Function of the pigeon nidopallium caudolaterale and hippocampus in context-dependent extinction learning under appetitive conditions.

Inaugural–Dissertation
zur
Erlangung des Grades eines Doktors der Naturwissenschaften
in der
Fakultät für Psychologie
der Ruhr–Universität Bochum

vorgelegt von

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Oktober 2014
“My first extinction curve showed up by accident. A rat was pressing the lever in an 
experiment on satiation when the pellet dispenser jammed. I was not there at the 
time, and when I returned I found a beautiful curve. The rat had gone on pressing 
although no pellets were received. [...] The change was more orderly than the 
extinction of a salivary reflex in Pavlov's setting, and I was terribly excited. It was a 
Friday afternoon and there was no one in the laboratory who I could tell. All that 
weekend I crossed streets with particular care and avoided all unnecessary risks to 
protect my discovery from loss through my accidental death.”

(Skinner, 1980)
Abstract

The majority of experiments investigating mechanisms of fear conditioning in rodents explore context-dependent extinction learning. Since mechanisms of appetitive and aversive learning seem to differ to some extent at the neuronal level, I sought to investigate extinction learning in an appetitive setting. Working with pigeons, I established a within-subject ABA renewal paradigm based on Rescorla (Q J Exp Psychol 61:1793) and combined it with pharmacological interventions during extinction. From the fear conditioning literature, it is known that both prefrontal cortex and the hippocampus are core structures for context-specific extinction learning. Accordingly, in the first set of experiments I transiently inactivated the nidopallium caudolaterale (NCL, a functional analogue of mammalian prefrontal cortex) and the hippocampus in separate experiments by intracranial infusion of the sodium-channel blocker Tetrodotoxin (TTX) immediately before extinction training. TTX in both structures non-specifically suppressed conditioned responding, as revealed by a reduction of response rate to both the extinguished conditioned stimulus and a control stimulus which remained reinforced throughout the experiment. Furthermore, TTX during extinction training impaired later extinction retrieval under drug-free testing conditions for spontaneous recovery. In a second set of experiments I assessed the role of N-methyl-D-aspartate receptors (NMDARs) for extinction learning in the NCL. I locally antagonized NMDARs through 2-Amino-5-phosphonovalerianacid (APV) or stimulated with D-cycloserine (DCS) during extinction learning in the established within-subject paradigm. APV-injection slowed extinction learning and disinhibited responding to a continuously reinforced control stimulus. Importantly, slowed extinction learning occurred independently of this concomitantly observed disinhibition of conditioned responding, as assessed through comparison with the non-extinguished control stimulus. However, context-dependent extinction retrieval was not affected. Contrary to expectations, administration of DCS did neither affect extinction learning nor extinction memory retrieval. In summary, the effects of APV as well TTX in the NCL or the hippocampus resemble those observed in studies of fear extinction with rodents, suggesting common neural substrates of extinction under both appetitive and aversive conditions and highlighting the similarity of mammalian prefrontal cortex and the avian caudal nidopallium despite 300 million years of independent evolution.
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1. Introduction

1.1. Extinction learning

A definition of extinction

Animals are confronted with an ever-changing environment and have thus evolved the ability to continuously learn and remember. However, an acquired association that was valid in the past might not hold anymore today. Therefore, learning also involves the ability to extinguish memorized associations. Generally, learning is initialized if a neutral conditioned stimulus (CS) is paired repeatedly with an unconditioned stimulus (US) and a conditioned response (CR) is established (Pavlov, 1927). This is called the ‘acquisition phase’ (Fig. 1). If the CS then occurs repeatedly alone the CR vanishes. This phenomenon is called ‘extinction’ (Fig.1). Extinction research was already conducted by Skinner (1980) and Pavlov (1927) and is nowadays conducted by a huge community of investigators. Since the 1960s, the number of publications about extinction increased exponentially (Delamater and Westbrook, 2014). This is due to the growing interest in the behavioral and neural processes of extinction learning, partly owing to the use of extinction in behavior therapy. The arguably biggest challenge of extinction research is to solve the problem of relapse – the reoccurrence of conditioned responding after extinction is completed (Fig. 1). Scientific findings suggest an important role of extinction in the treatment of human psychopathologies like anxiety disorder or drug addiction (Milad and Quirk, 2012; Rauch et al., 2006; Ressler and Mayberg, 2007). Therefore, making extinction memory persistent is one major goal of contemporary extinction research.
Fig. 1: Principle of acquisition, extinction learning and memory retrieval. During initial acquisition the response rate (e.g. freezing, lever-pressing for reward) increases. If the CS occurs frequently without the US, responses vanish. Subsequently, extinction memory is consolidated. Testing under distinct conditions (e.g. context change, single US presentation) could either generate low response recovery due to persistent extinction retrieval or behavioral reappearance of the previous response rate, that is, a failure to retrieve extinction memory (adapted from Quirk and Mueller, 2008).

**Extinction is context-dependent: context-CS-US (Rescorla) vs. CS-context-US (Bouton)**

Human as well as animal behavior is highly context-dependent, a fact which challenges efforts to make extinction memory persist. Context consists of exteroceptive as well as interoceptive factors. In animal experiments, conditioning chambers serve as distinct contexts. In an exteroceptive way they differ in visual, tactile or olfactory characteristics. Interoceptive contexts can include a drug-induced state, hormonal state, mood state, deprivation state, recent events, expectation of events or even time. It is clear that extinction is a novel learning event and does not erase the old trace in its entirety (Bouton, 2004; Pavlov, 1927). There are two predominating theories about how the extinction context controls extinction. In the first view, context directly indicates the conditions of CS-US association or CS-noUS (Rescorla, 1972). The theory, based on the Rescorla-Wagner-Model, holds that the discrepancy between the actual (US omission) and the predicted (US presentation) outcome produces a negative prediction error and induces a reduction of the associative strength between CS and US. That means that extinction learning is “unlearning”. However, as already recognized in Rescorla’s and Wagner’s seminal article (1972), this view is incomplete at best, since the extinguished response is prone to reappear, for example if it is tested in another context. The second (and
more common) point of view posits that the context operates as a “(negative) occasion setter”, and that the presence of the occasion setter is necessary to retrieve extinction memory (Bouton, 2004; Bouton and Ricker, 1994). Put differently, the extinction context acts as a gate which enables the CS to inhibit the CS-US association (Bouton and Nelson, 1994), thereby “setting the occasion” for the CS-US association (Bouton and Todd, 2014; Holland, 1992).

*The big three phenomena: Extinction is not forgetting*

The context specificity of extinction learning reveals that extinction is not forgetting and does not erase old memories. Three phenomena underscoring this claim are renewal, spontaneous recovery and reinstatement. If the CS is tested outside the extinction context, conditioned responding to the CS reappears – the CR is “renewed” (Bouton and Bolles, 1979). Testing in the initial acquisition context produces the greatest renewal effect. In cases where the test context is identical to the extinction context, testing after a period of time also reveals the return of conditioned responding – a phenomenon termed ‘spontaneous recovery’ (Pavlov, 1927). Responding during renewal testing is therefore an additive effect of spontaneous recovery and renewal because in most experiments, time elapses and context changes before retrieval is assessed. Reinstatement involves a reminder US presentation prior to the test phase. Subsequently, subjects show increased conditioned responding (Rescorla and Heth, 1975).

These different reasons for relapse clearly illustrate the interest in context dependent extinction learning. Psychopathological diseases often originate from inflexible, rigid and inappropriate behavior due to deficient interpretation of contextual information. For patients suffering from post-traumatic stress disorder, schizophrenia, depression or substance abuse disorder, for example, it would be desirable to control or even erase previously learned painful associations in any context. Here, extinction-based behavioral therapies and exposure therapies offer appropriate treatment (Milad and Quirk, 2012; Rauch et al., 2006; Ressler and Mayberg, 2007). Psychotherapeutic therapies aim at producing long-lasting and persistent extinction training to avoid relapse. Typically, the patient seeks a therapist, undergoes therapy, and subsequently returns into the environment where previous CS-US association took place or time merely elapses. In general, these are the most dangerous situations for
a patient to show a relapse, and this fact severely limits the efficacy of extinction-based behavioral treatment.

Vervliet (2008) reviewed a range of behavioral approaches to inhibit the recovery of the original memory trace. As might be expected, the number and/or time point of extinction trials and extensive extinction context training improve treatment success. Compound extinction training of previously extinguished CSs as well as ‘retrieval cues’ ensures extinction and spontaneous recovery could be avoided by spacing extinction trials out over a period of time. Human exposure therapy successfully applies D-cycloserine (DCS) to facilitate extinction (acrophobia, social anxiety) and prevent relapse (Hofmann et al., 2006; Ressler et al., 2004). DCS treatment is FDA-approved and previously served as medication for tuberculosis. Acute treatment has only mild side effects.

This indeed motivates scientists to extend the investigation of the neural foundation of extinction learning. Behaviorally based knowledge of extinction learning is now widely understood, making the underlying neural mechanisms of context-specific extinction learning even more relevant. Here, three principal approaches are available: Manipulation before extinction training to influence extinction learning directly, after training to influence extinction memory consolidation, or before testing to influence the retrieval process. Overall the original CS-US memory has to be weakened and the ‘new’ CS-noUS association has to be consolidated persistently and independently of context exposure.

1.2. Neural results in mammals

The neurobiological investigation of extinction learning began in the 1990s. The understanding of the behavioral and the neural basis of extinction learning relies mostly on rodent data obtained in fear conditioning procedures (Quirk and Mueller, 2007).

In the following paragraphs, I outline the general neural network for fear extinction memory before I highlight differences to appetitive extinction learning. As mentioned, extinction has an inhibiting function on the previous CS-US-association. Therefore, scientists search for an underlying inhibition network. Extinction is a learning process, and plasticity changes neural circuitries. Neurons constantly adapt their physiology
and their molecular structures. In the field of fear conditioning, three main brain structures play key roles: the amygdala, the prefrontal cortex (PFC) and the hippocampus (Milad and Quirk, 2012).

The amygdala

The amygdala is important for fear expression. Primary sensory cortical fibers project to the basolateral amygdala (BLA) or the intercalated cells (ITC). The BLA is divided into lateral (LA) and basal amygdala (BA). In the BLA the foundation for fear learning is processed that is the association of stimulus and shock is built. The BA and the ITC project to the central nucleus (CE), which is the output structure of the amygdala and forwards signals through descending projections (Davis, 2006; Paré et al., 2004; Phelps and LeDoux, 2005). Pharmacological interventions turned out to be the most useful method to study the amygdala circuitries. Intra-BLA infusion of NMDA receptor antagonist (Falls et al., 1992; Sotres-Bayon et al., 2007), metabotropic glutamate receptor blocker (Kim et al., 2007), muscimol (Akirav et al., 2006) or mitogen activated protein kinase blocker (Herry et al., 2006; Lin et al., 2003; Lu et al., 2001) all impaired extinction learning. Protein synthesis in the BLA was also tested (Lin et al., 2003) and shown to be involved in new learning and the formation of long-term memory (Berman and Dudai, 2001; Pedreira and Maldonado, 2003; Vianna et al., 2001). The 'new' memory trace, built during extinction learning, does not erase the acquisition memory trace, except for training within minutes after conditioning. Training directly after acquisition affects ongoing protein synthesis and can erase previously acquired memory (Myers et al., 2006). Consolidation of extinction memory depends on structural changes in the BLA. This has been tested by reducing brain-derived neurotrophic factor receptors (Chhatwal et al., 2006) or inhibiting of cell adhesive molecules (Bonfanti, 2006; Markram et al., 2007). Both procedures decrease plasticity, and subsequently impair the retrieval of extinction. GABAergic neurons modulate the expression of fear, and therefore stimulating or lesioning the BLA impairs extinction learning and subsequent retrieval (Blair et al., 2005; Harris and Westbrook, 1998; Müller and Fendt, 2006; Muller et al., 1997). Electrophysiological recordings from the LA reveal distinct spiking activity for extinguished responses of the CSs when tested either in the extinction or the acquisition context (Maren and Hobin, 2007).
The PFC

The second prominent structure for extinction learning is the PFC. The PFC feeds the amygdala with strong glutamergic excitatory projections (Brinley-Reed et al., 1995). First it was suggested that the ventromedial part (vmPFC) modulates fear expression in the amygdala. Early data refer to lesion studies of the vmPFC or the orbitofrontal cortex and essentially reported impaired extinction due to response perseveration during extinction of conditioned fear (Morgan et al., 1993; Sotres-Bayon et al., 2006). Later on, more sophisticated methods revealed that subregions of the vmPFC assume different functions (Morgan and LeDoux, 1995; Quirk et al., 2000). In rats, the prelimbic subregion (PL) subserves excitatory functions while the infralimbic subregion (IL) subserves inhibitory functions for extinction learning (Milad and Quirk, 2012). A range of manipulations with different agents suggests that the IL is less involved in the acquisition of extinction but highly important for extinction memory retrieval. Intra-IL infusion of Tetrodotoxin (a sodium channel blocker) (Sierra-Mercado et al., 2006), CPP (NMDAR antagonist) (Burgos-Robles et al., 2007), protein kinase inhibitor (Mueller et al., 2008), propranolol (beta-adrenergic blocker) (Mueller et al., 2008), MAPk inhibitor (Hugues et al., 2004), NMDAR blockers (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009) or anisomycin (protein synthesis blocker) (Santini, 2004) did not impair extinction learning per se but instead extinction memory retrieval. Additionally, single cell recording from the IL shows a correlation between increased bursting activity and the amount of extinction memory retrieval (Burgos-Robles et al., 2007). In contrast to IL, PL activity is high at the beginning of extinction if fear is high and decreases if fear decreases during extinction (Burgos-Robles et al., 2009). This is in line with a pharmacological inactivation study (via either Muscimol or Tetrodotoxin) where conditioned fear was reduced (Blum et al., 2006; Corcoran and Quirk, 2007a). Also, Vidal-Gonzalez et al. (2006) showed that fear expression is increased during PL microstimulation.

The hippocampus

The PFC receives its main input from the hippocampus, which primarily innervates the IL (Farinelli et al., 2006). The hippocampus is believed to process multimodal contextual and spatial representations (Fanselow, 1999; Fischer et al., 2004, 2007) and also supports retrieval of extinction memory. Corcoran and colleagues inactivated the hippocampus before retrieval testing and observed impaired renewal
(Corcoran and Maren, 2001, 2004; Hobin et al., 2006). The retrieval of extinction was reduced if the hippocampus was inactivated before extinction training (Corcoran et al., 2005). These results suggest the relevance of hippocampal processing for extinction consolidation and contextual control in fear conditioning. The results from PFC and hippocampus inactivation are similar because contextual signals pass the PFC before reaching the amygdala. If the PFC is manipulated, the passage of hippocampal signals is impaired as well (Corcoran and Quirk, 2007b; Hobin et al., 2003).

**Network amygdala – PFC – hippocampus**

Bringing the three structures together, the cortical input to the amygdala seems to be the origin for contextual, temporal and mnemonic components which finally determines the level of fear (Fig. 2). The amygdala in respect to fear conditioning mainly receives input from the PFC and some, but less, hippocampal input. The competing subregions IL and PL of the mPFC target distinct amygdala regions via glutamergic projections (Brinley-Reed et al., 1995). The IL projects via the ITC to the CE and the PL sends fibers into the BLA, which also terminate in the CE. GABAergic ITC cells are situated between the BLA and the central nucleus and inhibit the CE (Paré and Smith, 1993; Royer et al., 1999), thereby reducing the expression of fear. Due to chemical stimulation of the IL (Berretta et al., 2005) which increases c-Fos concentration in the ITCs the connectivity of IL and ITC cells was tested. Quirk and Mueller (2008) conclude that cortical structures activate the amygdala so that ITCs switch off the amygdalar output if the cortical input originates specifically from the IL. Fear is subsequently reduced and extinction is temporarily persistent (Maren and Quirk, 2004; Milad and Quirk, 2002; Milad et al., 2004; Quirk et al., 2003; Vidal-Gonzalez et al., 2006).

In contrast to IL, the activation of PL leads to the expression of fear. The PL innervates the BA (Gabbott et al., 2005; Vertes, 2004), whose activity evokes fear (Herry et al., 2008). Quirk et al. (1995) observed neural plasticity in the LA during fear conditioning. The LA innervates the BA, which finally excites the CE (Blair et al., 2001). Thus, PL activity increases CE output activity and thereby fearful behavior appears through projections to the hypothalamus and the brainstem (Hopkins and Holstege, 1978; LeDoux et al., 1988). For fear expression, the IL and the PL have competing functions and influence extinction learning and retrieval in an opposite
manner. The sites of extinction memory are mainly the mPFC and the amygdala. The enhancement of ITC-inhibition via the IL blocks fear evocation and the activation of the BLA via the PL induces fear expression. Amygdala-dependent fear memory is subsequently mediated by IL and PL. Contextual information is added via projections from the hippocampus (Bouton et al., 2006; Ji and Maren, 2007). In particular, the ventral hippocampus innervates the PL/IL as well as BLA (Hugues and Garcia, 2007).

![Fig. 2: Fear and drug seeking projection circuitry originate in the PFC.](image)

The prelimbic cortex (PL) of the mPFC activates the central nucleus of the amygdala (CE) via the basal amygdala (BA). Lateral amygdala (LA) input to the BA additionally supports fear expression. In contrast, the infralimbic cortex (IL) reduces fear expression and promotes extinction via intercalated cells (ITC), hence ITC GABAergic inhibitory neurons inhibit the CE. Under drug seeking conditions, the PL actives the core of the nucleus accumbens and promotes abuse. The IL leads to a shell activation which inhibits drug seeking. (Peters et al., 2009).

**Translation from rodent to human**

Modern methods allow translating results of the extinction circuitry from rodent to human. Here, the human amygdala is actively involved in extinction learning (Gottfried and Dolan, 2004; Knight et al., 2004; Milad et al., 2007; Phelps et al., 2004). Knight et al. (2004) mentioned as well that distinct areas of the amygdala either code for extinction learning or memory retrieval. However, the vmPFC is more important for retrieving the extinction memory later on (Kalisch et al., 2006; Milad et al., 2007; Phelps et al., 2004). In humans, the vmPFC is homologous to the rats' IL.
This means that the vmPFC increased its activity during extinction learning and in addition its activation is positively correlated with the magnitude of extinction memory retrieval (Milad et al., 2007). In addition, the vmPFC is active in recall of context-related associations and thus important for the renewal effect (Lissek et al., 2013). There is reason to believe that the subdivisions of the PFC, the anterior cingulate cortex, functionally resemble the rodents PL (Hariri and Holmes, 2006). As known from rodent experiments, the hippocampus is active during the recall of contextual extinction memory (Kalisch et al., 2006; Milad et al., 2007). The interconnectivity of these three areas remains partially unclear or even controversial. For instance, the nature of projections between PFC and amygdala for memory retrieval is still not well understood.

**Neural correlates for appetitive conditions**

Thus far, fear conditioning extinction data has been discussed. In contrast, there are fewer approaches which address the neural foundation of appetitive extinction learning. In parallel to fear conditioning, the BLA and the vmPFC are involved in appetitive extinction learning. Monkey data from Weiskrantz (1956) and rat data from Burns et al. (1999) showed that BLA lesions slow down appetitive extinction learning. In reference to instrumental (Knapska et al., 2006) and classical conditioning (Lee et al., 2005), the central nucleus of the amygdala mediates conditioned responding, similar to findings in fear conditioning. The manipulation of the vmPFC does not impair within-session extinction, but negatively influences later retrieval as indicated by increased spontaneous recovery (Maruki et al., 2003; Rhodes and Killcross, 2004) and renewal (Rhodes and Killcross, 2007). Kalivas et al. (2006) reported that the vmPFC is needed to modulate the return of drug-seeking behavior. This is again consistent with findings from fear conditioning. Several cocaine self-administration studies in rats showed increased relapse rates after PL inactivation (Capriles et al., 2003; McFarland and Kalivas, 2001; McFarland et al., 2004; McLaughlin and See, 2003; Di Pietro et al., 2006). The PFC is strongly innervated by dopaminergic midbrain neurons (Lewis et al., 1992; Puig et al., 2014) and blocking dopamine receptors within the PL prevents extinction memory recall (LaLumiere and Kalivas, 2008; Roger et al., 2008). However, pharmacological IL inhibition after extinction induces initial drug seeking behavior (Ovari and Leri, 2008; Peters et al., 2008a, 2008b), while pharmacological IL stimulation suppresses a relapse of drug-seeking
(Peters et al., 2008b). For instrumental appetitive extinction tasks, especially in drug-seeking paradigms, the nucleus accumbens, mediodorsal hypothalamus and paraventricular thalamus play a major role (Fig. 2; Marchant et al., 2012; Peters et al., 2009). Scientists analyzed in particular the fiber connectivity of projections into the nucleus accumbens for appetitive learning: The PL projects to the nucleus accumbens core and the IL projects to the nucleus accumbens shell (Fig. 2) (Brog et al., 1993; Sesack et al., 1989; Voorn et al., 2004). Further on, both subunits of the nucleus accumbens project via GABAergic fibers to the ventral pallidum where motor output is controlled (Heimer et al., 1991; Kalivas et al., 1999; Walaas and Fonnum, 1979; Zahm and Heimer, 1990).

Differences between appetitive and aversive learning are obvious for instance when blocking endocannabinoid CB1 receptors. This leads to a disruption of extinction for aversive memories, while leaving extinction under appetitive conditions intact (Hölter et al., 2005; Niyuhire et al., 2007). Some of the endocannabinoid effects on extinction are probably mediated by hippocampal and amygdalar mechanisms (Marsicano et al., 2002; de Oliveira Alves et al., 2005; Shiflett et al., 2004). Within the ventral tegmental area (which targets the three main extinction brain sites), dorsal and ventral dopamine neurons are inhibited or excited by noxious or appetitive stimuli, respectively (Brischoux et al., 2009). Thus, dopaminergic pathways are differently driven by appetitive and aversive events, although it is not known how this difference in subgroups of dopamine neurons translates to extinction learning. Similarly, the central nucleus of the amygdala shows increased activation after appetitive but not after aversive operant conditioning (Knapska et al, 2006). Taken together, there are good reasons to assume that a detailed analysis of the neural fundaments of appetitive extinction learning could uncover new insights. It is clear that the underlying mechanisms for fear extinction learning in general are understood. In contrast, the neural foundations of reward related extinction learning remains somewhat unclear.
1.3. The importance of avian model systems

Extinction research mostly relies on rat and human data. In terms of both appetitive conditioning and comparative neuroscience, the pigeon serves as a popular animal model. In reference to early approaches in understanding the principles of learning, the pigeon was introduced to experimental psychology (Brown and Jenkins, 1968; Epstein R et al., 1984; Skinner BF, 1948). Pigeons are avid workers; for instance, they can be trained to perform >1000 trials in a single session (Starosta et al., 2014). Accordingly, pigeons are an excellent model system for studying mechanisms of learning in the avian brain.

Since my laboratory has already published previous neural data of extinction learning in pigeons, further experiments are desirable (Lissek and Güntürkün, 2003, 2005; Rescorla, 2008; Stüttgen et al., 2013). The last decade has witnessed a major shift in our understanding of the bird brain and it is now well established that the pallium of birds and mammals are homologous (Jarvis ED et al., 2005; Reiner et al., 2004). While the avian hippocampus is one-to-one homologous to its mammalian counterpart, the nidopallium caudolaterale (NCL) in birds and the mammalian PFC are functionally similar, but constitute a case of evolutionary convergence without being homologous as a pallial field (Güntürkün, 2005; Kirsch et al., 2008). Regarding the hippocampus, Atoji’s and Wilds ‘s (2006) model of comparison of homology describes the birds V-shaped layer as being comparable with the dental gyrus, the dorsomedial complex comparable with the Ammon’s Horn, and the subiculum in a mosaics nature, and finally the dorsolateral complex is comparable with the entorhinal cortex. It remains unclear if direct connectivity exists between the hippocampal formation and the nidopallium (Atoji and Wild, 2006). Despite the lack of a laminated cortex, anatomical (Kröner and Güntürkün, 1999), neurochemical (Bast et al., 2002; Karakuyu et al., 2007), electrophysiological (Diekamp et al., 2002; Lengersdorf et al., 2014a; Rose and Colombo, 2005; Starosta et al., 2013) and behavioral studies underline the functional similarity of NCL and PFC. Both structures turned out to be crucially involved in the mediation of executive functions like strategy development and task-related planning. Additionally, these ‘prefrontal’ areas are necessary for working memory (Güntürkün, 2012) and behavioral flexibility. Both structures receive dense dopaminergic input from the ventral tegmental area and the substantia nigra (Wynne and Güntürkün, 1995). The NCL’s descending fibers target motor output structures, somatic and limbic striatal brain regions. With regard to
comparative neural circuitries between different species, the NCL also innervates the amygdala, nucleus accumbens, visceral structures and diverse chemically defined afferent systems (Kröner and Güntürkün, 1999). The dense concentration of NMDARs within the NCL as well as in the nidopallium caudocentrale (NCC) probably plays a key role in subserving behavioral plasticity (Herold et al., 2011). The NCC is the adjacent structure medioventral of the NCL. In contrast to the NCL, the NCC has rarely been investigated. As judged from the fiber connections (Atoji and Wild, 2009; Rehkämper and Zilles, 1991; Shimizu et al., 1995) and a single lesion study (Hartmann and Güntürkün, 1998) the NCC is sketched as a tertiary sensory limbic area. Additionally, Atoji and Wild (2009) reported projection to the arcopallium, which is assumed to be equivalent to the mammalian amygdala.

**Fig. 3: Schematic pigeon brain.** Color labeled areas represent NCL and hippocampus.
1.4. Aims and hypotheses

In the introduction I illustrated few appetitive paradigms to study the neural mechanisms of extinction learning. Under aversive and appetitive conditions neuroscientists revealed the context-specificity of extinction learning. Neural foundations within the scope of fear extinction learning remain dominant in neuroscience, whereas the need and relevance of research on appetitive extinction learning increase steadily. While most existing data thus far stems from human and rodent subjects, comparative approaches with other species could facilitate the understanding of more general underlying mechanisms. In reference to the history of behavioral research, my comparative research interests and outlined neuroscientific results reveal that the pigeon is an appropriate animal model. I focused on the hippocampal and ‘prefrontal’ involvement for context-specific extinction learning in the pigeon brain. Here, I raised the question of whether previously investigated structures involved in aversive learning conditions also play similar roles under appetitive conditions in the pigeon. Therefore I applied pharmacological manipulations during extinction learning to subsequently compare the results of pigeon with mammalian data. The approach required an adequate paradigm to access extinction learning, extinction memory consolidation, renewal, and spontaneous recovery. In order to do so, I adopted and established the within-subject renewal paradigm for pigeons from Rescorla in our lab (2008). Rescorla demonstrated context-specific extinction learning in pigeons under appetitive Pavlovian conditioning terms in a within-subject design. In his study, each bird acquired a response to CS1 in context A and to a CS2 in context B. Subsequently, responses to CS1 and CS2 were no longer reinforced when they appeared in their opposed context (CS1 in context B; CS2 in context A) during extinction. The final test then occurred in both contexts for both stimuli to test for renewal and spontaneous recovery. I extended the paradigm by adding a pharmacological manipulation of the named brain areas in a within-group design by intracranial injections. Within the first two studies, I manipulated the NCL and the hippocampus during extinction training with the sodium-channel blocker Tetrodotoxin (TTX). TTX transiently prevents cells from firing action potentials. Subsequently, my focus shifted to the involvement of NMDRs in the NCL. This was due to their importance for learning and their high density in the NCL. Therefore I injected either the NMDAR antagonist DL-2-amino-5-phosphonovaleric acid (APV) or the NMDAR-agonist D-cycloserine (4-amino-3-
isoxazolidinone; DCS) in the NCL before extinction learning. Previous studies have already shown that NMDARs in the NCL are required for reversal learning (Lissek and Güntürkün, 2003; Lissek et al., 2002) and that NMDAR blockade results in an inability to adjust responses to changing contextual circumstances (Lissek and Güntürkün, 2005). Within my experiment I focused in particular processing contextual extinction learning by means of a newly established within-subject design for pharmacological manipulations in the pigeon. Thus, the aim of my thesis was to further the understanding of the neural fundaments of extinction in birds using an appetitive task, and to compare these new findings with those known from fear conditioning in rodents and humans.
2. Methods

In total the project includes four experiments. For simplicity reasons, TTX injection either in the NCL or the hippocampus will be outlined as experiment 1 (study 1: NCL; study 2: hippocampus) and the manipulation of NMDARs in the NCL will be summed as experiment 2 (study 3: APV; study 4: DCS).

2.1. Subjects

In experiment 1, sixteen adult and experimentally naïve pigeons (Columba livia) served in the NCL experiment and twenty-four animals served in the hippocampus experiment. The APV group consists of 24 animals and the DCS group includes 16 animals. Birds were obtained from local breeders and housed in individual wire-mesh cages (30 cm x 30 cm x 45 cm) inside a colony room. Temperature and humidity as well as the 12-hr light-dark schedule were strictly controlled (lights on at 8 am). During the experiment animals were maintained close to 85% of their free feeding weight with additional free food on weekends. Water was available ad libitum. All experiments were approved by the national authorities of the state of North Rhine-Westphalia, Germany and carried out in accordance with the National Institute of Health Guide for Care for Laboratory Animals.

2.2. Surgery

The birds were chronically implanted with 26-gauge (8 mm) stainless steel guide cannulas (Plastics One Inc., Roanoke, USA). NCL TTX animals were implanted bilaterally with a single cannula in each hemisphere, and hippocampus animals were implanted with two cannulas per hemisphere. The same held truth for the NMDAR manipulations where animals were implanted with two cannulas in each hemisphere. For surgery, birds were premedicated with Dolorex (0.3 ml, 10 mg/ml, Butorphanol, Intervet, MSD Animal Health, Unterschleißheim, Germany) as painkiller and anesthetized with Isoflorane (Forane 100% (V/V), Mark 5, Medical Developments International, Abbott GmbH & Co KG, Wiesbaden, Germany). Small craniotomies were performed above the target areas, the dura mater was removed and cannulas were inserted slowly into the brain under visual control and targeted to the following...
coordinates: NCL (single cannulas): A +6.5 mm, L ±7.2 mm, V +1.8 mm; hippocampus: A +5.7 mm and +7.7 mm, L immediately lateral from the superior sagittal sinus, V +0.5 mm; NCL (double cannulas): A +5.25 mm, L ±5 mm and ±7 mm, V ≈ 1.1. Hippocampal and double NCL cannulas were inserted at an angle of 30° relative to the coronal plane (Karten and Hodos, 1967). Up to eight stainless steel microscrews (Small Parts, Logansports, USA) were attached to the bone to anchor the dental cement encasing the cannulas. Postoperatively, Carpofen (0.3 ml, 10 mg/ml, Rimaldyl, Pfizer GmbH, Münster, Germany) was applied twice daily as an analgesic. Pigeons were allowed to recover 7–10 days before initial training started.

2.3. Behavioral Apparatus

All subjects were trained in skinner boxes of similar shape (36 cm x 34 cm x 36 cm). The rear and side walls of the chambers were covered with colored wallpaper either by 2.5 cm wide vertical or horizontal tan stripes spaced 5 cm apart on red background or by yellow marbling pattern on white background (Rescorla, 2008). White noise and brown noise was provided (approximately 80 dB SPL) to increase distinctness of the environments. Boxes were housed in sound-attenuating cubicles to mask extraneous sounds. Altogether four chambers were used and randomly assigned with color, noise and stimuli and were named context A or B. The rear wall of one box was featured with two translucent rectangular pecking keys (2 cm x 2 cm; 12 cm above the floor), but only the right key was used in these experiments. A single pecking key (2 cm x 2 cm; 12 cm above the floor) was situated at the center of the rear wall in all of the other chambers. The boxes were illuminated with 6 W light bulbs placed either in the center of the ceiling or on the upper edge of the side wall. Stimuli were presented on LCD flat screen monitors (2x: Belinea Model No.: 101536; Philips Model No. 150S4 and Model No. 150P4CG/00) mounted against the outer back wall of the chambers. Overall, four different visual stimuli were simultaneously used and defined as target, non-target, conditioned stimulus 1 (CS1) and conditioned stimulus 2 (CS2) (example stimuli in fig. 4). Each effective key peck produced an audible feedback click. Otherwise, key pecks were inconsequential throughout the experiment. During acquisition, the target and the CS were followed by food reward delivered immediately at stimulus offset (5 s stimulus presentation time); the non-target was never followed by food. Food (grain) was delivered by a food hopper positioned at the lower middle of the rear wall. Whenever food was available, a
feeder light 2 cm above the food hopper was illuminated. The hardware was controlled by custom-written Matlab code (The Mathworks, Natick, MA; Rose et al., 2008).

![Example stimuli](image)

**Fig. 4:** Example stimuli.

### 2.4. Procedure

The experimental procedure consisted of five separate phases: Pretraining I and II, acquisition, extinction and test. They are detailed below and illustrated in Figure 5 and Table 1.

![Diagram of ABA renewal design](image)

**Fig. 5:** Depiction of the within-subject ABA renewal design. Single pictures show rear walls of the two different conditioning chambers A and B. The blue and orange squares with numbers 1 and 2 indicate the two different conditioned stimuli. Not shown are the target stimulus (present and reinforced in all sessions) and the non-target stimulus (present and non-reinforced in all sessions). Contexts, stimuli and injection sequences were balanced across subjects, hence this figures shows a single possible example.
2.4.1. Pretraining I

Initially, animals were submitted to a simple sign tracking procedure with a single stimulus (target). In each session in Pretraining I, the same target stimulus was presented 48 times for 5 s and immediately followed by 3 s access to grain. The intertrial interval was fixed at 45 s. Animals received two training sessions on each workday, one session in each of the two contexts, spaced 2 h apart and conducted in alternating succession. Once the learning criterion of 80% responses in both contexts on three consecutive days was reached, the animals entered the next phase of training, Pretraining II.

2.4.2. Pretraining II

Conditions were identical to those in Pretraining I except that the target stimulus was presented only 24 times per session, and a non-rewarded stimulus (non-target) was introduced (12 presentations per session). The non-target was shown for up to 5 s and was never followed by reward. In case the animal responded to the non-target, either the house light was turned off immediately for 3 s and a clearly audible tone (sawtooth wave at 1000 Hz) was presented in experiment 1 or responding remained inconsequent until presentation time expired in experiment 2. The order of stimulus presentation was randomized with the exception that each session started with two target presentations. A minimum of 80% correct responses for both stimuli in both contexts was required to enter the next phase of training, the Acquisition.

The inclusion of the target and the non-target stimuli was done to control for any non-systematic effects triggered by injecting a pharmacological substance (unspecific up-or downregulation of responding). Additionally, was reasoned that the occasional presentation of a non-rewarded stimulus would enhance the birds’ attention towards the stimuli. The same target and non-target stimuli were presented in both contexts.

2.4.3. Acquisition

In addition to the target and the non-target stimuli, CS1 was introduced in context A and CS2 was introduced in context B; both CSs were followed by food reinforcement after 5 s fixed stimulus appearance. Each stimulus was presented 12 times per session. Acquisition training was conducted for a minimum of six days with two
sessions per day – one in context A for CS1 and one in context B for CS2. Overall, pigeons had to reach a performance criterion of 80% correct responses for all stimuli across three consecutive days to be transferred to extinction training.

2.4.4. Extinction
Extinction took place on two days with one day without training in between to allow for complete washout of the drug. On days without training, animals were provided with 10 g of grain. During extinction training, non-reinforced CS1 trials were conducted in context B on one day and non-reinforced CS2 trials were conducted in context A on the other day, with half of the animals being exposed first to extinction in context A and the other half first to extinction in context B. All other conditions were identical to acquisition training, with the exception that the target and the CS were presented 24 rather than 12 times, respectively, whereby the number of non-target presentations (12 times) remained unchanged. Approximately 30 minutes before extinction training commenced, either 1 µl TTX (10 ng/µl, Tetrodotoxin citrate, Tocris) or 1 µl saline was infused bilaterally into the NCL or the hippocampus. In experiment 2 0.5 µl APV per cannula (5 µg/µl, Sigma-Aldrich) or 0.05 µl DCS per cannula (20 µg/µl, Sigma-Aldrich) respectively were applied approximately 10–15 min. before extinction training. Under control conditions 0.1 µl or 0.05 µl saline was injected per hemisphere. Hence a single animal received the drug in one extinction session and saline in the other in a within-subject design. TTX is completely washed out 48 h after injection (Freund et al., 2010). For APV and DCS no data were available.

2.4.5. Test
Finally, responding to CS1 and CS2 as well as target and non-target stimuli was observed in both contexts in balanced succession 48 h after the second extinction session. Each stimulus was presented 12 times, but only target presentations were followed by reinforcement.
Table 1: Overview experimental sequence

<table>
<thead>
<tr>
<th>phase</th>
<th>context</th>
<th>no. target</th>
<th>no. non-target</th>
<th>no. CS1 or CS2</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>acquisition</td>
<td>A</td>
<td>12x (+)</td>
<td>12x (–)</td>
<td>12x CS1 (+)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12x (+)</td>
<td>12x (–)</td>
<td>12x CS2 (+)</td>
</tr>
<tr>
<td>extinction</td>
<td>A</td>
<td>24x (+)</td>
<td>12x (–)</td>
<td>24x CS2 (–)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>24x (+)</td>
<td>12x (–)</td>
<td>24x CS1 (–)</td>
</tr>
<tr>
<td>test</td>
<td>A</td>
<td>12x (+)</td>
<td>12x (–)</td>
<td>12x CS1 (–) &amp; 12x CS2 (–)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12x (+)</td>
<td>12x (–)</td>
<td>12x CS1 (–) &amp; 12x CS2 (–)</td>
</tr>
</tbody>
</table>

2.5. Histology

After completion of the test session injections sites were verified with immunohistochemical techniques. Animals were euthanized with Equithesin (0.5 µl per 100 g body weight). Once the animal was deeply anesthetized and claw reflexes were totally absent, transcardinal perfusion with warm sodium chloride solution (0.9%, 38°C) and subsequently cold paraformaldehyde (4% in 0.12 M phosphate buffer pH 7.4, PBS, 4°C) was performed. The brain was removed via preparation from the scull and postfixed in 4% paraformaldehyde for 2 h. Then the brain was transferred to paraformaldehyde with additional 30% sucrose overnight for cryoprotection. Under freezed conditions the brain was sliced in 40 µm section with a microtome. For animals of the first experiment TTX was again injected before euthanasia so that immune-ABC technique (Freund et al., 2010) could be applied to stain TTX. Here in principle slices were incubated in a primary antibody (mouse-α-TTX, 1/200 in PBS+, Hawaii Biotech) and biotinylated with a secondary antibody (horse-α-mouse, 1/200 in PBS+, Vectastain, Vector, Camon). Subsequently a heavy metal-intensified 3.3-diaminobenzidine reaction (DAB, Sigma) visualized TTX. All of the sections, inclusive section of the second experiment, were finally stained cresyl violet to reveal anatomical structures. The positions of the cannulas were analyzed under the microscope by means of the brain atlas from Karten and Hodos (1967). Finalized reconstruction was done with schematic drawings in CorelDraw X5.

2.6. Data Analysis

The absolute number of responses during stimulus presentation was the main dependent variable. In addition, I computed the percentage of trials on which a conditioned response was observed (i.e. >1 key peck). I used two-way repeated
measures analysis of variance (RMANOVA) and paired-samples t-tests to analyze the response data.
CS responses during extinction training were normalized by multiplying the average number of responses in a given bin of four consecutive trials by the ratio of target responses under saline and drug in the same bin of four trials. Responding to the non-target was near or at zero throughout all experiments and was not affected by any experimental manipulation; therefore, I decided to omit this information from the results figures.
Analyses were conducted employing the Statistics Toolbox of Matlab R2012a (The Mathworks, Natick, USA).
3. Transient inactivation of the NCL and the hippocampus

3.1. Background

The first experiment focuses on the involvement of the NCL and the hippocampus in context-specific extinction learning. In general, both permanent brain lesions and drug injections (either systemically or locally) are sufficient to manipulate extinction learning processes. Lesions are relatively long lasting and due to neural plasticity, initial deficits can be compensated via other brain sites when time elapses. However, pharmacological interventions are rather temporarily transient and affect merely selected experimental phases. In the scope of extinction learning I assess the possibility to inactivate either the NCL or the hippocampus during extinction learning. By this method, extinction learning itself and possibly extinction memory consolidation could be affected. One highly effective strategy for inactivation is to suppress action potential firing via blockade of voltage-gated sodium channels in all types of cells. TTX is a selective sodium channel blocker which was originally found in the blowfish. In humans, several micrograms are lethal. For neuroscientific experiments, a small amount of TTX injected in brain regions of interest is a powerful tool. Our laboratory recently established TTX injections in the pigeon brain. Freund et al. (2010) analyzed the spread of the drug in the NCL after injection, and Helduser et al. (2012; 2013) applied TTX in behavioral experiments. During neuronal contamination, the rest of the brain does not receive information from the affected brain site, thus restricting information processing. Subsequent to the injection, the effect on specific behaviors can be observed.
3.2. Results

3.2.1. Inactivation of NCL during extinction learning

3.2.1.1. Histology

Overall, eight animals were included for analysis. Histological analysis (Fig. 6) showed bilateral well-centered cannulas within the NCL. One of the subjects was included even though one cannula was at the very anterior border within the NCL. The remaining subjects were excluded due to improper cannula positions (n=1), failing to reach the learning criteria (n=2) or complete behavioral suppression during extinction under TTX (n=5).

Fig. 6: Histology results depicting the position of cannulas within the NCL represented by black dots. Schematic slices base on Karten and Hodos (1967).
3.2.1.2. Acquisition
Conditioning went uneventful. Response rates for all reinforced stimuli in the last four sessions were similar (Fig. 7A; target vs. CS1: t(7)=−0.14; p=0.89; target vs. CS2: t(7)=−0.23; p=0.83; CS1 vs. CS2: t(7)=−0.15; p=0.88).

3.2.1.3. Extinction
Figure 7B depicts the time course of responding to each of the stimuli during extinction training. Obviously, responding to the CSs decreased over the course of the session both under TTX and under saline. Responding to the target was fairly stable, but TTX resulted in an initially decreased response rate early in the session. A two-way RMANOVA revealed a significant main effect of treatment (F(1,7)=5.7, p=0.049) but not of block (F(5,35)=1.2, p=0.308) along with a significant treatment x block interaction (F(5,35)=3.5, p=0.012), reflecting reduced response rates during target presentation under TTX early in the session.
Regarding the CS, a two-way RMANOVA yielded a significant main effect of block (F(5,35)=16.4, p<0.001) but not of treatment (F(1,7)=3.2, p=0.119) and a significant interaction of treatment and block (F(5,35)=4.9, p=0.002), again reflecting decreased response rates under TTX relative to the saline control, and this difference was again most pronounced in the first two blocks of the session.
At first glance, inactivation of the NCL seems to facilitate extinction learning: responding to the CS ceases earlier in the session under TTX than under saline. However, TTX treatment yielded reduced response rates to the target stimulus as well, so the reduced response rates to the CS under TTX cannot simply be taken to imply enhanced extinction. Indeed, when normalizing response rates relative to the target stimulus, the difference between TTX and saline disappears, suggesting a similar time course of extinction in both conditions (Fig. 7C; RMANOVA: block: F(5,30)= 18.1, p<10^{-7}; treatment: F(1,6)=0.2, p=0.646; interaction: F(5,30)=1.9, p=0.124).

3.2.1.4. Retrieval
Figure 7D shows response rates for the CSs and the target in the test sessions in both contexts. A two-way RMANOVA revealed no main effects for either context or treatment (F(1,7)=0.5, p=0.501 and F(1,7)=1.8, p=0.222, respectively), but a
significant interaction of the two factors F(1,7)=22.3, p=0.002). Pairwise comparisons showed no significant difference in ABA responding between treatment conditions (t(7)=0.1, p=0.947) but a trend towards a statistically significant difference in ABB responding (t(7)=2, p=0.092). Importantly, the latter effects were significant when taking the percentage of trials with conditioned responses as dependent variable (Fig. 7E; ABA: t(7)=0.36, p=0.732; ABB: t(7)=2.5, p=0.042), constituting evidence that ABB responding was indeed affected by NCL inactivation.

As response rates were quite high in the ABA condition after both treatments, it could be that differences in responding went undetected because of a potential ceiling effect. However, responses to the CS extinguished under saline during ABA retrieval were still significantly reduced compared to target responding (t(7)=2.7, p=0.032).

To sum up, NCL inactivation during extinction unspecifically suppressed conditioned responding without clear evidence of affecting the speed of learning. However, there was some evidence that extinction learning was actually impaired by NCL inactivation, signified by enhanced conditioned responding to the extinguished CS in the context of extinction (ABB) after TTX as compared to saline treatment.
Fig. 7: Results of NCL inactivation. (A) Mean response rates (+/−SEM) in the last four acquisition sessions for target and conditioned stimuli. (B) Mean response rates (+/−SEM) during extinction training, shown separately for target and conditioned stimuli and in TTX and saline conditions. (C) Normalized response rates for the conditioned stimulus reveals comparable extinction dynamics. (D) Mean response rates (+/−SEM) in the retrieval sessions in contexts A and B. (E) Relative response (+/−SEM) rate indicate increased spontaneous recovery for TTX treatment. For simplicity, I collectively refer to ABA/BAB and ABB/BAA as ABA and ABB, respectively.

3.2.2. Inactivation of hippocampus during extinction learning

3.2.2.1. Histology
Overall, response data of 13 animals was deemed suitable for analysis (see Fig. 8 for histology). In all remaining cases, the positions of the cannula were either not within the hippocampus (n=5), subjects did not respond during extinction training (n=5) or did not satisfy the learning criterion (n=1).
3.2.2.2. Acquisition

Conditioning went uneventful. Response rates for all reinforced stimuli in the last four sessions were similar (Fig. 9A; target vs. CS1: t(12)=-0.05; p=0.96; target vs. CS2: t(12)=-0.15; p=0.88; CS1 vs. CS2: t(12)=-0.16; p=0.88).

3.2.2.3. Extinction

Figure 9B shows response rates during extinction training separately for each stimulus type and treatment condition. Response rates to the target remain on a relatively stable level and CS responses decrease during extinction training. Similar
to the NCL data, responding to all conditioned stimuli was reduced under the influence of TTX. There was a significant effect of treatment for target responses (F(1,12)=13.8; p=0.003), along with a marginally significant effect of block (F(5,60)=2.3, p=0.06) but no interaction (F(5,60)=1.1, p=0.352).

Responding to the CS decreased over the course of the session, as evidenced by a significant effect of block (F(5,60)=24, p<10^{-12}). Additionally, there was a main effect of treatment (F(1,12)=5.6, p=0.035) and a significant interaction of the two factors (F(5,60)=2.6, p=0.034).

Again, normalized response rates did not differ significantly across treatment groups (Fig. 9C), as shown by a two-way RMANOVA (block: F(5,50)=10.9, p<10^{-6}; treatment: F(1,10)=0.7, p=0.425; interaction: F(5,50)=1.8, p=0.129).

3.2.2.4. Retrieval

A two-way RMANOVA did not show significant effects for context or treatment (F(1,12)=0.1, p=0.709 and F(1,12)=2.9, p=0.116) but a significant interaction of the two factors (F(1,12)=28.1, p<10^{-3}). In contrast to the NCL inactivation testing under ABB conditions reveal significant differences already for absolute CS responding (ABB, t(12)=2.5, p=0.028) while testing in the acquisition context significance remain absent (ABA, t(12)=1.1, p=0.3) (Fig. 9D). Analyzing the fractional response rate, once more the results after NCL inactivation is mirrored. There was no significant difference between treatment groups when testing retrieval of extinction in the acquisition context (ABA, t(12)=1.5, p=0.16) but when testing in the context of extinction (ABB, t(12)=2.6, p=0.022) (Fig. 9E).

To exclude the possibility that the absence of a difference in ABA was due to a ceiling effect (see above), I tested whether responding to the CS extinguished under saline was reduced compared to responding to the target, and that was indeed the case (t(12)=1.5, p=0.001), speaking against a ceiling effect masking differences in extinction retrieval when testing in the context of acquisition.

In conclusion, transient inactivation of the hippocampus unspecifically reduced overall response rate during extinction, similar to inactivation of the NCL. During retrieval of extinction, responses to the CS extinguished under TTX were increased when testing in the context of extinction (ABB) but not the context of acquisition (ABA).
Fig. 9: Results of hippocampus inactivation. (A) Mean response rates (+/−SEM) in the last four acquisition sessions for target and conditioned stimuli. (B) Mean response rates (+/−SEM) during extinction training, shown separately for target and conditioned stimuli and in TTX and saline conditions. (C) Normalized response rates for the conditioned stimulus reveals comparable extinction dynamics. (D) Mean response rates (+/−SEM) in the retrieval sessions in contexts A and B. (E) Fractional response rates (+/−SEM) reveal the same response pattern as absolute responses.
3.3. Discussion

The first experiment shows that pharmacological inactivation of the pigeon NCL or hippocampus during extinction training impaired subsequent retrieval of extinction memory when tested in the context of extinction (spontaneous recovery, ABB) but not in the context of acquisition (renewal, ABA). Additionally, I observed that TTX injection in both areas respectively resulted in a general suppression of conditioned responding, while the principal extinction dynamics were not affected. Thus, my data show that both the 'prefrontal' NCL as well as the avian hippocampus play an important role in the encoding of extinction memory.

3.3.1. NCL inactivation

Multiple sources of evidence point to an involvement of the PFC in recall of extinction memory. Here, especially the infralimbic prefrontal cortex of rats seems to be relevant. Lesions or pharmacological inactivation of this area do not affect extinction learning per se but subsequent extinction memory retrieval (Milad and Quirk, 2012; Morgan and LeDoux, 1995; Quirk and Mueller, 2007; Quirk et al., 2000). Additionally, more specific neural manipulations with e.g. NMDA- (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2009) or mGlu5-receptor blockers (Fontanez-Nuin et al., 2011) before or subsequent to extinction learning in the infralimbic prefrontal cortex impair extinction retrieval on the next day. Sepulveda-Orengo et al. (2013) observed that prefrontal mGluR5 activation promotes consolidation of fear extinction by regulating the intrinsic excitability of infralimbic neurons. Electrophysiological data revealed that the degree of infralimbic burst firing is correlated with extinction retrieval (Santini et al., 2008). Human studies revealed ventromedial prefrontal activity in a predictive learning task during extinction memory retrieval (Lissek et al., 2013). Thus, prefrontal neurons seem to be active during extinction learning, readjust their synaptic weights in the hours subsequent to extinction and then play a critical role when extinction memory is retrieved (Milad and Quirk, 2012). Such an interpretation is in line with our findings of TTX-injections into the pigeon NCL. Transient inactivation of the NCL during extinction did not alter extinction learning dynamics but seemed to prevent proper extinction memory consolidation at the 'prefrontal' level. Consequently, in
subsequent sessions an increased response rate to the previously extinguished CS was observed.

Taken together, the avian NCL assumes a similar function in the expression of extinction learning as subcomponents of the PFC (infralimbic PFC in rats, ventromedial PFC in humans). This interpretation is in line with previous studies showing that antagonizing NMDA-receptors in the pigeons NCL affect extinction and context integration (Lissek and Güntürkün, 2003, 2005).

3.3.2. Hippocampus inactivation

As for the NCL, also TTX infusions into the hippocampus did not affect the principal dynamics of extinction learning but compromised extinction retrieval in the ABB test. Indeed, several strands of evidence show that the mammalian hippocampus plays a major role in extinction memory retrieval. Diverse studies documented that alteration in hippocampal activity alters extinction learning (de Carvalho Myskiw et al., 2013; Corcoran et al., 2005; Psotta et al., 2013). Extinction learning is also accompanied by changes of theta oscillation coupling between the hippocampus, the amygdala and the prefrontal cortex in mice (Lesting et al., 2011). Contextual extinction learning is represented by hippocampal activity as well and here the activity level correlates with the predicted impact of the renewal effect in human (Lissek et al., 2013). Overall, the hippocampus is involved in all kinds of context-dependent conditioning tasks (Bouton et al., 2006; Hobin et al., 2006; Ji and Maren, 2007; Milad and Quirk, 2012). Transient hippocampal inactivation during extinction learning results in poor extinction retrieval (Corcoran et al., 2005; Sierra-Mercado et al., 2011). The results of Bast et al. (2001) indicate that rats injected with TTX into the ventral hippocampus immediately before extinction were neither able to decode contextual nor tone cues in a retrieval test. Enhanced spontaneous recovery for TTX-treated pigeons in my study therefore indicates that only memory of extinction learning is disrupted. This is in line with a conditioned magazine approach from Campese and Delamater who showed that muscimol inactivation of the hippocampus did not impair ABA renewal (Campese and Delamater, 2013) but spontaneous recovery (Campese and Delamater, 2014). Hence I conclude that the hippocampus is involved in building context-specific extinction memory. Beyond extinction learning as a specific learning procedure, the hippocampus is responsible for the transfer and consolidation of short-term memory to long-term memory stored in the pallium. Thus, hippocampal
TTX-injections could prevent the consolidation of extinction memory, thus increasing spontaneous recovery in the ABB design. Indeed, TTX concentrations remain high within the tissue for at least 4 h after injection (Freund et al., 2010) and could therefore affect the consolidation process after extinction training. For both NCL and hippocampus ABA retrieval remains unaffected and a ceiling effect could be excluded. This underlines the assumption that impaired extinction learning was entirely linked to the context of extinction.

3.3.3. General reduced motor behavior after TTX injection

During extinction learning I observed a reduction of conditioned responses for animals with TTX-injections both into NCL and hippocampus. Due to our within-subject design and the addition of the target stimulus, it was possible to show that this response reduction was not specific for the conditioned stimulus but reflected a general behavioral inhibition. Presently I can only speculate about the reasons for this effect. The NCL is critical for response selection (Helduser and Güntürkün, 2012; Helduser et al., 2013; Lissek and Güntürkün, 2004) and response timing (Kalenscher et al., 2003). Helduser et al. (2013) reported that NCL-inactivated pigeons frequently fail to initiate trials and explained these failures by attentional deficits. Single unit recordings show that some NCL-neurons are associated with learned response patterns (Koenen et al., 2013; Lengersdorf et al., 2014a; Scarf et al., 2011; Starosta et al., 2013). This is similar to studies in the mammalian PFC that describe prefrontal neurons which code for learned targets, conditioned movements and the selection of responses depending on the history of reward within the task (Lennert and Martinez-Trujillo, 2013; Warden et al., 2012). Consequently, prelimbic PFC-lesions in rats also reduce responding in appetitive tasks during extinction learning, possibly by affecting a differential outcome-mediated initiation and selection of learned responses (Corbit and Balleine, 2003).

Alternatively, the reduction in response rate could be due to a reduction in the hedonic value of the rewards. The NCL is heavily implicated in reward processing (Koenen et al., 2013; Lengersdorf et al., 2014a; Starosta et al., 2013), and therefore its inactivation might affect the subject’s incentive motivation to perform the sign-tracking response. Furthermore, appetitive extinction is used as a model for depressive disorders, and the response decline in appetitive extinction is slowed down by the administration of antidepressant drugs (Huston et al., 2012, 2013).
However, it is difficult to reconcile this interpretation with the context-specificity of extinction retrieval impairment. Thus, while the overall response reduction might be attributed to decreased incentive motivation, an additional interpretation in terms of context-specific extinction memory consolidation is required.

Regarding TTX-injections into the hippocampus, I also observed an overall reduction in response rate. Again, this is similar to the mammalian hippocampus in which infusions of TTX into the ventral hippocampus before fear extinction conditioning also results in hypoactivity (Bast et al., 2001). Similarly, hippocampal ablation in pigeons before acquisition training also results in reduced conditioned responding (Richmond and Colombo, 2002). While the latter authors did not find impaired contextual transfer in hippocampus-ablated pigeons, this apparent discrepancy could be readily explained by a crucial difference in procedure: they tested for transfer of responding of a ‘first-learned’ association (the sign-tracking response), while I tested for transfer of a ‘second-learned’ association (extinction memory), which is well known to be much more context-specific (e.g. Bouton, 2004).

Importantly, the overall response strength during extinction could affect subsequent retrieval due to a reduction of response-outcome pairings during extinction and a concomitant reduction of the efficacy of extinction training (Rescorla, 2001, 2003). Indeed, Krupa and Thomson (2003) found that preventing conditioned responding during extinction results in an impairment of extinction learning. However, this interpretation is at odds with our finding that extinction retrieval is unaffected when tested in the context of acquisition. Although I could not find statistical support for this interpretation (see results), I am still inclined to believe that such accounts of associative learning could explain at least a smaller part of the result pattern.

To summarize, the first experiment showed that the NCL and the hippocampus of pigeons are involved in context-dependent encoding of extinction memory in an appetitive conditioning paradigm, demonstrating the involvement of ‘prefrontal’ and hippocampal areas in appetitive extinction learning. Subsequent in the second experiment selective manipulation of NMDA-receptors will elucidate more specific mechanisms of context-specific extinction learning in bird and provide further insights into its neural network.
4. Manipulation of NMDARs in the NCL

4.1. Background

The second part of the thesis addresses the role of NMDARs for extinction learning. The NMDAR is a glutamate receptor and is well known to play a fundamental role for associative learning (Redondo and Morris, 2011). One instance of associative learning goes back to the Hebb rule, which implicates that if two neurons are active at the same time, their connection is strengthened. In the brain, this rule is implemented through the activity of NMDARs and subsequent protein synthesis processes. The activation of a neuron due to an action potential induces the release of glutamate from the presynapse into the synaptic cleft. Glutamate opens AMPA-receptors within the postsynaptic membrane and sodium (Na⁺), potassium (K⁺) and calcium (Ca²⁺) diffuse into the postsynapse and depolarizes the membrane. Indeed this signal evokes a weak excitatory postsynaptic potential. NMDARs are also integrated in the postsynaptic membrane. However, this type of channel is impermeable for Na⁺, K⁺ and Ca²⁺ as long as a magnesium ion (Mg²⁺) blocks the channel. The activation of the channel requires either a high frequent stimulation or two contemporary (for instance CS and US activation) activations of synapses to release the magnesium ion. Subsequently the channel is permeable for Na⁺, K⁺ and Ca²⁺. First, the membrane is depolarized and through further or additional stimulation the magnesium block is removed. The increased concentration of Ca²⁺-ions in the postsynapse leads to several modifications – for instance the activation of intracellular kinases, insertion of AMPA-receptors, increase neurotransmitter release from the presynapse, and especially increase synaptic arborizations. Lastly, this leads to increased synaptic sensitivity and generates long-term-potentiation (LTP). In reference to behavioral learning procedures it is obvious that simultaneous presentation of a CS and a UCS strengthen a weak synapse as well and generated long-term-potentiation.

The manipulating of NMDARs by stimulating or blocking them provides an excellent opportunity to explore their function within the brain of different species during learning. Pharmacological manipulations are transient, so defined learning stages can be influenced. Two widely established drugs are available which agonize and
antagonize NMDARs, respectively. DCS is a selective partial agonist of the NMDR which affects the glycine binding site. The amino acid glycine has a stimulating function when binding at the NMDAR. DCS therefore benefits the Ca$^{2+}$-influx into the dendrite. In contrast, APV inhibits the NMDAR glutamate binding site in a competitive way and hence acts as an antagonist. Glutamate is restricted from contact with NMDAR and the channel membrane remains inactive.

Since NMDARs are densely distributed in the pigeon NCL, injection of DCS or APV before extinction learning should severely change learning dynamics and subsequent extinction memory consolidation.

4.2. Results

4.2.1. Blocking NMDA-Receptors in the NCL with APV

4.2.1.1. Histology

I tested 21 subjects. Two animals were excluded due to improper cannula position, failed performance criterion (n=2) or a training mistake by the experimenter during extinction (n=1) (Fig. 10). In total, data from 16 subjects were analyzed. Regarding cannula position, I included subjects if the tip of the lateral cannulas was positioned in the NCL and the medial cannula was either in the NCL or the nidopallium caudocentrale (NCC). Overall 36 cannulas were found to be within the NCL and 28 cannulas were placed in the NCC. The NCC is adjacent to the NCL. As judged from the fiber connections (Atoji and Wild, 2009; Husband and Shimizu, 1999; Rehkämper and Zilles, 1991) and a lesion study (Hartmann and Güntürkün, 1998) the NCC appears to function as a tertiary limbic area. Consistent with Herold et al. (2011) the NMDAR density within the NCC is comparably high. The reported effects therefore result from manipulations of both areas.
4.2.1.2. Acquisition

Mean response rates for individual stimuli during acquisition over the last four sessions were similar (Fig.11A) and did not differ significantly (target vs. CS1: t(15)=1.1; p=0.294; target vs. CS2: t(15)=1; p=0.322; CS1 vs. CS2: t(15)=0.03; p=0.980).

4.2.1.3. Extinction

Fig. 11B illustrates the mean response rates to the target and the CS under saline and drug conditions during extinction. A two-way RMANOVA for target responses between the two conditions revealed no treatment (F(1,15)=1.9, p=0.188) but a block effect (F(5,75)=5.7, p<10^{-3}), as well as a significant interaction of treatment and block
Follow-up RMANOVAs indicated that target responses under APV increased significantly (RMANOVA: F(5,75)=10, p<10\(^{-6}\)), while target responses under saline were unaffected (RMANOVA: F(5,75)=1.7, p = 0.143).

For CSs responses, a two-way RMANOVA yielded both significant treatment (F(1,15)=13.1, p=0.003) and significant block effects (F(5,75)=14.6, p<10\(^{-9}\)), accompanied by a significant interaction (F(5,75)=2.8, p=0.021). Follow-up RMANOVAs revealed significant decreases in response decrements to the CS in both conditions (CS\(_{APV}\): F(5,75)=3.5, p=0.007; CS\(_{sal}\): F(5,75)=16, p<10\(^{-10}\)).

These results suggest that blocking NMDA-receptors of the NCL delays extinction learning. However, APV injection also increased responding to the (non-extinguished) target, indicating that the drug effect was not specific to the CS. In order to factor out this non-specific effect on responding and to isolate potential effects of APV on CS responding, I calculated normalized response rates to the CS by multiplying CS responses by the ratio of target responses under saline to target responses under APV (Fig. 11C). Importantly, even when the non-specific increase in responding as measured by increased target responses is factored out through normalization of CS responses, differences between APV and saline remain: while the time course of the response decrement is highly similar between conditions, responding under APV is stronger than under saline, as indicated by a significant treatment effect (two-way RMANOVA: F(1,15)=10, p=0.006; block: F(5,75)=14.3, p<10\(^{-9}\); interaction: F(5,75)= 0.3, p=0.919). Consequently, the data support the hypothesis that APV, in addition to an unspecific enhancement of conditioned responding, also specifically delays extinction learning.

4.2.1.4. Retrieval

Retrieval of extinction memory was tested by presenting all stimuli in both contexts (Fig.11D). A two-way RMANOVA showed no effect of treatment (F(1,15)=3, p=0.105) or interaction (F(1,15)=0.1, p=0.774), but for context of testing (ABA vs. ABB; F(1,15)=37, p<10\(^{-4}\)). Responding to the CS extinguished under saline was not significantly different from responding to the CS extinguished under APV when tested in the context of acquisition (ABA; t(15)=1.1, p=0.297) or when tested in the context of extinction (ABB; t(15)=1.6, p=0.132). However, the latter comparison does reach
statistical significance when taking relative rather than absolute response counts (ABB: \(t(15)=2.5, p=0.025\); ABA: \(t(15)=1.7, p=0.111\)) (Fig. 11E). Thus, relative response counts again turned out to be more sensitive for detection of pharmacological manipulation, as was found in the first experiment (study I).

To disentangle whether increased spontaneous recovery originates from impaired extinction training, or if extinction memory retrieval was affected, I compared the normalized response difference between APV and saline within the last four extinction trials and the normalized response difference between APV and saline in the first block of ABB testing. I calculate this comparison upon normalized data to exclude the unspecific effect of APV treatment during extinction but statistical analysis revealed no significant effects (\(t(15)=0.5, p=0.659\)).

Unimpaired renewal testing with absolute counts could be due to a ceiling effect, i.e. that animals responded maximally during ABA testing under both drug and saline and therefore masking an effect. Indeed, the difference in ABA responding to the CS extinguished under APV compared to target responding just missed the significance threshold (target vs. CS\textsubscript{APV} in ABA: \(t(15)=2.12, p=0.051\)), which may be taken to indicate that, at least for renewal, a ceiling effect might have contributed to masking differential responding between APV and saline treatments in ABA testing.
Fig. 11: Results from APV injections. (A) Mean response rate (± SEM) within the last four acquisition sessions of the target and the two conditioned stimuli. (B) Mean response (± SEM) rate for stimuli during extinction learning. Separate graphs show rewarded target and non-rewarded CS responses for APV and saline conditions. (C) Normalized extinction data reveal prolonged extinction dynamics for APV treated subjects. (D) Mean responses (± SEM) of the entire test phase under conditions of spontaneous recovery (ABB) and renewal (ABA). (E) Significant fractional responses (± SEM) for the ABB conditions. Differences for renewal testing under APV and saline did not reach significance.

Taken together, these results suggest that blockade of NMDAR in the NCL delays extinction learning, which is concomitant with an unspecific increase of responses toward the target stimulus. There are some indications that this delay is carried over into the retrieval phase two days later.
4.2.2. Stimulating NMDAR in the NCL with DCS

4.2.2.1. Histology

Sixteen animals entered the experiment. One animal was excluded due to failing the learning criterion. Histological analysis of the remaining 15 subjects resulted in 14 animals with well-centered cannulas. These subjects had lateral cannulas in the NCL (n=41) and medial cannulas in the NCC (n=14), a single cannula was positioned at the border of the mesopallium (n=1) (Fig. 12). Hence, as the majority of cannulas targeted the NCL, the results primarily rely on NCL manipulation.

Fig. 12: Histology analysis of DCS injection sites. Dots reveal the tips of the implanted cannulas in the NCL and NCC. Pictures taken from the atlas of Karten and Hodos (1967).
4.2.2.2. **Acquisition**
As shown in fig. 13A, the response rate for all stimuli did not differ during acquisition (target vs. CS1: $t(13)=0.9$; $p=0.404$; target vs. CS2: $t(13)=0.1$; $p=0.928$; CS1 vs. CS2: $t(13)=0.8$; $p=0.418$).

4.2.2.3. **Extinction**
Figure 13B depicts data from extinction training under saline or DCS treatments. A two-way RMANOVA revealed that target responses were not influenced by drug administration and remained stable over time (treatment: $F(1,13)=0.0$, $p=0.932$; block: $F(5,65)=1$, $p=0.411$; interaction $F(5,65)=2$, $p=0.087$).

Similarly, a two-way RMANOVA of responding to the conditioned stimuli indicated no significant effects of treatment ($F(1,13)=0.0$, $p=0.835$) but for the block comparison ($F(5,65)=25.3$, $p<10^{-13}$) without a significant interaction of these factors ($F(5,65)=1.3$, $p=0.275$). Results were similar for normalized data (Fig. 13C) (two-way RMANOVA, treatment: $F(1,13)=0.2$, $p=0.671$; block: $F(5,65)=28$, $p<10^{-14}$), although a trend towards a significant interaction was visible ($F(5,65)=2.3$, $p=0.051$).

To sum up, stimulating NMDA receptors during extinction learning through DCS did not result in any statistically significant effects.
Fig. 13: Results for testing NMDAR agonist in the NCL. (A) During acquisition, mean response rates (± SEM) for target and CSs show no differences. (B) Response data (± SEM) from DCS manipulations during extinction learning. (C) Normalized data for DCS treated animals. (D) Mean response rates (± SEM) for testing target and CSs in context A and B. (E) Fractional responses rate (± SEM) under both test conditions reveal the same pattern as in (D).

4.2.2.1. Retrieval

Statistical analysis of the data presented in figure 13D showed that the pharmacological manipulation with DCS extinction did not result in altered responding during retrieval tests, regardless of the test context. The two-way RMANOVA did not yield significant effects of treatment (F(1,13)=0.6, p=0.458), but of condition (F(1,13)=39.9, p<10^{-4}) without indication of an interaction (F(1,13)=0.3, p=0.621). Analysis of relative response counts showed the same pattern (fig. 13E, ABA: t(13)=0.83, p=0.43; ABB: t(13)=0.15, p=0.885).
Overall, these results do not support the hypothesis that stimulating of NMDARs with DCS during extinction has an effect on acquisition, consolidation, or retrieval of extinction memory.
4.3. Discussion

In the first study, I reported that transient ‘prefrontal’ NCL inactivation with TTX during extinction learning impairs extinction memory consolidation. The second experiment aimed to examine the role of NCL NMDARs for extinction learning and memory retrieval by pharmacologically modulating these receptors with the antagonist APV or the agonist DCS during extinction training. In APV-injected subjects, extinction slowed down, accompanied by behavioral disinhibition as evidenced through enhanced responding to the consecutively rewarded target stimulus. Testing retrieval in the extinction context (ABB) suggested an increased response probability for APV treated subjects when relative response counts were taken as a dependent measure. At the same time, ABA-renewal of responding was not affected. The injection of the agonist DCS during extinction training had no effect on extinction learning and did not affect extinction memory retrieval either in the ABA or in the ABB condition.

4.3.1. Blocking NMDAR impairs extinction learning

The present findings for blocking NMDAR with APV fall in line with previous work from our laboratory. That is, in a color reversal learning paradigm, APV-NMDAR-blocking of the pigeon NCL preserved the response rate after reversed contingencies (Lissek et al., 2002) as the course of extinction was prolonged. In another experiment, Lissek and Güntürkün (2003) trained pigeons in a go-noGo task and demonstrated that APV-injections into the NCL before extinction retard extinction learning as well. In my current within-subject paradigm, extinction learning was impaired in the same way. Together, these results reveal that NMDAR blocking plays a crucial role in the NCL for processing extinction learning. I conclude that NMDARs are crucial to adopting behavior during extinction training which fits with the role NMDARs play in processing learning.

Prolonged extinction learning, however, could appear due to disinhibition. To disentangle impaired extinction learning and disinhibition, Lissek and Güntürkün (2003) introduced a non-rewarded stimulus during the entire experiment as I did with the non-target. Results from both studies showed that APV treatment does not increase responding to the unrewarded stimulus (noGo-stimulus or non-target). Lissek and Güntürkün (2003), as well as me concluded from this finding that
NMDARs modulate extinction learning without affecting disinhibition with respect to a never rewarded stimulus.

My present data draw a distinctly more detailed picture, because slowed extinction learning is accompanied with an increased target response rate. By adding a rewarded control stimulus (target) in addition, I could reveal that blocking NMDARs in the NCL increased responding explicitly for the stimulus that has a reward history and remains rewarded during extinction. Thus, NCL NMDARs do participate in controlling inhibition to rewarded stimuli. Disinhibition is often accompanied with increased PFC activity, in particular without inhibition of pyramidal cells in mammals. Indeed, disinhibition induced via systemic NMDAR antagonist MK-801 injections in rats have resulted in impaired working memory and increased motor activity which correlates with firing rate potentiation and burst activity reduction in the PFC (Jackson et al., 2004). MK-801 seems to decrease GABAergic interneuronal activity. As result, these fast spiking interneurons project onto pyramidal cells, and their blockade prevents the inhibition of pyramidal neurons. This then causes disinhibition and increased pyramidal cell activity which impairs cognitive PFC-dependent functions like working memory and motor control (Homayoun and Moghaddam, 2007). This is in line with results of Wen et al. (2010), which demonstrated that preventing stimulation of GABAergic interneurons in the PFC which lead to an impaired working memory and hypoaclivity in a automated radial arm maze task. When taking the APV-injections in my experiment, this possibly increased the excitability of the caudal nidopallium. Principal neurons, due to disinhibition, increased their output from the NCL and disturb downstream structures activity. The NCL is one of the largest hubs of the bird forebrain, and is connected to a very large number of sensory-associative, limbic and motoric areas (Shanahan et al., 2013) – for instance the amygdala. Possibly, an APV-induced increase of excitation in the nidopallial principal neurons interferes with extinction learning in this wide forebrain network, resulting in slowed down extinction.

It remains unclear why the response rate for the target increased, but not for the non-target. I assume that this results from a floor effect, i.e. responding to the non-target was suppressed to such an extent that behavioral disinhibition through APV was not sufficient to elicit conditioned responding. Additionally, the value of a rewarded stimulus is naturally more relevant to focus on while there is no benefit to attending to non-rewarded stimuli. Thus, goal-directed behavior is guided more by rewarded
associations. This is indeed visible on the cellular level, in particular where stimuli associated with food consumption increases the dopamine concentration within the PFC (Ahn and Phillips, 2002; Bassareo and Di Chiara, 1997; Robbins, 2000). Dopamine in turn interacts with NMDARs, in increases trafficking of NMDAR subunits in the dendrites, and therefore supports neural plasticity (Gao and Wolf, 2008). The modulation of the NCL by Dopamine was shown by Güntürkün and Durstewitz (1999), and Diekamp et al. (2000) revealed that D1 receptor blockade in the NCL reduces the capacity for reversal learning.

The neural concept of disinhibition and extinction learning discussed here merely relies on anatomical rodents and humans data. But are there comparable projections in the avian brain? Wynne and Güntürkün (1995) reported an extensive dopaminergic input into the NCL. Electrophysiological and morphological analyses of NCL neurons indicate the existence of fast spiking neurons that resemble GABAergic interneurons of the mammalian telencephalon (Kröner et al., 2002), which in turn projects on principal neurons. Thus, the present findings lend further support to the functional and microanatomical similarity of NCL and PFC.

Furthermore, I analyzed the impact of APV treatment on memory retrieval both in the extinction and the acquisition context. Pharmacological APV-manipulation during extinction significantly increased spontaneous recovery when using relative rather than absolute response rates. This effect is readily explained by the impairment of extinction learning under APV. The fact that relative, but not absolute response rates, yielded significant effects (although analysis using the latter measure pointed into the same direction) was already observed in the first experiment using TTX inactivation of the NCL. This is somewhat puzzling, since absolute response counts reflect the subject’s valuation of a given CS (Honig, 1962; Starosta et al., 2013; Kasties et al., in preparation), while relative response counts largely omit this information by reducing a continuum of responding to a dichotomous measure. This could result from a high variability in valuing the rewarded stimuli such that absolute responses turned out to be noisier. In that case, the dichotomical analysis would reduce that variability. The response frequency at the end of extinction is also higher for APV treated animals. It appears that subjects retrieve the memory of the final extinction response level when tested in the extinction context. Extinction memory is not subsequently impaired, but rather transferred to the retrieval session. As such, renewal remained unaffected and
the pharmacological manipulation did not impair the context as an occasion setter which gives the CS its meaning. Lissek and Güntürkün’s study (2003) also yielded no deficit of extinction memory recall as well. A crucial difference between these studies is the number of extinction trials (220 trials (Lissek) vs. 24 trials in the current study) resulting in different response levels at the end of extinction training. This might have masked the detection of impaired spontaneous recovery in their experiment. Thus, the number of extinction trials has an impact on memory consolidation and subsequent extinction memory recall (cf. Denniston, Chang and Miller, 2003).

The avian data presented here once more reflects a functional equivalence of the NCL and the PFC. In rodents, chronic alcohol abuse generally results in slower extinction and impaired memory consolidation, which is correlated with a reduced IL bursting during extinction learning (Holmes et al., 2012). The IL usually projects to the amygdala via inhibitory fibers so that reduced projections increase the anxiety, such that fear is retrieved (Falls et al., 1992). The effect of alcohol abuse therefore mimics the antagonistic output of APV which has a major impact on neural plasticity and extinction learning consolidation (Holmes et al., 2012). Systemic MK-801 turned out to impair fear extinction learning (Lee et al., 2006) dose-dependently (Baker and Azorlosa, 1996), while systemic CPP-extinction showed a trend for extinction retardation (Santini et al., 2001). However, while CPP intra-vmPFC injections were ineffective for extinction (Burgos-Robles et al., 2007). These colleagues did not differentiate between IL and PL, so the effect could be covered by interaction of these brain sites. Blocking the mPFC via APV in an amphetamine conditioned place preference task before extinction impairs extinction learning as well (Hsu and Packard, 2008). Overall, these data suggest that blocking NMDARs results in a retardation of extinction learning. In all of the previously cited experiments, it was shown that either extinction consolidation or retrieval was impaired if APV had been injected previously. Indeed, these results agree with my findings for context-specific extinction learning in reference to the pigeon forebrain, and indicate analogies to the infralimbic part of the PFC. Additionally, I showed that NMDARs control inhibition of responses to reward-associated stimuli, and that NMDARs in the NCL probably do not only guide context-specific processes.
4.3.2. **Stimulating NMDAR during extinction is ineffective**

The last experiment focused on NMDAR stimulation during extinction learning. DCS treatment prior to extinction had no effect on extinction learning or retrieval. In light of the well-known properties of DCS, this result was unexpected. Most of the rodent studies reported a facilitation of extinction and generalized extinction memory if DCS was systematically injected (Bouton et al., 2008; Langton and Richardson, 2010; Quartermain et al., 1994; Richardson et al., 2004; Walker et al., 2002). But a very relevant parameter for reduced memory retrieval is the injection time point. While immediate DCS treatment after extinction (<4h) leads to enhanced extinction memory, delayed injection relapses original behavior (Langton and Richardson, 2010; Ledgerwood et al., 2003). In contrast, Langton and Richardson (2010) treated rats systemically immediately prior to extinction training and revealed no pharmacological effect for extinction and retrieval. In a cocaine self-administration paradigm Torregrossa and Taylor (2010) also reported negative results for systemic DCS administration after extinction under ABA condition. Woods and Bouton (2006) explored in particular the effect of two separate DCS doses (15 vs. 30 mg/kg). Surprisingly, the previously well-established lower concentration yielded no effect for spontaneous recovery or renewal. Instead, the higher dose facilitated extinction learning but remained less context specific due to a renewal effect. From this perspective, it is clear that the dose of DCS plays a key role for the effectiveness of systemic administration. My chosen dose for intracranial injections was adapted from applications into rats and mice (Bolkan and Lattal, 2014; Ren et al., 2013) because to my knowledge no comparable data for birds is available, and most notably I was the first to injected DCS in the pigeon brain. Chan and Maren (2011) injected DCS in the IL of rats in an auditory fear conditioned paradigm. Immediately after acquisition, DCS was injected intra-IL and subsequently extinction training was started. On the next day, rats were tested under drug-free conditions. The acquisition context was different from the extinction and re-extinction context on the second day (ABB condition). Similar to my results, extinction learning was unimpaired. 24 hours later, subjects were re-extinguished and the freezing level did not differ between groups within the first five trials but then DCS treated animals showed a facilitated extinction rate. These data perfectly mirror my results when activating the pigeons’ NCL NMDARs with DCS before extinction learning. First, extinction learning was unaffected. Second, extinction memory was unimpaired. But I should take into
account that extinction learning under appetitive conditions is relatively slow, and differences within the retrieval test (i.e. “re-extinction”) remain undetected if the number of trials or the sessions are restricted. Consequently, for future experiments the number of test trials or test days should be increased. Additionally, there is no measurable parameter to reveal that the subjects could learn faster during extinction training. Because of this, I cannot exclude a floor-effect i.e. subjects reach their maximum for extinction learning.

One purely speculative explanation comes from a strict behavioral perspective of Bouton (2002). He explains extinction learning as a context-modulated CS-inhibition. The memory of the original CS-US association competes with the context-dependent and new CS-noUS during extinction learning. Extinction training leads to an inhibition of previous CS-US memory while finally the context predicts the CS-noUS memory. This was previously defined as negative occasion setter. This ‘normal’ context-modulated CS-inhibition is thought to be promoted via DCS injection and results in enhanced extinction learning. Subsequently, testing spontaneous recovery and reinstatement reveal a lasting inhibition effect for the context-CS-noUS association (Richardson et al., 2004; Woods and Bouton, 2006), while renewal remains unaffected. In contrast, my results neither show increased contextual inhibition learning nor affect on extinction memory consolidation. Hence I conclude that DCS injections may have no additional inhibitory effect on the extinction learning. This could result from different methodological procedures (e.g. appetitive vs. aversive, training schedules) or even differences between species. Finally, more research is required to disentangle these diverging results.

On the whole, the second experiment demonstrates that NMDARs in the pigeons “prefrontal cortex” are necessary for extinction learning, and in particular act on reward driven response patterns. The comparative approach underlines the equivalent functionality of the NCL and the prefrontal areas of mammals although a few aberrations have to be clarified in further experiments.
5. Overall Discussion

Four studies, presented in the current thesis, assessed how extinction learning is processed in the pigeon forebrain. The underlying neural mechanisms of context-dependent extinction learning in the pigeon are not well understood and rarely investigated. To achieve general insight into the extinction network, I started with inactivation of entire structures hypothesized to be crucially involved in extinction learning on the basis of previous work. On the basis of existing rodent data, I decided to manipulate the ‘prefrontal cortex’ (pigeon NCL) and the hippocampus. Although in mammals the most complex structure for fear learning is the amygdala, I assessed the ‘prefrontal cortex’ and the hippocampus of the pigeon because most of the existing pigeon literature refers to results from these brain sites. Since my results of inactivation were similar for the NCL and hippocampus I decided to focus more intensively on the NCL. Previous data of the pigeon NCL indicated a high density of NMDARs (Herold et al., 2011). These receptors are known to play a central role for neural plasticity during learning. In comparison to the entire inactivation in my first experiments, the manipulation of this single receptor type revealed a more detailed picture of the functionality of the NCL within the extinction learning network.

Neither transient inactivation of the NCL nor the hippocampus affected extinction learning as such, but it seemed that extinction memory was not properly consolidated in a context-specific way. I conclude that the NCL and the hippocampus encode the CS-(no)US association and the context, and are thus involved in the consolidation of memory for the extinction-relevant contingencies. However, NMDARs of the NCL seem to be indirectly involved in extinction learning, as indicated by a slowed decrease in responding. Since unorganized output activity due to NMDAR blocking disrupts proper learning processing in downstream structures, I assume that the NCL is not the initial relay of learning. On the other hand, the stimulation of NMDAR was ineffective, and therefore other ways of enhancing extinction learning remain to be identified.

These main conclusions also result from two important (side) effects: Blockade of neural activity in the NCL and hippocampus leads to response rate reduction while blocking NMDARs in the NCL leads to an unspecific response rate increase. It is reasonable that the suppressed motor output during TTX inactivation is produced
from general inactivation, not only of interneurons, but also from projection neurons which in turn control the motor output. For NMDAR blockade, the general disinhibition towards reward-associated stimuli likely resulted from reduced interneuronal inhibition of principal neurons, which is discussed elsewhere. This disinhibition disrupted the appropriate innervations of related brain sites involved in extinction learning, and therefore learning capacity was impaired. As the inactivation of the NCL impaired later (drug-free) extinction memory retrieval, I conclude that the NCL mainly stores memory and does not per se process learning since the NCL-output was blocked. The hippocampus probably adds additional contextual information. Other inactivation studies indeed showed that extinction learning can likely proceed without prefrontal involvement (Burgos-Robles et al., 2007; Milad and Quirk, 2012). These authors also assume that learning per se is processed in various downstream neural structures. Sierra-Mercado et al. (2006) for instance concludes that the access of prefrontal areas to stored memory for recall is mandatory because its inactivation prevents memory saving. The PFC modified its synaptic contacts with neurons that had undergone extinction learning (Herry et al., 2008; Milad et al., 2007; Vertes, 2004), and this in turn can be assumed for synapses in the NCL as well.

From the comparative neuroscientific point of view, the results from TTX- and APV-injections underpin that the NCL is the functional homolog of the PFC in general and additionally rather assumes infralimbic PFC functions. The hippocampus copes with processing contextual informations and these results overlap with rat and human data.

There are additional factors that may have influenced the main effects. For example, hedonic values and accordingly motivation could have been manipulated either negatively or positively by inactivation or receptor antagonization. The suppressed behavior due to inactivation may result from devaluated stimulus associations while the disinhibition during NMDAR blockade revalues the stimulus association. In accordance with the surprising but for the most part clarified differences between study I and III, it has to be mentioned that TTX was injected via a single cannula while APV was administered via double cannulas. Pharmacological infusions via a single cannula probably did not spread into the entire target area, so that potential effects could be masked by remaining neural activity. In addition, the double cannula
injection of APV on the other hand also affected the NCL adjacent NCC, which may have had an effect.

Alongside the neural results presented here, I should mention the new establishment of an experimental within-subject design suitable for pharmacological manipulations. All of the acquired data were conducted with the same within-subject experimental procedure and thus the data were perfectly comparable. One of the advantages of the within-subject procedure is that the number of applied animals is reduced and at the same time the relatively small number of animals reveals high statistical power. Although the experimental design is somewhat complex and Rescorla (2008) at his time established the paradigm for behavioral research in the pigeon, my studies demonstrated the successful adaptation of the paradigm for pharmacological experiments to explore neural correlates of extinction and renewal.

Results from rodents and birds serve to aid our understanding of the principles of extinction learning, and will eventually improve applied science in human therapies. Frequently, scientists exploring extinction learning aim to treat for instance post-traumatic stress disorder or relapse after drug withdrawal. The investigation of extinction learning aims to make extinction long-lasting. Behavioral animal studies try to imitate these conditions and via manipulations on the neuronal or the behavioral level, extinction strategies can be assessed. In reference to the current study, DCS is already applied to humans suffering from schizophrenia. Hence DCS is an approved treatment in human medicine and thus offers an opportunity to apply it for extinction learning procedures as well. There exist some studies in which oral treatment facilitated extinction learning and reduced relapse probability in drug-addicted humans (Hofmann et al., 2006; Kalisch et al., 2009; Ressler et al., 2004). Aside from DCS treatments, neither APV which is reducing the success of extinction nor TTX which is highly toxic in small oral dosages, are proper treatments for humans. But it remains very challenging to assess extinction learning processes in animal models with pharmacological methods. This will extend our knowledge about responsible brain sites and improve the understanding of the underlying mechanisms in the human brain.
6. Outlook

The established experimental procedure offers a powerful paradigm for future work to investigate extinction learning circuitries in the avian brain. Several essential experiments could be conducted to benefit our knowledge. In a first step, the non-results for DCS treatment have to be clarified by examining different doses of DCS. Since the NCL contains a high concentration of GABA-receptors (Herold et al., 2011) this may be a reasonable experiment to shed light on underlying projection leading to the inhibited or disinhibited output as mentioned before. Additionally, NMDAR manipulation within the hippocampus would be interesting. The pharmacological investigation of the third important structure, the avian amygdala is currently running in our laboratory. In parallel, our lab is in the progress of establishing an animal fMRI scanner method to investigate floating brain activity within behavioral experimental procedures.

In reference to many mammalian studies, the time point of injection is a crucial variable for affecting extinction learning. Postextinction or even before retrieval testing, drug infusion would be an approach to find the involved structures for extinction memory consolidation and extinction memory retrieval.

The avian research community aims to examine indications that the mammalian PFC and the NCL are functionally homologous structures. Here, an understanding of the adjacent structure NCC would be desirable. This would be interesting for the exploration of comparable structures in the pigeon – such as IL and PL – already known in rodents. Independent of the NCL, the hippocampus, and the amygdala, the nucleus accumbens is a prominent structure that seems to be involved in drug-seeking behavior. Several manipulations, including the manipulation of the nucleus accumbens, can provide insights into appetitive extinction learning in the pigeon.

Extinction research aims to find a treatment to avoid relapse of previously learned behavior, so that the worthwhile result would be a drug-free treatment. Monfils et al. (Monfils et al., 2009) showed that a single retrieval trial before fear extinction learning reduced the amount of relapse. But is the underlying mechanism similar in different species, and does it also appears under appetitive conditions? This is probably true for humans (Schiller et al., 2010) and rats (Schiller et al., 2010, but see Xue et al.,
2012) and I plan to examine this claim using the within-subject design in the pigeon. In conclusion, there are many questions to answer, and powerful methods available to tackle upcoming problems in the future.
7. Bibliography


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8. Appendix

8.1. Acknowledgement

All experiments were supported by the DFG through the FOR 1581: Extinction learning: Neural Mechanisms, Behavioral Manifestations and Clinical Implications.

8.2. Personal Acknowledgement

Vielen Dank Maik, dass du mir immer wieder geholfen hast und mir so viel beigebracht hast, was ich zum guten wissenschaftlichen Arbeiten benötigt habe.

Vielen Dank Onur, dass du mir die Promotion in der Biopsychologie ermöglicht hast und mich durchgängig unterstützt und motiviert hast.

8.3. List of Publications

The first experiment is published in Behavioral Brain Research:


The second experiment was submitted to Behavioral Brain Research:

**8.4. Curriculum Vitae**

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**Biographicals**

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**Education**

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(Prof. Dr. Dr. hc. Onur Güntürkün, Dr.Maik C. Stüttgen)

2009 -2011

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8.5. Statement

I certify herewith that the dissertation was completed and written independently and without assistance. The “Guideline for Good Scientific Practice” according to §9, Sec. 3 was adhered too. This work has never been submitted in this or a similar form at this or any other domestic or foreign institution of higher learning as a dissertation.

________________________________
Daniel Lengersdorf

Bochum, 29.10.2014