Neurobiology of Learning and Memory 158 (2019) 50–59

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Transient inactivation of the visual-associative nidopallium frontolaterale (NFL) impairs extinction learning and context encoding in pigeons

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ARTICLE INFO

Keywords:
Nidopallium frontolaterale
Visual-associative area
Extinction learning
Context encoding
Avian

ABSTRACT

Extinction learning is a fundamental learning process that enables organisms to continuously update knowledge about their ever-changing environment. When using visual cues as conditioned stimuli (CS), visual cortical areas of mammals are known to participate in extinction learning. The aim of the present study was to test whether similar processes can also be observed in birds. With pigeons as an animal model, we therefore investigated the role of the nidopallium frontolaterale (NFL), a key avian visual associative area, in an extinction learning task. We adopted a within-subject extinction task design with context manipulation, and tested the animals for extinction memory retention and renewal. Before extinction, the NFL was transiently inactivated by intracerebral tetrodotoxin (TTX) injections. Our data suggest that inactivation of NFL indeed produces a slowing of extinction learning. Importantly, NFL also plays a key role in context encoding, as indicated by an abolishment of the renewal effect. This is not due to an overall perceptual decrement, since the ability to distinguish between the different visual stimuli was unaltered, but might be caused by an impaired formation of the context-CS-configuration during extinction. Taken together, our experiment not only reveals similarities of neural substrates of extinction learning in birds and mammals, but also provides strong evidence for a specific contribution of the NFL in context encoding.

1. Introduction

Extinction learning refers to the ability to adapt to the changing situations through a learning process, in which the previously learned behavior is partly suppressed and partly erased (Bouton, Westbrook, Corcoran, & Maren, 2006; Rescorla, 2004). It usually involves a procedure in which organisms acquire a conditioned response (CR) towards a previously neutral conditioned stimulus (CS) after its repeated pairing with a biologically potent unconditional stimulus (US). When the CS is unexpectedly and repeatedly presented in the absence of the US, the previously established CR decreases. This process is the hallmark of extinction learning. Importantly, the reduction of the CR during extinction is not permanent and can be restored in many ways, such as by changing the context of stimulus presentation (renewal), or by testing the organisms after a time interval (spontaneous recovery). Numerous studies on the return of responding provide convincing evidence that extinction involves a formation of a new CS-NoUS association (Bouton et al., 2006), and in parallel, a partial erasure of the old memory trace (e.g. An et al., 2017; Rescorla, 2004).

In recent years, there has been increasing interest in the underlying neural mechanisms of extinction learning due to the awareness of its clinical relevance in several human psychopathologies, such as anxiety disorders, substance abuse and posttraumatic stress disorder. Thanks to the deciphering of fear-learning circuits during the 1980–1990s (Fendt & Fanselow, 1999; Ledoux, 2000), extensive knowledge is obtained in respect of the extinction network (Kim & Richardson, 2010; Pare & Duvarci, 2012) in rodent models on fear conditioning tasks, which comprises primarily the amygdala, the prefrontal cortex (PFC), and the hippocampus. During the acquisition of fear, the basolateral amygdala (BLA) receives input from the lateral amygdala (LA), forms the CS-US association and activates the central nucleus of the amygdala (CeA), which initiates the fear responses (Ciocchi et al., 2010; Fernando, Murray, & Milton, 2013; Haubensak et al., 2010; Marek, Strobel, Bredy, & Sah, 2013). After extinction training, activation from the infralimbic area (IL) within the PFC targeting the intercalated cells (ITC) in the amygdala (Milad & Quirk, 2012) produce a feedforward inhibition onto CeA, resulting in a reduced fear output (Amano, Duvarci, Popa, & Pare, 2011; Marek et al., 2013). Furthermore, the ventral part of hippocampus projects directly to the IL and BLA, and therefore is able to modulate the CR in different contexts (Hugues & Garcia, 2007; Inoue,

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https://doi.org/10.1016/j.nlm.2019.01.012
Received 25 October 2018; Received in revised form 7 January 2019; Accepted 15 January 2019
Available online 18 January 2019
1074-7427/

Kamiyama, Matsumoto, Yanagawa, & Hiraide, 2013).

Before the current focus of extinction research shifted to the amygdala-PFC-hippocampus network, the sensory cortices had received much attention as possible candidates to modulate memory retrieval of conditioned fear. Usually, fear conditioning involves the transmission of the CS and US information to the LA, which occurs in two ways in mammals. One is directed from the sensory thalamus to the LA (auditory: e.g. LeDoux, Farb, & Ruggiero, 1990; Ledoux, Ruggiero, & Reis, 1985; visual: e.g. Doron & Ledoux, 1999; Graybiel, 1972; Heath & Jones, 1971; Lin, May, & Hall, 1984; Pessoa & Adolphs, 2010) whereas the other involves multi-synaptic pathways from the sensory thalamus via the sensory cortex and then to the LA (auditory: e.g. Mascagni, McDonald, & Coleman, 1993; Romanski & Ledoux, 1993; visual: e.g. Graybiel, 1972; Heath & Jones, 1971; Lin et al., 1984; Pessoa & Adolphs, 2010). Interestingly, either pathway is sufficient for acquisition of conditioned fear (Boatman & Kim, 2006; Menzel, 2012; Romanski & LeDoux, 1992a; Song, Boatman, Jung, & Kim, 2010; Teich et al., 1989). However, the thalamo-cortico-amygdala pathway is suggested to be the principle pathway, because post-acquisition lesions in this pathway completely abolish the fear response during CS presentations, while lesions in the thalamo-amygdala pathway only partially reduce the fear reaction (Boatman & Kim, 2006).

In addition to the amygdala-PFC-hippocampus network, there is evidence that the sensory cortices could be an important storage site for fear memories. Indeed, lesions of the auditory cortex in fear extinction experiments prevent the occurrence of extinction to auditory stimuli and thus result in persistent fear responses (Song et al., 2010; Teich et al., 1989). The case for similar studies using the visual system are much more controversial. LeDoux, Romanski, and Xagoraris (1989) showed delayed fear extinction after conditioned lick suppression to a visual stimulus. In contrast, Falls and Davis (1993) reported no effect in the extinction of fear-potentiated startle when the visual cortex was ablated. One possible reason for the controversy lies in the different contexts used during the extinction training in the latter study (Falls & Davis, 1993), which will be discussed later. Nevertheless, these findings suggest some role of the sensory cortices in fear extinction, without being able to clearly specify in detail the contribution of these areas (LeDoux et al., 1989).

In order to uncover the variant and the invariant neural properties of extinction learning across classes (mammals vs. birds) and in different conditions (appetitive vs. aversive), we adopted pigeons as an animal model using an appetitive extinction task. Pigeons have the ability to work on large sets of visual stimuli in parallel and are highly sensitive to alterations of reward contingencies (Güntürkün, Stüttgen, & Manns, 2014). The last ancestor of todays' birds and mammals lived ca. 300 million years ago, so that members of these two vertebrate classes assumed a considerably different brain organization (Jarvis et al., 2005). There are a few brain regions that are one-to-one homologous to those of mammals, such as the hippocampus and some amygdalar nuclei. At the same time, there are also many non-homologous, but functionally equivalent structures, such as the nidopallium caudolaterale (NCL) which is comparable to the mammalian PFC (Güntürkün & Bugnyar, 2016; Waldmann & Güntürkün, 1993). Recently, studies with pigeons under appetitive conditions have begun to reveal the extinction network in the avian forebrain. These studies suggest that the avian hippocampus and the premotor arcopallium are important in the consolidation of extinction memory (Gao, Lengersdorf, Stüttgen, & Güntürkün, 2018; Lengersdorf, Stüttgen, Uengoer, & Güntürkün, 2014). Blocking the N-methyl-D-aspartate receptors (NMDARs) in the 'prefrontal' NCL and the amygdala resulted in impaired extinction acquisition (Gao et al., 2018; Lengersdorf, Marks, Uengoer, Stüttgen, & Güntürkün, 2015; Lissek & Güntürkün, 2003) without affecting consolidation of extinction memory (Gao et al., 2018; Lengersdorf et al., 2015). In addition, NMDARs in pigeon's NCL are also involved in contextual processing in a conditional discrimination task (Lissek & Güntürkün, 2005). Taken together, the avian neural network of extinction learning shows comparable characteristics to those in mammals, although both systems evolved independently since 300 million years.

In the present study, we examined the nidopallium frontolaterale (NFL), a visual associative area of the avian brain, which, according to studies with mammals (LeDoux et al., 1989), may also play a role in extinction learning. Similar to inferior temporal (IT) cortex in primates, the NFL is involved in more complex forms of visual processing, like motion and color processing (Stacho, Ströckens, Xiao, & Güntürkün, 2016), image categorization (Koenen, Pusch, Bröker, Thiele, & Güntürkün, 2016), and processing and memorizing of color cues during working memory tasks (Johnston, Anderson, & Colombo, 2017). It receives projections from the two visual processing pathways, the thalamofugal and tectofugal pathways, of the avian brain. Both of these two pathways have a relay-station in the visual thalamus, with projections running from there via the primary sensory areas, and ending in NFL and a few further associative visual structures (Mouritsen, Heyers, & Güntürkün, 2016). Anatomically, the NFL is reciprocally connected to the posterior amygdala (PoA; Atoji, Saito, & Wild, 2006; Kröner & Güntürkün, 1999) which resembles the mammalian lateral amygdala by its connectivity and neurochemistry (Atoji et al., 2006; Wynne & Güntürkün, 1995). This visual circuitry encompassing the thalamus-NFL-PoA in pigeons is therefore similar to the thalamo-cortico-amygdala pathway in mammals. However, there is no evidence for a direct anatomical projection from the visual thalamus to the avian amygdala, indicating no comparable visual circuitry in birds to the mammalian thalamo-amygdala pathway. Facing this anatomical pattern, the aim of the present study was to investigate whether the visual NFL in birds is involved in extinction learning. For this purpose, we transiently inactivated the NFL with tetrodotoxin (TTX) and adopted a within-subject sign-tracking design (Lengersdorf et al., 2014, 2015). By locally injecting TTX in the avian NFL, we were able to reveal a delayed extinction acquisition to a visual CS under appetitive conditions and an abolished renewal effect during retrieval of extinction memory.

2. Materials and methods

2.1. Subjects

25 adult homing pigeons (Columba livia) obtained from local breeders were used in the experiment. The animals were housed in individual wire-mesh home cages (40 × 40 × 45 cm) in a colony room. The temperature, humidity and the 12-h-light-dark circle were strictly controlled (lights on at 8 am). All animals went through food deprivation and maintained at 80–90% of their normal weight. They received additional free food on weekends. Water was provided ad libitum in their home cages. With approval by the national authorities of the state of North Rhine-Westphalia, Germany, the experiment was carried out strictly 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 (6 mm) stainless steel guide cannulas (Plastics One Inc., Roanoke, USA). For anesthesia, 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 injected i.m. with a dosage of 0.070 ml for each 100 g body weight. An additional application of Isoflurane anesthesia was applied (Forane 100% (V/V), Mark 5, Medical Developments International, Abbott GmbH & Co KG, Wiesbaden, Germany) through a mask to maintain the anesthetized state during surgery.

As soon as the animals no longer responded to stimulation, they were secured to a stereotaxic frame. Body temperature was maintained using a warming plate. With one incision in the skin, the skull was

exposed for the subsequent implantation. Two craniotomies were performed on both sides. One cannula per hemisphere was implanted vertically, targeting the NFL using the following coordinates based on the pigeon brain atlas (Karten & Hodos, 1967): AP + 12 mm, ML ± 5.5 mm, DV + 1.8 mm. Four stainless steel micro-screws (Small Parts, Logansports, USA) around each cannula were fixed to the skull as anchors prior to craniotomies. Finally, the application of dental cement secured the cannulas to the implanted positions. After 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 received 7-day recovery with free food and water access until two days before the behavioral training.

2.3. Behavioral apparatus

Behavioral training took place in four skinner boxes with similar shapes (36 × 34 × 36 cm), housed in sound-attenuating cubicles (80 × 80 × 80 cm). 6 W light bulbs or LED bands at the ceiling illuminated each Skinner box. 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 on the floor underneath the pecking key. One LCD flat screen monitor (either Belinea Model No.: 10 15 36 or Philips Model: Brilliance17S1/00) was secured behind the rear wall. The animals could see the stimulus through the pecking key as presented on the monitor screen. Every effective key peck produced a feedback sound.

The skinner boxes were grouped in two distinct contexts. Contexts were differentiated by differently colored wallpapers covering the rear and side walls (either with 2.5 cm wide vertical brown stripes spaced 5 cm apart on red background or marbling pattern on turquoise background) and additionally by different masking noise, either white or brown noise (80 dB SPL) in the training chamber. Four well-distinguishable visual stimuli were used in the experiment (see Section 2.4). The hardware was controlled by custom-written Matlab code (The Mathworks, Natick, MA) using the Biopsychology-Toolbox (Rose, Otto, & Dittrich, 2008)

2.4. Behavioral procedure

The detailed training procedure was described in Lengersdorf et al. (2014, 2015) and in Gao et al. (2018). Briefly, training consisted of five separate phases: pretraining I, pretraining II, conditioning, extinction and test. Except for extinction training, animals were trained with one session in each context, two sessions per day spaced 2 h apart (Fig. 2). Prior to extinction training sessions, animals were injected either with TTX or saline (described later), while conditioning and test were conducted drug free.

2.5. Pretraining I & II

Each session in the pretraining I consisted of 48 trials (Table 1), where a stimulus ("target") was presented for 5 s and followed by 3 s food reward with grain provided by the food hopper. The target was always rewarded regardless whether the pigeons responded or not. The inter-trial-interval (ITI) was 48 s. After achieving the learning criterion (consistent pecking response in 80% of the trials in both contexts on three consecutive days), the animals entered the pretraining II. Here, another control stimulus ("non-target") which was never rewarded, was introduced. Each session consisted of 24 trials of target and 12 trials of non-target presentations (5 s each) in both contexts. The ITI was reduced to 35 s. A minimum of 80% correct responses (pecking to the target and no pecking to the non-target) to both stimuli in both contexts were required to enter the conditioning phase. The "target" remained rewarded, while the "non-target" not rewarded, throughout the whole experiment. These stimuli were used to control for possible non-specific effects triggered by injection of a pharmacological substance.

2.6. Conditioning

In the conditioning phase, an additional CS was added to each context. CS-1 was added in context A and CS-2 in context B (Fig. 1 & Table 1). Both CS and target presentation were followed by 3 s of food reward. Each of the three stimuli (target, non-target and the corresponding CS) was presented 12 trials per session for a fixed presentation time of 5 s. Specifically, the number of conditioning sessions depended on how long the pigeons needed to achieve the learning criterion with 80% correct responses for all stimuli across three consecutive days.

2.7. Extinction

In order to examine the context-specificity of extinction learning, we adopted a within-subject ABA-ABB design, where the animals received the extinction training sessions for each CS in the opposite contexts. The extinction phase consisted of four days in total. The two extinction sessions were placed 48 h apart (Figs. 1 and 2). The pigeons received an extinction session in each context where the corresponding CS was no longer paired with food reward. Approximately 30 min before extinction training, the pigeons were microinfused bilaterally with either 1 µl of 10 ng/µl TTX dissolved in saline (Tocris Cookson Ltd. Bristol, United Kindom) or 1 µl pure saline (B. Braun Melsungen AG, Germany). After infusion, the injecting cannulas stayed in place for another 15 min to ensure a maximum absorbance of the injected substances by the brain tissue. The order of injections (TTX 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. Extinction sessions took place in the two contexts with one session in each context (Fig. 1): the CSs were presented without reward in the other context in which they had not been presented in the conditioning phase (Fig. 1 & Table 1). In each extinction session, animals received the corresponding CS- (24 trials), target (24 trials) and non-target (12 trials). Reward only occurred after presentations of the target. The order of contexts was randomized across subjects.

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).

Phase	Context	# Target (T)	# Non-target (NT)	# CS-1 or CS-2
Pretraining I	A	48 × T (+)	–	–
	B	48 × T (+)	–	–
Pretraining II	A	24 × T (+)	12 × NT (−)	–
	B	24 × T (+)	12 × NT (−)	–
Conditioning	A	12 × T (+)	12 × NT (−)	12 × CS-1 (+)
	B	12 × T (+)	12 × NT (−)	12 × CS-2 (+)
Extinction	A	24 × T (+)	12 × NT (−)	24 × CS-2 (−)
	B	24 × T (+)	12 × NT (−)	24 × CS-1 (−)
Test	A	12 × T (+)	12 × NT (−)	12 × CS-1 (−) & 12 × CS-2 (−)
	B	12 × T (+)	12 × NT (−)	12 × CS-1 (−) & 12 × CS-2 (−)

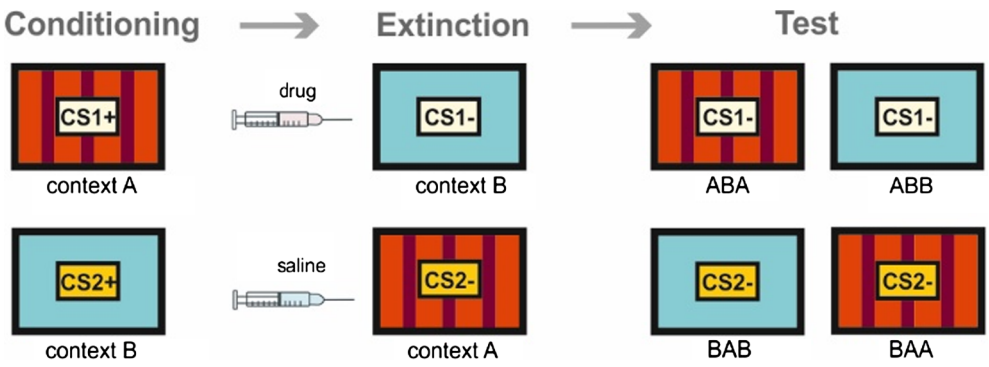

Fig. 1. Schematic representation of the within-subject design in the experiment. Pictures show rear walls of the two training chambers A and B. The squares with labels CS1 and CS2 indicate CS1 and CS2, respectively. The '+' indicates food reward and the '−' shows no reward. Not shown are the target stimulus (rewarded in all phases) and the non-target stimulus (never rewarded). In the experiment, contexts, stimuli and injection sequences were balanced across subjects. This figure shows only one possible example of (a) the sequence in which animals were exposed to contexts A and B, and (b) the sequence in which they received saline and TTX infusions, all of which were counterbalanced across animals.

2.8. Test

In the final testing phase, responses to all four stimuli were tested under drug-free conditions 48 h after the second extinction session (Fig. 2 & Table 1). Each stimulus was presented for 5 s and for 12 times in each context with 2 h between the two testing sessions. One session contained 48 trials in total, only target was rewarded.

Overall, this within-subject design (Fig. 1) allows each pigeon to be compared with itself for two different conditions with 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 the extinction memory retention can be tested 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 TTX or saline in the corresponding contexts. Therefore, the effect of TTX on extinction memory retention can be examined by comparing condition ABB and BAA within one pigeon. Also, by comparing ABA and BAB, it revealed how TTX affected the renewal.

2.9. Histology

Subsequent to the behavioral study, histology was conducted to verify whether the cannulas were positioned in the NFL. Before the perfusion procedure, animals were injected i.m. with 0.1 ml heparine (Rotexmedica GmbH, Trittau, Germany) dissolved in 0.1 ml of 0.9% NaCl to prevent blood clots. 15 min. later, equithesin (0.55 ml/100 g body weight) was injected i.m. for anesthetization. After the animal was nonresponsive to physical stimulation, the animal's circulatory system was transcardially flushed with 500 ml of 0.9% saline (40 °C). Subsequently animals were perfused with 1000 ml 4% paraformaldehyde (VWR Prolabo Chemicals, Leuven, Belgium). Then the brain was dissected and post-fixed for 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 (1967) were used to identify the positions of cannulas.

2.10. Data analysis

Response strength was assessed by counting the number of pecks on the pecking key. The main dependent variable was the pecking rate during 5 s stimulus presentation. We computed the mean response rates for target, non-target, CS_{TTX} and CS_{Saline}. Since different injections were conducted before extinction training, the two CSs were processed under different pharmacological conditions. Therefore, $CS\text{-}_{TTX}$ refers to the CS under the effect of TTX in extinction without food reward. $CS+_{TTX}$ is used to refer to the CS responses in conditioning phase and $CS\text{-}_{TTX}$ in testing phase. The same applies for CS_{Saline}, accordingly. We will use these terms when discussing results from the conditioning phase and retrieval tests to indicate each stimulus' treatment history, although neither saline nor TTX were infused prior to these training sessions in conditioning and testing.

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 subjects which failed to extinguish responding under the control saline condition.

The data from the last three training sessions in the conditioning phase were included for statistical analysis. The extinction session was rebuilt into six blocks with four consecutive trials constituting one block. Similarly, the test session was reconstructed into 3 blocks with four trials in one block. As mentioned in Section 2.4, data from ABA condition and BAB were pooled together and labeled as ABA for simplification purpose. And the ABB and BAA conditions were summarized

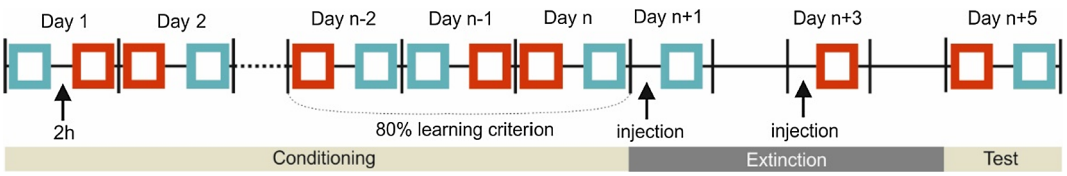

Fig. 2. Schematic representation of the training phase. This depiction shows only one possible example, and pretraining I and II are not included. Squares indicate a single training session in one corresponding context (depicted in blue or red). The black vertical bars separate one workday from the other. The black arrows on days n + 1 and n + 3 indicate the injections of different substances either TTX or saline 15 min before extinction training. In the conditioning phase, 2 sessions were 2 h apart on every workday. The conditioning phase was at least 6 days. The specific duration (n) depended on how long the pigeons needed to achieve the learning criterion. During the extinction phase on day n + 1 and n + 3, the animals were trained with one extinction session per day. There was no training on the day after the injection to ensure that injected agents were completely washed out. Subjects were tested in each context on day n + 5.

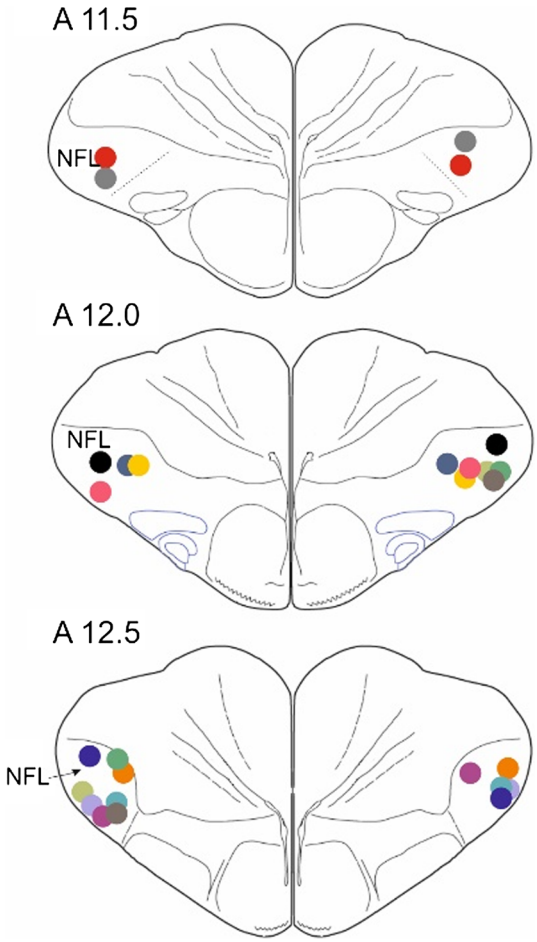

Fig. 3. Schematic sections of the pigeon brain showing TTX injection sites. Dots represent cannula tips. Identical colors represent the two cannulas of a single pigeon. There were 14 pigeons in the experiment. NFL: nidopallium frontolaterale. We adopted the brain atlas by Karten and Hodos (1967).

as ABB. Normal 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 the sphericity, the Greenhouse-Geisser and Huynh-Feldt correction was applied. Importantly, the post hoc tests were conducted in case of significant factor effects. For extinction phase, we also included simple linear regression to analyse the learning dynamics of extinction.

3. Results

3.1. Histology

In total, 25 pigeons participated in the experiment. 11 pigeons were excluded: 3 animals failed to learn the task; 8 pigeons had incorrect cannula implantations. Thus, data from the remaining 14 pigeons were used for analysis. All 14 pigeons had the cannulas implanted in the NFL in both hemispheres. The exact positions of the cannulas are depicted in Fig. 3.

3.2. Training histories

The pretraining I took on average 7.6 (± 3.0, standard deviation) days, while pretraining II took around 6.9 (± 3.7) training days to complete. The pigeons needed 10.6 (± 4.3) days of training on average in the conditioning phase to achieve the learning criterion.

3.3. Conditioning

In the last three sessions of conditioning, the mean response rates to the target (7.3 ± 1.0; mean ± sem), the $CS+_{TTX}$ (7.4 ± 11) and the $CS+_{saline}$ (8.0 ± 1.0; Fig. 4A) did not differ significantly between each other (paired sample *t*-test: target vs. $CS+_{TTX}$: $t_{(13)} = 0.1$, $p = 0.955$, Cohen's $d = 0.01$; target vs. $CS+_{saline}$: $t_{(13)} = 1.6$, $p = 0.130$, Cohen's $d = 0.17$; $CS+_{TTX}$ vs. $CS+_{saline}$: $t_{(13)} = 1.0$, $p = 0.353$, Cohen's $d = 0.15$; Fig. 4A). The mean response rates to non-target remained at zero (0.08 ± 0.02) at the end of conditioning.

3.4. Extinction

Two-way RMANOVA for both target and CS responding were conducted with two factors, block and injection (TTX or saline).

No effect of injection for the mean response to the target (two-way RMANOVA, $F_{(1, 13)} = 0.1$, $p = 0.719$, $\eta_p^2 = 0.01$; Fig. 4B) was observed, indicating that the response rates to target under TTX and saline did not differ from each other during extinction. Moreover, there was no block effect ($F_{(5, 65)} = 1.1$, $p = 0.344$, $\eta_p^2 = 0.08$). The target pecking response remained constant under both conditions across six blocks (one-way RMANOVA, $target_{saline}$: $F_{(5, 65)} = 1.8$, $p = 0.121$, $\eta_p^2 = 0.12$; $target_{TTX}$ with Greenhouse-Geisser correction: $F_{(2.3, 30.0)} = 0.7$, $p = 0.515$, $\eta_p^2 = 0.05$; Fig. 4B). In addition, there was no significant injection × block interaction ($F_{(5, 65)} = 1.1$, $p = 0.385$, $\eta_p^2 = 0.07$). The results imply that responses of the pigeons to the target were not affected by injections and stayed constant across the six blocks during extinction. Thus, this control stimulus helped us to ensure that TTX in NFL caused no motor deficits.

For responding to the non-target, two way RMANOVA (injection × block) indicated no effect of injection ($F_{(1, 13)} = 3.2$, $p = 0.099$, $\eta_p^2 = 0.20$), no block effect (Greenhouse-Geisser correction, $F_{(1.0, 13.4)} = 3.8$, $p = 0.071$, $\eta_p^2 = 0.23$) and no interaction (Greenhouse-Geisser correction, $F_{(1.0, 13.9)} = 2.7$, $p = 0.121$, $\eta_p^2 = 0.17$). The response rates to non-target remained close to zero both for saline (mean ± sem, 0.06 ± 0.03) and TTX (0.48 ± 0.26) conditions throughout extinction sessions.

For pecking responses to the CS, there was a strong effect of block (two-way RMANOVA, Greenhouse-Geisser correction, $F_{(2.7, 35.2)} = 18.3$, $p < 0.001$, $\eta_p^2 = 0.58$; Fig. 4B). Specifically, the CS-related pecking response dropped significantly both with saline (one-way RMANOVA, Greenhouse-Geisser correction, $F_{(2.5, 32.4)} = 21.162$, $p < 0.001$, $\eta_p^2 = 0.62$) and TTX ($F_{(5, 65)} = 4.8$, $p = 0.001$, $\eta_p^2 = 0.27$; Fig. 4B). Consequently, no effect of injection was found (two-way RMANOVA, $F_{(1, 13)} = 0.004$, $p = 0.950$, $\eta_p^2 < 0.01$). However, there was a significant interaction of injection × block ($F_{(5, 65)} = 3.2$, $p = 0.013$, $\eta_p^2 = 0.20$), pointing to a shallower extinction process in the TTX condition (Fig. 4B). Subsequent post hoc tests revealed medium to large effect sizes of the $CS\text{-}_{saline}$ and $CS\text{-}_{TTX}$ differences in the first ($CS\text{-}_{saline} > CS\text{-}_{TTX}$, $p = 0.085$, $\eta_p^2 = 0.21$), second ($CS\text{-}_{saline} > CS\text{-}_{TTX}$, $p = 0.114$, $\eta_p^2 = 0.16$) and sixth ($CS\text{-}_{saline} < CS\text{-}_{TTX}$, $p = 0.097$, $\eta_p^2 = 0.20$) blocks. In addition, in order to check whether the significant interaction between injection and block was caused by the different decreasing rate of responding under the saline and the TTX conditions, a simple linear regression was calculated to predict the CS-responding under saline and TTX conditions based on the factor block. A significant regression was found for saline condition with a slope of −1.53 ($R^2 = 0.375$, $F_{(1, 82)} = 49.2$, $p < 0.001$) and for TTX with a slope of −0.77 ($R^2 = 0.092$, $F_{(1, 82)} = 8.3$, $p = 0.005$). The slopes of linear regression lines for the $CS\text{-}_{TTX}$ were smaller compared to that for the $CS\text{-}_{saline}$ (paired *t*-test: $t_{(13)} = -3.4$, $p = 0.005$, Cohen's $d = 0.97$). In order to rule out the possibility that this significant effect might be induced by the higher pecking rate of $CS\text{-}_{saline}$ in comparison to $CS\text{-}_{TTX}$ in the first two blocks of extinction, the linear regression analysis was conducted with data from blocks 3 to 6. Results suggested a significant regression for $CS\text{-}_{saline}$

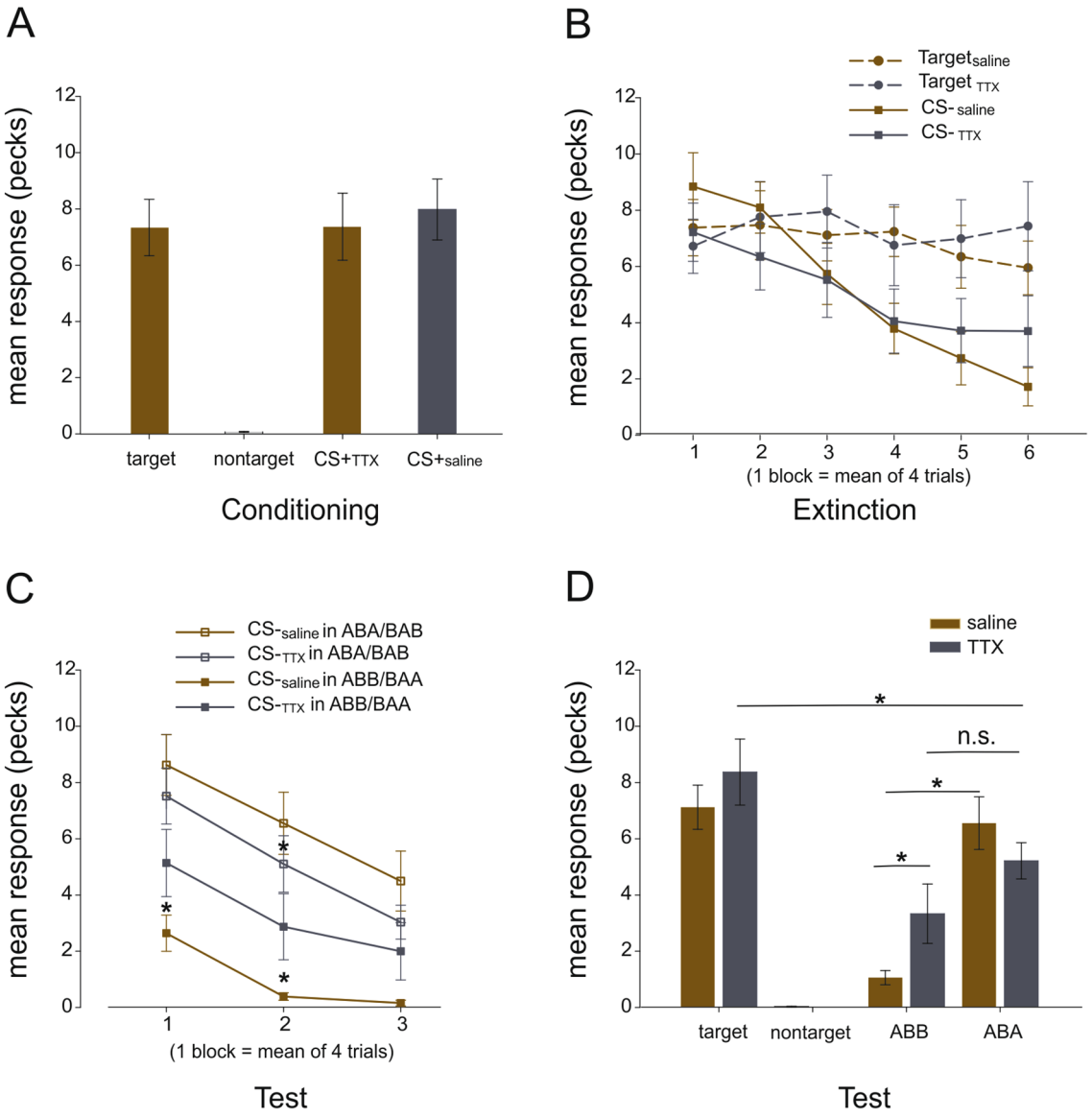

Fig. 4. Mean response rates for different stimuli in different experimental phases (N = 14). Y axis indicates the mean number of pecks during the 5 s stimulus presentation. (A) Mean response rates (± sem) for the three stimuli were calculated in the last three conditioning sessions. (B) Mean response rates (± sem) of the target and CS are shown for the six blocks under TTX (dark blue) and saline (brown) in extinction. Dashed and solid lines indicate target and CS, respectively. (C) Mean response rates (± sem) of CS are depicted for the three blocks under TTX (dark blue) and saline (brown) in the test. Solid lines with full-squares are the ABB/BAA condition while the solid lines with empty-square refer to responses to the CS in ABA/BAB. For simplification purposes, the ABB and BAA conditions were summarized as ABB, while the ABA and BAB was simplified as ABA. (D) Mean response rates (± sem) for the stimuli through all three blocks in the test. brown and dark blue indicates saline and TTX, respectively.

with a slope of −1.31 (R^2 = 0.162, $F_{(1, 54)}$ = 11.5, p = 0.001) and a non-significant regression line for TTX condition with a slope of −0.58 (R^2 = 0.005, $F_{(1, 54)}$ = 1.3, p = 0.268). The lack of significance for the TTX condition indicated that the responding to $CS\text{-}_{TTX}$ was random and did not follow a deceasing or increasing trend. Then we computed for each animal the slopes for $CS\text{-}_{saline}$ as well as for $CS\text{-}_{TTX}$. And in comparison to the CS-responding under saline, the slopes for the $CS\text{-}_{TTX}$ were smaller, with a moderate to large effect size and a trend for statistical significance (paired *t*-test: $t_{(13)}$ = −2.1, p = 0.057, Cohen's d = 0.61). In a nutshell, response rates to $CS\text{-}_{saline}$ were higher but not significant in the first two blocks, and then decrease faster than the $CS\text{-}_{TTX}$. This resulted in a lower tendency in pecking rate to $CS\text{-}_{saline}$ than to $CS\text{-}_{TTX}$ at the end of extinction. Taken together, the significant interaction effect indicated a slower decrease of responding to CS under TTX conditions, meaning a TTX-induced decrement of extinction learning.

In order to rule out a visual deficit due to the NFL inactivation, we also compared the pecking response to CS- with that to the target under both TTX and saline conditions. Two-way RMANOVA with the factor stimuli (CS- vs. target) and blocks revealed a significant effect of stimuli for the TTX (two-way RMANOVA: $F_{(1, 13)}$ = 10.6, p = 0.006, η_p^2 = 0.45) and the saline condition ($F_{(1, 13)}$ = 6.6, p = 0.024, η_p^2 = 0.34). The results indicated that the animals could clearly differentiate between target and CS- under the effect of both saline and TTX injections. Since the pecking to non-target was zero, animals clearly differentiated well also between CS- and non-target. Therefore, TTX injection in the NFL did not induce any visual deficit in stimulus discrimination.

3.5. Retrieval test

In the test phase, there were no differences between response rates

to $target_{saline}$ and $target_{TTX}$ (paired sample *t*-test: $t_{(13)} = 1.5$, $p = 0.162$, Cohen's $d = 0.35$; Fig. 4D), ruling out any residual motor effects. The pecking rates to non-target were at zero under conditions of saline (mean ± sem: 0.02 ± 0.01) as well as TTX (0.01 ± 0.01) and did not differ from each other ($t_{(13)} = 1.4$, $p = 0.190$, Cohen's $d = 0.15$).

For the mean response rates to the CS, a three-way RMANOVA with the factors of injection (TTX or saline), context (ABA and ABB) and block was conducted (Fig. 4C). The analysis indicated significant effects of context ($F_{(1, 13)} = 48.9$, $p < 0.001$, $\eta_p^2 = 0.79$; Fig. 4C), and block ($F_{(2, 26)} = 25.8$, $p < 0.001$, $\eta_p^2 = 0.67$), but not of injection ($F_{(1, 13)} = 1.7$, $p = 0.218$, $\eta_p^2 = 0.11$; Fig. 4C). In addition, a significant interaction was observed only between context and injection ($F_{(1, 13)} = 6.9$, $p = 0.021$, $\eta_p^2 = 0.35$). For the others, no significant effects were found (context × block: $F_{(2, 26)} = 2.6$, $p = 0.091$, $\eta_p^2 = 0.17$; injection × block: $F_{(2, 26)} = 0.2$, $p = 0.788$, $\eta_p^2 = 0.02$; context × injection × block: $F_{(2, 26)} = 0.1$, $p = 0.897$, $\eta_p^2 < 0.01$). The results indicated that pigeons responded to a significant extent differently to the stimuli in different contexts during testing.

Subsequently, follow-up tests revealed how pigeons responded in different contexts. Because of the training histories, the animals were expected to respond more in the conditioning context (ABA) than in the extinction context (ABB). This is known as the renewal effect. Accordingly, we observed significant renewal for the CS extinguished under saline (paired sample *t*-test, $CS\text{-}_{saline}$ in ABB vs. ABA: $t_{(13)} = -7.2$, $p < 0.001$, Cohen's $d = 2.23$; Fig. 4D), but not for the CS extinguished under TTX ($CS\text{-}_{TTX}$ in ABB vs. ABA: $t_{(13)} = -2.0$, $p = 0.071$, Cohen's $d = 0.60$; Fig. 4D). The absence of renewal for the pecking responses to $CS\text{-}_{TTX}$ indicates a possible interference of the retrieval of the conditioning context due to TTX injection prior to extinction.

We then carried out separate analyses for ABA and ABB conditions to further scrutinize this TTX effect. In the extinction context (ABB), the animals responded significantly more to $CS\text{-}_{TTX}$ than to $CS\text{-}_{saline}$ ($t_{(13)} = 2.6$, $p = 0.021$, Cohen's $d = 0.82$; Fig. 4D). In the conditioning context (ABA), however, pigeons responded equally but with a tendency of responding less to $CS\text{-}_{TTX}$ than to $CS\text{-}_{saline}$ ($t_{(13)} = 2.0$, $p = 0.071$, Cohen's $d = 0.46$). We also conducted follow-up tests for the individual blocks during testing. In the ABA condition in the second block a significant higher pecking response to $CS\text{-}_{saline}$ was found (ABA, $p = 0.041$, $\eta_p^2 = 0.28$) but not in the first and third blocks (1st block: $p = 0.356$, $\eta_p^2 = 0.07$; 3rd block: $p = 0.205$, $\eta_p^2 = 0.12$). In the ABB condition, we observed a higher response rate to $CS\text{-}_{TTX}$ than $CS\text{-}_{saline}$ in all blocks with the exception of the last block, when response rates approached 0 (1st block: $p = 0.007$, $\eta_p^2 = 44$; 2nd block: $p = 0.036$, $\eta_p^2 = 0.30$; 3rd block $p = 0.080$, $\eta_p^2 = 0.22$). To sum up, in the extinction context (ABB), animals generally responded more to the $CS\text{-}_{TTX}$ than to $CS\text{-}_{saline}$. On the contrary, animals responded equally to the two CSs in the conditioning context (ABA), which led to the absence of renewal after TTX injection.

To examine whether the higher responses to $CS\text{-}_{TTX}$ than $CS\text{-}_{saline}$ in ABB testing was a result of delayed extinction learning or deficits in memory consolidation, we compared the CS responses in the last block of extinction with that in the first block of the test in the extinction context (ABB). Results indicated no significant changes of pecking response to $CS\text{-}_{TTX}$ (paired sample *t*-test: $t_{(13)} = 1.3$, $p = 0.224$, Cohen's $d = 0.32$) and to $CS\text{-}_{saline}$ ($t_{(13)} = 1.3$, $p = 0.225$, Cohen's $d = 0.38$; Fig. 4B and C), implying that the pigeons responded equally from the end of extinction to the beginning of testing in the extinction context two days later. Therefore, the consolidation of the extinction memory was not affected by the TTX injection. Thus, the response difference between $CS\text{-}_{TTX}$ and $CS\text{-}_{saline}$ in ABB, both in individual blocks and across all blocks, was due to the impaired extinction acquisition under TTX which resulted in an incompletely extinguished CR to $CS\text{-}_{TTX}$.

Finally, to detect possible ceiling effects for renewal, responses to CS in ABA was compared with target pecking for both TTX and saline conditions (Fig. 4D). There were no significant differences between $CS\text{-}_{saline}$ and $target_{saline}$ (paired sample *t*-test, $t_{(13)} = 0.2$, $p = 0.428$, Cohen's $d = 0.18$; Fig. 4D), although responding to the former was not reinforced in the testing session. However, a significant difference between $CS\text{-}_{TTX}$ and $target_{TTX}$ ($t_{(13)} = 3.89$, $p = 0.009$, Cohen's $d = 0.92$; Fig. 4D) was observed, indicating no ceiling effect for $CS\text{-}_{TTX}$ in ABA but a ceiling effect for $CS\text{-}_{saline}$ with a small effect size when the pigeons were tested in the conditioning context (ABA).

4. Discussion

The present study investigated the role of the NFL, a key avian visual associative area, in extinction learning. We pharmacologically inactivated the NFL with TTX during extinction. Our results showed a slower decrease in pecking rate to $CS\text{-}_{TTX}$ in comparison to $CS\text{-}_{saline}$ during extinction, which indicate a moderate deficit in extinction learning after transient NFL inactivation. There was no interference with subsequent memory consolidation induced by TTX, which can be observed in the comparable response rates to $CS\text{-}_{TTX}$ in the end of extinction and in the start of test. Most importantly, TTX-injections into NFL abolished the renewal effect with no significant differences in pecking to the $CS\text{-}_{TTX}$ between ABA and ABB conditions, which possibly indicates that under normal conditions NFL is part of a system that codes and memorizes the context during extinction. We will now discuss these points, one by one.

4.1. NFL participates in extinction learning

NFL inactivation slowed down the pace of extinction learning (Fig. 4B), which can be observed through a significant interaction effect as well as a shallower slope of the decrease in pecking to the $CS\text{-}_{TTX}$ than $CS\text{-}_{saline}$ during extinction. Unexpectedly, we also observed a trend of lower responding to the $CS\text{-}_{TTX}$ than the $CS\text{-}_{saline}$ in the beginning of extinction. This could be caused by the improper transfer of CS-responding to a different context. Similar observations of a delayed extinction were also reported by Song et al. (2010) after auditory cortex lesions and by LeDoux et al. (1989) after visual cortex lesions (LeDoux, Romanski, & Xagoraris, 1989). These results and our findings contrast with those reported by Falls and Davis (1993) who did not report any interference of visual cortex lesions on learning an extinction to a visual CS during a fear-potentiated startle test. Falls and Davis (1993) argued that the involvement of visual cortex during extinction depends on the demand for visual discrimination of subtle contextual cues. For subsequent renewal to occur, the animal is required to discriminate the conditions under which the CS will or will not be followed by the US (Bouton, 2004). Thus, well-distinguishable contextual cues help the animals to discriminate between these conditions (e.g. Bouton & Bolles, 1979a, 1979b; Bouton & King, 1983; Swartzentruber & Bouton, 1986). In the absence of salient contextual visual cues, the animal is forced to make efforts to detect subtler details, during which a fully functional visual system is required. In rodents, the extrageniculate visual system can partly compensate the lesion-induced loss of primary visual cortex functions (Legg & Cowey, 1977; Tohmi, Meguro, Tsukano, Hishida, & Shibuki, 2014). This could explain the absence of effects of lesioning the primary visual cortical in the study of Falls and Davis (1993), who conducted their conditioning and extinction training in novel and easily distinguishable contexts. This is different to our study, where both contexts were equally familiar to the pigeons and had acquired the same associative learning histories during initial training (Lengersdorf et al., 2015, 2014; Rescorla, 2008). Importantly, each context was supposed to modulate only the corresponding CS-US association, requiring the formation of separate conditional relations between familiar contexts and familiar CS but with different rewarding contingencies. The complexity of these kinds of associations seems to require in pigeons a higher-order visual associative area like the NFL. As a result, inactivating the NFL resulted in a prolonged acquisition of extinction.

The more than 300 million years since the partition of extant birds

and mammals has produced a mixture of both similarities and differences in their neural systems. In mammals, fear conditioning involves the direct thalamo-amygdala and the thalamo-cortico-amygdala routes that transmit sensory information about relevant stimuli to the amygdala. Probably due to the direct input from the visual/auditory thalamus to the lateral amygdala, mammals can compensate lesions of primary and associative visual/auditory cortices in fear conditioning tasks (Boatman & Kim, 2006; Falls & Davis, 1993; LeDoux, Romanski, & Xagoraris, 1989; Romanski & LeDoux, 1992a, 1992b). In birds, however, there is yet no clear evidence for a direct visual thalamo-amygdala projection (for PoA: Atoji et al., 2006; for nucleus taeniae of amygdala: Balthazart & Absil, 1997; for subpallial amygdala: Wild, Arends, & Zeigler, 1990). Thus, the cortex-equivalent visual areas seem to be necessary to relay CS and US information to the amygdala in both the thalamofugal and the tectofugal pathways. NFL has reciprocal projections with PoA (Atoji et al., 2006), and projects to both the 'prefrontal' NCL (Kröner & Güntürkün, 1999) and the striatum (Veenman, Wild, & Reiner, 1995). Since previous experiments with birds indicated that the NCL (Lengersdorf et al., 2015), the amygdala (Gao et al., 2018), and the striatum (Gao, Pusch, & Güntürkün, in prep) are key structures in encoding of extinction memory, this connectional pattern of the NFL may also contribute to its relevance for acquisition of the extinction memory.

4.2. NFL participates in context encoding during extinction

Transiently inactivating the NFL during extinction abolished subsequent renewal. This effect was not mediated by a general visual deficit during extinction, since the animals could still perfectly differentiate between target, non-target and CS and acted accordingly during extinction. We therefore assume that the abolishment of renewal was caused by an impaired integration of contextual cues into extinction memory, rendering extinction less context-specific. It is conceivable that, without a functional NFL, the pigeons were only able to attend to the specific visual CS- at the expense of memorizing the visual extinction context. As a result, pecking to the $CS-_{TTX}$ in the extinction context (ABB) during testing was perceived as equal to the conditioning context (ABA) (Fig. 4D). Thus, context changes lost their ability to drive the retrieval of specific extinction memories. This finding implies that the associative visual NFL was especially involved in encoding and memorizing the association and/or configuration of visual contextual cues with visual CSs (Pearce, 1994). In contrast, the association of visual CSs with the presence or absence of reward could be processed independently in other neural structures.

With regard to the mechanisms underlying renewal following extinction, Rescorla and Wagner (1972) assumed that, according to the presence or absence of the US, the contexts of conditioning and extinction acquire direct excitatory and inhibitory properties, respectively. This inhibitory nature of extinction context prevents the CS from a complete loss of its excitatory associative strength. Therefore, renewal occurs because of the remaining excitatory strength of the CS in the absence of the inhibitory extinction context. Later, Pearce's configural theory (1994) postulated that a specific combination of context and CS results in one unitary representation that develops an association to the US. According to Pearce, both conditioning and extinction are therefore context-specific. Since the combination of conditioning context and CS was associated with the presence of US, it should result in responding to the CS during memory retrieval testing in the conditioning context, hence the renewal effect. More recently, Bouton (2004) proposed that extinction involves a second-learned inhibitory CS-NoUS association that contains information on the extinction context. Thus, retrieval of extinction memory implies the retrieval of context information, while the first-learned excitatory association is context-independent.

In the present experiment, the two contexts have the same learning histories (Rescorla, 2008; see also Lengersdorf et al., 2014, 2015), and the excitatory and inhibitory properties of contexts are therefore equal. However, we still observed the ABA renewal due to context changes under normal saline conditions. This speaks against the Rescorla-Wagner model. Furthermore, Starosta et al. (2016) and Starosta, Bartetzko, Stüttgen, and Güntürkün (2017) used a similar paradigm as in the present study and tested whether the conditioning and extinction contexts can be specifically encoded in the memory traces formed during different training stages. Their results suggest that both conditioning and extinction can be context-specific, which also opposes Bouton (2004) but endorses Pearce (1994). It is highly likely that Pearce's configural theory also operates as a key mechanistic explanation of our finding. In the present study, NFL possibly participated in encoding information of the context and of the CS into a specific configuration. Depending on the associative value of the context-CS configuration with the US, which was established across different training phases, NFL is also able to influence indirectly the response selection during testing for memory retrieval in different contexts. Transiently inactivating the NFL with TTX may have disturbed the formation of this configuration during extinction, which lead to the impaired memory retrieval in the two contexts at test thus resulting in an abolished renewal effect.

5. Conclusion

In conclusion, we provide experimental data able to show that the visual processing area NFL is involved in extinction acquisition and plays a crucial role in context encoding during extinction. The functionality of NFL in context encoding might operates through integrating the context and CS into a specific configuration and associating this configuration with the occurrence or absence of the US, thus modulating memory retrieval in different test conditions. Taken together, our experiment not only reveals similarities of neural substrates of extinction learning in birds and mammals, but also provides strong evidence for a specific contribution of the NFL in context coding.

Acknowledgments

This work was funded by the German Research Foundation [FOR 1581] and [SFB 1280]. The funding agency had no role in study design, collection, analysis or interpretation of the data, in writing the manuscript or the decision to submit the paper for publication. The authors also declare no conflict of interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2019.01.012.

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