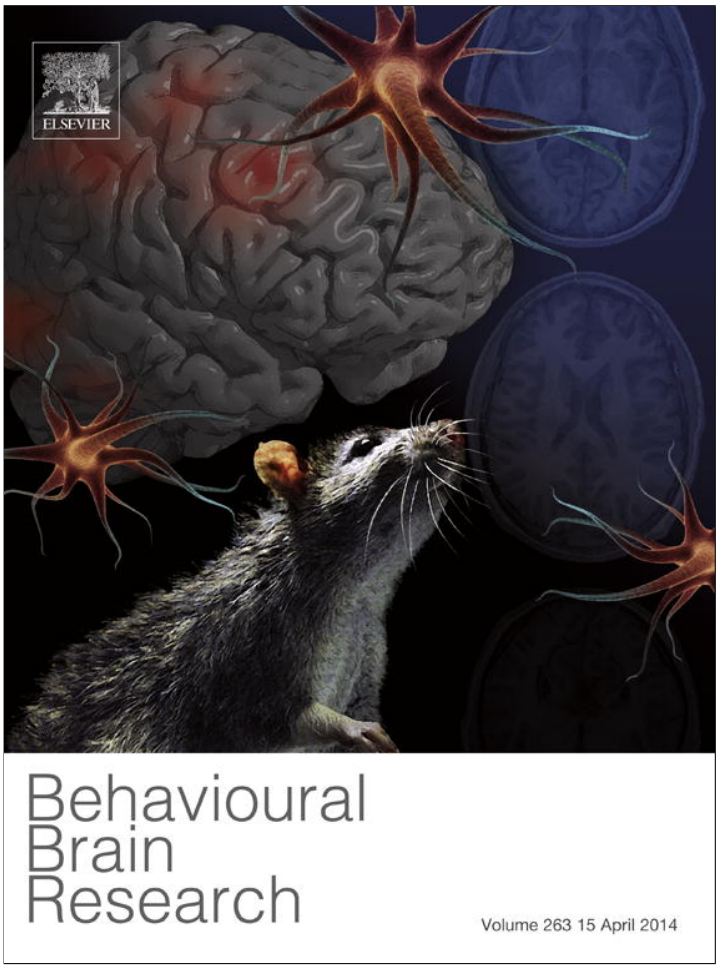

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Short communication

The putative pigeon homologue to song bird LMAN does not modulate behavioral variability

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HIGHLIGHTS

- The oscine song system may have evolved from a motor system for sequence generation.
- The pigeon as a non-song bird has brain circuits resembling the song system.
- A common origin suggests that homologous components exert similar functions.
- The pigeons NIML is the putative homologue to oscine LMAN.
- We found that NIML does not generate behavioral variability in contrast to LMAN.

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ABSTRACT

The active generation of behavioral variability is thought to be a pivotal element in reinforcement based learning. One example for this principle is song learning in oscine birds. Oscines possess a highly specialized set of brain areas that compose the song system. It is yet unclear how the song system evolved. One important hypothesis assumes a motor origin of the song system, i.e. the song system may have developed from motor pathways that were present in an early ancestor of extant birds. Indeed, in pigeons neural pathways are present that parallel the song system. We examined whether one component of these pathways, a forebrain area termed nidopallium intermedium medialis pars laterale (NIML), is functionally comparable to its putative homologue, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) of the song system. LMAN conveys variability into the motor output during singing; a function crucial for song learning and maintenance. We tested if NIML is likewise associated with the generation of variability. We used a behavioral paradigm in which pigeons had to find hidden target areas on a touch screen to gain food rewards. Alterations in pecking variability would result in changes of performance levels in this search paradigm. We found that pharmacological inactivation of NIML did not reduce pecking variability contrasting increases of song stereotypy observed after LMAN inactivation.

Comparative neuroscience is a pivotal tool to gain insights into how our brains work. Bullock [1] states that "[..] we cannot expect truly to comprehend either ourselves or how the nervous system works until we gain insight into [the diversity] of nervous systems". Birds and mammals, e.g., have developed very similar brain regions that underlie higher cognitive abilities [2]. A comparison of these structures–the prefrontal cortex in mammals, and the caudolateral nidopallium in birds–reveals which structural and anatomical features seem to be a requirement for higher cognitive functions [3]. Here we apply a comparative neuroscientific approach to gain inside into the evolution of vocal learning. Pigeons are no song birds but they possess brain circuits that strikingly resemble the song system of oscine birds like zebra finches [4]. The dominant hypothesis about the origin of the song system states that it developed from a pre-existing motor system [5,6]. Indeed, the song system is embedded in areas that are active during general body movements in song birds as well as non-vocal learners [7]. If these motor areas in the pigeon are indeed homologous to the song system, they probably exert similar functions for general motor behavior as well. In this study we focused on a forebrain area in the pigeon, termed nidopallium intermedium medialis pars laterale (NIML) and compared its function to the putatively homologous lateral magnocellular nucleus of the anterior nidopallium (LMAN) of the song system.

LMAN plays a pivotal role during song learning [8,9] and is associated with variability generation in the motor output [10,11].

Abbreviations: AFP, anterior forebrain pathway; CV, coefficient of variance; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NIML, nidopallium intermedium medialis pars laterale; TTX, tetrodotoxin.

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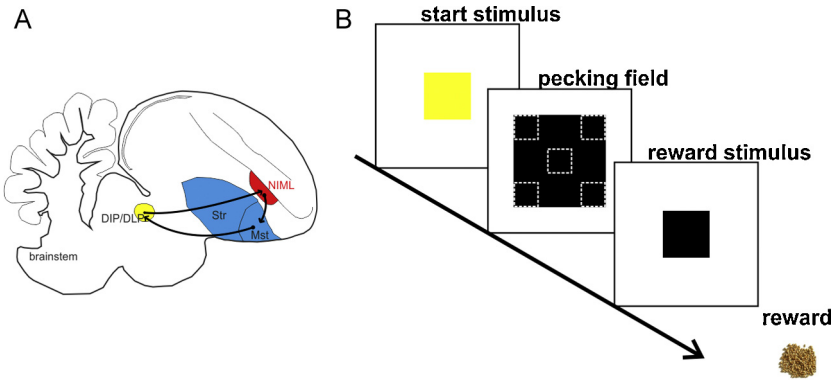

Fig. 1. Connectivity of NIML and schematic of the search paradigm. A: NIML is integrated into a basal-ganglia pathway that resembles the oscine anterior forebrain pathway (AFP). B: Each trial started with the presentation of the start stimulus. A peck to this stimulus initiated the trial. The pecking field was presented in which the pigeons had to search for the invisible target area. The target area was randomly placed at one of five possible locations (marked by dotted frames). Upon one peck to the target area, the reward stimulus was presented. A food reward was gained by pecking the reward stimulus. DIP: nucleus dorsointermedius posterior thalami; DLP: nucleus dorsolateralis posterior thalami; Mst: medial striatum; NIML: nidopallium intermedium medialis pars laterale; Str: striatum.

Active generation of variability is believed to be critical for successful song learning [12,13] and song maintenance [14,15]. Variability is necessary for exploring motor space during trial-and-error based sensorimotor learning to find the optimal motor state [12] and is modulated to reduce errors [16].

LMAN is part of the anterior forebrain pathway (AFP), a basal-ganglia circuit with a high degree of homology with the mammalian direct basal-ganglia pathway [5,17,18]. In pigeons, NIML is anatomically similar to LMAN [19] and is integrated into a basal-ganglia pathway resembling the AFP to a high degree [19–23] (Fig. 1A). Previous studies showed that NIML is associated with the execution of learned sequences [10,24]. Hence, NIML functionally differs from LMAN, which is not associated with production of learned song [11,12]. Yet, this finding does not rule out that NIML may play a role in variability generation, thus contributing to sensorimotor learning analogous to LMAN. Therefore, we devised a novel paradigm (Fig. 1B, Supplementary video) that allowed us to assess changes of pecking variability after pharmacological inactivation of NIML in pigeons. In short, pigeons had to search for hidden target areas on a touch screen. Since the locations were selected randomly in each trial, the pigeons had to vary peck locations to maximize their reward.

See Supp Figure S1 as supplementary file. Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.01.019.

In our study we used 7 adult homing pigeons (*Columba livia*) of unknown sex. 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 80–90% 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).

The experiments were carried out in a custom made operant chamber ($38 \times 38 \times 42\ cm^3$) 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. Controlling the set-up and recording the data was done in Matlab (R2006b, The MathWorks) applying functions of the Biopsychology Toolbox [25].

Initially, the pigeons were trained in an autoshaping procedure to peck a yellow stimulus (3 cm × 3 cm) centrally presented on the touch screen. As soon as the animals started to respond to the stimulus they were transferred to an FR1 schedule.

Upon responding in more than 80% of trials the "pecking field" was introduced. The pecking field was represented by a 5 cm × 5 cm black square in the center of the screen. In the first training step, the pecking field was subdivided into quarters of 2.5 cm × 2.5 cm size that were chosen randomly as the rewarded target areas. A trial began with the presentation of a "start stimulus" (yellow stimulus, 5 cm × 5 cm). One peck on the start stimulus initiated the trial. The onset of the presentation of the pecking field was marked by briefly flickering the field and a buzzer sound. The target area in the respective trial was marked yellow. There was no limitation of pecks within the pecking field. To gain a reward one peck to the target area was required. Trials in which the target area was pecked were counted as correct. Upon pecking the target area, the pecking field was extinguished and the "reward stimulus" (a black stimulus, 3 cm × 3 cm) was presented. Pecking the reward stimulus activated the feeder and a small amount of mixed grain was delivered as reward.

In the following training step, the size of the target areas was reduced to 1 cm × 1 cm. The position of the target field was randomly selected from a set of five possible positions: the four corners and the center of the field. Subsequently, the visibility of the target areas was reduced by stepwise increasing the transparency (60%, 80%, 90%, and 95%). Finally, the target areas were not marked anymore, so that the pigeons had to search for them. During the training the pigeons were transferred to the next training steps when the performance was above 85% successful trials in two subsequent training sessions. The criterion for the final step was lowered to 50% because of the difficulty of the task.

As soon as the pigeons reached the criterion in the final training step, the animals were implanted with cannulas (C315G 8 mm, Plastic One) at the following coordinates: AP: 9.5 mm; ML: 3.5 mm; DV: 3.7 mm according to the atlas of [26]. During the surgery, the pigeons were deeply anesthetized with isoflurane (Forene®, Abbot). During the perioperative period the animals were treated with Butorphanol (Dolorex®, Intervet) for analgesia. For long-term analgetic treatment the pigeons received carprofen (Rimadyl®, Pfizer) for 3 days after the surgery.

After the recovery period of at least one week, the animals were retrained until they reached pre-surgery performance before test sessions were started. The pigeons received bilaterally either 1 μL tetrodotoxin (TTX, 10 ng/μL, tetrodotoxin citrate, Tocris) for transient inactivation of NIML or vehicle (Saline) 30 minutes before a test session. The details of the injection procedure were described

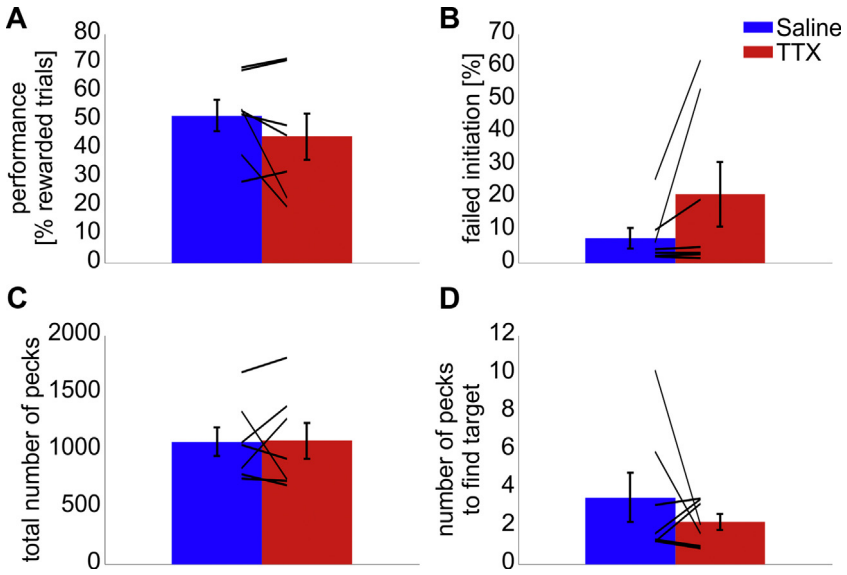

Fig. 2. General performance was not affected by NIML inactivation. Four parameters were measured to assess general performance of the animals; A: overall performance level, B: number of failed trial initiations, C: number of pecks per session and D: number of pecks to find the target area were not changed significantly by bilateral TTX injections into NIML ($p > 0.05$, Wilcoxon signed rank test). Presented are means ± s.e.m.

previously [20]. In total there were three sessions with TTX and three sessions with Saline injections for each pigeon. TTX and Saline sessions were alternated. Between subsequent sessions lay a period of 48 h. The order of sessions was balanced between pigeons. In all seven pigeons the correct positioning of the cannulas was verified after completion of the experiments.

Our paradigm is designed to induce highly variable pecking behavior. If the pigeons nonetheless behaved habitually, the first pecks of each trial should be focused on one location. To quantify the amount of variability, the first peck of each trial was attributed to one of the four quadrants of the entire touch screen and the probability $p(i)$ of the first peck hitting quadrant i was estimated. To access the randomness of the location of the first peck, entropy was computed according to $S\,(\text{first peak}) = -\sum_i p(i)\log_2(p(i))$. High entropy reflects high randomness thus a high degree of variability. Entropy was high compared to the theoretical maximum (Supplementary Fig. 1). Furthermore, we estimated the conditional probability $p(i|j)$ of the pigeon pecking into quadrant i having pecked into quadrant j throughout time and trials. Start and end of a trial were attributed to a fifth state. Here, entropy was measured according to $S\,(\text{transitions}) = -\sum_i p(i|j)\log_2(p(i|j))$. Average entropy was rather high compared to the theoretical maximum (Supplementary Fig. 2). A comparison between control- and TTX-condition revealed no difference for both entropy measures ($S\,(\text{first peck}) : p = 0.3$; $S\,(\text{transitions} : p = 0.735)$), Wilcoxon signed rank test, used for all statistical analyses if not denoted otherwise), suggesting that NIML inactivation did not affect behavioral variability. We conducted a detailed analysis on different aspects of the pigeons' behavior to validate this finding.

See Supp Figure S1 as supplementary file. Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2014.01.019.

First, we analyzed general performance measures (Fig. 2) that could indicate, on the one hand, changes in behavioral variability and, on the other hand, might show unspecific motor deficits that could also impair expression of variable behavior. There was no deficit observed after NIML inactivation (Fig. 2A); performance levels did not differ between Saline and TTX sessions ($p = 0.176$). However, there was a trend for an increased number of failed trial initiations, i.e. a reduction of pecking the start stimulus ($p = 0.063$, Fig. 2B). Contrary, neither the total number of pecks per session (Fig. 2C) nor the average number of pecks to find the target areas (Fig. 2D) were affected ($p = 0.866$ and $p = 0.499$). In addition, overall the pattern of peck locations did not show any obvious differences between NIML inactivation and control (Fig. 3). Hence, we can rule out strong motor deficits, though the moderate increase of failed trial initiations points out a mild impairment to initiate behavior or reduced attention.

Next, we analyzed parameters and measured their variability by means of the coefficient of variance (CV) that may reveal more subtle behavioral changes. We analyzed the latency and distance between subsequent pecks as well as pecking speed (Fig. 4A–C). None of these parameters or their variability was significantly affected by NIML inactivation ($p > 0.05$, each). Moreover, we analyzed the peck directions (Fig. 4D). Therefore, we computed the angle between the peck directions of subsequent pecks and compared the mean angles and circular variance [27]. Mean directions lie between 90 and 150 degrees showing that the pigeons usually vary the direction of subsequent pecks (180° opposite directions, 0° same direction). There was no difference in the mean angle between TTX and control condition ($p = 0.291$, Watson-Williams

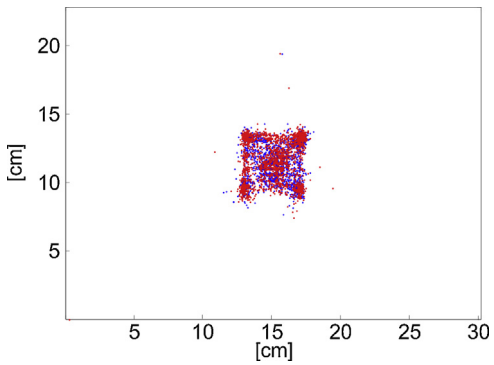

Fig. 3. Peck locations do not differ after NIML inactivation. The figure displays the peck locations of pigeon 882 as a typical example. Locations from control- and TTX-condition overlap. Blue: saline; red: TTX (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

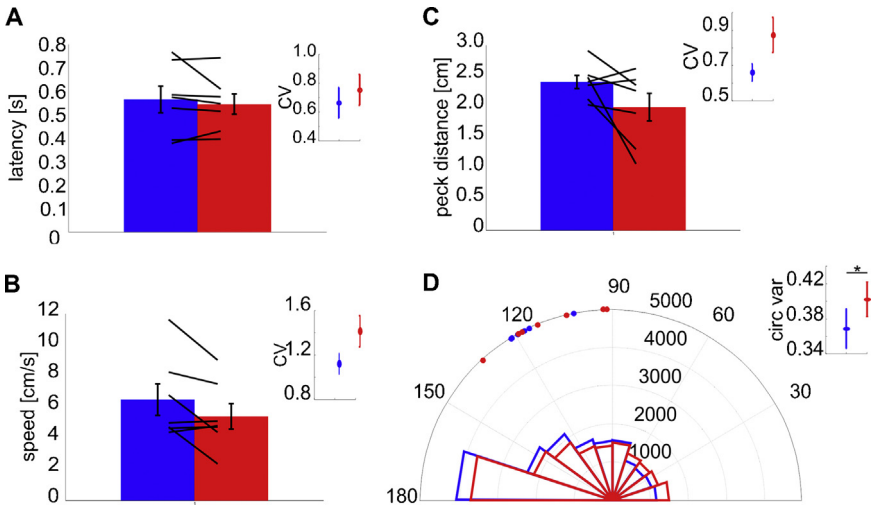

Fig. 4. Effects of NIML inactivation on pecking variability. Four measures were analyzed that describe the pecking behavior of the pigeons; A: peck distance; B: peck latency and C: pecking speed. To assess the variability of these measures the coefficient of variation was computed (insets). None of these parameters was affected by NIML inactivation ($p > 0.05$, Wilcoxon signed rank test). D: Distribution of angles between subsequent pecks. The majority of pecks are directed at angles bigger than 90°, i.e. pigeons usually change their peck directions. There was no difference ($p > 0.05$, Watson-Williams test) of mean directions between saline (blue marks) and TTX condition (red marks). An increase of circular variance was apparent after NIML inactivation ($p < 0.05$, Wilcoxon signed rank test) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

test) but a slight increase in variance ($p = 0.018$). An increase in the variance of peck directions may be indicative of impairments in fine motor control resulting in less precise pecks. A similar reduction of peck precision was already reported in a previous study [20].

In conclusion, we found that NIML is not associated with variability generation in the pigeon's pecking behavior. There are obvious differences in the requirements of vocal learning and the paradigm applied here, e.g., the behavioral context and different sets of muscles that are involved in the execution of the behaviors. Moreover, the contribution of LMAN to behavioral variability is reduced during the development of juvenile birds [8]. Nevertheless, taken together with previous findings, our results add support to the view that the two (most likely) homologous structures evolved different functions. Previous studies [20] revealed an association of NIML with sequential behavior. Hence, on a very general level, this accords with LMAN since bird song is an example of sequential behaviors. However, on a closer look substantial differences are evident. In the song system the pathways for learning and production of song are separated. LMAN is part of the AFP which is not required for production of learned song while the pigeon's NIML does contribute to production of learned sequences. In addition, here we showed that in contrast to LMAN, NIML does not generate behavioral variability. Thus, these results suggest that a separation of learning and production pathways and the ability of LMAN to generate and modulate variability in the motor output probably evolved *de novo* as adaptations to the complex demands of vocal learning. Apomorphies, i.e., the development of novel traits, likewise evolved in the production pathway of song birds as vocal learning developed. One pivotal feature of vocal learners is a direct connection of forebrain areas to brainstem nuclei controlling the vocal organs which is not present in most non-song birds [28]. Similarly, comparable cortico-bulbar projections are only found in humans but not other primates that do not learn vocalizations [29]. Moreover, mice that also may have limited vocal learning abilities possess this direct projection [30]. Thus, if not lost in the evolution of pigeons, variability generation in the AFP is likely a novel trait that evolved with vocal learning. In contrast, less complex forms of sensorimotor learning may not require fine tuned variability generation.

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