Extinction learning is an essential mechanism that enables constant adaptation to ever-changing environmental conditions. The underlying neural circuit is mostly studied with rodent models using auditory cued fear conditioning. In order to uncover the variant and the invariant neural properties of extinction learning, we adopted pigeons as an animal model in an appetitive sign-tracking paradigm. The animals firstly learned to respond to two conditioned stimuli in two different contexts (CS-1 in context A and CS-2 in context B), before conditioned responses to the stimuli were extinguished in the opposite contexts (CS-1 in context B and CS-2 in context A). Subsequently, responding to both stimuli was tested in both contexts. Prior to extinction training, we locally injected the N-methyl-D-aspartate receptor (NMDAR) antagonist 2-Amino-5-phosphonovaleric acid (APV) in either the amygdala or the (pre)motor arcopallium to investigate their involvement in extinction learning. Our findings suggest that the encoding of extinction memory required the activation of amygdala, as visible by an impairment of extinction acquisition by concurrent inactivation of local NMDARs. In contrast, consolidation and subsequent retrieval of extinction memory recruited the (pre)motor arcopallium. Also, the inactivation of arcopallial NMDARs induced a general motoric slowing during extinction training. Thus, our results reveal a double dissociation between arcopallium and amygdala with respect to acquisition and consolidation of extinction, respectively. Our study therefore provides new insights on the two key components of the avian extinction network and their resemblance to the data obtained from mammals, possibly indicating a shared neural mechanism underlying extinction learning shaped by evolution.

1. Introduction

There has been growing interest in the phenomenon of extinction learning in recent years. This is partly due to a better understanding of the neural mechanisms underlying especially fear conditioning \[1,2\], as well as the awareness of the clinical relevance of extinction learning in several human psychopathologies, such as anxiety disorders, substance abuse and post-traumatic stress disorder. In Pavlovian conditioning tasks, the conditioned response (CR) can be acquired after repeated pairings of an initially neutral conditioned stimulus (CS) with a biologically potent unconditioned stimulus (US). During the extinction phase, the repeated presentation of the CS without US results in a reduction of the CR. However, the CR decrement is not permanent and responding can be restored in various ways, like the passage of time (spontaneous recovery), or a context change from the extinction phase to testing (renewal). Numerous experimental investigations on the recovery of responding to an extinguished CR have given rise to the notion that extinction involves partial erasure of the original learning \[3\], and at the same time, the formation of a new memory trace \[4\].

In parallel, numerous studies were conducted to investigate the neural circuits of extinction learning. Rodent models of fear conditioning strongly suggest that amygdala, hippocampus and prefrontal cortex (PFC) constitute the core extinction network \[5,6\]. Current studies show that CS- and US-related signals are associated in the basolateral complex of the amygdala (BLA) during the acquisition of fear, while the central nucleus of the amygdala (CeA) initiates the fear responses \[7–10\]. After the extinction phase, the infralimbic area (IL) within the PFC exhibits an increased activation and acts directly on the GABAergic intercalated cells (ITC) of the amygdala \[11\]. Also, inputs from the BLA as the second source of activation, together with the inputs from ITC, produce a feedforward inhibition onto the CeA, resulting in an increased inhibition and thus a reduced fear output \[10,12\]. Besides, the increased interaction between hippocampus and PFC during extinction is believed to underlie the consolidation of extinction memory. The dependence on contextual factors during extinction memory retrieval indicates a key role of hippocampus \[13\].

Keywords:
Amygdala
Arcopallium
NMDA receptor
(pre)motor
Extinction learning
Avian

**A B S T R A C T**

Extinction learning is an essential mechanism that enables constant adaptation to ever-changing environmental conditions. The underlying neural circuit is mostly studied with rodent models using auditory cued fear conditioning. In order to uncover the variant and the invariant neural properties of extinction learning, we adopted pigeons as an animal model in an appetitive sign-tracking paradigm. The animals firstly learned to respond to two conditioned stimuli in two different contexts (CS-1 in context A and CS-2 in context B), before conditioned responses to the stimuli were extinguished in the opposite contexts (CS-1 in context B and CS-2 in context A). Subsequently, responding to both stimuli was tested in both contexts. Prior to extinction training, we locally injected the N-methyl-D-aspartate receptor (NMDAR) antagonist 2-Amino-5-phosphonovaleric acid (APV) in either the amygdala or the (pre)motor arcopallium to investigate their involvement in extinction learning. Our findings suggest that the encoding of extinction memory required the activation of amygdala, as visible by an impairment of extinction acquisition by concurrent inactivation of local NMDARs. In contrast, consolidation and subsequent retrieval of extinction memory recruited the (pre)motor arcopallium. Also, the inactivation of arcopallial NMDARs induced a general motoric slowing during extinction training. Thus, our results reveal a double dissociation between arcopallium and amygdala with respect to acquisition and consolidation of extinction, respectively. Our study therefore provides new insights on the two key components of the avian extinction network and their resemblance to the data obtained from mammals, possibly indicating a shared neural mechanism underlying extinction learning shaped by evolution.
Pharmacological studies show that inactivation of hippocampus directly before testing prevented renewal [14–16]. Similar effects were also found for mPFC [17] suggesting that the mPFC may be an important target of hippocampal projections for contextual modulation of extinction retrieval [18,19].

Neural substrates of extinction learning in aversive conditions described above are well understood thanks to deciphering of the fear circuit in rodents. Although highly comparable results are obtained from the aversive and appetitive paradigms at the behavioral level, differences concerning the mediating neural mechanisms still exist. In our lab, we have been examining the neural structures underlying extinction using appetitive tasks with pigeons. The reason for using pigeons as a model system is that pigeons represent an excellent model for learning and memory, especially for visual stimuli. A wealth of knowledge on the mechanisms of learning was gained by studies using this model organism. Pigeons can work on large sets of visual stimuli with different reward contingencies in parallel, and adapt their behavior accordingly [20]. In addition, birds evolved in parallel to mammals since ca. 300 million years [21]. As a result, birds have a quite different brain organization that harbors some one-to-one homologies to mammals like the hippocampus but also many non-homologous, but functionally equivalent structures like the nidopallium caudolaterale (NCL) that is comparable to the mammalian PFC [22]. As a result, studying the neural basis of extinction learning in pigeons can uncover some invariant properties of extinction that is shared by evolutionary distant animals.

Recently, Lengersdorf et al. [23] showed that transient inactivation of NCL and hippocampus impairs context-specific consolidation of extinction memory in appetitive conditioning. They also indicated that the involvement of the NCL in extinction learning is specifically mediated by the N-methyl-D-aspartate receptors (NMDARs). Based on the observation of the high density of NMDARs in the NCL [24], injections of the NMDAR antagonist, 2-Amino-5-phosphonovaleric acid (APV), into the NCL resulted in impaired extinction learning of an appetitive task [25] without affecting consolidation of extinction memory [26]. In addition, NMDARs in pigeon’s NCL are involved in conditional processing in a conditional discrimination task [27]. Taken together, these results suggest that the NCL and hippocampus in birds have comparable functions to those in mammals during extinction, shaped during 300 million years of independent evolution.

In the present study, we examined the avian amygdala and the avian arcopallium, which seems to play a role in motor behavior. According to the meanwhile outdated nomenclature of Karten and Hodos [28], this ventroposterolateral part of the bird pallium was named archistriatum and was suggested to be partly comparable to the mammalian amygdala and partly to be of motor nature [29]. Based on a large number of neuroscientific evidences, the avian nomenclature forum [21,30] accepted the dual nature of the archistriatum and its subnuclei were subsequently identified as being of amygdaloid or of somatomotor nature. While the first group is collectively called “amygdala”, the second group constitutes the “arcopallium”.

This view was not unanimously accepted by the entire scientific community. Especially neurogenetic studies suggested the existence of a tetrapartite pallial model that is based on field homologies of pallial divisions [31]. According to this view, neurogenetic markers make it unlikely that the arcopallium contains structures that are homologous to cerebral cortex [32]. The tetrapartite model is in flux and was recently expanded to include six divisions [33]. As important as these discoveries are, they are in conflict with various connectional, neurochemical, physiological, and even other neurogenetic studies. For example, Belgard et al. [34] compared expression patterns of more than 5000 orthologous genes and did not find evidence for the proposed homologies of the tetra- or hexapartite model. Further neurogenetic studies, some of them using thousands of genes, could also not verify these models and instead argued that the arcopallium is characterized by expression patterns that resemble neurons of cortical layer V [35–37] and/or pre/motor cortex [38]. Further neurogenetic studies confirmed the limbic nature of the amygdaloid substructures as defined by the nomenclature forum [39,40].

Also, connectional analyses show that the arcopallium shares similar connectivity patterns with the mammalian pre/motor areas, while the limbic nuclei display connections resembling the mammalian amygdala [29,30,41–47]. This is nicely shown in the connectome analyses of the avian telencephalon [48]. Using a graph theoretical analysis that is theory-free with regard to the above referred discussion, the pigeon amygdala and arcopallium turned out to constitute entirely different viscero-limbic and premotor modules, respectively. In addition, the recent study of Herold et al. [49] analyzed binding sites of 12 ligands using quantitative in vitro receptor autoradiography combined with a detailed cyto- and myeloarchitectural analysis. They revealed a clear parcellation between a limbic component that shares patterns with the mammalian amygdala and an arcopallial entity that resembled cortical systems. This separation is also true for functional, pharmacological and electrophysiological studies in various bird species that tested visual, vocal, auditory, and emotional learning, fear and reproduction behavior as well as neuroendocrine control and homeostasis. These studies also testify a functional division between a limbic (amygdaloid) and a sensorimotor (arcopallial) complex [50–59]. Based on these evidences, we depart from the assumption that amygdala and arcopallium are closely located clusters that are nevertheless differentially embedded in limbic and pre/motor circuits, respectively. It is important to note that we do not claim a homology of the arcopallium to premotor and motor cortices, but depart from a functional comparison.

The anterior and intermediate parts of this complex, incorporating the anterior (AA), dorsal (AD), and intermediate (AI) arcopallium, are considered non-limbic because of their sensory afferents and their descending motor telencephalothalic efferents [29,60]. They are seen as the premotor and motor structures that innervate pallial, diencephalic, and brainstem structures, possibly even down to cervical spinal levels [29]. On the other hand, the posterior and ventral part of the complex, mostly the posterior pallial amygdala (PoA), is regarded as visceral and limbic in its connections and is thus amygdaloid in its nature [30]. Together with the nucleus taenia of amygdala (TnA) and subpallial amygdala (SpA), PoA is currently recognized as one important part of amygdaloid complex in birds [30].

Until now, to the best of our knowledge, the involvement of arcopallium and amygdala in extinction learning in birds has not been investigated. Therefore, the aim of the present study was to investigate the role of avian arcopallium and amygdala in appetitive extinction behavioral tasks. For this purpose, we selectively blocked NMDARs in the amygdala and arcopallium [24], and adopted the within-subject sign-tracking design which has been established by Lengersdorf et al. [23]. By locally injecting APV bilaterally in the amygdala and arcopallium before extinction, we were able to demonstrate that the blockade of NMDARs in the amygdala impaired the acquisition of extinction learning, while the arcopallium plays a role in the consolidation and/or expression of extinction memory.

2. Materials and methods

2.1. Subjects

40 experimentally unsexed adult homing pigeons (Columba livia) from local breeders were used as subjects. The animals were housed in individual wire-mesh home cages (40 × 40 × 45 cm) in a colony room where temperature, humidity and the 12 h-light-dark circle were strictly controlled (lights on at 8 a.m.). All pigeons were maintained at 80%–90% of their normal body weight with additional free food on weekends. Water was freely available in their home cages. The experiment was approved by the national authorities of the state of North Rhine-Westphalia, Germany, and was carried out in accordance with
the National Institute of Health Guide for Care for Laboratory Animals.

2.2. Surgery

The pigeons were chronically implanted bilaterally with 26-gauge (8 mm) stainless steel guide cannulae (Plastics One Inc., Roanoke, VA, USA). Before surgery, the feathers on top of the skull were cut. A 7:3 mixture of Ketamine (100 g/ml; Pfizer GmbH, Berlin Germany) and Xylazine (20 mg/ml Rompun, Bayer Vital GmbH, Leverkusen Germany) was prepared. To induce the anesthesia, 0.075 ml of the mixture was injected i.m. for each 100 g body weight of the pigeon. To maintain the anesthetized state during surgery, an additional application of gas anesthesia was adopted with Isoflorane (Forane 100% (V/V), Mark 5, Medical Developments International, Abbott GmbH & Co KG, Wiesbaden, Germany).

As soon as the claw responses disappeared, animals were fixed onto a stereotactic device. One incision on the skin was performed to expose the skull, and stainless steel micro-screws (Small Parts, Logansports, USA) were fixed to the skull as anchors. Two craniotomies were performed to expose the brain tissue. One cannula was inserted vertically into each hemisphere under visual control at the following coordinates: For the amygdala group: AP + 4.5 mm, ML ± 6.4 mm, DV + 5.3 mm were taken to target the PoA; AP + 6.1 mm, ML ± 5.2 mm, DV + 4.2 mm were adopted for TnA and SpA. For the arcopallium were taken to target the PoA; AP + 6.1 mm, ML ± 5.2 mm, DV + 4.1 mm were used [28].

Finally, dental cement was applied around the screws and the cannulas in order to fix them to the implanted positions. Following surgery, 0.5 ml 10 mg/ml Rimaldyl (Pfizer GmbH, Münster, Germany) was applied twice daily on three consecutive days as an analgesic. The animals were allowed for recovery with free food and water access until two days before the behavioral training.

2.3. Behavioral apparatus

The behavioral training took place in four Skinner boxes with similar shapes (36 × 34 × 36 cm.), housed in sound-attenuating cubicles (80 × 80 × 80 cm). Additional white or brown noise was played (80 dB SPL) to mask extraneous sounds. Each Skinner box was illuminated by 6 W light bulbs or LED bands at the ceiling. A transparent rectangular pecking key was placed in the center of the rear wall (2 × 2 cm; 12 cm above the floor) with a food hopper positioned at the bottom center directly underneath the pecking key. An LCD flat screen monitor (either Belinea Model No.: 10 15 36 or Philips Model: Brilliance 17S1/00) was fixed behind the rear wall, so that the animals could see the stimulus presented on the monitor screen through the pecking key. Every effective key peck produced an audio feedback sound.

The Skinner boxes were grouped in two distinct contexts, by covering the rear and side walls with different colored wallpapers (either with 2.5 cm wide vertical brown stripes spaced 5 cm apart on red background or yellow marbling pattern on white background) and also by providing either white or brown noise in the training chamber. Four well distinguishable stimuli were used in each experiment (see Section 2.4.2). The hardware was controlled by custom-written Matlab code (The Mathworks, Natick, MA; [97]).

2.4. Procedure

The experimental procedure encompassed five separate phases: pretraining I, pretraining II, conditioning, extinction and test. Details are described below and are also summarized in Figs. 1A & 2 and Table 1.

2.4.1. Pretraining I

The behavioral training adopted a sign-tracking procedure. A stimulus (“target”) was presented for 5 s and followed by 3 s reward time, during which grain was provided by the food hopper. The target was always followed by reward regardless whether the pigeons responded or not. Based on the previous studies [23,26,61], the animals exhibited continuous responding also during fixed inter-trial-intervals (ITI), therefore, we adopted a fixed ITI at 45 s. Each session consisted of 48 target presentations and animals were trained with one session in each context per day. The two sessions were spaced 2 h apart and conducted in an alternating succession (Fig. 2). After the achievement of the learning criterion, consistent pecking response in 80% of the trials in both contexts on three consecutive days, the animals entered the next phase.

2.4.2. Pretraining II

In Pretraining II, an additional stimulus (“nontarget”) was introduced which was never rewarded when the animals pecked at it. Each session consisted of 24 trials of target and 12 trials of nontarget presentations. As before, two sessions were conducted per day, one in each context. The inter-trial-interval was reduced to 35 s. Each session started with two target trials, and for the remaining trials the order of stimulus presentation was randomized. A minimum of 80% correct responses (pecking response to the target and no response to the non-target) to both stimuli in both contexts were required to enter the next phase of training.

The rewarding contingencies for target (rewarded) and nontarget (non-rewarded) remained unchanged throughout the whole experiment to serve as control stimuli. These stimuli were used to examine possible non-specific effects triggered by injection of a pharmacological substance. In addition, the nontarget served to discourage pigeons from pecking indiscriminately to all visual stimuli. It is also likely that the occurrence of a non-rewarded stimulus enhances the birds’ overall attention towards the critical stimuli [96].

2.4.3. Conditioning

In addition to target and nontarget, an additional CS was introduced into each context. CS-1 was added in context A and CS-2 in context B (Fig. 1A). Both CS and target presentation were followed by 3 s of food reward. Each of the three stimuli (target, nontarget and the corresponding CS) was presented 12 trials per session for a fixed presentation time of 5 s.

Conditioning was conducted at least for six days with two sessions per day – one in context A for CS-1 acquisition and one in context B for CS-2 (Fig. 1A). Specifically, the duration of conditioning depended on how long the pigeons needed to achieve the learning criterion with 80% correct responses for all stimuli across three consecutive days.

2.4.4. Extinction

The duration of the extinction phase was four days in total (Figs. 1A and 2). The pigeons received an extinction session in each context where the CS was no longer paired with food reward. The two extinction sessions were 48 h apart. 15 min before extinction training, the pigeons were microinjected bilaterally with either 1 μl of 5 μg/μl APV (Sigma-Aldrich Co. St. Louis, USA) dissolved in saline or 1 μl pure saline (B. Braun Melsungen AG, Germany). The order of injections (APV or saline) was randomized across subjects and contexts. There was one day free after each extinction session to ensure a complete wash out of the injected substances from the body. On the day off subjects received the amount of grain they usually received during daily training.

Extinction sessions took place in the two contexts with one session in each context (Fig. 1A): the CSs were presented without reward in the other context in which they had not been presented in the conditioning phase. That is, the CS-1 which was presented in context A in the conditioning, was presented for 24 trials without reward in context B during extinction, together with target (24 trials) and nontarget (12 trials) stimuli. The CS-2, used previously in context B during the conditioning phase, was presented in context A during extinction without reward for 24 trials. The order of contexts was randomized across subjects.
2.5. Histology

Subsequent to the behavioral study, histology was conducted to examine the positions of the cannulas. Animals received 0.1 ml i.m. injection of Heparine (Rotexmedica GmbH, Trittau, Germany) dissolved in 0.1 ml of 0.9% NaCl to prevent blood clots during perfusion. 15 min. later, Equithesin (0.55 ml/100 g body weight) was injected i.m. for injection of Heparine and for cannula fixation.

Overall, the experiment constitutes a within-subject design (Fig. 1A). Each pigeon can be compared with itself for different pharmacological manipulations. For example, CS-1 was acquired in context A, extinguished in context B, and tested in both A and B. Thus, we had two conditions, ABA and ABB. Renewal can be observed in the ABA condition, while spontaneous recovery is visible in ABB. For CS-2 the BAB was the same as ABA, and the BAA equaled ABB. As described above, the two CSs were trained under the effect of either APV or saline in the corresponding contexts. Therefore, the effect of APV on spontaneous recovery can be examined by comparing the CS-APV with CS-saline in the condition ABB/ABA within one pigeon. And by comparing the CS-APV with CS-saline in ABA/BAB, it revealed how APV affected renewal.

2.4.5. Test

Responses to all four stimuli were tested under drug free conditions 48 h after the second extinction session (Fig. 2). Each stimulus was presented 12 times in each context with 2 h between the two testing sessions. One session contained 48 trials in total, and only the target stimulus was followed by reward.

As described above, the two CSs were trained under the different pharmacological conditions. Therefore, the CS-APV refers to the CS responding during extinction under the effect of APV, and the CS-saline indicates no reward following the CS presentation in extinction. Although conditioning and testing sessions were conducted drug-free, we still adopted the CS+APV to indicate the responses to CS in conditioning, and CS-APV in test for clarification. The same applies for CS+saline and CS-saline accordingly. In the experiment, contexts, stimuli and injection sequences were balanced across subjects. The figures show only one possible example. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 1. Schematic representation for the within-subject design. Pictures show rear walls of the two training chambers A and B. (A) The squares with numbers 1 and 2 indicate CS-1 and CS-2. The ‘+’ indicates that the CS was rewarded, and the ‘−’ indicates that the CS was not rewarded. Not shown are the target stimulus (rewarded) and the nontarget stimulus (not rewarded). (B) The yellow and blue rectangles depicted with ‘CS+APV’ and ‘CS+saline’ refer to the CS-1 and CS-2 in (A), respectively. Since different injections were conducted before the extinction training, the two CSs were processed under different pharmacological conditions. Therefore, the CS+APV refers to the CS responding during extinction under the effect of APV, and the CS+saline indicates no reward following the CS presentation in extinction.

Fig. 2. Schematic representation of the training phase. This depiction shows only one possible example, and pretraining I and II are not included. Squares indicate a single training session in one corresponding context (depicted in yellow or red). The black vertical bars separate one workday from the other. The black arrows on day n + 1 and n + 3 indicate the injections of different substances either APV or saline 15 min. before extinction training. In the conditioning phase, 2 sessions were 2 h apart on every workday. The conditioning phase lasted at least 6 days. The specific number of days (n) depended on how long the pigeons needed to achieve the learning criteria. During the extinction phase on days n + 1 and n + 3, the animals were trained with one extinction session per day. There was no training the day after the injection to ensure the complete wash out of injected agents from the body system. The subjects were tested in each context on day n + 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Table 1: Overview of the experimental procedure. ( (+ ) = rewarded stimulus; (−) = non-rewarded stimulus; CS-1 = conditioned stimulus 1; CS-2 = conditioned stimulus 2; − = no stimulus presentation).  

<table>
<thead>
<tr>
<th>Phase</th>
<th>Context</th>
<th># Target</th>
<th># Nontarget</th>
<th># CS-1 or CS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretraining I</td>
<td>A</td>
<td>48x (+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>48x (+)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pretraining II</td>
<td>A</td>
<td>24x (+)</td>
<td>12x (−)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>24x (+)</td>
<td>12x (−)</td>
<td>–</td>
</tr>
<tr>
<td>Conditioning</td>
<td>A</td>
<td>12x (+)</td>
<td>12x (−)</td>
<td>12 x CS-1 (+)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12x (+)</td>
<td>12x (−)</td>
<td>12 x CS-2 (+)</td>
</tr>
<tr>
<td>Extinction</td>
<td>A</td>
<td>24x (+)</td>
<td>12x (−)</td>
<td>24 x CS-2 (−)</td>
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<tr>
<td></td>
<td>B</td>
<td>24x (+)</td>
<td>12x (−)</td>
<td>24 x CS-1 (−)</td>
</tr>
<tr>
<td>Test</td>
<td>A</td>
<td>12x (+)</td>
<td>12x (−)</td>
<td>12 x CS-1 (−) &amp; 12 x CS-2 (−)</td>
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<td></td>
<td>B</td>
<td>12x (+)</td>
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<td>12 x CS-1 (−) &amp; 12 x CS-2 (−)</td>
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</table>
anesthetization. Once the animal was nonresponsive to physical stimulation, the animal’s circulatory system was transcardially flushed with ca. 500 ml of 0.9% saline (40°C). Subsequently animals were perfused with 1000 ml 4% paraformaldehyde (WWR Prolabo Chemicals, Leuven, Belgium). Hereafter the brain was dissected and post-fixed for a period of at least 2 h in paraformaldehyde and 30% sucrose at 4°C. Afterwards it was transferred in 30% sucrose diluted in 0.12 M PBS for 24 h for cryoprotection.

Finally, the brains were embedded in 15% Gelatine (Merck KGaA, Darmstadt, Germany) dissolved in 30% sucrose for an overnight fixation in 4% paraformaldehyde, and subsequently preserved in the solution of 30% sucrose and 0.12 M PBS. For the last steps of histology the brains were cut frontally into 40 μm slices with a microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and then stained with cresyl violet to reveal the brain structures. The atlas of the pigeon brain from Karten and Hodos [28] were used for identifying the positions of cannulas.

2.6. Data analysis

Responses were assessed by counting the number of pecks on the pecking key. The main dependent variable was the pecking rate during 5 s stimulus presentation. To this end we computed the mean response rates for target, nontarget, CS+APV and CS+saline (Fig. 1B). Since different injections were conducted before the extinction training, the two CSs were processed under different pharmacological conditions. Therefore, CS+APV refers to the CS under the effect of APV with the ‘-’ indicating no reward following the CS presentation in the extinction training (Fig. 1B). Although conditioning and testing sessions were conducted drug-free, CS+APV is also used to refer to the CS responses in conditioning with reward and CS+APV in testing phases without reward. The same applies for CS+saline and CS+target accordingly (Fig. 1B).

Statistical analysis was conducted with IBM SPSS Statistic (Version 21, IBM Corp. USA) and Matlab. For pre-processing, we adopted the one-way repeated measure ANOVA (RMANOVA) to screen out the factors of the treatment (APV or saline), the context (ABA and ABB) and block was conducted (Fig. 5C). To simplify, ABA and ABB were included for statistical analysis. The extinction session was divided into 3 blocks with four trials each. Data from the ABA and BAB conditions were summarized together and were simplified for statistical analysis. The extinction session was included for statistical analysis. The extinction session was divided into 3 blocks with four trials each. Data from the ABA and BAB conditions were summarized together and were simplified as ABA. Similarly, the data for ABB and BAA were presented as ABB. Normality of data distribution was evaluated by Kolmogorov-Smirnov test. Then data sets were analysed with Repeated Measure ANOVA (RMANOVA). Mauchly’s test was conducted to validate the data sphericity, on occasion of violation of sphericity, the Greenhouse-Geiser or Huynh-Feldt correction was applied. Importantly, the post hoc tests were conducted in case of significant main or interaction effects.

3. Results

3.1. Histology

In total, 40 pigeons participated in the experiment. 23 pigeons were excluded due to various reasons: 12 animals failed to learn the task, 3 animals showed no response to either CS or target after the injection of APV. In addition, 8 pigeons were excluded due to incorrect implantation of the cannulas. Thus, data from the remaining 17 pigeons were included for analysis.

The amygdala group consisted of 10 pigeons with the cannulas located in the positions depicted in Fig. 3. Of the 20 cannulas in total, 11 had their tips in the posterior pallial amygdala (PoA), 4 cannulas in arcopallium ventrale (AV), 3 in nucleus tenniae of the amygdala (TmA), 1 in subpallial amygdala (SpA) and 1 in arcopallium mediale (AM). All structures are considered amygdaloid based on anatomical, embryonal, neurochemical and connectivity studies [29,30,39,43,44,62,63]. The arcopallium group consisted of 7 pigeons with the cannulas placed in the positions depicted in Fig. 4. All 14 cannula tips were located in the pigeons’ dorsal and intermediate arcopallium (AD and AI; Fig. 5B). AD and AI belong to the premotor/motor areas of the pigeons’ brain [48].

Considering the 3 mm spread of pharmacological agents in all directions after injection [95], the pigeons with cannula tips located closely above or adjacent to the target areas were also included into the data analysis (Figs. 3 and 4).

3.2. Amygdala group

3.2.1. Conditioning

The mean response rates did not differ significantly between the target (8.0 ± 1.3; mean ± sem), CS+APV (8.1 ± 1.5) and CS+saline (8.5 ± 1.2; Fig. 5A) in the last three sessions of acquisition (paired sample t-test: target vs. CS+APV: t (9) = 0.3, p = 0.779; target vs. CS+saline: t (9) = 1.4, p = 0.207; CS+APV vs. CS+saline: t (9) = 0.4, p = 0.686; Fig. 5A). Since responding to the nontarget did not differ from zero in all phases, comments on the nontarget are omitted in the following sections.

3.2.2. Extinction

Two-way RMANOVA for both target and CS pecking rates were conducted with two factors, the block and the treatment (APV or saline).

There was no effect of treatment for the target response rate (two-way RMANOVA, F (5, 45) = 0.5, p = 0.671; Fig. 5B): the pecking rates to target under APV and saline did not differ from each other in extinction sessions. And there existed no block effect (F (5, 45) = 0.9, p = 0.514): Pecking rates remained constant under both conditions across six blocks (one-way RMANOVA, saline: F (5, 45) = 1.0, p = 0.499; APV with Greenhouse-Geiser correction: F (2.4, 21.3) = 1.6, p = 0.232; Fig. 5B). Additionally, no interaction effect was observed (two-way RMANOVA, F (5, 45) = 2.0, p = 0.092). The results imply that the target pecking responses were not affected by the treatment, and served as an excellent control stimulus.

As for the CS responses, there was a strong effect of block (two-way RMANOVA, Greenhouse-Geiser correction F (2.5, 22.9) = 8.5, p = 0.001; Fig. 5B). Analysis with one-way RMANOVA indicated that the CS responses dropped significantly under saline (one-way RMANOVA with Greenhouse-Geiser correction, F (2.3, 20.6) = 12.9, p < 0.001), but stayed constant under APV (F (2.3, 20.8) = 1.6, p = 0.224; Fig. 5B), indicating a treatment effect (two-way RMANOVA, F (1, 9) = 7.5, p = 0.023; Fig. 5B). In addition, a trend towards an interaction effect of treatment × block was found (Greenhouse-Geiser correction, F (2.2, 19.8) = 2.7, p = 0.085). Post hoc comparisons with Sidak correction were conducted, revealing significant differences between CS+APV and CS+saline in the third block (p = 0.012) and mild differences in the fourth (p = 0.076), fifth (p = 0.068) and the last (p = 0.087) blocks. Overall, the results indicated that extinction learning was delayed due to the injection of APV.

3.2.3. Retrieval test

In the test phase, there was no difference between pecking response to target in saline and APV contexts (paired sample t-test: t (9) = 0.1, p = 0.890).

For the mean response rates to CS, the three-way RMANOVA with the factors of the treatment (APV or saline), the context (ABA and ABB) and block was conducted (Fig. 5C). To simplify, ABA and ABB were used to indicate ABA/BAB and ABB/BAA, respectively. The analysis indicated a significant effect of context (three-way RMANOVA, F (1, 9) = 18.3, p = 0.002; Fig. 5C) and block (F (2, 18) = 46.0, p < 0.001) and a strong trend for treatment (F (1, 9) = 4.7, p = 0.059; Fig. 5C). No interaction effects were found (context × treatment: F (1, 9) = 1.6,
The results indicated that pigeons responded significantly different to the stimuli in different contexts during testing. Post hoc tests were conducted to reveal how differently the subjects responded in ABA and ABB conditions. For both CS-APV and CS-saline, the animals responded significantly more in the conditioning context (ABA) than in the extinction context (ABB) (CS-APV in ABB vs. ABA: \( p = 0.021 \); CS-saline in ABB vs. ABA: \( p = 0.001 \), Fig. 5D) showing a renewal effect due to the context change. Separately for the ABB and ABA conditions, in ABA the responses for CS-APV and CS-saline did not differ from each other when taking all blocks together (\( p = 0.347 \); Fig. 5D) nor in each of the three blocks (first block: \( p = 0.920 \); second block: \( p = 0.335 \); third block: \( p = 0.222 \), Fig. 5C). Yet, in ABB, there was a significant difference between CS-APV and CS-saline (\( p = 0.034 \), Fig. 5D) especially in the first (\( p = 0.046 \)) and third block of the test (\( p = 0.028 \), Fig. 5C).

We then compared the CS pecking rate in the last block of extinction with the first block of the test under ABB condition. There were no significant changes of pecking to CS-APV (paired sample \( t \)-test: \( t_{(9)} = 0.2, p = 0.841 \)) and to CS-saline (\( t_{(9)} = 1.4, p = 0.191 \), Fig. 5B and C), which indicated that the pigeons responded during the start of the test phase as they did at the end of the extinction phase. The absence of a significant spontaneous recovery was possibly due to the short retention period between test and extinction (e.g. Leising et al. [64]).

3.3. Arcopallium group

3.3.1. Conditioning

There were no significant differences between the target (9.3 ± 1.1), CS+APV (10.2 ± 1.1) and CS+saline (8.6 ± 1.9; mean ± sem; Fig. 6A) in the conditioning phase (paired sample \( t \)-test: target vs. CS+APV: \( t_{(6)} = 0.7, p = 0.528 \); target vs. CS+saline: \( t_{(6)} = 0.6, p = 0.605 \); CS+APV vs. CS+saline: \( t_{(6)} = 0.7, p = 0.538 \), Fig. 6A).

3.3.2. Extinction

Two-way RMANOVA for both target and CS pecking rate were conducted with two factors; the block and the treatment (APV or saline).

There was no effect of treatment for pecking responses towards the target (two-way RMANOVA, \( F_{(1, 6)} = 0.9, p = 0.376 \); Fig. 6B) but a strong block effect (\( F_{(5, 30)} = 5.0, p = 0.002 \); one-way RMANOVA, APV: \( F_{(5, 30)} = 4.5, p = 0.004 \); saline: \( F_{(5, 30)} = 1.6, p = 0.193 \);
difference in pecking responses between target\textsubscript{APV} and target\textsubscript{saline} in the fifth block (p = 0.033).

As for the CS, there was a strong effect of block (two-way RMANOVA, F(5, 30) = 22.5, p < 0.001; Fig. 6B). Follow-up analysis with one-way RMANOVA indicated that the CS pecking rate dropped significantly under APV (F(5, 30) = 13.7, p < 0.001) as well as under saline (F(5, 30) = 8.4, p < 0.001; Fig. 6B). Neither a treatment effect (F(1, 6) = 0.05, p = 0.834) nor an interaction effect of treatment \times block (Greenhouse-Geisser correction: F(1.4, 8.6) = 0.5, p = 0.553) was found.

Since APV had an unspecific effect on the pecking response in general, we normalized the CS response rates in the APV condition by multiplying an index $\frac{Tar\text{saline}}{Tar\text{APV}}$ (see Eq. (1.1)), which represents the ratio of target response rates under saline to that under APV.

$$\text{normalisedCS}_{\text{APV}} = \frac{Tar\text{saline}}{Tar\text{APV}} \times \text{CS}_{\text{APV}}$$

This parameter corrects the CS pecking performance and indicates how the pecking response should manifest without the unspecific effect induced by APV. This enables us to detect the effect of APV on extinction learning dynamics. A two-way RMANOVA was conducted with the normalized CS\textsubscript{APV} responses rates to CS\textsubscript{saline} to investigate whether the two factors, injection and block, had an effect on learning dynamics in extinction training. A strong block effect was observed (two-way RMANOVA, F(5, 30) = 12.6, p < 0.001, Fig. 6C), indicating that the normalized CS\textsubscript{APV} pecking rates decreased significantly and similarly as CS\textsubscript{saline}. Neither a treatment (F(1, 6) = 0.03, p = 0.870) nor an interaction (treatment \times block) effect (Greenhouse-Geisser correction: F(2, 11.8) = 0.4, p = 0.708, Fig. 6C) was found. So, the analysis indicated that APV did not affect learning dynamics in the extinction training but produced a mere motoric side effect that was detected by our target stimulus control procedure.

### 3.3.3. Retrieval test

In the test phase, there were no differences between pecking rates to targets in saline and APV conditions (paired sample t-test: t(6) = 1.5, p = 0.273).

For the mean response rates to CS, the three-way RMANOVA with the factors of treatment (APV or saline), context (ABA and ABB), and block was conducted (Fig. 6D). For simplification purpose, the ABA and ABB were adopted to indicate ABA/BAB and ABB/BAA, respectively. The analysis indicated a significant effect of context (three-way RMANOVA, F(1, 6) = 23.2, p = 0.003; Fig. 6D) and block (F(2, 12) = 12.8, p = 0.001), but no effect of treatment (F(1, 6) = 1.4, p = 0.278). Only an interaction effect between treatment and block was found (F(2, 12) = 5.6, p = 0.020).

Post hoc tests showed, that the animals responded in general significantly more often in the conditioning (ABA) than in the extinction context (ABB) (p = 0.003, Fig. 6D and E). Taking the CS\textsubscript{APV} and CS\textsubscript{saline} separately, pecking rate to CS\textsubscript{APV} was significantly higher in ABA than in ABB (p = 0.003, Fig. 6E). CS\textsubscript{saline} results exhibited the same pattern (p = 0.030, Fig. 6E). These results indicate a clear renewal effect on the pecking response in the first block of test (p = 0.006; Fig. 6D) and a trend in the second block (p = 0.052).

In order to detect the influence of APV on the consolidation or retrieval of extinction memory, we analyzed the transition from extinction to testing in the extinction context (ABB). The response rate to CS in the last block of extinction training was compared with the first block of testing. No significant changes of CS\textsubscript{saline} pecking rate were found (paired sample t-test: t(6) = 1.4, p = 0.220). But a significant increase occurred under APV (t(6) = 3.5, p = 0.013, Fig. 6D) from the end of

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**Fig. 4.** Schematic sections of the pigeon brain of the arcopallium group depicting APV injection sites. Dots represent the tips of the injection cannulas. Each color represents the two cannulas of one pigeon. There were 7 pigeons in the arcopallium group. AD: arcopallium dorsale, AI: arcopallium intermediate. Pictures are based on the brain atlas by Karten and Hodos [28] and Herold et al. [49] on the receptor distribution in the pigeon’s arcopallium and amygdala. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
extinction to the beginning of test in ABB, which indicated that the extinction memory may not be properly consolidated under the effect of APV, since APV might stay effective for at least 1 h after extinction training was finished [65].

In order to detect the ceiling effect for the renewal, responses to CS in ABA was compared with target response for both APV and saline across all trials in the test (Fig. 6E). There was no difference between CS_{saline} and target_{saline} (paired sample t-test, \( t(6) = 0.8, p = 0.118 \); Fig. 6E) and between CS_{APV} and target_{APV} (\( t(6) = 0.07, p = 0.882 \), Fig. 6E), indicating a strong ceiling effect on CS pecking behavior when tested in the conditioning context.

4. Discussion

This study aimed to examine the role of NMDARs in the amygdala and the (pre)motor arcopallium of pigeons for extinction learning. To this end, NMDARs were pharmacologically blocked with APV during extinction. We observed a double dissociation of deficits in acquisition and consolidation of extinction in amygdala and arcopallium, respectively. We will discuss our findings, one by one.

4.1. The avian amygdala in extinction learning

Given the highly conserved structure of the amniote amygdala [66], its homology between birds and mammals [30], and the comparable overall connectivity pattern of the avian and the mammalian amygdala [29,44,48], it is conceivable that similar limbic mechanisms might operate in pigeons and rodents. This assumption is also substantiated by a detailed comparison of the targeted amygdala subnuclei of the current study. The TnA is considered to resemble the medial amygdala of mammals, and the SpA, the central medial nucleus, is similar to the mammalian extended amygdala [39,67]. This study aims to examine the role of NMDARs in the amygdala and the (pre)motor arcopallium of pigeons for extinction learning. This study aims to examine the role of NMDARs in the amygdala and the (pre)motor arcopallium of pigeons for extinction learning. To this end, NMDARs were pharmacologically blocked with APV during extinction. We observed a double dissociation of deficits in acquisition and consolidation of extinction in amygdala and arcopallium, respectively. We will discuss our findings, one by one.
induced a disinhibition resulting in deteriorated CR reduction, i.e. encoding of extinction memory during extinction. Further research is needed to clarify if this hypothesis holds. It is worth noting that the possible impairment of extinction learning occurred under appetitive conditions. Likewise, the BLA of rodents is integrally involved in appetitive extinction learning [71]. For instance, BLA lesions [72,73] or local inhibition of BLA [74] lead to impaired extinction in appetitive food-seeking behavior. Specifically, NMDAR inactivation in BLA blocks acquisition of operant responding for food reward [92] and further disrupts consolidation of stimulus-reward memory as well as extinction learning during reinstatement of cocaine-seeking behavior [75]. Consequently, potentiation of NMDARs systemically through a partial agonist D-cycloserine (DCS) enhances the persistence of extinction in drug-seeking tasks [76–78].

4.2. The avian arcopallium in extinction learning

In the arcopallium group, our injection sites were mostly located in the avian arcopallium (Fig. 6).
the pigeons’ AD and AI, which, as outlined above are considered components that subserve pre/motor functions [29,48]. APV injections in the arcopallium did not affect the extinction dynamics but disturbed the motor output in general. Additionally, an increased spontaneous recovery was observed under APV, when tested in the extinction context (ABB). We did not observe an effect of APV on renewal in ABA. However, pecking rate in ABA was strongly constrained by a ceiling effect. We therefore assume that this ceiling obstructed the occurrence of a similar effect in ABA.

The increased spontaneous recovery under APV in ABB can be ascribed to impaired consolidation of extinction memory. Indeed, APV is effective for several hours after injection and, as shown by Tronel and Sara [81], APV can induce amnesia in an odor-reward associative learning in rats even when injected after learning. Thus, under normal conditions, the consolidation of extinction memory possibly alters synaptic dynamics in a network of structures of which the arcopallium is one component. When arcopallial NMDARs are blocked during extinction learning and for several hours thereafter, the arcopallium fails to properly consolidate the respective memory components. Since this (pre)motor structure constitutes a key telencephalic downstream component of action generation [29,48,60], our pigeons were not able to properly retrieve all components of extinction memory and started to peck vigorously at the CS under ABB conditions.

The arcopallium in birds consists of the arcopallium anterius (AA), AD and AI. It projects to the striatum, the ventral pallidum and connects through the tractus occipito-mesencephalicus with the sensory and motor structures of the diencephalon, and the brain stem [43,82]. This connectivity pattern of the arcopallium suggests an essential role for sensorimotor control of various tasks [63]. Letzner et al. [83] demonstrated that a small arcopallial and amygdaloid cluster of neurons constitute the avian commissura anterior and enable interhemispheric exchange of sensorimotor and limbic information. Earlier evidence also indicated a role of arcopallium in learning processes. Lowndes and Davies [84] demonstrated that bilateral lesions of the arcopallium lead to an impairment of learning in avoidance tasks. Also, lesions of the arcopallium cause a learning deficit during training without showing any impairment in pecking accuracy in an aversive task [84]. Importantly, electrophysiological studies of Aoki et al. [51] provided evidence on diverse neural codes in the AI and AD for distinct aspects of sensorimotor transfer in a color discrimination in domestic chicks. Some arcopallial neurons selectively responded to the occurrence of the CS+, others fired for reward delivery, while a third group responded to the acoustic cue prior to movement onset. Taken together, arcopallial cells code for the full sensorimotor circle that represents a memorized CS-US association [51]. This is in full agreement with our findings that not the acquisition, but a motor output and the consolidation of previously learned sensorimotor associations was perturbed after blocking of arcopallial NMDARs. This differentiation could help to re-interpret the result patterns of some previous learning-related studies on the avian arcopallium (e.g. Lowndes and Davies [84]). In addition, our findings could spark new interest in the role of the mammalian motor areas during extinction learning.

Actually, the participation of the (pre)motor area in human has been shown in various neuroimaging studies on fear conditioning [93,94,85] and it is part of the fear network (see review Selhmyer et al. [86]). In a pain-related fear conditioning task with visual CSs and rectal distensions as pain inducing US, differential activation in response to the CS- was observed in the primary motor cortex during extinction and reinstatement [87]. In another pain anticipation study with visual CSs and uncomfortable air puff as US, activation in supplementary motor area was observed both during conditioning and extinction phase [88]. Similar results were also reported by LaBar et al. [89]. In an animal study with a rodent model, the motor cortex has been found to display elevated metabolism in relation to extinction of conditioned fear, indicating that the animals with higher neural activity in these areas were more successful at inhibiting their conditioned freezing response to tone during extinction [90]. Unfortunately, none of these studies can properly dissociate between motor and retrieval effects, but it is surely worth re-visiting the mammalian motor areas, including the motor cortices, in the context of extinction research with an improved behavioral design.

4.3. Dissociation between the avian amygdala and the arcopallium

As mentioned above, we have observed a functional double dissociation of the avian amygdala and the arcopallium with respect to different components of extinction behavior. Similarly, Saint-Dizier et al. [55] described a differential involvement of the PoA and the anterior arcopallium in aversive emotional processing in Japanese quail. Lesions damaging the PoA significantly increased fear responses, whereas lesions in the anterior arcopallium reduced the overall fear behavior [55]. In addition, Xin et al. [59] reported the distinctive functions of the domestic chick’s amygdala and the arcopallium in the social foraging process. Lesions of arcopallium, including the AI, and the lateral arcopallium, disturbed social facilitation of foraging efforts [59] and induced a handling-cost aversion in a binary choice task [91], whereas the amygdaloid nuclei, including the TnA and AM, were not involved [59]. Taken together, like its mammalian homologue, the avian amygdala plays a key role in extinction acquisition. The avian arcopallium is not involved in the process of learning the extinction task. It is, however, affected by the system’s changes pertinent to the establishment of extinction memory. Therefore, the blockade of arcopallial NMDARs during extinction learning can profoundly affect extinction memory retrieval.

5. Conclusions

To summarize, the current study demonstrated a double dissociated role of the avian amygdala and the (pre)motor arcopallium of pigeons for extinction learning in a within-subject appetitive conditioning paradigm. On the one hand, the NMDARs in the avian amygdala impaired the acquisition process of extinction learning but did not affect the consolidation and/or retrieval of the CS-(no)US association during extinction. This is in line with data obtained in rodent studies and further provides evidence for a significant role of amygdala both in appetitive and aversive conditions. On the other hand, the NMDARs of the arcopallium contributed to the motor aspects of the task as well as to the consolidation and/or expression of extinction memory indicating a critical role in associative learning. Our results broadened the view of the neural circuits underpinning extinction learning in the avian brain, a process that involves the NCL [23,26], the hippocampus [23], and also amygdala, and arcopallium. The similarities between our results and those from mammals indicate a shared neural mechanism underlying extinction learning shaped by evolution.

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