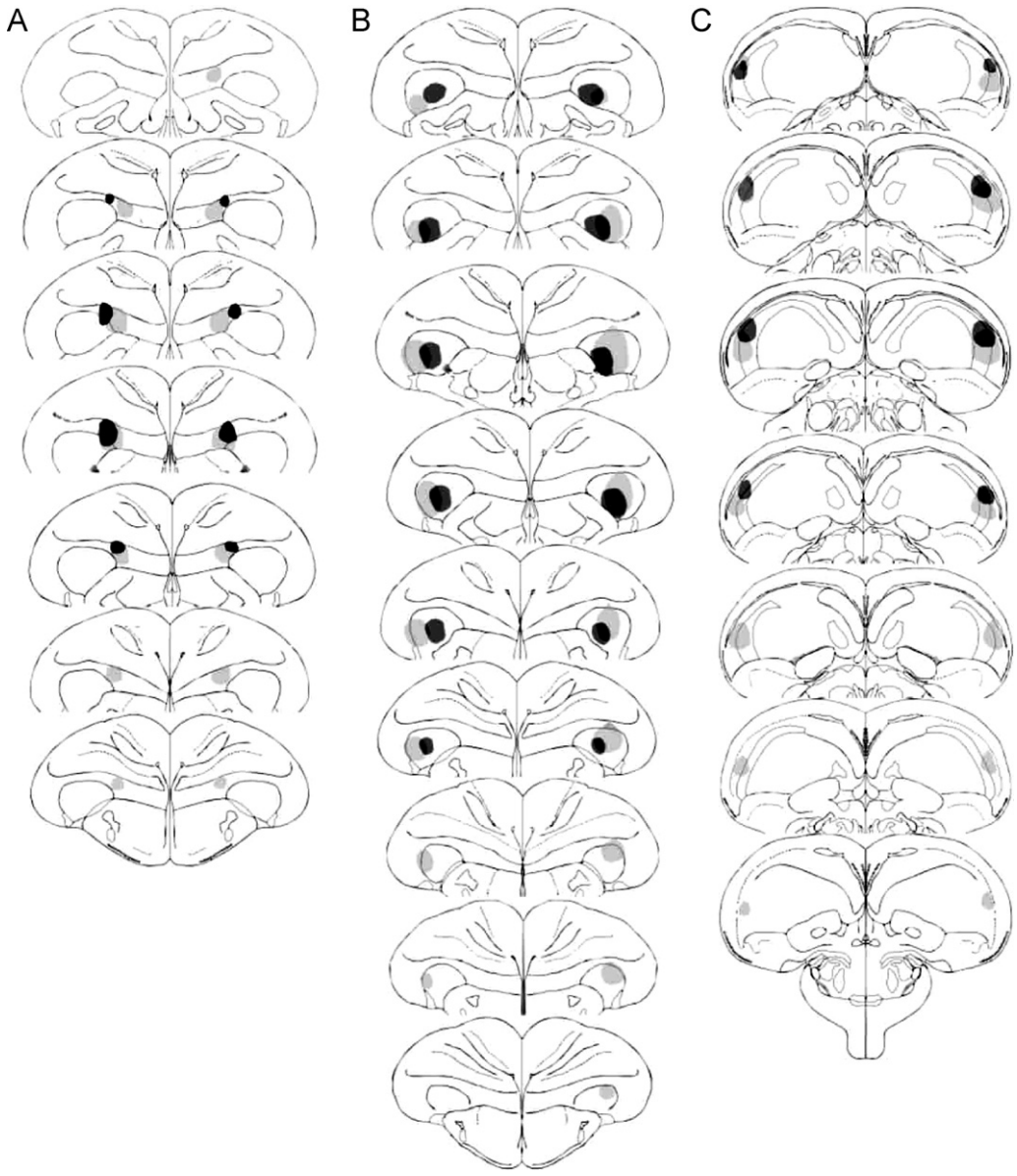

Fig. 3. Schematic frontal sections illustrate TTX spread. The figure is based on the brain atlas by Karten and Hodos [37]. Light gray shaded areas show the maximal spread of TTX and dark areas the minimal spread of TTX found in the different injection groups. (A) NIMl, (B) entopallium, (C) NCL.

medial most portion of the entopallium. Thus, some of the deficits after NIMl-injections could have resulted from blocking entopallial activity. To control for this possibility, we created a third experimental group of pigeons with cannula into the entopallium. The TTX spread in this entopallium group was confined to the entopallium between AP 9.25 mm to 11.25 mm. In no case was there a spread of TTX into NIMl (Fig. 3B). The maximal spread of TTX in the NCL group was located between AP 5.75 mm to 7.25 mm (Fig. 3C). The staining was visible directly beneath the lateral ventricle within the borders of the NCL defined by its dopaminergic innervation [38] and its receptor architecture [39].

3.2. Sequences were successfully learned

In order to test whether the pigeons did acquire knowledge about the occurrence of a sequence in the SRTT, a sequence violation test was introduced (see Section 2). The RT to respond to a sequential position was compared between the normal and the violated sequence separately for each injection group (NIMl, entopallium, NCL) and treatment (Saline, TTX). For each group a significant increase of RT was apparent at the violated positions 5, 6 and 7 both after Saline and TTX injections ($p < 0.05$), indicating that the pigeons had indeed learned the sequence and needed to adapt if a key was indicated unexpectedly. For all other sequential positions there was no significant difference between violated positions and the normal sequence. Fig. 4 shows the results for all three injection groups in the Saline and TTX condition.

3.3. The first peck is the slowest

Pigeons sometimes missed the onset of trial since they had just turned their back to the monitor. Thus, the first peck of a trial had a conspicuously longer RT. Therefore, the RT for the first pecks of a trial was analyzed separately and compared with subsequent pecks (correct and incorrect ones) both after Saline as well as after TTX injections (Fig. 5). Friedman analysis of variance revealed a

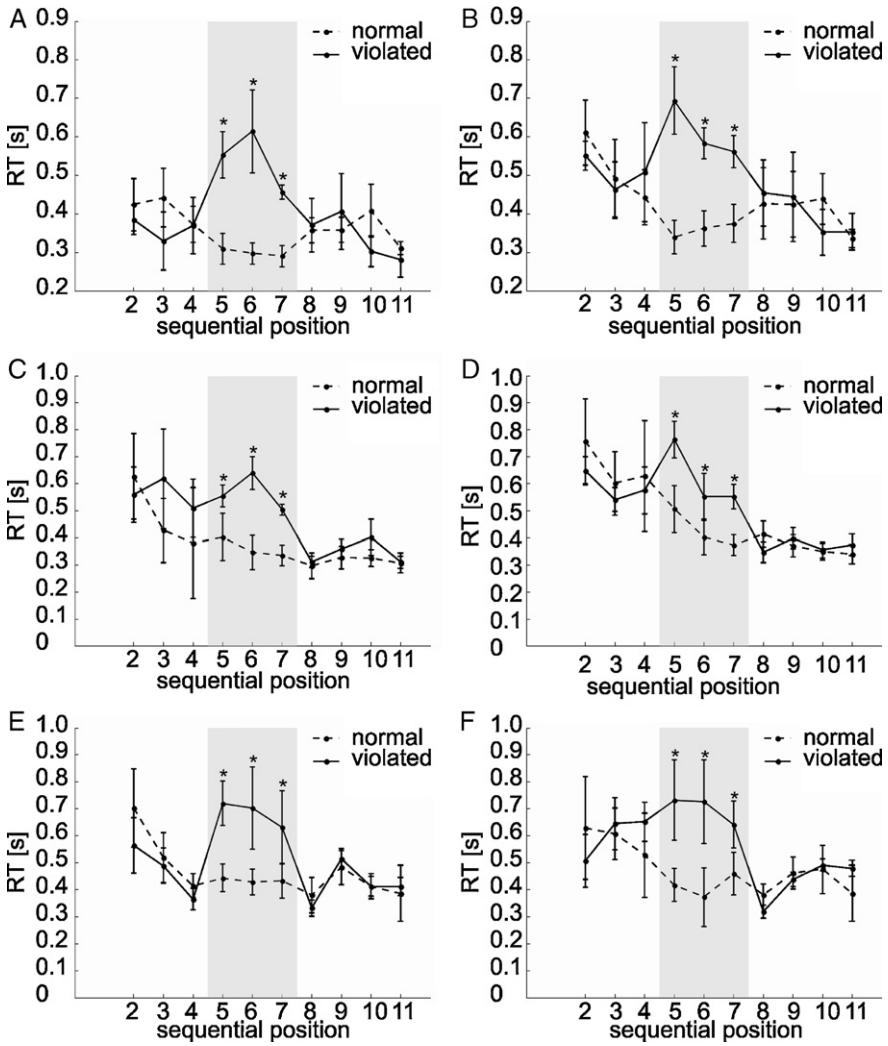

Fig. 4. Increase of RT at violated positions. In a sequence violation test the RT at violated positions (gray shaded area) increased significantly compared to the normal sequence ($p < 0.05$. Wilcoxon signed rank test). The results for the three injection groups are shown for both conditions: (A) NIMl, Saline; (B) NIML, TTX; (C) entopallium, Saline; (D) entopallium, TTX; (E) NCL, Saline; (F) NCL, TTX. Depicted are means ± SEM for sequential positions 2–11. Asterisks indicate significant differences.

significant difference of RT between first pecks, correct pecks and incorrect pecks in all conditions ($p < 0.05$). Post hoc comparisons revealed that this was due to a significant RT-difference of first pecks compared to RT of correct and incorrect pecks ($p < 0.05$) in all conditions but the TTX injection into the NIMl. Here, the statistical difference between faster correct and slower first pecks only revealed a trend ($p = 0.083$). We therefore did not include RT-values of the first peck into subsequent analyses.

3.4. RT-effects after inactivation of different brain regions

The RTs for correct pecks and incorrect pecks were analyzed for each injection group. There was a significant RT-increase of 82 ms after the inactivation of the NIMl compared to the Saline condition (Saline: 308 ± 24 ms, TTX: 390 ± 22 ms; $p = 0.016$). No other significant differences between Saline and TTX injections were observed.

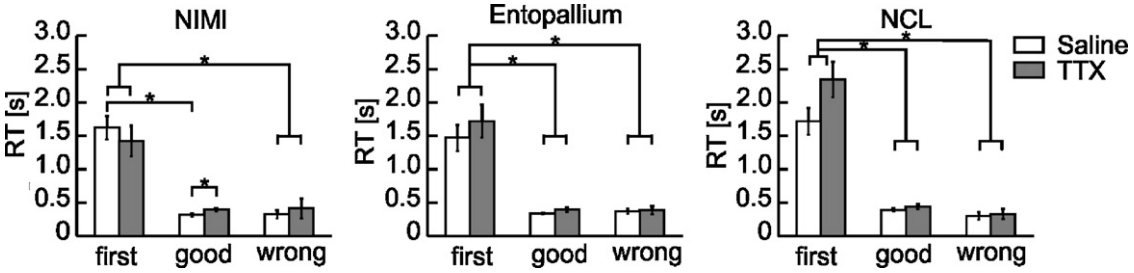

Fig. 5. Reaction times during the different conditions. The reaction times (RT) are compared within the injection groups between Saline and TTX condition (means ± SEM). RT were evaluated separately for the first pecks of a sequence (first), the correct pecks (good) and erroneous pecks (wrong). In the NIMl group a significant increase of RT of correct pecks under TTX can be seen. The first pecks have much longer RT than the following pecks.

3.5. Inactivating different brain regions yields specific error types

Two pigeons had to be excluded from the error analysis from each the entopallium and the NCL group since these pigeons had omission rates above 90% of trials after TTX injections – i.e. pigeons did not start to peck at the beginning of a new trial. Thus, these animals did not produce enough data for a meaningful analysis. Therefore, the NIMl group comprised 7, the entopallium group 6 and the NCL group 6 pigeons, respectively. The error rates are expressed as percentage of pecks. Data points from individual pigeons are averages from two test sessions in the Saline and TTX condition, respectively. The order of Saline and TTX injections was balanced between groups. For statistical analysis Wilcoxon signed rank tests were applied. Kruskal–Wallis one-way analysis of variance was used for between group comparisons.

An overview of the inactivation effects is depicted in Fig. 6 as transition probabilities between the pecking keys. It is evident that blocking of all three brain areas increased pecking variability, since percentage of correct transitions decreased while more alternative transitions occurred. This effect was apparently more prominent in the NIMl and NCL groups than in the entopallium group. The diagrams indicate that in particular skipping errors (red arrows) increased after inactivation of both NIMl and NCL, in contrast to the entopallium where such was not observed. The inactivation effects on the different error categories are described in detail below.

3.5.1. Sequence specific errors

Moving backwards a step in the sequence, skipping one position as well as perseveration on one key were considered as sequence specific errors since they signify a violation of the succession of sequential items. That means there occurs no progression to the following item, but either a previous item is pecked (backwards moving), one item is left out (skipping) or the pigeon sticks to the already finished position (perseveration). Sequence specific errors had a low incidence (Fig. 7A); nevertheless, both for NIMl and NCL a significant increase of skipping errors (both $p < 0.05$) as well as perseverations (both $p < 0.05$) in comparison to Saline injections were observed. Blocking the entopallium with TTX did not cause any statistically significant changes of sequence specific errors.

A comparison of the error rates between injection groups revealed a significant difference of the amount of skipping errors in the TTX condition ($p < 0.05$). Post hoc pairwise comparisons show that the rate of skipping errors was higher after NIMl than entopallium inactivation. For perseveration between group comparison showed a trend in the TTX condition ($p = 0.06$) as well as for backwards error in the Saline condition ($p = 0.06$).

3.5.2. General performance

There were two error types that were classified as measures of general performance. These were trial abortions and trial omissions. Trial abortions reflect the inability of the pigeons to continue their behavior in the SRTT, although the trial had been started properly. Both blocking the NIMl ($p = 0.047$) as well as the NCL ($p = 0.031$) resulted in an increase of trial abortions (Fig. 7B). The inactivation of all three target brain areas resulted on average in a substantial increase of trial omissions, i.e. the failure to start a trial. This was significant for NIMl and entopallium ($p < 0.05$) and revealed a strong trend for NCL ($p = 0.055$; all pigeons were included in this analysis). There were no significant differences between the groups.

3.5.3. Visuomotor deficits

Two kinds of errors were defined that are supposed to reflect visuomotor deficits, i.e. errors en passant and undirected pecks somewhere on the screen. Both errors cannot be explained within the logic of the sequence. Rather, both reflect possible visual and/or motor deficits. After the inactivation of NIMl, an increase of the proportion of undirected pecks was observed ($p = 0.043$; Fig. 7C). In Fig. 8A typical example of the distribution of peck locations is depicted. There are apparently more pecks within the area between the keys and also farther away from the region of the keys on the touch screen in the TTX condition (red marks) than the Saline condition (blue marks). As a comparison, Fig. 8B shows an example of the NCL group were such a pattern of dispersed peck locations did not occur. In contrast, TTX injections into the entopallium only lead to an increased rate of errors en passant ($p = 0.031$; Fig. 6C). No visuomotor deficits were observed after NCL inactivation (Fig. 7C).

There was a significant difference between injection groups for the en passant error. Post hoc tests revealed a significant difference between NIMl and entopallium in the Saline condition ($p = 0.04$). Differences of the rates of undirected pecks were almost significant in the TTX condition ($p = 0.053$).

3.6. Between group comparison of relative changes of error rates

In order to compare the amount of change of the error rates between the injection groups, a difference index (DI) was calculated (see Section 2, Fig. 9). The strongest relative increase of errors en passant was observed after inactivation of the entopallium ($DI = -0.5 \pm 0.08$). In contrast, fewer en passant errors happened if the NIMl was blocked ($DI = 0.25 \pm 0.13$). The DI value for the NCL group was close to zero ($DI = -0.03 \pm 0.29$). Post hoc comparisons showed that there was a significant difference between the NIMl and the entopallium group ($p < 0.05$). No other comparisons of the DIs yielded significant group differences.

4. Discussion

We have investigated the role of three brain regions of the pigeon – the NCL, the NIMl as well as the entopallium as a control structure – for the production of sequential behavior in a visually cued sequence task. Reversible inactivation both of NIMl and NCL resulted in the increase of sequence specific errors. This was not observed after inactivation of the entopallium, a visual processing area (cf. [40]) that is situated adjacent to NIMl. Hence, our results indicate a function of both areas in the processing of motor sequences.

4.1. Comparative studies as a point of departure

To our knowledge, this is the first study that aims to identify neural systems for visuomotor sequential behavior in a bird. In mammals, cortico-striato-thalamo-cortical loops are crucial for sequential action patterns [1,41,42]. In songbirds, the anterior forebrain pathway (AFP), has a similar connectivity (although some differences at the level of the striatum are present; [43]) and plays a role in song learning [44]. Since song is a learned sequence of vocalizatory elements (cf. [45,46]), we used the song system as one point of departure for our study in pigeons. Most importantly, an AFP-like system exists in pigeons and chicks [17,47], although physiological differences between chick and songbird spiny striatal neurons were noted [48]. The oscine AFP is formed by striatal area X, the dorsolateral anterior thalamic nucleus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (cf. [30]). The AFP receives input from HVC [24] and its output is conveyed by projections from LMAN to RA [27] (Fig. 1B).

In the pigeon, the NIMl is integrated in a circuit that resembles the AFP and projects to the medial striatum (MSt) [17] (Fig. 1A). Connections of the MSt to the nucleus dorsomedialis anterior and posterior (DMA, DMP), nucleus dorsointermedius posterior (DIP) and nucleus dorsolateralis posterior (DLP) have been described [17,49,50]. The NIMl receives thalamic input from DIP, DLP, and DMP [17,51].

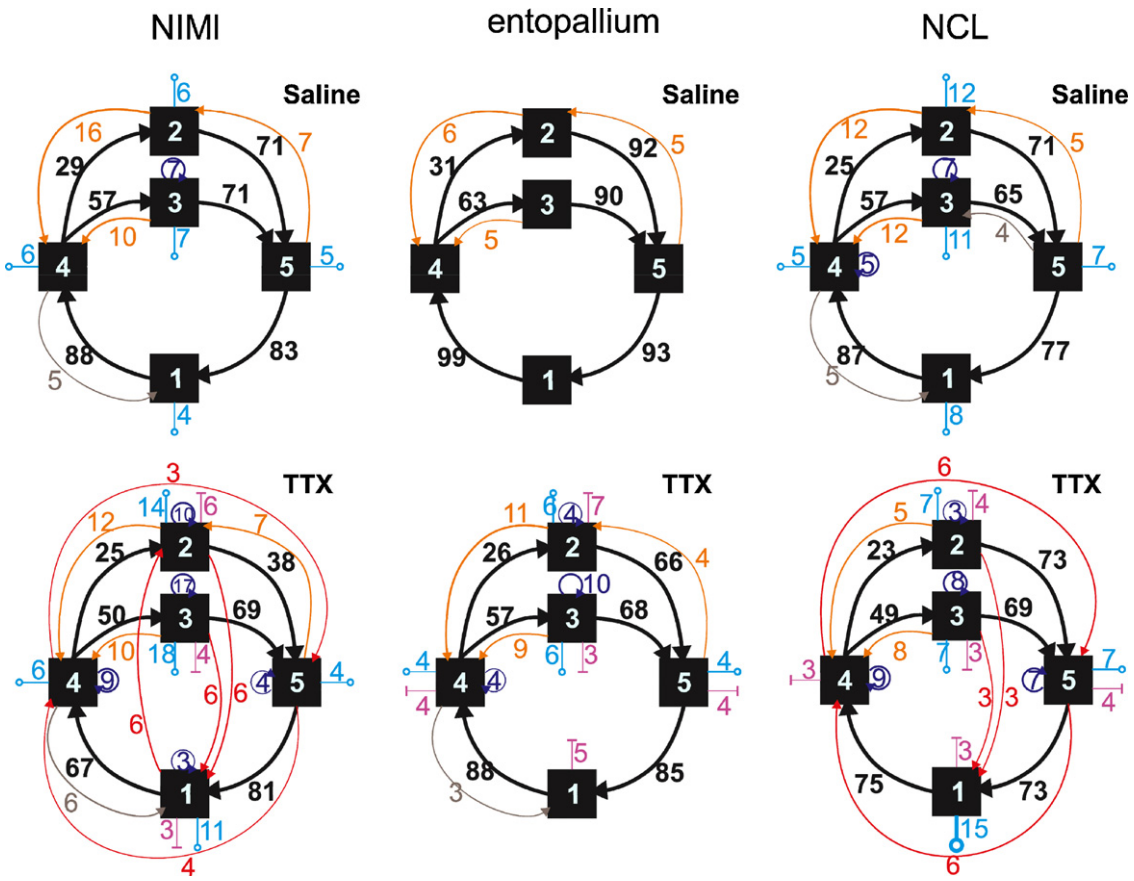

Fig. 6. Changes in transition probabilities following TTX injection. The averaged transition probabilities between the pecking keys are shown for Saline and TTX injections for the NIMl, the entopallium and NCL group, respectively. Black squares represent the pecking keys. Arrows show the direction of transitions (black: correct; red: skip error; orange: en passant error; brown: backwards error; blue circular: perseveration). Cyan colored marks represent undirected pecks and magenta trial abortions. Numbers adjacent to the arrows indicate the probability of this transition expressed as percent of all transitions originating from the respective key. Transitions with a probability of less than 3% are not depicted for clarity. TTX injection in each brain structure increased the variability of transitions. NIMl as well as NCL inactivation especially yielded the addition of skipping errors. In the entopallium group there was an increase of en passant type errors apparent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

On the other hand, we chose the second critical structure of our study – the NCL – based on parallels to the mammalian PFC. The PFC receives dense innervations by dopaminergic afferents from the ventral tegmental area and the substantia nigra [52]. The same holds true for NCL [15,53]. Moreover, there are striking similarities on the functional level between PFC and NCL. For instance, the PFC is associated with working memory functions [54–56] and behavioral scheduling [57,58]. Similarly, NCL is also active during working memory tasks [20,59]. Moreover, the PFC mediates sequential behavior [7,9–14]. Therefore, we hypothesized that NCL could have a function for sequence execution, as well.

Additionally, one can draw parallels to the song system to a certain extent, too. In chicks and pigeons, the NCL projects to the arcopallium and the medial striatum [17,60]. This connectivity is reminiscent of the HVC → RA and HVC → area X projections in the posterior pathway of the song system (Fig. 1). Since the posterior pathway is required for song production [24,61,62], the comparison of NCL to HVC provides further support for a possible role of NCL in a neural system for sequential behavior. Based on those arguments, we planned to test the role of NCL and NIMl for sequential visuomotor behavior.

4.2. Pigeons learn the sequence within the SRTT

In order to perform the SRTT the animals do not need to know the underlying sequence, since they are always cued by the visual stimulus. Nevertheless, it is well documented in human as well as animal experiments that knowledge about the occurrence of a sequence is acquired during training with a SRTT (cf. [32,33,35]). Here we have applied a sequence violation test as it was introduced by Domenger and Schwarting [35]. Similar to the performance of the rats in the Domenger and Schwarting [35] study, our pigeons correctly pecked at the cued positions in the violated sequence, however, with a clear RT increase. This indicates that the animals indeed had anticipated the next sequential position based on past sequence learning.

4.3. Are effects of NIMl inactivation confounded by TTX spread into the entopallium?

Since the NIMl is situated adjacent to the entopallium, which is the primary projection area of the tectofugal visual pathway [63], and a slight spread of TTX into this region could not entirely be prevented, we introduced TTX injections into the entopallium as a control group. If the deficits after NIMl injections were due to visual deficits mediated by the entopallium, we would expect that entopallium and NIMl-injections produce overlapping results.

The error patterns after entopallium inactivation were visuomotor and thus differed markedly from those caused by TTX injections into NIMl (Fig. 10). While NIMl inactivation resulted in a significant increase of sequence specific errors, this did notably not occur after blocking the entopallium. In contrast, errors en passant were

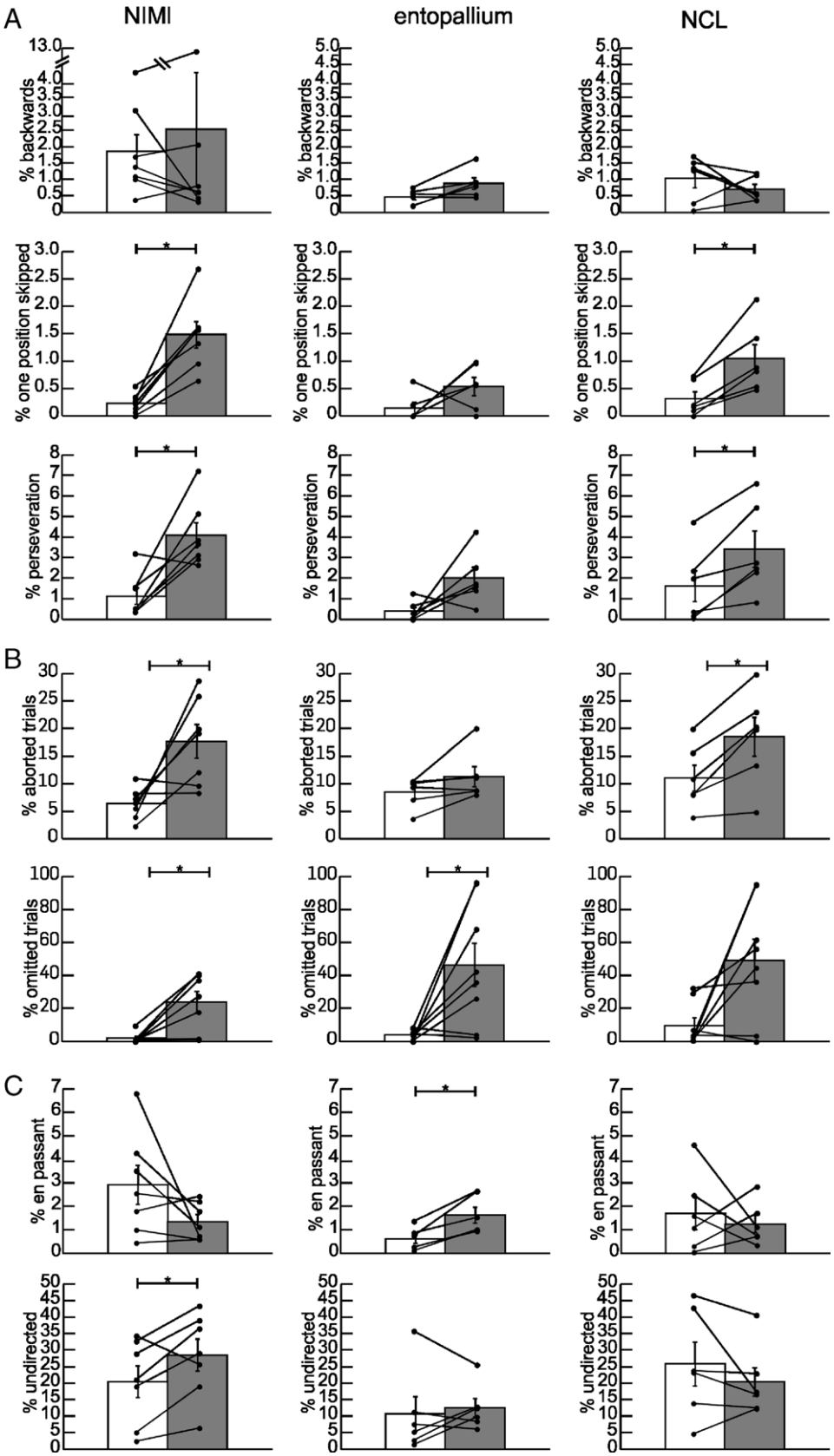

Fig. 7. Effect of TTX injections on the occurrence of different types of errors during the performance of the SRTT. (A) Errors reflecting sequence specific deficits. Skipping and perseverations increased significantly after inactivation of NIMl and the NCL. (B) General performance. A significant increase of the percentage of aborted trials was observed after the inactivation of NIMl as well as NCL. The amount of omitted trials increased significantly after injection of TTX into NIMl and the entopallium. (C) Visuomotor deficits are reflected by errors en passant and undirected pecks. The increase of undirected pecks after inactivation of NIMl as well as the occurrence of errors en passant after injection of TTX into the entopallium was statistically significant. Depicted are means ± SEM. White bars and gray bars show results after Saline and TTX injections, respectively. Lines depict results from individual pigeons. Asterisks indicate significant differences.

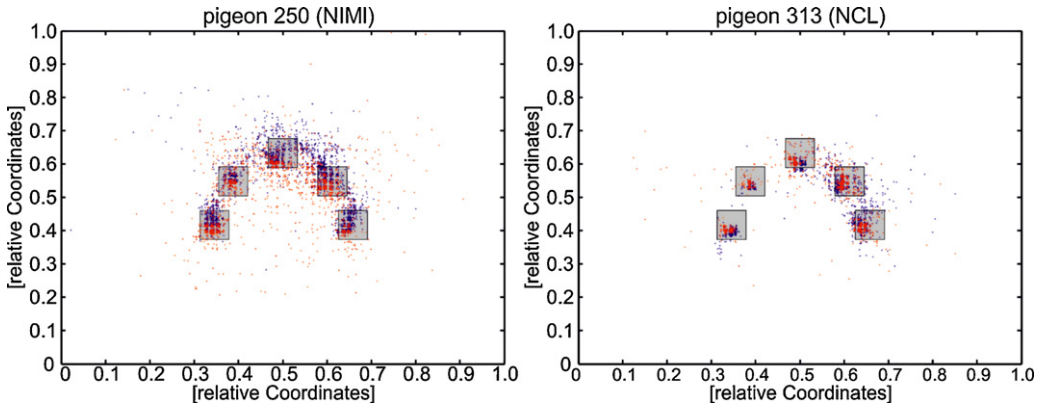

Fig. 8. Peck locations on the touch screen in Saline and TTX condition. The peck locations of two pigeons are exemplarily shown; one pigeon from the NIMI group (left panel) the other from the NCL group (right panel). The pecks are mainly clustered within the area of the keys (gray shaded areas) both in the Saline (blue marks) and TTX (red marks) condition. However, the pigeons also pecked at other locations of the touch screen (undirected pecks). The number of undirected pecks increased after NIMI but not NCL or entopallium (not shown) inactivation. The diagrams represent the full extent of the touch screen in relative coordinates. One equals 30.4 cm and 22.8 cm on the *x*-axis and the *y*-axis, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

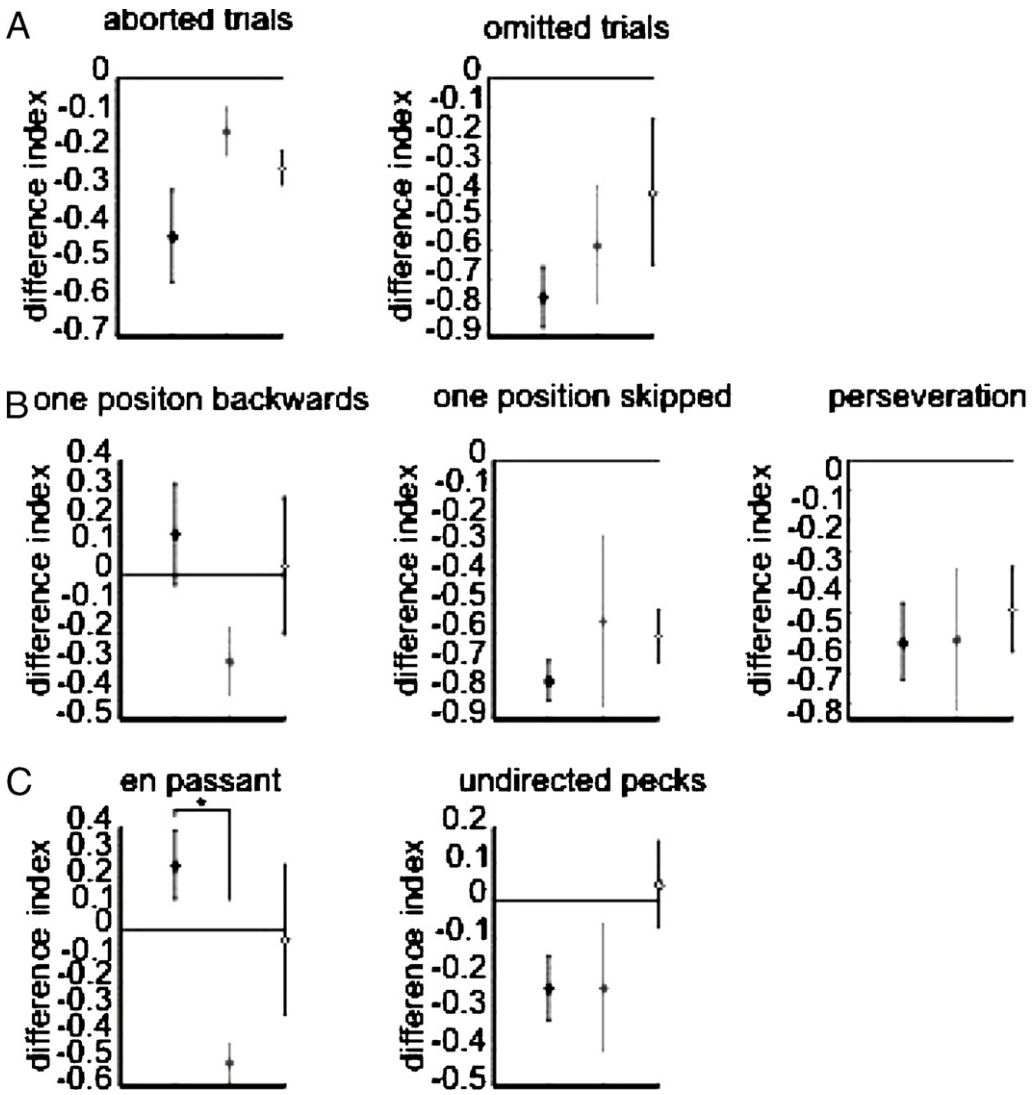

Fig. 9. Between group comparison of relative changes of error rates. To compare the relative changes a difference index was calculated (see Section 2). Positive values indicate a decrease and negative values an increase after TTX injection. (A) General performance. The relative changes of trial abortions and omissions were not significantly different between the groups. (B) There were no significant differences between the injection groups concerning sequence specific errors. (C) Difference Indices for visuomotor deficits. The highest increase of errors en passant occurred after inactivation of the entopallium. There was a decrease of this error after TTX injection into NIMI; while in the NCL there was no significant change of the error rate. The difference between the NIMI and the entopallium group is significant. Shown are means ± SEM. Black: NIMI, grey: entopallium, white: NCL.

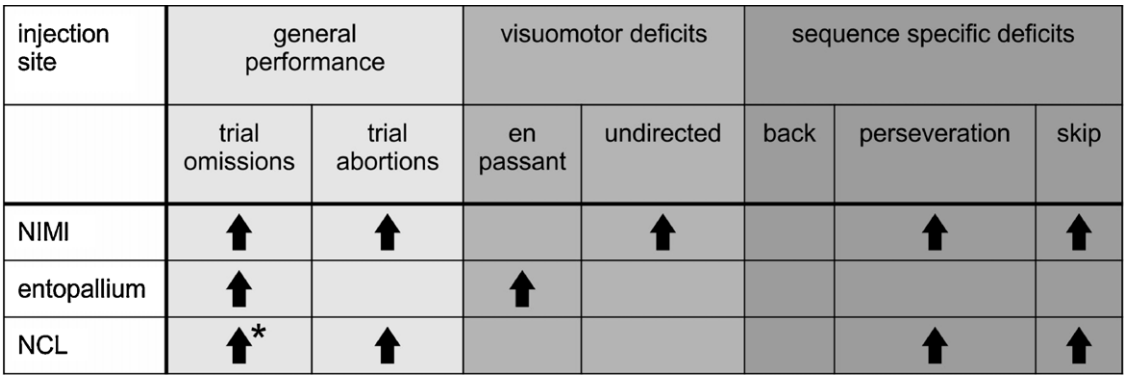

Fig. 10. Summary of significant effects within each group on error rates after TTX inactivation. Black arrows indicate a significant ($p < 0.05$) increase (upwards) or decrease (downwards) of the error after TTX injection compared to the Saline condition. *The increase of omissions after NCL inactivation shows a strong trend that virtually reaches significance ($p = 0.055$). Note that this figure does not imply significant between group differences. See text for details.

significantly increased in the entopallium but not in the NIMl group. Only trial omissions were significantly increased in both entopallium and NIMl groups. As outlined before, general sequence errors like trial omission can result from a deficit in a sequence controlling system, but could also result from purely perceptual problems. In case of the entopallium a visual deficit could likely produce a failure to see the visual stimuli at trial onset. In case of the NIMl, it is also conceivable that sequential memories could not be sufficiently activated, resulting in trial omissions.

However, though the pattern of error increases is different, between group comparisons of error rates and relative changes of error rates were mostly non-significant. Therefore, a reliable separation of the effects of NIMl, entopallium and NCL injections is not possible. Yet, the incidence of skipping was significantly higher after TTX injection into NIMl than into the entopallium. Additionally, there was a significant difference in the relative change of en passant errors between NIMl and entopallium. There was an increase of en passant errors in the entopallium group while NIMl inactivation yielded a decrease of this error. But there was also a significantly higher occurrence of en passant errors in the NIMl group in the control condition (Saline). Hence, differences in relative changes of the error rate could be attributed to this fact.

In conclusion, the data patterns imply that NIMl and entopallium inactivation result in different deficits. Moreover, the amount of TTX spread into the entopallium from NIMl injections was minimal (cf. Fig. 3) making it very unlikely that the effects in the NIMl group were substantially confounded by this. But the lack of robust inter group differences does not permit a differentiation of the inactivation effects of the three injection groups. Thus, a confounding effect, though unlikely, cannot be ruled out.

4.4. Both NCL and NIMl contribute to sequential behavior

The inactivation of NIMl and NCL yielded very similar patterns of error increases. Remarkably, both NIMl- and NCL-injections resulted in a significant increase of sequence specific errors, namely the skipping of one position and perseverations (Fig. 9). This could suggest an impairment of motor sequence processing that results in a failure to activate the next sequential unit and thus results in the perseveration on one position or in triggering an element further downstream in the sequence. Strikingly, this happens albeit the animals' behavior is mainly cue guided, as we have discussed above. This makes it likely that the behavior is partly automated and the underlying representation within the motor system can overrule the instructive perceptual signal. Possibly, the simultaneous presence of the cue is the reason why sequential errors occur overall in small numbers although they are on average tripled in NCL and NIMl pigeons.

Moreover, there was a significant RT increase to perform correct pecks after NIMl inactivation. This speed reduction was specific for correct pecks and was not apparent for the first peck after trial onset or for incorrect pecks. Thus, NIMl-animals were not slowed down in an overall fashion but had a deficit in anticipating the next position of the sequence. Taken together, the results support our general hypothesis that both the NCL and NIMl play a role in mediating sequential behavior.

4.5. Contribution of the NIMl and the NCL to general sequential components of behavior

General sequence deficits as discussed here were defined as failures to activate a sequential behavior or append the following sequence elements of an already started sequence. These kinds of deficits could reflect an overall problem to perform a sequence task but could also be produced by sensory deficits. We observed an increase of trial abortions and trial omissions in NIMl and NCL animals. Since NIMl and NCL animals evinced no sensory deficits, it is conceivable that these errors result from deficits of sequence processing. Trial abortions could reflect the failure of the motor system to trigger the upcoming sequential unit preventing a continuation of the behavior. Trial omissions might be caused by a failure to initiate the representation of the sequence and hence the pigeons fail to respond. Also attentional impairments are a likely explanation for both errors. This possibility needs to be taken into account especially for the NCL which is thought to be a functional equivalent to the mammalian prefrontal cortex [22]. The PFC is known to control attention and lesions of rat PFC or application of dopaminergic antagonists affect performance in a non-sequential version of the SRTT which is discussed to reflect impaired attention [64,65].

The significant increase of undirected pecks after TTX injections into NIMl was not expected and could imply that this pallio-striato-thalamo-pallial loop is to some extent also involved in the spatial tuning of motor acts.

4.6. Is the song system of oscines an adequate framework to study neural pathways of sequence behavior in the pigeon?

We chose to study the neural control of sequence behavior in the pigeon's NCL and NIMl in part because we drew an analogy to the song system of oscines. Although, the requirements of the applied visuomotor task are very different from song, basic principles of sequence control could be common to both behaviors.

Do our results validate these analogies? Indeed, our approach to select the target areas for this study based on parallels to the song system proved to be successful. At the level of a hypothesis assuming the general involvement of NCL and NIMl in sequence control, we could indeed show that both structures make similar and mostly specific contributions to sequence behavior. Blocking each of these structures produces deviations of the order of sequential choices. Also deficits to initiate a sequence or to go on with an already started sequence were observed. Thus, at this overall level, our hypothesis could be confirmed.

In conclusion, the present study is the first to investigate the neural basis of sequence production in the pigeon. The results reveal that both NIMl and NCL are associated with the control of a motor sequence in a SRTT. Future experiments investigating the function of NIMl and NCL in learning and production of motor sequences will further elucidate the possibly differential role of both structures for sequential behavior.

Acknowledgments

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