Predator odor-induced fear in rats: a behavioral characterization and neural substrate analysis

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Diese Doktorarbeit ist Teil eines vom *„Center for Behavioral Brain Sciences"* (CBBS) geförderten NeuroNetzwerkes, d.h. einer Kooperation zwischen dem Leibniz Institut für Neurobiologie (Dr. Jürgen Goldschmidt, M.Sc. Daniel Vincenz-Zörner), dem Institut für Anatomie (Dr. Wolfgang D'Hanis, Dipl. Biologin Silke Storsberg) und dem Institut für Pharmakologie und Toxikologie (Prof. Dr. Markus Fendt, M.Sc. Kerstin Wernecke). Ziel dieses NeuroNetzwerkes war es, mit einer Kombination verschiedenster verhaltens-pharmakologischer und neuroanatomischer Methoden, die neuronalen Grundlagen angeborenen Angstverhaltens in Laborratten weiter zu identifizieren und zu charakterisieren.

I CONTENT

II LIST OF ABBREVIATIONS

ACoA	cortical nucleus of the amygdala, anterior part
ACTH	adrenocorticotropin
AHN	anterior hypothalamic nucleus
AHiAL	amygdalo-hippocampal area, anterolateral part
AHiPM	amygdalo-hippocampal area, posteromedial part
ANOVA	analysis of variance
AOC	amygdalo-olfactory cortex
APir	amygdalo-piriform cortex
BLA	basolateral nucleus of the amygdala
BLP	basolateral nucleus of the amygdala, posterior part
BMP	basomedial nucleus of the amygdala, posterior part
BNST	bed nucleus of the stria terminalis
CBBS	center for behavioral brain sciences
CoA	cortical nucleus of the amygdala
DMP	2,6-dimethylpyrazine
EDMP	3-ethyl-2,5-dimethylpyrazine
EPM	elevated plus-maze
fr	fasciculus retroflexus
$GABA_A$	*gamma*-aminobutyric acid receptor A
GAD	generalized anxiety disorder
Hb	habenula
HPA	hypothalamic-pituitary-adrenal axis
IBI	inter-bout interval
ICD	international statistical classification of diseases and related health
ICI	inter-call interval
IF	interfascicular nucleus
IPN	interpeduncular nucleus
L-felinine	2-amino-7-hydroxy-5,-dimetyl-4-thiaheptanoic acid
LHb	lateral nucleus of the habenula
LS	lateral septum

MEA	medial nucleus of the amygdala
MHb	medial nucleus of the habenula
NAc	nucleus accumbens
PAG	periaqueductal grey
PEA	2-phenylethylamine
PLCo	cortical nucleus of the amygdala, posterolateral part
PMCo	cortical nucleus of the amygdala, posteromedial part
PMd	dorsal premammillary nucleus
PVP	posterior paraventricular thalamic nucleus
PW	pellet weight
PW50	half maximal pellet weight
rCBF	regional cerebral blood flow
SBT	2-sec-butyl-4,5-dihydrothiazoline
SEM	standard error of the mean
SPECT	single-photon emission computed tomography
TMP	trimethylpyrazine
TMT	2,4,5-trimethylthiazoline
USV	ultrasonic vocalization
VMHdm	ventromedial nucleus of the hypothalamus, dorsomedial part
VTA	ventral tegmental area
WHO	world health organization

1. GENERAL INTRODUCTION

1.1. Normal fear and pathological fear

Fear is an adaptive response to a potentially dangerous or life-threatening event and a basic "emotion" which is shared by humans and other animals (Sotnikov et al., 2011; Ohl et al., 2008). From an evolutionary viewpoint, fear can be conceptualized as a biological warning and survival circuit that supports the recognition of danger and that contributes to the survival and well-being of the organism by triggering a range of appropriate autonomic and species-specific defensive responses (Misslin and Ropartz, 1981; Staples, 2010; LeDoux, 2012; Fendt and Fanselow, 1999). Many fear states are also caused by the anticipation of danger (Sartori et al., 2011; Gelfuso et al., 2014). In this context, some psychological theories have distinguished between fear and anxiety, even if both terms are often interchangeably used. Fear is generally defined as the response to an explicit and immediate threatening stimulus (Adolphs, 2013; Sartori et al., 2011). Anxiety, in contrast, is considered as a more general state of distress and a response to an undetermined, more generalized dangerous stimulus (Sartori et al., 2011; Lang et al., 2000) or in anticipation of danger (Davis et al., 2010; Ohl et al., 2008). An appropriate level of fear/anxiety is protective in many situations, directing the individual's attention to the danger and motivating coping behaviors in stressful challenges. Therefore, a certain degree of fear/anxiety as the situation requires, is a normal part of everyday life (Morschitzky, 2002).

However, when fear becomes excessive, persistent, uncontrollable, and is expressed in the absence of true danger and lacks any adaptive value, it is considered as severe psychiatric illness classified as anxiety disorders. According to the "International Statistical Classification of Diseases and Related Health" (ICD) from the World Health Organization (WHO), anxiety disorders exhibit a broad range of symptoms and various degrees of severity, and can therefore be classified into different nosological categories (ICD-10, Chapter V: Mental and behavioural disorders, subchapter F40 – F48: Neurotic, stress-related and somatoform disorders): agoraphobia (with and without panic disorder)(F40.0), social phobia (F40.1), specific phobias (F40.2), panic disorder (F41.0) and generalized anxiety disorder (GAD)(F41.1). Since anxiety disorders share high co-morbidity with other mental illnesses, particularly major depressive disorder, compulsive disorder or certain personality disorders, an extra group of mixed anxiety disorders (F41.2 – F41.8) was defined in addition.

Exaggerated fear learning has long been accepted as a central feature in the development of pathological anxiety in the human. However, more recent findings point out that patients suffering from anxiety disorders might also have altered innate fear processing (Lissek et al., 2005; Rosen et al., 2015) and that innate predispositions for certain threats might have stronger etiological influences on, for instance specific phobias, than classical fear conditioning processes (Rosen et al., 2008; Rosen et al., 2015). Therefore, animal models of innate fear such as the ones used in the present doctoral thesis, might be also effective in mimicking aspects of specific anxiety states in the human and might contribute to a fuller understanding of their neurobiological mechanisms (Blanchard and Blanchard, 1989b; Rosen et al., 2008). In the following, the different types of anxiety disorders together with their most manifested symptoms will be briefly presented.

In general, all phobic anxiety disorders are characterized by the intense and persistent fear of certain well-defined situations or specific stimuli that are usually not dangerous resulting in a compelling urge to avoid these situations or these stimuli (Morschitzky, 2002). In the case of social phobia which is one of the most common anxiety disorders, the fear is specific to a particular social situation (such as eating or speaking in public) with patients having fear to be humiliated or to act inadequately in public. Sufferers know that their fears are irrational and exaggerated, but will still try to avoid social encounters which often leads to complete social isolation. The exposure to the social situation usually evokes immediate fear responses and accompanying somatic symptoms such as blushing, sweating, or palpitations (Emilien et al., 2002). In contrast, specific phobias are developed towards highly specific objects, such as the fear of spiders, snakes, heights, closed spaces, dentistry, or the sight of blood (Emilien et al., 2002; Morschitzky, 2002). The fear of being in a place or situation where escape to a safe place (usually home) is difficult and where help may be unavailable determines the symptoms of agoraphobia. Patients suffering from agoraphobia often experience secondary phobias including fears of leaving home, e.g., the fear of crowds, public places or traveling alone in buses or planes which in severe cases may end in complete houseboundness.

People with panic disorder, in contrast, have sudden and unpredictable attacks of intense terror accompanied by severe autonomic symptoms, like sweating, trembling, dizziness and shortness of breath that last for several min reaching a maximum state in less than 10 min. While having a panic attack, people experience the fear, for instance of losing control, dying or having a heart attack, while in periods without panic attacks they worry

about the attack's potential implications and future attacks (Morschitzky, 2002; Emilien et al., 2002).

Contrary to all other types of anxiety disorders, the fear of patients with GAD is not restricted to any particular situation but generalized and pervasive. In turn, people become overly concerned with everyday matters. They cannot relax or concentrate, startle easily and are persistently nervous. Even if the increased anxiety level usually does not evoke panic attacks, it is still linked with physical symptoms such as lightheadedness, muscular tensions, fatigue, nausea, trembling or sweating (Emilien et al., 2002; Morschitzky, 2002).

Nowadays, anxiety disorders are among the most common mental disorders in the USA, Europe and Germany affecting both children and adults. At any age, woman are more affected than men (Emilien et al., 2002). Due to their high prevalence in the western countries, anxiety disorders also represent a major health problem causing high and enduring economic costs for both the social and healthcare system (Robert Koch Institute, Berlin, Germany, DEGS: Studie zur Gesundheit Erwachsener in Deutschland, 2013).

1.2. Animal models of innate fear

In preclinical research, animal models of neurobehavioral disorders are usually described as experimental paradigms developed in a nonhuman species in order to artificially replicate certain pathophysiological or behavioral states in the human, since studying these states and processes in the human is difficult due to ethical reasons (Steimer, 2011). Animal models are developed and utilized for mainly two purposes: (1) as simulation devises to improve the knowledge of the mechanisms and the etiology underlying the disorder and (2) as screening tool in the search for novel medications (Kumar et al., 2013; Rodgers et al., 1997). Importantly, the development and usage of each single animal model relies on the notion that the process of interest is sufficiently similar between the model organism and the human. Often, three types of validity criteria are applied to validate animal models: face validity, construct validity and predictive validity. Face validity refers to phenotypical similarities meaning that the responses observed in the model organism should be similar to those observed in the human. Construct validity is fulfilled when the neural mechanisms underlying disorder-related behavioral or physiological changes are the same in the animal model and the human. Lastly, predictive validity implies that the animal model should be sensitive to clinically effective pharmacological treatments (Bourin et al., 2007; Kumar et al., 2013).

As previously described, “anxiety disorders” encompass a heterogeneous group of disorders, each of them is in itself complex. Consequently, it is comprehensible that one single animal model cannot cover all features of human fear not to speak of its pathology. Instead, different animal models should be used to mimic distinct endophenotypes such as behavioral responses to acute fear-inducing stimuli. Although humans and rodents may look totally different, at a physiological and anatomical level they are remarkably similar. They still share more than 90 % of their genes (the rodent and the human genome diverged approximately 75 million years ago) and possess a number of neuroanatomical and neurochemical commonalities (Kumar et al., 2013). Moreover, typical signs of rodent fear such as fight/flight responses, avoidance, attention/vigilance and the release of corticosteroids are strongly conserved throughout evolution and are presently viewed as essential precursors to human fear and anxiety reactions (Sartori et al., 2011; Kumar et al., 2013; Steimer, 2011; Rodgers et al., 1997). As such, an understanding of the neurobiology of defensive fear responses in rodents may also extend our knowledge about some aspects of human anxiety and the underlying biological mechanisms.

According to the nature of the fear stimulus and whether the evoked behavioral response is innate or learned, animal models of anxiety/fear can be grouped into two main classes (see also Tab. 1). The first group includes animal models that assess the animal’s learned response to aversive stimuli. Such animal models (e.g., fear-potentiated startle test, active/passive avoidance test) use fear conditioning procedures in which a neutral stimulus is associated with an aversive unconditioned stimulus (e.g., foot shock, loud noise) in order to become a conditioned stimulus. Following association, the originally neutral stimulus serves as a conditioned stimulus and will elicit anticipatory fear responses that help the organism to cope with the expected aversive event (Davis, 1992). Besides the usage of electric foot shocks as unconditioned stimuli some conditioned fear models also require food or water deprivation and extensive training sessions which might be disadvantageous under certain circumstances (Davis, 1992; Rodgers et al., 1997).

Animal models of the second group include more ethologically-based animal tests focusing on the animal’s spontaneous and natural reaction to aversive stimuli and do not involve learning processes. These animal models do not explicitly involve pain, but attempt to mimic more natural stimulus conditions in which evoked fear responses are more likely to be the ones expressed by rodents in their natural environment (e.g., exposure to novel environments, heights or to predator odors) (Kumar et al., 2013; McGregor et al., 2002;

Litvin et al., 2008; Bourin et al., 2007). Most of these animal models, such as the elevated plus-maze (EPM) test or the open-field test, are based on the confrontation of the animals with unfamiliar and/or unprotected environments and measure changes in exploratory behaviors (Litvin et al., 2008). To which degree an animal will explore such environments has been shown to be the consequence of an inner conflict between the natural drive of rodents to explore (motivated by curiosity) and their inborn aversion (motivated by fear) to elevated, open or brightly lit spaces (Bourin et al., 2007; Sotnikov et al., 2011; Kumar et al., 2013). Usually the higher the anxiety level, the less the animal will show exploratory behaviors (Gelfuso et al., 2014).

Another class of more ethological animal models is based on defensive responses evoked by the interaction of rodents with predators (separated by a wire mesh or anesthetized) or predator-related stimuli such as their odors (Kumar et al., 2013; Litvin et al., 2008). Exposure to a live predator inevitably includes the presentation of the full complement of multimodal threat stimuli making research on the neural system from the sensory input to the brain output centers relative difficult. An easier possibility to study innate fear responses in the context of predator-prey interactions is the usage of only a single sensory modality such as the odor of predators. A summary of the predator odors most commonly used in behavioral research on laboratory rats and mice will be given in the following chapter.

Table 1 Conditioned and innate animal models of fear/anxiety (Steimer, 2011; Kumar et al., 2013).

Animal models of learned fear/anxiety	**Animal models of innate fear/anxiety**
Fear-potentiated startle test	Open-field test
Active/Passive avoidance test	Elevated plus-maze (zero-maze/T-maze) test
Vogel water-lick conflict test	Light-dark box test
Four-plate test	Hole-board test
Conditioned taste aversion test	Acoustic startle response test
	Separation-induced ultrasonic vocalization test
	Predator/Predator odor exposure test
	Staircase test

1.3. Predator odors used in recent research

A plethora of studies has demonstrated that predator-naive rodents display a broad array of defensive fear responses accompanied by autonomic changes and the activation of the hypothalamic-pituitary-adrenal axis (HPA) when being exposed to predator odors (Apfelbach et al., 2005; File et al., 1993; Blanchard et al., 1998). Such odors that harm the interests of the releaser while being beneficial for the receiving animal (of another species) are called kairomones (Rajchard, 2013; Fortes-Marco et al., 2013). Over the last several decades, there have been significant advances in understanding how kairomones affect behavior. However, there are still a number of unresolved issues that we do not yet understand. For instance: Is the urine of each predator species fear-inducing in rats? Do rodents communicate predation threats signaled by predator odors? Which brain regions decide when to choose an active or passive fear response strategy? Some of these open questions will be addressed in the present doctoral thesis. A summary of the physiological and behavioral consequences of predator odor exposures in rodents will be given in chapter 3.1. Chapter 4.1 provides an overview about the neural basis of predator odor-induced innate fear known so far.

Considering both laboratory and field studies, many different animal species have served as “predators” ranging from domestic cats/dogs over different mustelid species and larger carnivores (e.g., foxes, wolves, dingos, bobcats, lions) to snakes (Papes et al., 2010; Rosell, 2001; Staples and McGregor, 2006; Ferrero et al., 2011; Masini et al., 2005; Blanchard et al., 2003c; Osada et al., 2013; Farmer-Dougan et al., 2005; Swihart, 1991; Parsons et al., 2007; Ramp et al., 2005; Zhang et al., 2003). Moreover, various odor sources have been used including odors from the fur or skin, urine, feces or anal gland secretions of a predator (Apfelbach et al., 2005). In the laboratory, the predator odors most frequently employed to investigate defensive responses in rodents are cat fur odors principally obtained from worn cat collars or cloths rubbed against the fur of domestic cats (e.g., Takahashi et al., 2005; Dielenberg and McGregor, 2001) and 2,4,5-trimethylthiazoline (TMT), a component of red fox feces (e.g., Fendt et al., 2005a; Wallace and Rosen, 2000). In the last few years, an increasing number of studies has now been performed exposing laboratory rodents to predator urine samples (Feoktistova et al., 2003; Ferrero et al., 2011; Osada et al., 2013; Funk and Amir, 2000; Voznessenskaya et al., 2003; Fendt, 2006).

Both fur odors and predator urine samples have clear disadvantages in that its usage cannot be well controlled. This is mainly because the active chemical compounds that

actually give rise to the fear responses are widely unknown. This makes it almost impossible to work with odor samples identical in intensity or quality across experiments. An additional drawback of fur- and urine-derived odors is that their compositions might be dependent on the gender, age and diet of the donator animal (e.g., Berton et al., 1998; Nolte et al., 1994). In general, it is assumed that elevated levels of sulfur- and nitrogen-containing compounds in the secretions of predators, as typical metabolites of meat digestion, may be responsible for the aversive qualities of predator odors. According to this hypothesis, the urine of coyotes maintained on a meat diet has been shown to have a greater repellent effect to mountain beavers than urine of coyotes on a fruit diet or coyote urine with all sulfur compounds being chemically removed (Nolte et al., 1994; Apfelbach et al., 2005). Up to now, a number of single molecule components of natural predator odor blends that effectively induce innate fear in rats or mice has been identified. In the following, the most investigated single predator odor components are presented (reviewed in Apfelbach et al., 2015).

The best investigated individual odor component is TMT which was originally extracted from red fox feces. TMT has not been found in dog feces (Arnould et al., 1998) or in anal gland secretions of the dog and coyote (Preti et al., 1976) which suggests that TMT may be a specific odor of foxes but not of other predators. TMT has strong fear-producing properties (Galliot et al., 2012; Endres and Fendt, 2009; Fendt et al., 2005a; Rivard et al., 2014; Staples and McGregor, 2006; Hacquemand et al., 2013; Wallace and Rosen, 2000). However, up to this date, there is an enduring debate on whether TMT really represents a biologically-relevant predatory signal (Fendt and Endres, 2008; McGregor et al., 2002) since TMT does not activate the hypothalamic defensive circuit (Staples et al., 2008), an highly connected brain system which is strongly implicated in the processing of defensive behavior towards a live cat or cat odor (Canteras et al., 1997; Pagani and Rosen, 2009; Dielenberg and McGregor, 2001; Markham et al., 2004). Furthermore, TMT in high concentrations has a repugnant and acrid irritating smell. This raises the possibility that the observed behavioral effects should rather be attributed to the noxious nature of TMT than to its specific fear-inducing properties. Arguments against the view that TMT primarily works through nociceptive mechanisms originate from recent studies showing that olfactory bulb ablation completely blocked the freezing response of rats to TMT whereas trigeminal nerve transection did not affect TMT-induced freezing (Ayers et al., 2013; Taugher et al., 2015). Notably, the freezing response to butyric acid, a pungent but certainly non-predatory odor, was significantly reduced following trigeminal nerve transection but not following olfactory

bulb removal. In this regard, TMT also increased blood pressure in mice as a physiological measure of fear, whereas butyric acid was almost ineffective in doing so (Brechbühl et al., 2013; Horii et al., 2010).

In 2011, Ferrero and colleagues isolated another single predator odor component from the urine of several predator species, the biogenic amine 2-phenylethylamine (PEA). PEA has also been found in urine samples of different herbivores, however, in more than 3,000-fold lower amounts. Of importance, PEA triggered hard-wired avoidance behavior in rats and mice as similarly observed in response to coyote or lion urine samples. Interestingly, the fear-inducing properties of lion urine were lost when lion urine was PEA-depleted and fully restored when the lion urine sample was again respiked with PEA (Ferrero et al., 2011). Consequently, PEA has been discussed as a key component of a predator urine blend that induces innate defensiveness in rodents. Unfortunately, no further experiments have been performed so far using PEA as fear-evoking stimulus in rodents.

Pyrazine and pyridine analogues represent a different group of predator urine components that might be important for predator-prey signaling (Osada et al., 2013; Brechbühl et al., 2015). For instance, Osada and colleagues (2013) showed that pyrazine analogues found in urine samples of wolves mimicked the ability of original wolf urine samples to induce freezing behavior in mice. Notably, the strongest fear-inducing effects were observed when mice were exposed to a mixture of different analogues, namely 2,6-dimethylpyrazine (DMP), trimethylpyrazine (TMP) and 3-ethyl-2,5-dimethylpyrazine (EDMP) (Osada et al., 2013). Interestingly, Brechbühl et al. (2013) almost simultaneously identified a volatile mouse alarm pheromone, 2-sec-butyl-4,5-dihydrothiazoline (SBT), that is released by both male and female mice under different alarm conditions in order to warn other conspecifics. SBT shares many chemical features of the sulfur-containing pyrazines present in predator urine samples. Accordingly, it has been speculated whether heterocyclic sulfur-containing compounds might represent chemical basic elements for signaling danger to prey animals, both in terms of intra-species and inter-species communication.

Another odor component specific to all members of the felidae family has been reported to be 2-amino-7-hydroxy-5,-dimetyl-4-thiaheptanoic acid (L-felinine) and its derivatives. L-felinine is a sulfur-containing amino acid and is considered to be a putative precursor molecule of 3-marcapto-3-methylbutan-1-ol, a cat pheromone that is important for territorial behaviors (Hendriks et al., 1995b; Hendriks et al., 1995a; Miyazaki et al., 2006). Exposure to L-felinine significantly suppressed reproduction in rats and mice as indicated by

an increased pregnancy block, lower litter size, a significant drop of the weight of offsprings and a skewed sex ratio in favor of males (Voznessenskaya and Malanina, 2011; Voznessenskaya et al., 2003; Voznessenskaya, 2014).

2. AIM OF THE STUDY

In contrast to acquired fear through learning, predator odors have been suggested to recruit “learning-independent” systems with animals being innately sensitive to the threatening stimulus. In the field of animal research, most studies unraveling the neural basis of fear and its pathology used conditioned fear paradigms. Studying the response to conditioned fear stimuli is certainly important, however, more recent findings showed that some anxiety disorders might be also caused by deficits in innate fear processes. Therefore, animal models of innate fear might be also effective in mimicking aspects of specific anxiety states in the human and might contribute to a fuller understanding of their neurobiological mechanisms. The present doctoral thesis further investigates innate fear processes in rodents. Different research aims were pursued: The first aim was to further characterize the behavioral consequences of predator odor exposures in rats. Secondly, different experiments were performed to better understand the neural substrates underlying innate fear. To reach these aims, different behavioral and pharmacological techniques were used. The doctoral project was divided into three research parts:

Part 1: The characterization of predator odor-induced behavioral changes.

Part 2: The identification of the neural basis of predator odor-induced fear.

Part 3: The establishment of the olfactory hole-board test to simultaneously assess behavioral responses to different odors.

Part 1 of this thesis had the aim to further characterize the effects of predator odors on both defensive and non-defensive behavioral responses in rats. To this end, different animals were tested in three single experiments. In *Experiment 1,* the effectiveness of different predator urine samples and the single predator urine component PEA to induce avoidance behavior in rats, was assessed. *Experiment 2* investigated food-carrying behavior of rats in a “high risk” testing condition by presenting a fox urine sample next to the food source. Lastly, *Experiment 3* was performed to study whether exposure to different predator odor samples evokes aversive 22-kHz alarm calls in rats.

The experiments included in Part 2 were part of a CBBS-funded NeuroNetwork that was aimed at elucidating the neural circuit underlying predator odor-induced fear behavior in rodents. Together with Dr. Jürgen Goldschmidt and M.Sc. Daniel Vincenz-Zörner (Leibniz

Institute of Neurobiology) different brain sites activated or inactivated during exposure to fox urine were identified using single-photon emission computed tomography (SPECT)-imaging of the regional cerebral blood flow (rCBF). Of all identified brain regions, the amygdalo-olfactory cortex (AOC, *Experiment 1*) and the habenula-interpeduncular nucleus axis (Hb-IPN axis, *Experiment 2*) were selected to further investigate their relevance in innate fear processes. Local muscimol injections as well as electrolytic lesions were applied to temporary or permanently inactivate these target regions. Effects of these inactivations on predator odor-induced defensive behaviors were assessed.

Research focused on the effects of odors on rats' behaviors often include multi-trial paradigms where animals experience a sequence of single odor exposures as this was also the case in the experiments of Part 1. Such test designs are usually time-consuming and risk the possibility that animals could get habituated to predator odors. In Part 3 of the doctoral project, a new behavioral paradigm to assess aversion and preference responses to biologically-relevant odors was introduced. The so called olfactory hole-board test is unique in that it allows studying behavioral responses to different odors at the same time, in single trials. Four experiments have been conducted to further describe and validate this paradigm.

3. PART 1: PREDATOR ODOR-INDUCED BEHAVIORAL CHANGES

3.1. Introduction

Predator odors are biologically-relevant chemosignals that if recognized by prey animals signal the potential presence of a predator. Thereby, they alert the prey animal and facilitate its survival. Even though predator odors do not signal actual predatory presence but only a possibility of danger, they are effective stimuli for eliciting an array of different physiological and anti-predatory behavioral responses in rodents (reviewed in Dielenberg and McGregor, 2001; Takahashi et al., 2005; Apfelbach et al., 2005; Fendt et al., 2005a).

The most often observed physiological response associated with fear induced by predator odors is the activation of the HPA. For instance, exposure to cat fur odor (File et al., 1993; Blanchard et al., 1998b; Day et al., 2004), TMT (Day et al., 2004; Kobayakawa et al., 2007; Morrow et al., 2000) or the fur of ferrets (Masini et al., 2005) has been shown to increase the secretion of adrenocorticotropin (ACTH) or corticosterone. A transient rise in blood pressure of rats has also been observed following cat odor and TMT exposure (Dielenberg et al., 2001a; Brechbühl et al., 2015).

Besides physiological changes, rodents also choose from a repertoire of species-specific behavioral responses to defend themselves from a predation threat. The occurrence of specific defensive behaviors appears to be largely modulated by external factors including the context in which the predation threat is encountered and the exact nature of the threat (Yang et al., 2004; Blanchard et al., 2003b; Blanchard and Blanchard, 1989b). Earlier experiences might play a role, too (Fendt and Endres, 2008). Determined by these factors, the animal will select the behavior that will be the most effective one for the particular circumstances and that offers the highest probability of survival. This shows that the defense system of rodents is not simply reflexive but rather flexible and adaptive (Fendt and Endres, 2008).

In the literature, usually two major defensive responses of rats expressed in the presence of predator odors are described: avoidance/hiding behavior and freezing (e.g., Fendt and Endres, 2008; Staples, 2010; Wallace and Rosen, 2000). Avoidance responses are usually accompanied by risk assessment behaviors that orient the animal towards the threat stimulus and allow cautious analysis of the potential threat and the situation in which the threat is encountered (Staples, 2010; Ohl et al., 2008). The animal may even approach the

odor source. While approaching, rats usually adapt a low-back or stretched-approach posture interspersed with periods of immobility and avoidance behavior (McGregor et al., 2002). Slowly movements of the head from side to side in order to catch further visual or auditory cues as well as directed sniffing are additional characteristics of risk assessment (Blanchard et al., 2011; Blanchard et al., 2003b). When rats have the opportunity to hide, they display so called "head-out behavior" where the individual stands in a stretched-attended posture with the head and shoulders protruding to the unprotected area while the rest of the body is still concealed in the hiding possibility (Staples, 2010; McGregor et al., 2002). When retreat to a secure place is not possible or the threatening stimulus is very intense (e.g., high concentration of TMT), freezing is the predominant response (Rosen et al., 2008; Wallace and Rosen, 2000). Freezing is defined as an immobility state in which all movements except those required for respiration are reduced (Blanchard and Blanchard, 1969). During freezing animals usually adopt a sitting or crouching posture. Freezing in an upright or rearing posture has been observed, too (Blanchard et al., 1990).

In addition to eliciting robust anti-predatory responses, predator odors also change locomotor activity and suppress ongoing non-defensive behaviors such as grooming, sexual behaviors or playing behaviors (Apfelbach et al., 2005; Staples and McGregor, 2006; Siviy et al., 2010b). Furthermore, Sundell et al. (2004) observed that free-living bank voles forage more in a low risk situation relative to a high risk situation where a tube with a live weasel inside was situated next to a food patch. Therefore, predatory threats are influencing also food intake and foraging behaviors (Lima and Bednekoff, 1999; Lima and Valone, 1986; Brown and Kotler, 2004).

Like many other rodents, rats also incorporate ultrasonic communication into their defensive response patterns. 22-kHz ultrasonic vocalization (USV) has been observed to be emitted in a variety of aversive situations probably in order to warn relatives of external danger. For instance, Wöhr and colleagues (e.g., in Wöhr et al., 2005; Borta et al., 2006) observed that rats emit 22-kHz USV during fear conditioning when an aversive stimulus (e.g., electric food shock) is repeatedly paired with a formerly neutral stimulus (e.g., light, tone). Moreover, 22-kHz USV occurred during withdrawal from drugs such as opiates and cocaine (Mutschler and Miczek, 1998; Vivian and Miczek, 1991; Covington and Miczek, 2003) or in aggressive inter-male interactions (Lore et al., 1976; Thomas et al., 1983). The first study showing that rats emit 22-kHz USV during confrontation with a predator, namely a cat, had been performed by Caroline and Robert Blanchard using the visible burrow system, a semi-

natural environment that provides tunnels and burrow systems as well as “surface areas” for mixed-set rat colonies (Blanchard et al., 1991).

Part 1 of this thesis includes three experiments that were performed to assess how predator odors affect rats’ behaviors. Since the experiments vary in terms of their pursued research aims and methodologies (measurement of avoidance behavior, food-carrying-behavior or USV), short introductions for each experiment will be given separately.

3.2. General Materials and Methods

3.2.1. Subjects

95 experimentally naive male Sprague-Dawley rats, 2-3 months old at the start of testing were used. Rats were bred and reared at the local animal facility (original breeding stock: Taconic, Denmark). They were housed in groups of 5-6 rats in standard Macrolon Type IV cages (55 cm x 33 cm x 20 cm) in temperature- (22 ± 2 °C) and humidity- (50-55 %) controlled rooms under a 12 h light/dark cycle (lights on at 6:00 am). For all animals, tap water was available *ad libitum*. Rats used in *Experiment 2* were maintained at about 85 % of their free-feeding body weight by providing a limited amount of 12 g standard laboratory rodent chow (Sniff Spezialitäten GmbH, Soest, Germany) per rat per day. All other rats received food *ad libitum*. The experiments were conducted during the light phase between 8:00 am and 4:00 pm.

All experiments were carried out in accordance with the international ethical guidelines for the care and use of laboratory animals for experiments (2010/63/EU) and were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42505-2-1172 UniMD).

3.2.2. Odor Samples

Urine samples of foxes (*Vulpes vulpes*), wolves (*Canis lupus*), bobcats (*Lynx rufus*), pumas (*Puma concolor*) and coyotes (*Canis Iatrans*) were purchased from Maine Outdoor Solutions Inc. (Hermon, ME, USA). Urine samples of lions were obtained from the local zoo (Zoologischer Garten Magdeburg, Germany). Female rat urine (*Rattus norvegicus*) was self-collected by placing adult female Sprague-Dawley rats (n = 18, 3-6 months old) individually in a metabolic cage (Tecniplast, Hohenpeißenberg, Germany) for ca. 30 min on consecutive days. Female urine samples of individual animals were mixed up to ensure that urine from all estrus cycle phases was present. All urine samples were aliquoted into 1 ml portions and stored at -18 °C until usage. Furthermore, rats were exposed to different concentrations (0.04; 0.4; 4; 40 µmol/ml) of PEA (Sigma Aldrich, Steinheim, Germany) or to pure TMT (Contech Enterprises Inc., Victoria, Canada). Common tap water served in each experiment as control odor.

3.3. Experiment 1: Predator odor-induced avoidance behavior

During the last decades, predator odors have extensively been used to study innate fear responses of rodents in the laboratory. The typical sources of such odors are manifold and range from skin and fur odors to urine, feces or anal gland secretions (see chapter 3.1). However, it is still unclear whether odors of different origin and from different species have comparable predatory qualities? Up to now, most comparative studies compared the behavioral effects induced in rodents by cat fur odor exposure with those observed upon exposure to the synthetic predator odor TMT (Staples et al., 2008; Fendt, 2006; McGregor et al., 2002; Masini et al., 2005) thereby assessing predator odors differing in both their odor source (fur odor vs. feces odors) and donor species (cat vs. fox).

Previous studies further showed that the diet of a predator strongly affects the aversiveness of its odor (Epple et al., 1993; Nolte et al., 1994; Berton et al., 1998). Accordingly, urine of meat-fed coyotes have been demonstrated to have greater repellent effects than the urine of fruit-fed coyotes (Nolte et al., 1994). On the basis of this, many scientists followed the hypothesis that especially metabolites in secretions that reflect increased meat digestion (nitrogen- or sulfur-containing compounds) might be critically important for the aversive effects of predator odors. In search of such ”meat-eater cues” (Nolte et al., 1994), Ferrero et al. (2011) identified PEA, a product of protein metabolism from the urine of several predators. In a series of behavioral experiments, they showed that rats and mice clearly avoided the smell of PEA. Interestingly, rats failed to show full avoidance behavior when being exposed to PEA-depleted lion urine. Unfortunately, no further studies have been performed supporting the view that PEA is a key trigger for avoidance behavior.

Thus, the present experiment had the straightforward aim (1) to assess the relative effectiveness of different predator urine samples (from fox, wolf, bobcat, puma) to induce avoidance behavior in rats and (2) to compare the avoidance responses evoked by natural urine samples with those evoked by different concentrations of PEA (0.04; 0.4; 4; 40 µmol/ml). Considering PEA as an universal component of predator urine blends, rats should avoid PEA and natural predator urine samples to similar degrees.

3.3.1. Materials and Methods

Testing apparatus

Testing took place in one of four identical testing boxes (45 cm x 45 cm x 30 cm) constructed from opaque polyvinyl chloride. The boxes were placed inside an animal detection frame (TSE Systems, Bad Homburg, Germany) with infrared light sensors (14 mm distance between two sensors) which enables automatic measurement of the locations of the rats and thereby their movements. Each box was located in a sound-attenuating measurement chamber provided with four light sources (illumination: < 10 lux) and an observation camera (CCTV-Camera Miniature-CCD-Camera, RS Components GmbH, Mörfelden-Walldorf, Germany). Four small glass bowls (4 cm outer diameter, 2.5 cm height) were placed and fixed in each corner of the testing box by using double-side adhesive tape (Fig. 1.).

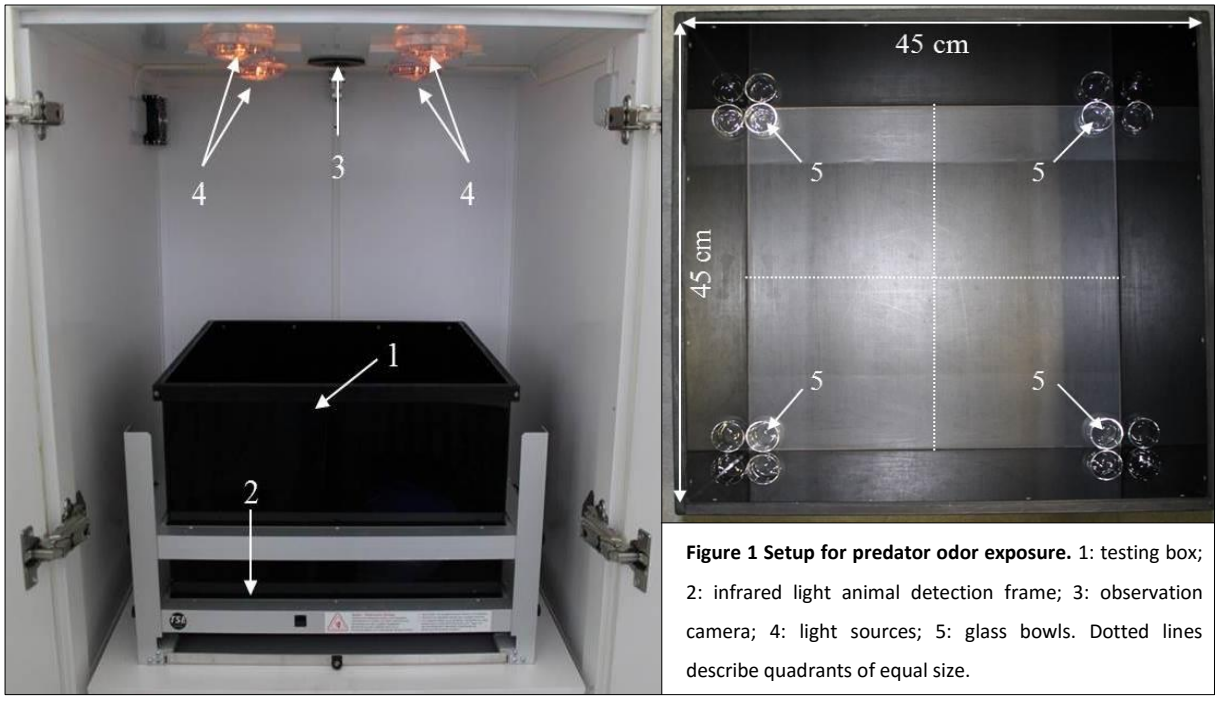

Figure 1 Setup for predator odor exposure. 1: testing box; 2: infrared light animal detection frame; 3: observation camera; 4: light sources; 5: glass bowls. Dotted lines describe quadrants of equal size.

Behavioral testing procedure

Rats were initially given three 10-min habituation sessions without odor to familiarize the rats to the testing boxes and the glass bowls (once per day on consecutive days). On the following testing days, each animal was exposed to one odor sample. Each rat was tested once per day and in seven odor conditions in total with the corner and order of odor sample presentation pseudo-randomized. Briefly, four different cohorts of rats were tested (n = 12; n = 11; n = 16; n = 12). In each cohort, all animals were exposed to water, female rat urine, at

least one type of carnivore urine (fox, wolf, puma or bobcat urine) and at least three concentrations of PEA (0.04; 0.4; 4; 40 µmol/ml).

Rat behavior was monitored for 10 min by the infrared detectors (sample rate: 100 Hz) and videotaped as described above. The TSE software automatically calculated the rat's location for a predefined time interval. Avoidance behavior was measured for each min by calculating the mean percentage of time the rat spent in the quadrant (8 x 8 sensors) containing the odor sample. The testing boxes were thoroughly cleaned with soapy water after each test and ventilated with clean air.

Exclusion criteria

Analysis of locomotor activity from all animals revealed that some few rats showed a high level of inactivity. The analysis of avoidance behavior requires a certain level of locomotor activity. Therefore very inactive animals were excluded from further analysis. The exclusion criterion was reached if animals spent more than 75 % of the test time in only one quadrant of the testing box under control conditions (exposure to water).

Statistical analysis

Behavioral data were expressed as means ± standard errors of the mean (SEMs). To analyze avoidance behavior, one sample t-tests (with Bonferroni correction) were carried out comparing the mean percentage of time rats occupied the particular odor quadrant (total testing time and for each two min-block) with chance level (25 %). Non-normally distributed data (D'Agostino and Pearson omnibus test) were analyzed by the Wilcoxon signed rank test (with Bonferroni correction). To assess differences in the time course of avoidance behavior evoked during exposure to urine sample of either canids (fox, wolf) or felids (puma, bobcat), data for each animal were grouped according to the sample origin (canid urine, felid urine, control). A two-way repeated measures analysis of variance (ANOVA) using odor and time as within-subject factors was conducted. Post-hoc analysis was performed using Tukey's multiple comparisons tests. For all statistical evaluations, a $p < 0.05$ was considered statistically significant (GraphPad Prism 6.00, GraphPad Software Inc., La Jolla, USA).

3.3.2. Results

Avoidance behavior

One animal was excluded due to the above mentioned criterion. Figure 2 A depicts the mean percentages of time (total testing time) rats occupied the odor quadrants containing the particular odor samples. Typical examples of motion traces of individual rats during exposure to the water control (upper panel) and the fox urine sample (lower panel) are shown in Figure 2 B.

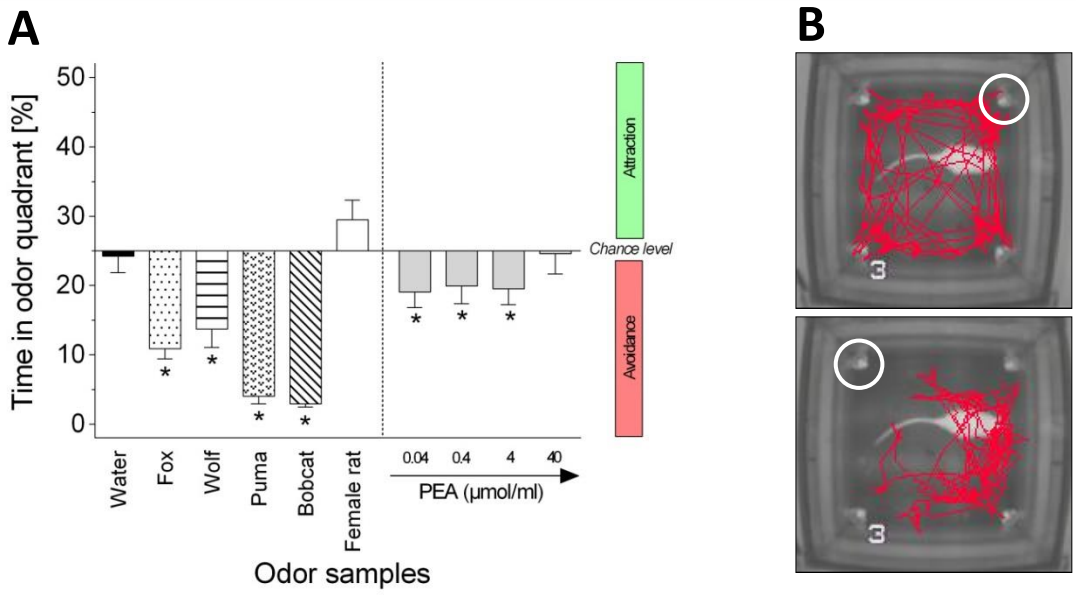

Figure 2 Rats avoided predator urine samples and PEA to different extents. (A) Represented are the mean percentages of time (± SEM) rats occupied the quadrant, defined as 25 % of the testing arena, containing test stimuli indicated (n = 50 for water, female urine, PEA (0.4; 4; 40 µmol/ml); n = 38 for PEA (0.04 µmol/ml); n = 28 for fox urine; n = 12 for puma urine; n = 11 for wolf urine, bobcat urine). *p < 0.05 comparison with 25 % chance level (Wilcoxon signed rank test/One-sample t-test). **(B)** Motion tracks (red) of an exemplary rat during exposure to water (upper) and fox urine (lower). White circles indicate the location of the odor samples.

The type of odor sample strongly affected the occupancy of rats in the odor quadrant. Importantly, animals did not display a spatial preference for any quadrant of the testing box during exposure to water revealing no statistically significant difference to chance level (25 %, Wilcoxon signed rank test: p = 1.635). However, rats spent significantly less time in the odor quadrant during exposure to fox urine (One sample t-test: p < 0.001), wolf urine (Wilcoxon signed rank test: p = 0.048), puma urine (One sample t-test: p < 0.001) and bobcat urine (One sample t-test: p < 0.001), i.e. each predator urine sample was strongly avoided. The urine of female rats did not affect the percentage of time spent in the odor quadrant when compared to chance level (One sample t-test: p = 0.856). Significant avoidance responses were also observed to odor quadrants containing different concentrations of PEA

(Wilcoxon signed rank test: p = 0.018 (0.04 µmol/ml); p = 0.036 (0.4 µmol/ml); p = 0.005 (4 µmol/ml)), whereby only the highest concentration of PEA did not induce avoidance behavior (Wilcoxon signed rank test: p = 3.014).

Time course of avoidance behavior

In Figure 3 the expression of avoidance behavior during the course of odor exposures is shown in two min-blocks. Notably, rats tested under control conditions, i.e. during exposure to water spent constantly about 25 % of each time block in the odor quadrant indicating that water is neither avoided nor preferred by the rats (each time block: p > 0.05). There was a continuously increase in avoidance behavior till time block four (min 7-8) followed by a slight decline in avoidance behavior when rats were exposed to fox urine (block 1: p = 0.003; block 2: p = 0.008; block 3: p < 0.001; block 4: p < 0.001; block 5: p < 0.003). Exposure to wolf urine evoked avoidance behavior only in time block 4 (block 1, 2, 3, 5: p > 0.05; block 4: p = 0.02). In comparison, puma and bobcat urine induced much stronger levels of avoidance behavior (< 10 % of the total testing time in the odor quadrant) from the beginning on. This avoidance response did not strongly intensify across the course of the experiment (puma: each time block: p < 0.003; bobcat: each time block: p < 0.005) as it was the case for fox and wolf urine. When compared to chance level, rats spent longer times in the quadrant containing female rat urine during the first time block (p = 0.004). Afterwards, behavior became indistinguishable from chance level (block 2, 3, 4, 5: p > 0.05). In comparison to the natural predator urine samples, exposure to different concentrations of PEA induced only moderate avoidance behavior in rats (ca. 15-20 % of the total testing time in the odor quadrant) that did not seem to largely change in intensity during the course of the experiment (PEA 0.04 µmol/ml: block 1,4: p > 0.05; block 2,3: p = 0.006; block 5: p = 0.21; PEA 0.4 µmol/ml: block 1,5: p > 0.05; . block 2: p = 0.02; block 3: p = 0.005; block 4: p = 0.04; PEA 4 µmol/ml: block 1,2: p > 0.05; block 3: p = 0.04; block 4: p = 0.004; block 5: p = 0.002). The highest concentration of PEA induced avoidance behavior in none of the time blocks (each time block: p > 0.05).

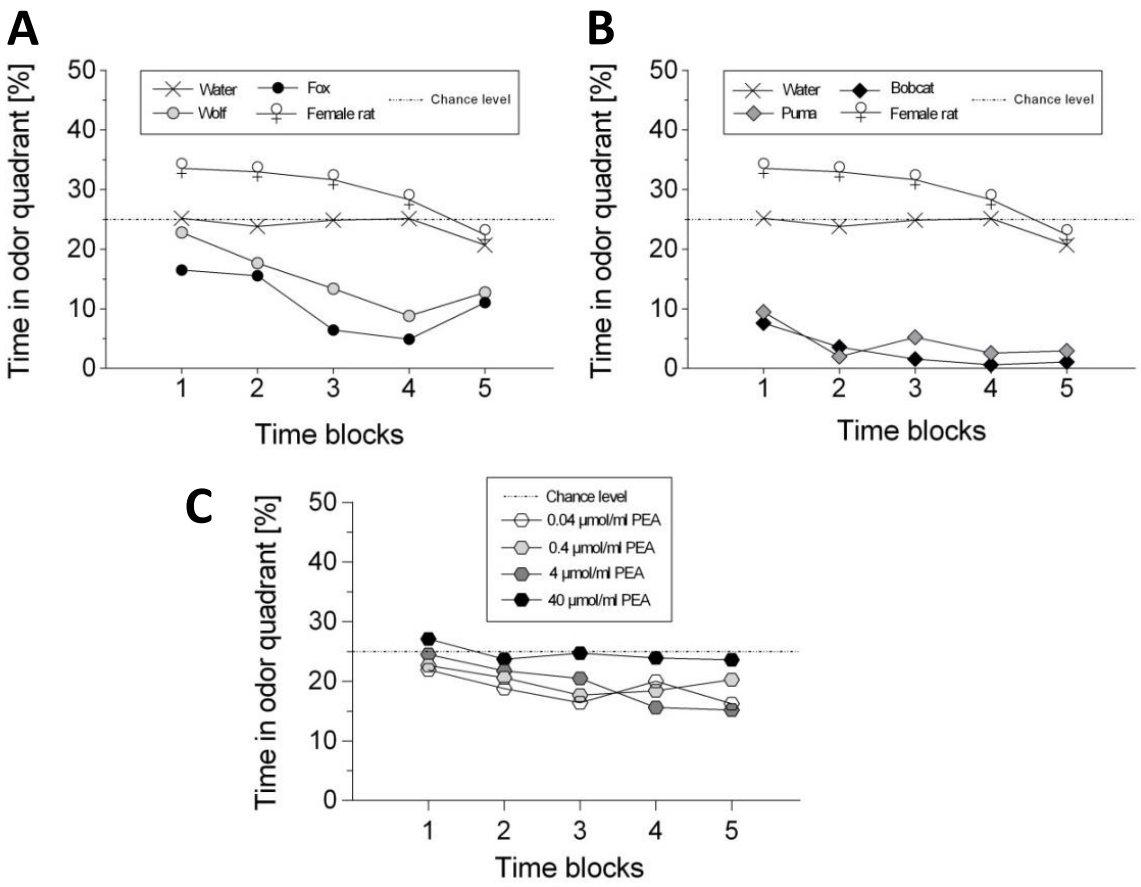

Figure 3 Time course of avoidance behavior. Time course (in two-min blocks) of the mean percentage of time rats spent in the odor quadrant during exposure to the odor samples as indicated (**A**–**C**). The horizontal dashed line represents chance level (25 %). Significant differences to the 25 % chance level were not shown.

Avoidance behavior in dependence on the sample origin (canids vs. felids)

To further investigate the influence of predator urine origin (either from canids or felids), behavioral data of rats during exposure to fox and wolf urine and those during exposure to puma and bobcat urine have been pooled together. A two-way repeated measures ANOVA revealed a significant effect for the factor odor ($F_{(2,436)}$ = 22.81; $p < 0.001$) and the factor time ($F_{(4,436)}$ = 3.10; $p = 0.016$). However, the interaction between odor and time clearly failed to reach statistical significance ($F_{(8,436)}$ = 1.613; $p = 0.12$). Post-hoc pairwise comparisons showed that rats avoided the quadrant containing urine of felids across the whole duration of testing (Tukey's test: water vs. felines: $p < 0.05$ for each time block). In contrast, when exposed to urine of canids, avoidance behavior of rats was only observed in time block 3 and 4 (Tukey's test: water vs. canids: $p < 0.001$) but not during time block 1, 2 and 5 (Tukey's test: water vs. canids: $p > 0.05$). Notably, during time block 2 rats spent significantly less time in the odor quadrant containing felid urine than when exposed to urine from canids (Tukey's test: canids vs. felids: $p = 0.016$).

3.3.3. Discussion

The aim of the present experiment was to assess whether urine samples of different predator species (from foxes, wolves, bobcats and pumas) possess comparable fear-inducing properties in rats. Moreover, avoidance behavior induced by these natural urine samples was compared with that evoked by PEA, a recently identified single molecule component of predator urine samples (Ferrero et al., 2011).

The first finding of the present experiment is that all predator urine samples robustly induced avoidance behavior in rats (see Fig. 2), i.e. rats spent significantly less than 25 % of the testing time (chance level) in the quadrant containing a particular predator urine sample. Similar fear-inducing properties of predator urine samples have been observed elsewhere in both laboratory-reared and free-living rodent species (Osada et al., 2013; Funk and Amir, 2000; Nolte et al., 1994; Rosell, 2001; Swihart, 1991; Xu et al., 2012). In nature, the urine of predators (e.g., dingo, coyote, bobcat, wolf) also has been proven to be very effective repellents protecting forestry and agricultural areas from feeding-related damage (Sullivan et al., 1985a; Rosell, 2001; Swihart, 1991; Nolte et al., 1994; Parsons et al., 2007; Apfelbach et al., 2005; Bramley and Waas, 2001). These findings together with the present results indicate that predator urine samples function as full predatory signals that reliably induce fear behavior in rodents. This is in contrast to the wide-spread suggestion that urine- or feces-derived predator odors are poor predictors for the proximity of a predatory threat and evoke less clear and no robust behavioral signs of fear (Masini et al., 2005; Blanchard et al., 2003a; Nielsen et al., 2015).

Despite the overall effectiveness of the predator urine samples to induce avoidance behavior, differences in the strength of their avoidance-evoking properties are clearly recognizable (see Fig. 2). The most intense avoidance response (less than 5 % of the total testing time in the odor quadrant) was expressed in rats when exposed to urine of the puma or bobcat, two representatives of the felidae family. Urine from species of the canidae family, the fox and wolf, was also clearly avoided (ca. 10-15 % of the total testing time in the odor quadrant) by rats, albeit not as strongly and more variable as in response to puma or bobcat urine. Why odors of the felidae family are stronger avoided by rats, is not clear. Considering the development of avoidance behavior during the course of odor exposure might provide further insights into this issue. Notably, avoidance behavior of rats to fox urine became more intense during the course of odor exposure (see Fig. 3) with the strongest avoidance effect being observed at time block 3 and 4. The avoidance response

towards the wolf urine-containing quadrant is much more variable and only statistically different to chance level at time block 4. A likely explanation for the expression of less or no avoidance behavior to fox and wolf urine during the first min of testing is that rats might have been more engaged in risk assessment behaviors at this time. Risk assessment behaviors are usually expressed to create a first impression of the threat stimulus and the situation in which the threat is encountered. Besides approach in a stretched-attended posture, risk assessment behaviors also include direct contacts with the odor source (Blanchard et al., 2011), both in turn would increase the time spent in the odor quadrant. Unfortunately, in the present study risk assessment behavior was not measured. Therefore, the previous conclusions need to be considered with caution. When risk assessment confirms the danger, rats switch to a more effective defense pattern (Wallace and Rosen, 2000; Blanchard et al., 2011), for instance to intense avoidance behavior as this can be observed in the present study during exposure to urine of canids. In contrast, strong avoidance behavior towards the puma and bobcat-containing quadrant was already evident from the first time block on. This suggests that urine samples of canids and felids may not necessarily differ in their capacity to induce fear but maybe trigger different defensive responses in rats with fox/wolf urine inducing avoidance and risk assessment behaviors while bobcat/puma urine is predominately avoided.

In general, two detection mechanisms are conceivable allowing prey animals to eavesdrop on predator species. On the one hand, rats may sense predatory threats by detecting common urinary molecules that are produced by all predators probably through shared carnivore metabolic pathways. Alternatively or in addition, predator detection may involve more complex sensory mechanisms where prey species detect a wide range of species-specific predator signals (Ferrero et al., 2011; Papes et al., 2010). In 2011, Ferrero and colleagues identified PEA to be a predator urine-derived kairomone that activates neural circuits in the rodent brain important for innate avoidance responses. PEA is a common metabolite that can be found in the urine of different mammalian orders. However, its production has been shown to be elevated in carnivores possibly as a direct consequence of increased levels of dietary protein in meat-eating species. Interestingly, screening more than 100 urine samples, Ferrero et al. (2011) showed that only low concentrations of PEA have been identified in the urine of smaller carnivores (e.g., ferret, fox, cat, human) while higher amounts of PEA are present in the urine of larger feline carnivores (e.g., tiger, lion, jaguar). Differences in defensive behaviors evoked in response to urine samples of canids or felids

might therefore reflect different amounts of PEA in the respective urine samples. Thus, ongoing research should determine PEA contents in the urine samples used.

To test whether rats avoid PEA to similar degrees as natural predator urine samples, in the present experiment rats were additionally exposed to different concentrations of PEA. The range of concentrations was chosen because of statements made in the manuscript of Ferrero et al. (2011). Subsequent behavioral analysis revealed that all PEA concentrations (0.04; 0.4; 4 µmol/ml) except for the highest concentration (40 µmol/ml) were avoided by the rats supporting the suggestion that PEA acts as an individual predator odor component. However, in contrast to the findings of Ferrero et al. (2011) who observed strong avoidance responses of rats to PEA that were comparable with those induced by coyote and lion urine exposure, in the present study the avoidance responses towards the PEA-containing odor quadrants were only modest (approximately 20 % of the total testing time in the odor quadrant). Although a plausible explanation for the different study results is still missing, the present data suggest that the full expression of fear behavior to predator odors might require the detection of additional kairomones (e.g., pyrazine analogues as identified by Osada et al., 2013) and support the view that PEA is only one, but not an exclusive component of a predator urine blend. Interestingly, in the present experiment only the lower concentrations of PEA were avoided assuming that higher concentrations of PEA would be not of physiological relevance and no longer fear-evoking in rats. Indeed, the highest level of PEA was found in lion urine (645.3 µMol, lion 3, Capron Park Zoo, MA, USA) being almost 100 fold weaker concentrated than the highest PEA concentration used in the present study.

In the current experiment, rats were tested in a rather simple odor exposure paradigm in order to study behavioral effects of mainly aversive odors. Appetitive odors, in contrast should induce attraction behavior (Achiraman and Archunan, 2006; Achiraman et al., 2010; Hosokawa and Chiba, 2005), i.e. rats should remarkably spent more time in the odor quadrant. To check this, rats tested in *Experiment 1* were also exposed to female rat urine samples. Contrary to our expectation, the statistical analysis did not reveal a clear preference response towards the female rat urine sample (at least for the total testing time). Different studies revealed that male rats are more attracted to odors of estrous females than to that of non-estrus females (Achiraman and Archunan, 2006; Hosokawa and Chiba, 2005; Achiraman et al., 2010). In the present study, urine of females was collected regardless of the female's estrus cycle stage. Therefore, different amounts of estrus urine in

the different odor samples may serve as a likely explanation for the weak effectiveness of female rat urine to attract male rats.

Summarizing, *Experiment 1* showed that natural predator urine samples robustly induced avoidance behavior in laboratory rats. However, despite this overall effectiveness to evoke avoidance responses, urine of canids appeared to be less strongly avoided by rats and might induce also higher levels of risk assessment than urine of felids which was predominantly avoided. Moreover, PEA, a natural component of a predator urine blend induced only mild avoidance behavior in rats suggesting that probably a whole bouquet of urine components is required to elicit a full fear response in rats.

3.4. Experiment 2: Effects of fox urine on food-carrying behavior

Wild rats have to leave their protective nests to search for food in order to cover their energy demands. However, being outside the nest foraging is also connected to the risk of being detected and attacked by a predator. Both foraging and predation risk avoidance are clearly essential for the survival of all prey species. However, in nature, maximization of feeding efficiency while simultaneously minimizing predation risk is impossible (Lima and Valone, 1986; Lima et al., 1985). Consequently, prey animals try to optimize the trade-off between foraging behavior and risk avoidance (Lima and Valone, 1986; Lima et al., 1985; Onuki and Makino, 2005).

Upon encountering a food source, rats face a so called “food-handling conflict”, where they have to decide on whether to eat a piece of food at the food source or to transport it to the safe nest (food-carrying behavior) to eat it there (Nakatsuyama and Fujita, 1995; Lima and Valone, 1986; Lima et al., 1985; Leaver and Daly, 2003). Eating food where it was found directly reduces hunger, whereas carrying food allows eating to occur in a safe place, although carrying food wastes time and requires energy for transportation (Lima and Valone, 1986). Such decisions on where to eat are known to be dependent on different incentive factors (Leaver and Daly, 2003; Nakatsuyama and Fujita, 1995; Onuki and Makino, 2005). For instance, it has been shown that sated rats carry more food to the nest, while hungry rats eat more food at the food source indicating that the feeding status modifies food-carrying behavior (Onuki and Makino, 2003). If hunger alters the food-handling decision in favor of immediate food intake to maximize feeding efficiency, then foraging rats should likewise increase food-carrying behavior when the foraging situation becomes more risky. In most field and laboratory studies investigating food-carrying behavior in rodents, predation risk was defined as a function of the time spent away from a protective cover (Leaver and Daly, 2003; Nakatsuyama and Fujita, 1995; Lima et al., 1985). The lack of protective cover, however, represents only one possibility of danger and not the imminence of a direct threat. To increase the aversiveness of a foraging situation, Onuki and Makino (2005) tested food-carrying behavior of rats while presenting a conditioned fear stimulus as a risk-approaching signal. Presentation of this conditioned stimulus increased food-carrying behavior.

In contrast to stimuli with acquired fear properties, there are also stimuli that innately induce fear in prey animals (McGregor et al., 2002; Fendt, 2006; Rosen et al., 2006). Being primarily olfactory oriented, rats as previously mentioned, can recognize predation threats in their environment by detecting predatory olfactory cues (Fendt and Endres, 2008).

Predator odors can induce an array of different anti-predatory responses (reviewed in Apfelbach et al., 2005; Dielenberg and McGregor, 2001; Takahashi et al., 2005). For example, using odor exposure tests, we and others (Ferrero et al., 2011; Fendt, 2006) previously reported robust avoidance behavior of laboratory rats in response to urine samples from predators (see *Experiment 1*). In addition, predator odors have been proven to be effective repellents protecting forestry and agricultural areas from feeding-related damage (Nolte et al., 1994; Bramley and Waas, 2001; Parsons et al., 2007). Whether predator odors during foraging can modulate food-carrying behavior in rats has not been investigated so far.

Therefore, the aim of the present study was to examine whether the presentation of predator odors increases food-carrying behavior in laboratory rats. Animals were first trained to travel along an alley to find a food source at the end of the alley. Since food-carrying behavior has been shown to be also dependent on the food size and/or weight (Onuki and Makino, 2005; Lima et al., 1985), food pellets of different sizes and weights were used. Food-carrying behavior was measured under the following two conditions: (1) in a "low risk" testing condition when a water sample was presented next to the food source and (2) in a "high risk" testing condition by presenting a fox urine sample next to the food source.

3.4.1. Materials and Methods

Testing apparatus

Both training and testing took place in a straight alley with closed side walls made of grey polyvinyl chloride (Fig. 4). The apparatus consisted of a start box (31 cm x 31 cm x 30 cm) fitted with some fresh and home cage bedding materials, and an alley (15 cm x cm x 28 cm). Via a small opening (11 cm x 10 cm) in the start box, rats were allowed to enter freely both compartments of the testing apparatus. At the distal end of the alley was a 1 cm deep notch, which served as a food well. Beside the food well, a glass bowl (4 cm outer diameter, 2.5 cm height) was fixed. The experimental room was only dimly illuminated (start box: ca. 30 lux, alley at food well: ca. 100 lux) by an indirect light source. During testing, the rats' behaviors were observed by an experimenter standing calmly next to the start box.

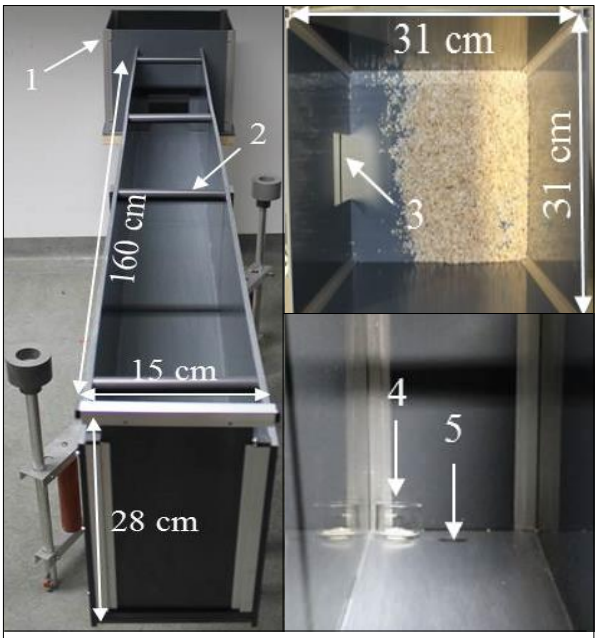

Figure 4 Setup for testing food-carrying behavior in rats. 1: start box; 2: run alley; 3: opening; 4: glass bowls; 5: food well.

Food pellets preparation

During training, 45 mg casein pellets (Dustless Precision Pellets; Bio-Serv Inc., Frenchtown, New York, USA) were used. Food-carrying behavior was tested using casein pellets of six different weights and sizes (45 mg, 180 mg, 360 mg, 540 mg, 720 mg, 990 mg). The five larger pellet sizes were produced by compressing a corresponding amount of 45 mg food pellets in a self-made pill press. All pellets (Fig. 5) were stored dry and cool at ca. 4 °C until usage.

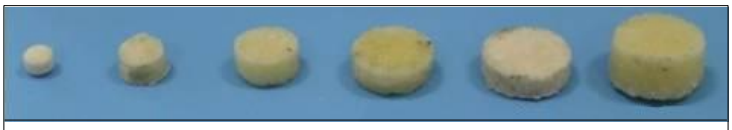

Figure 5 Food pellets of different sizes used in the food-carrying experiment. From left to right: 45, 180, 360, 540, 720 and 990 mg. For training, only 45 mg pellets were used.

Behavioral testing procedure

I. Training

On the first day, rats were placed into the start box, first in groups, then individually to familiarize them for 10 min to the testing apparatus without any casein pellets presented. Over the next days, rats were trained to run along the alley till the food well to get the

casein pellet there, and to return to the start box. For this, a trace of 45 mg casein pellets was placed along the alley with at least 5 cm distance between two pellets. Once rats improved their performance, the trace of casein pellets was shortened step-by-step until only one casein pellet was left in the food well. Each rat was trained two times per day for at least 15 min. As soon as the rats were able to run directly to the food well five times in succession, rats were tested in the food-carrying test. After each training session, rats were removed from the testing apparatus and returned to their home cage. For the following tests on food-carrying behavior, only those animals that express food carrying behavior were used (n = 21 out of 24).

II. Food-carrying test

Food-carrying behavior was tested using six different sizes of casein pellets. To manipulate the risk-level during the foraging situation, either 1 ml fox urine ("high risk") or 1 ml water ("low risk") was pipetted into the glass bowl next to the food well. Urine samples of foxes (*Vulpes vulpes*) were purchased from Maine Outdoor Solutions Inc. (Hermon, ME, USA). All urine samples were aliquoted into 1 ml portions and stored at -18 °C until usage. During the experimental sessions, the fox urine sample was exchanged after every 10^{th} trial.

In general, a food-carrying test session consisted of six single trials per rat in which each of the six different casein pellet sizes was presented in a pseudo-randomized order. In each trial, the rat was placed into the start box with the head of the animal pointing in direction of the alley. Usually, the animal traveled till the end of the alley and then decided whether to eat the casein pellet at the food well or to carry it back to the start box and eat it there. Each rat was allowed to finish eating the pellet and was then returned to its home cage. There was an interval of 10 min between subsequent trials of the same rat, in which two different rats of the same "risk level" condition were tested. Each rat was tested on two different testing days, once in the "high risk" and once in the "low risk" testing condition (balanced order).

The food-handling decision was recorded by the experimenter in a protocol. The testing apparatus was cleaned with soapy water after each training and test session and the experiment room was sufficiently ventilated after usage of the fox urine samples.

Statistical analysis

Food-carrying behavior was expressed as the percentage of animals that carried the casein pellet to the start box for the particular pellet sizes and for both the “low risk” and “high risk” testing condition. For analysis, sigmoid curve fits for both testing conditions were calculated (GraphPad Prism 6.00, GraphPad Software Inc., La Jolla, USA). The curve fits had the constraints of 0 % for the bottom and 100 % for the top. Using the Extra-sum-of-squares F test (GraphPad Prism 6.00), we then compared the slopes and the PW50 values of the two sigmoid curve fits. The PW50 value is the calculated food pellet weight where 50 % of the rats carried the pellet to the start box. A $p < 0.05$ was considered statistically significant.

3.4.2. Results

The percentages of rats that carried food pellets to the start box as a function of the food pellet weight is depicted for both testing conditions (“high risk”, “low risk”) in Figure 6. Furthermore, sigmoid curve fits (low risk: $R^2 = 0.98$, high risk: $R^2 = 0.99$) for the two risk conditions and the PW50 values ($PW50_{Low\ risk} = 396.2$, $PW50_{High\ risk} = 284.5$) are shown. For both testing conditions, food-carrying behavior in rats was increased with increasing casein pellet weight. All animals (n = 21) ate the 45 mg pellets at the food well, whereas the 990 mg pellets were always carried to the start box. Notably, food-carrying behavior for all middle pellet weights was increased in the “high risk” condition (180 mg: 28.6 vs. 9.5 %, 360 mg: 57.1 vs. 47.6 %, 540 mg: 85.7 vs. 66.7 %, 720 mg: 95.2 vs. 76.2 %). A statistical comparison of the two curve fits showed that the PW50 value was significantly increased in the “high risk” testing condition (Extra-sum-of-squares F test: $F_{(1,8)} = 12.39$, $p = 0.008$), whereas the slopes of the curve fits (2.50 vs. 2.59) were not different ($F_{(1,8)} = 0.03$, $p = 0.87$).

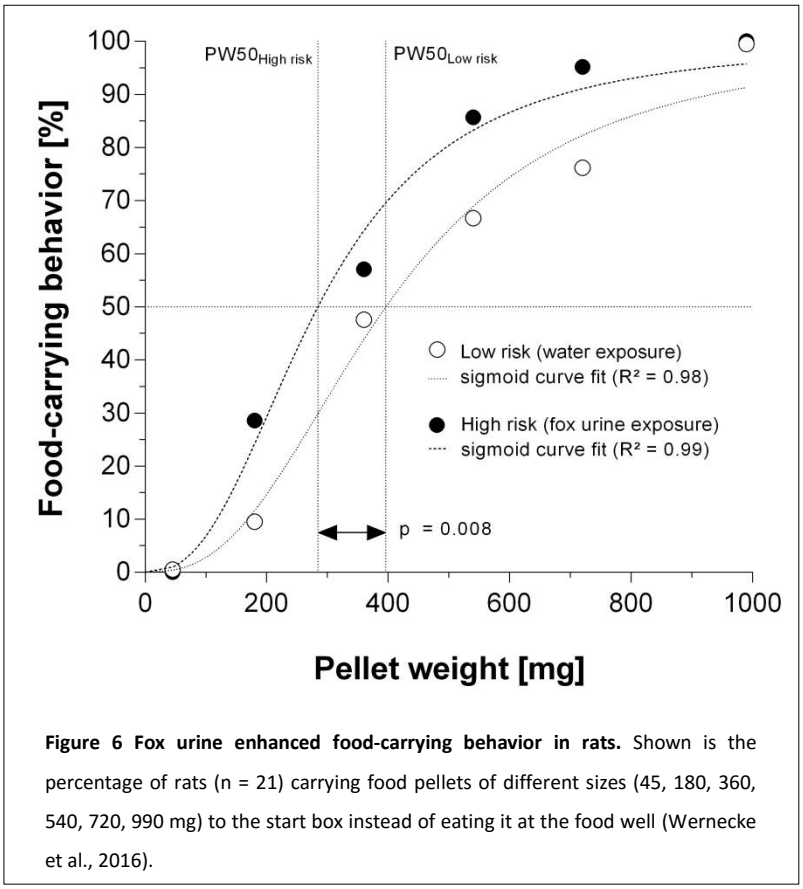

Figure 6 Fox urine enhanced food-carrying behavior in rats. Shown is the percentage of rats (n = 21) carrying food pellets of different sizes (45, 180, 360, 540, 720, 990 mg) to the start box instead of eating it at the food well (Wernecke et al., 2016).

3.4.3. Discussion

During foraging, animals are potentially easy prey for predators. Therefore, the decision of where to eat a found food piece has to be regarded as a motivational conflict in which animals need to trade-off maximization of food intake against predation risk avoidance (Onuki and Makino, 2005; Nakatsuyama and Fujita, 1995). Findings from field and laboratory studies showed that the probability of carrying food to a safe place (i.e., the nest) increases the more risky the foraging situation is (Lima and Valone, 1986; Nakatsuyama and Fujita, 1995; Dringenberg et al., 1994; Onuki and Makino, 2005).

The first finding of the present laboratory study is that rats generally tend to eat smaller food pellets at the food well while they carry larger food pellets to the start box. This supports previous literature findings (Nakatsuyama and Fujita, 1995; Whishaw and Tomie, 1989) showing that there is clearly more food-carrying behavior with increasing food size. Whereas rats eat smaller food pellets (≤ 200 mg) within 3-10 sec (representative measurements in the present study and (Onuki and Makino, 2003)), eating larger food pellets (≥ 750 mg) takes considerably longer, i.e. 40-60 sec. Consequently, to avoid spending a long time at the “high risk” food well, rats preferred to eat larger food pellets in the start

box which is in line with and can be explained by the predation risk-foraging efficiency-trade-off theory (Lima and Valone, 1986; Lima et al., 1985).

In contrast to other studies investigating food-carrying behavior in laboratory rats (Onuki et al., 2005; Onuki and Makino, 2005; Dringenberg et al., 1994), we observed both behavioral extremes, namely the complete absence and the exclusive expression of food-carrying behavior with the smallest and the largest pellet weight used in our study. Having these extremes facilitates the finding of optimal curve fits for the measured behavior and thereby also the statistical analysis. During pilot experiments, we modified different features of our setup until we were able to observe these extremes. In detail, we used (1) an alley with side walls whereas others used no walls, (2) a slightly different illumination of the setup, and (3) familiar bedding material in the start box, whereas other used an empty box (Onuki et al., 2005; Onuki and Makino, 2005; Dringenberg et al., 1994).

The second and more important finding of the present study is that food-carrying behavior was increased when a predator odor was presented next to the food well. In nature, predator odors provide a useful tool for prey animals to assess predatory threats in their environment. In general, predator odors are known to induce different defensive responses such as avoidance or freezing behavior. Predator odors also reduce non-defensive behaviors such as grooming and notably, feeding behavior (Apfelbach et al., 2005; Endres et al., 2005; Wallace and Rosen, 2000; Blanchard and Blanchard, 1989a). Strong repellent effects of predator odors on the feeding behavior of prey animals have been repeatedly demonstrated (Sullivan et al., 1985a; Bramley and Waas, 2001; Parsons et al., 2007; Rosell, 2001; Swihart, 1991; Melchiors and Leslie, 1985) with most of this work originating from the group of Thomas Sullivan (Sullivan et al., 1985a; Sullivan et al., 1988b; Sullivan et al., 1985b; Sullivan and Crump, 1984; Sullivan, 1986; Sullivan et al., 1988c; Sullivan and Crump, 1986). For instance, he observed that the mustelid anal gland secretion compound 3-propyl-l,2-dithiolane clearly suppressed feeding of snowshoe hares on lodgepole pine seedlings (Sullivan and Crump, 1984) and reduced vole feeding damage on apple trees (Sullivan et al., 1988a). Furthermore, different predator urine samples (mink, bobcat, coyote or domestic dog) reduced feeding of mountain beavers (1993).

In our study, predator odor (fox urine samples) decreased the PW50 value from 396 mg to 284 mg, i.e. by approximately one third. The PW50 value can be considered as the hypothetical pellet weight at which the perceived risk of being attacked during eating at the food source is equally high as the perceived risk of being attacked during carrying the pellet

to a safe place and eating it there (here in the start box). Eating at the safe place should only be connected to a minimal risk and can therefore be neglected. Since the time needed for eating is usually much longer than the time needed to carry the pellet to the start box (ca. 2-3 sec), the perceived risk during eating at the food source mainly guides the decision on whether to eat the pellet at the food source or to carry it to a safe place. According to these arguments, the here observed PW50 decrease indicates that the predator odor increased the perceived risk of being attacked. Notably, the here observed PW50 decrease was very similar to the PW50 decrease observed by Onuki and Makino (2005). In their study, rats were submitted to a classical fear conditioning paradigm, i.e. they learned that a tone predicts an aversive electric stimulus. When the animals were then tested in the presence of the tone (now a learned fear stimulus), food-carrying behavior was increased. The PW50 under control conditions was approximately 320 mg (calculated by using the reported values in (2005)) and during the presentation of the learned fear stimulus approximately 200 mg (i.e. a decrease by approximately one third). Both studies together strongly suggest that food-carrying behavior under such risky conditions can be regarded as a type of pre-encounter defensive response that is expressed in order to prevent being further exposed to a potential threat (Fanselow, 1994; Fanselow et al., 1988). It seems that the perceived risk in the two studies was very similar despite Onuki and Makino used a learned fear stimulus in their study (2005), whereas we used a predator odor that is innately recognized (Fendt, 2006; Rosen et al., 2006; Buron et al., 2007). However, such a comparison has to be done with caution since different rat strains, different setups and different pellets were used.

Taken together, the present study demonstrates for the first time that food-handling decisions (“eating” or “carrying”) are modulated by the presence of a predator odor. This decision is a trade-off between energy efficiency and predation risk avoidance and is also influenced by the size of the food pellet. In our experiment, food-carrying behavior was generally increased in the presence of a predator odor which strongly indicates that predator odor increases the perceived risk for rats. This is in line with the predation risk-foraging efficiency-trade-off theory (Lima and Valone, 1986; Lima et al., 1985).

3.5. Experiment 3: Predator odor-induced ultrasonic vocalization

Besides using the olfactory sense as important tool for both intra- and inter-species communication, rodents also communicate via acoustic signals. Rats and mice vocalize in the ultrasonic range between 20- and 70-kHz. Depending on the environmental situation and the age and sex of the emitter, different types of USV are distinguished with each call type serving different communicative purposes (Portfors, 2007; Wöhr and Schwarting, 2013). So called alarm calls, USV of 22-kHz, are usually emitted in aversive situations in order to warn group members about external danger, for instance of the appearance of a predator or in anticipation of inescapable aversive stimuli (Endres et al., 2007; Wöhr and Schwarting, 2013; Portfors, 2007). The hypothesis that 22-kHz USV functions as conspecific-specific alarm signal was firstly discussed in studies by the Blanchards using the visible burrow system, a semi-natural environment that provides tunnels and burrow systems as well as "surface areas" for mixed-set rat colonies (Blanchard and Blanchard, 1989a; Blanchard et al., 2001). Upon presentation of a cat at the "surface area" of the visible burrow system and following retreat to the burrows, usually the alpha rat will start to emit 22-kHz alarm calls. Following this, also other members of the rat colony that have not seen the cat themselves will initiate different anti-predatory responses like withdrawal to the burrows, the inhibition of ongoing behaviors, increased risk assessment or the repetition of alarm calls. 22-kHz USV has been shown to persist until about 30 min after the cat was removed (Litvin et al., 2007; Blanchard and Blanchard, 1989a; Endres et al., 2007). Whether this is an after-effect of the real cat encounter or rather a consequence of responding to remaining predatory cues for instance the cat's odor, has never been proven in a scientific study.

To assess whether the odor of a particular predator is sufficient to evoke 22-kHz USV in rats, in *Experiment 3* ultrasonic communication of rats in response to predator odors was further investigated. To optimize odor effects as well as recordings of 22-kHz USV, testing of rats was performed in a small mouse cage where rats were able to run around but where an escape from the odor or a clear avoidance response was not possible. Due to this, it was suggested that the presentation of predator odors in this test configuration would rather suppress general locomotor activity and increase the time spent immobile as previously observed in studies using similar small and confined test chambers (Wallace and Rosen, 2000). Moreover, differences in the number of contacts with the odor source should likely to be observed. In this experiment, rats were exposed to samples of fox urine, lion urine and TMT in order to test whether the odor samples induce 22-kHz USV and whether its

expression correlates with the expression of immobile behaviors and the number of odor contacts.

3.5.1. Materials and Methods

Testing apparatus

Testing took place in a standard Macrolon Type III cage (37.5 cm x 22.0 cm x 15.5 cm) constructed from transparent Plexiglas and equipped with a glass bowl (4 cm diameter, 2.5 cm height), placed and fixed at one side of the testing cage. Testing was performed under a fume hood with dimmed white lighting (illumination: ca. 30 lux). Behavior of the animals was recorded by a video camera (ABUS TVCC50011 WD DNR Tag/Nacht 650 TVL Camera, ABUS Security-Center GmbH & Co. KG, Affing, Germany) from about 48 cm above the floor of the testing cage (Fig. 7).

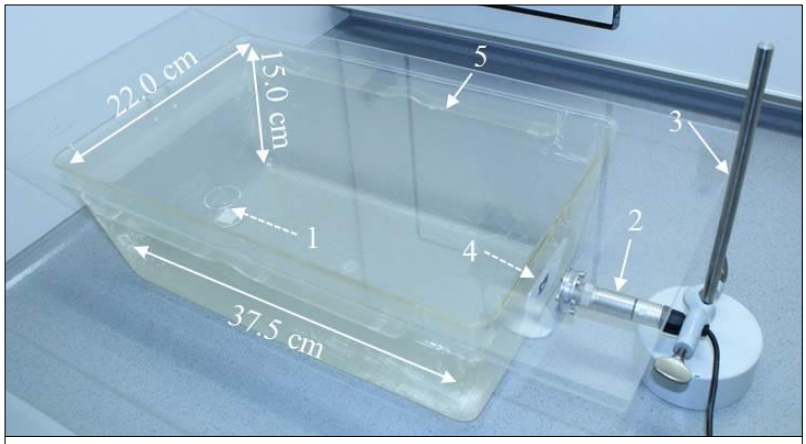

Figure 7 Setup for testing USV upon predator odor exposure. 1: glass bowl; 2: microphone; 3: microphone holder; 4: hole in the testing cage; 5: transparent cover. An observation camera is hanging above the testing cage.

Behavioral testing procedure

To familiarize the rats with the testing cage, on the first day each rat was singly placed into the cage for 10 min without an odor sample. On the following days, each animal was exposed for 10 min to 1 ml water, fox urine, lion urine or 20 µl TMT in a pseudo-randomized order. After preparing the odor sample, a transparent cover was immediately placed onto the top of the testing cage to prevent suction of the odor through the fume hood. Each rat was tested once per day and four times in total with the order of odor sample presentation being counterbalanced within and across days. During the time of testing, no other rats used in this experiment were present in the testing room.

A video tracking system (EthoVision XT, Version 10, Noldus Information Technologies, Wageningen, The Netherlands) was used to measure immobility (absence of movements) in sec. Immobility threshold was set at 2 % mobility (averaged over five samples), based on pilot studies showing that this threshold produced immobility scores that were concordant with manually-scored immobility. Numbers of contacts with the glass bowl containing the odor sample were scored manually (as a measure of risk assessment behavior) using recorded video files. The testing cage was thoroughly cleaned with soapy water after each test and ventilated with clean air.

Recording and analysis of ultrasonic vocalization

Potential ultrasonic calls emitted by the rat during testing were monitored by a UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) positioned next to a hole (1.5 cm diameter) on one side of the testing cage at a height of 6 cm. The microphone was connected via an Avisoft UltraSoundGate 416 USB audio devise (Avisoft Bioacoustics, Berlin, Germany) to a personal computer running the Avisoft-Recorder software (Version 4.2., Avisoft Bioacoustics, Berlin, Germany). For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (Version 4.2., Avisoft Bioacoustics, Berlin, Germany) and a fast Fourier transformation was conducted to obtain high resolution spectrograms. 22-kHz calls were scored manually off-line from the spectrograms. Several USV parameters were analyzed: numbers of 22-kHz USVs, numbers of bouts, total calling time [sec], calling latency [sec], shortest call length [sec], longest call length [sec], mean call length [sec], mean peak frequency [Hz].

Exclusion criterion

One rat vocalized during all odor exposure tests. Therefore, behavioral data of this animal were excluded from all statistical analyses.

Statistical analysis

All behavioral data are expressed as means + standard errors of the mean (SEMs). Immobility data and the number of odor contacts were first tested on normal distribution (D'Agostino and Pearson omnibus test). Normally distributed data were analyzed using a one-way repeated measures analysis of variance (ANOVA) followed by Dunnetts' multiple

comparison test. Non-normally distributed data were analyzed using the Friedman test followed by Dunn's multiple comparison test. A $p < 0.05$ was considered statistically significant. Analysis was carried out using GraphPad Prism (Version 6, GraphPad Software Inc., La Jolla, USA).

3.5.2. Results

Figure 8 A illustrates the effects of the different odor exposures on immobility in rats (n = 19). A non-parametric repeated measures ANOVA showed that the time rats spent immobile was significantly affected by the odor samples (Friedman test: Q = 12.62, p = 0.006). Post-hoc pairwise comparisons revealed that rats exposed to TMT spent longer time immobile than when exposed to the control odor water (Dunn's test: p = 0.001). Notably, exposure to fox urine and lion urine did not affect immobility of rats when compared to the control condition (Dunn's test: p = 0.45 (fox); p = 0.21 (lion)). There was also an effect of predator odor on the number of contacts with the odor source (Fig. 8 B, ANOVA: $F_{(3,54)} = 7.89$, p = 0.0003). When exposed to TMT, rats significantly less often contacted the glass bowl containing the odor sample in comparison to the control test with water exposure (Dunnett's test: p = 0.0013). Neither fox urine nor lion urine affected the number of odor contacts.

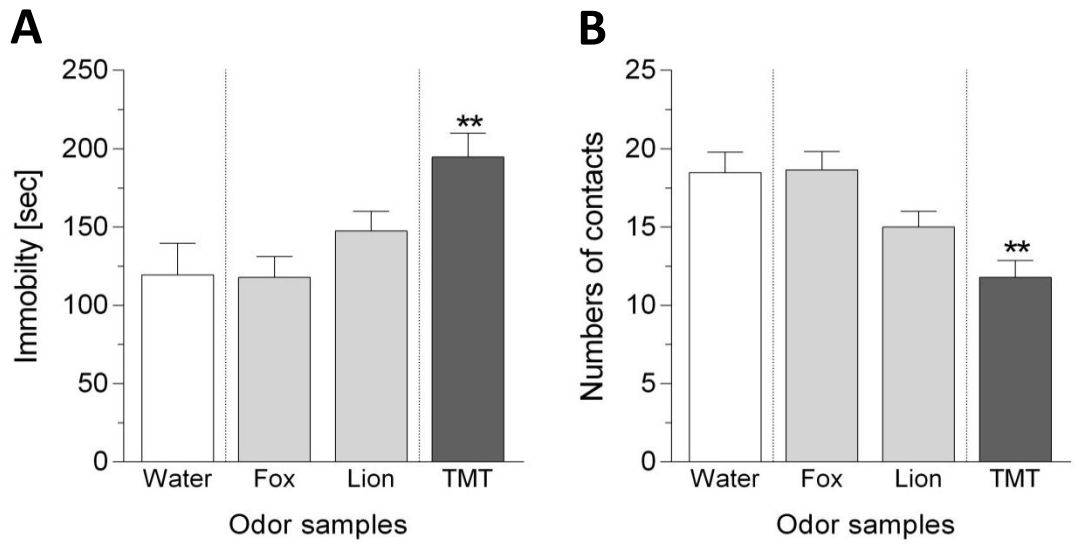

Figure 8 TMT increased immobility and reduced the number of contacts with the odor source in rats. (A) Time rats (n = 19) spent immobile (means + SEMs) during exposure to the odor samples as indicated. **(B)** Numbers of contacts (means + SEMs) with the odor samples. ** p < 0.01 comparison to water (Dunn's/Dunnett's multiple comparison test after significant main effects in the Friedman test/ANOVA).

Figure 9 A shows the number of rats emitting 22-kHz calls during testing in the different exposure conditions. No animal vocalized during exposure to water or TMT. Only one rat emitted 22-kHz USV when being exposed to fox urine. Moreover, four of the 19 rats emitted 22-kHz calls during exposure to lion urine. The total number of 22-kHz alarm calls (including the rats that vocalized when exposed to fox and lion urine) ranged between 16 and 154 calls (Fig. 9 B, Table 2) with a mean of 77 calls.

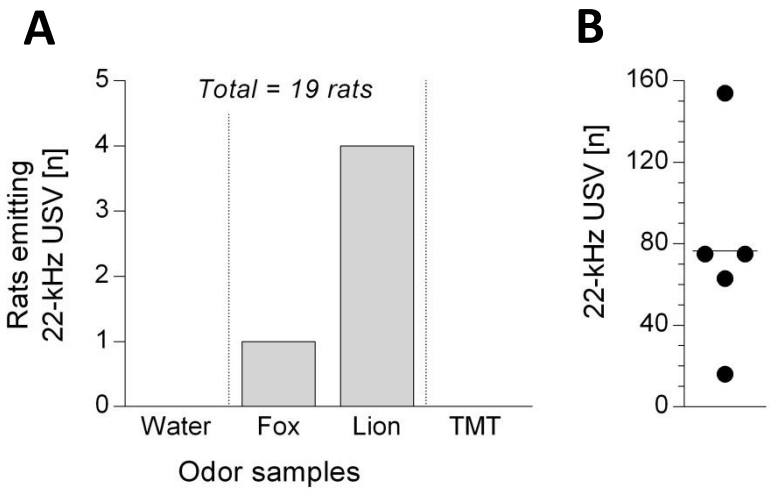

Figure 9 Fox and lion urine but not TMT evoked 22-kHz alarm calls. (A) Numbers of rats emitting 22-kHz alarm calls during odor exposures. **(B)** Scatter plot depicting the total numbers of 22-kHz alarm calls for all vocalizing animals (n = 5) with the horizontal line describing the mean.

A typical spectrogram of exemplary 22-kHz calls is depicted in Figure 10. Based on the interval between subsequent calls, calls were divided into single calls, calls that start a bout and calls within a bout. A bout is a number of calls which was separated from other calls by intervals longer than 320 msec (termed inter-bout interval, IBI). Shorter intervals were defined as inter-call interval (ICI)(according to Wöhr et al., 2005). Table 2 gives an overview about several other analyzed USV parameters such as call length and call frequency.

Table 2 Inter-individual differences in USV during exposure to lion urine (Rat No. 1-4) or fox urine (Rat No. 5).

	Rat No. 1	**Rat No. 2**	**Rat No. 3**	**Rat No. 4**	**Rat No. 5**	**Mean ± SEM**
No. of USV calls	75	154	63	16	75	76.6 ± 22.2
No. of bouts	28	34	35	8	31	27.2 ± 5.0
Total calling time (sec)	86.7	86.6	36.1	14.5	79.6	60.7 ± 14.9
Calling latency	72.3	344.7	186.6	301.8	224.3	226 ± 47.4
Shortest call length (sec)	0.29	0.03	0.16	0.32	0.17	0.2 ± 0.1
Longest call length (sec)	1.99	1.63	1.00	1.88	1.79	1.7 ± 0.2
Mean call length (sec)	1.16	0.56	0.57	0.91	1.06	0.9 ± 0.1
Mean peak frequency (Hz)	21860	20406	25033	23150	21780	22445 ± 779

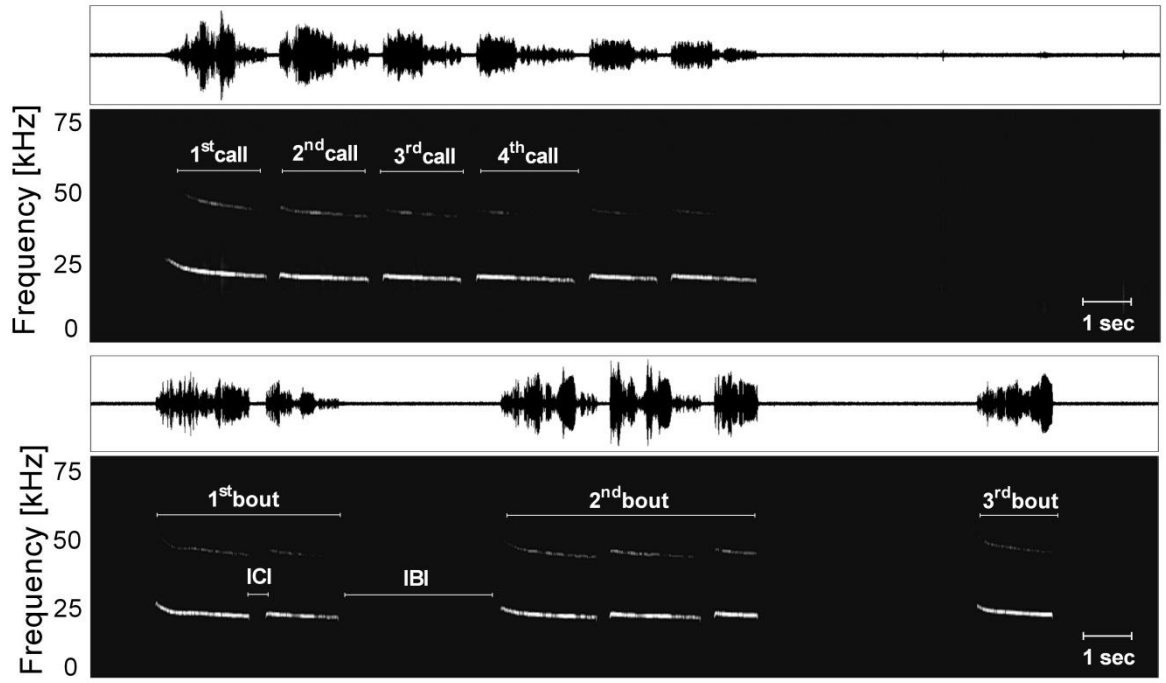

Figure 10 Exemplary aversive 22-kHz USV emitted by a rat exposed to lion urine. Upper panels display waveforms (amplitude) of successive USV. Lower panels depict the corresponding frequency spectrogram. Given are examples for calls, bouts, the inter-call-interval (ICI) and inter-bout-interval (IBI).

3.5.3. Discussion

Depending on a wide range of factors including the intensity of the threat and the testing environment, rats express diverse defensive responses to defend themselves from a predatory threat (Fendt and Endres, 2008; Wallace and Rosen, 2000). Freezing behavior appears to be the predominant defense reaction if the environment does not allow a flight response or the threat stimulus is inescapable (Wallace and Rosen, 2000; Rosen et al., 2008). In the present experiment, a rather small testing cage was used to optimize recordings of USV and to increase the aversiveness of the testing situation. Freezing is typically defined as an immobility state in which all movements cease except those necessary for breathing (Blanchard and Blanchard, 1969).

In the present study, exposure to TMT significantly increased the time rats spent immobile in the testing cage. An increase in immobility upon exposure to TMT has also been reported by others using both different rat (Endres and Fendt, 2009; Rosen et al., 2006; Wallace and Rosen, 2000; Morrow et al., 2000) or mouse strains (Buron et al., 2007; Janitzky et al., 2009) indicating that TMT has strong fear-inducing properties in rodents. Contrary to our expectations, fox and lion urine did not increase immobility in rats. However, taking into account the results of the previous experiments, it is extremely unlikely that the used

predator urine samples might lack any fear-inducing qualities since fox urine and urine samples of other predators induced robust avoidance behavior in these tests. As mentioned before, freezing behavior and thereby immobility is principally expressed when the threat is very close and intense. In comparison to the pungent and acrid smell of TMT, the smell of natural predator urine samples is less intense and irritating. Consequently, fox and lion urine might not reach the threat intensity required for the initiation of freezing behavior. This in turn may result in the expression of less manifested fear responses. Notably, in the present experiment only TMT but neither fox urine nor lion urine reduced the number of contacts with the glass bowl. This suggests that rats exposed to fox or lion urine might have been more engaged in the direct investigation of the threat stimulus possibly to better assess the risk of danger.

Alternatively, the used TMT concentration might probably not correspond to the intensity of a natural predator odor sample since in the present study rats were exposed to undiluted TMT. This nicely fits to previous findings by Buron and colleagues (2007) who directly compared fear behaviors observed in response to TMT and natural fox feces in mice. They observed that fear responses induced by natural fox feces were best comparable with those shown by mice during exposure to 10 % diluted TMT. Whether the same findings also apply to rats is not known. However, applied to the results of *Experiment 3*, this might suggest that the intensity of TMT might be considerably higher and almost incomparable with those of natural predator urine samples and raises the question that the TMT-induced increase in immobility results from its pungent properties (since undiluted TMT was presented) rather than its specific predatory qualities. To address this issue, experiments with concentrations of TMT in a physiological range should be conducted.

As previously mentioned, 22-kHz vocalization is believed to reflect a negative affective state akin to anxiety and fear (Wöhr and Schwarting, 2013) since 22-kHz USV was mostly observed in stressful and aversive situations such as during the presentation of foot shocks or during fear conditioning (Wöhr et al., 2005; Nobre and Brandão, 2004). To the best of our knowledge, this is the first study showing that exposure to predator odors evokes 22-kHz USV in rats indicating that the presence of a predator odor signal a potential threat to rats. Interestingly, 22-kHz vocalization was only emitted in response to fox and lion urine, but not to TMT suggesting that the “internal fear states” induced by natural predator urine samples and TMT were different thereby recruiting different defense pattern.

Notably, only a small number of rats (5/19) vocalized in response to natural predator urine samples. Further, more animals vocalized during exposure to lion urine than during exposure to fox urine hypothesizing that rats considered the presence of lion urine as more dangerous and risky. Why the majority of animals did not vocalized when being exposed to predator urine is not clear. However, USV emitted by only a small percentage of animals had also been observed by Wöhr et al. (2005). In their study, rats were tested in a classical fear conditioning test design in which foot shock intensities have been varied between different cohorts of rats (0 – 1.1 mA). During the conditioning phase, i.e. when a neutral tone stimulus was paired with an aversive electric foot shock, only 18 out of 33 rats vocalized. Interestingly, the likelihood of USV occurrence during fear conditioning was shown to be highly correlated with the intensity of the foot shocks. More specifically, only one out of seven rats vocalized during exposure to foot shocks of 0.2 mA. A foot shock intensity of 0.5 mA evoked USV in five out of seven rats, while each rat vocalized when tested with foot shocks of a higher intensity (0.8 mA: 7/7, 1.1 mA: 5/5). Based on the finding that the emission of 22-kHz USV appears to depend on the aversiveness of the situation, we here suggest that the presentation of a predator urine represents a relative mild aversive situation to rats that is comparable to electric foot shocks of lower intensity (e.g., 0.2 mA) (Wöhr et al., 2005).

Furthermore, the variability of USV occurrence in rats of the present study can also be explained by differences in individual dispositions to display fear-related behaviors (Borta et al., 2006). Evidence for this comes from a previous study showing that rats previously screened in the EPM to possess a high trait anxiety, are more likely to vocalize during fear conditioning testing than animals considered to be less anxious in the EMP test. Combining the assumptions that the variability of individual dispositions declines with the aversiveness of the test situation and that predator urine is only mild aversive to rats, it can be suggested that USV during predator odor exposure might be largely determined by individual traits of anxiety.

Lastly, by using natural urine samples of unknown chemical composition, we cannot exclude differences in the quality of urine samples. As stated in the materials and methods, lion urine samples were obtained from the local zoo. Zookeepers collected urine samples on days on which lions were fed or not fed with fresh meat (lions were fed on a feeding/fasting schedule). Currently, there is the assumption that typical metabolites of meat digestion (sulfur or nitrogen-containing compounds) may be responsible for the aversive qualities of

predator odors (Nolte et al., 1994; Apfelbach et al., 2005; Ferrero et al., 2011). According to this hypothesis, the urine of lions from fasting days should be less repellent than urine from days fed with meat. Similar influences of predator diet on urine aversiveness to prey animals have previously been discussed and reported (Berton et al., 1998; Nolte et al., 1994). Unfortunately, a shortage of sample availability forced us to use all available lion urine samples regardless of the feeding day. Different amounts of sulfur- or nitrogen-containing compounds in the different lion urine samples might therefore explain why only a few animals vocalized during testing.

In the present experiment, the mean call length was below one sec with even two animals evoking 22-kHz calls shorter than 0.6 sec. This is very surprising since 22-kHz calls of rats of the same rat strain and age but tested during fear conditioning with electric foot shocks in our laboratory, were observed to be considerably longer (mean call length: 1.5 sec). What such differences in call characteristics might mean is not clear. One explanation might be that rats could use such different call types in order to communicate different threat situations or the severeness of the endangerment.

In line with a putative alarm function, Blanchard and colleagues (Blanchard et al., 1991) were the first demonstrating that the production of 22-kHz calls in response to a predator is dependent on the presence of conspecifics indicating that 22-kHz USV has communicative functions. In *Experiment 3*, rats were tested individually with cage mates being located outside the testing room. However, despite litter mates being not visible to the testing animal, this does not exclude the rat's awareness of the conspecifics presence in the nearby since olfactory cues of cage mates might still be present in the testing room or testing cage. On the other hand, rats also emitted 22-kHz USV when being handled for the first time by an experimenter or when individually tested in an acoustic startle test (Borta et al., 2006; Brudzynski and Ociepa, 1992; Kaltwasser, 1990). In addition, there is no evidence for an audience effect, i.e. the potentiation of USV by the close presence of a familiar conspecific, in a fear conditioning study (Wöhr and Schwarting, 2008). Notably, social isolation itself induced aversive 22-kHz calls (Francis, 1977) presuming that the emission of 22-kHz USV does not necessarily require the presence of conspecifics but might possibly be enhanced by it.

Summarizing, *Experiment 3* demonstrates for the first time that aversive 22-kHz calls in rats are induced by predator odors. The emission of aversive 22-kHz vocalization might be restricted to predator odor samples of a physiologically relevant intensity since rats did not

vocalized in response to undiluted TMT. However, only TMT increased immobility and reduced the number of odor contacts in rats. This suggests that TMT and natural predator urine samples are signaling different threats to rats thereby inducing different behavioral responses.

3.6. Summary Part 1 and Conclusion

The aim of Part 1 of this doctoral thesis was to characterize the effects of predator odor exposure on rats' behaviors. For this, three experiments focusing on different types of behaviors were performed. Summarizing all study results, we here demonstrate that predator urine has distinctive behavioral effects in laboratory rats and that such odor samples have also an influence on the expression of non-defensive responses. This suggests that predator urine samples function as real threat stimuli for rats. But how does an individual rat decide on which defensive response pattern to select?

It is widely accepted that prey species adapt their behavioral responses with respect to environmental constraints (Blanchard and Blanchard, 1989b; Yang et al., 2004). For instance, it is only useful to express a flight response when an escape route exists in close proximity. In *Experiment 1,* rats were tested in rather simple, middle-sized testing boxes that do not offer any possibility to hide or to escape. When tested in these testing boxes, rats robustly avoided the odor quadrant containing predator urine, but constantly explored the odor quadrant when they were exposed to water. We further observed that these avoidance responses were occasionally interrupted by approach movements and direct contacts with the odor source which probably serves as a kind of risk assessment. In contrast, avoidance behavior could not be observed in *Experiment 3* when rats were tested in a smaller testing box that was additionally covered by a transparent plate to prevent evaporation of the odor samples. Consequently, the intensity of the predator odor samples should have been higher in this testing apparatus than when using the larger testing boxes of *Experiment 1*. This makes it highly unlikely that rats would be able to find a position within the smaller testing box where the odor is less intense and can be avoided. Instead, rats displayed longer periods of inactivity in response to TMT, a highly repugnant predator odor. Very interestingly, a few animals also started to emit 22-kHz vocalizations when fox or lion urine was presented, but not when they were exposed to TMT. This suggests that particular features of the testing environment such as the size of the testing box critically determine the fear response of rats to predator odors. In this regard, larger testing boxes offer rats the possibility to express different fear responses, while smaller testing boxes where the threat source is indispensable, gives way to the expression of freezing behavior and USV.

Besides the expression of important anti-predatory defensive responses in order to defend themselves from the threat, predator odors also appear to change non-defensive behaviors (Apfelbach et al., 2005; Fendt, 2006). Regardless of whether rats leave their

protective nest to search for food or potential mates, they always run the risk of being themselves hunted by predators. In *Experiment 2*, food-carrying behavior of rats for six different food pellet sizes was measured in a “low risk” and a “high risk” testing condition by presenting a water or a fox urine sample, respectively, next to the food source. For both testing conditions, food-carrying behavior of rats increased with increasing food pellet weight. This suggests that food-handling decisions can be interpreted in terms of a motivational conflict where prey animals need to trade off maximization of food intake against risk avoidance. Importantly, the proportion of food-carrying rats was remarkably higher during exposure to fox urine than when rats were tested with the water control. These results demonstrate that food-carrying behavior of rats is increased by the detection of a predator odor. This precautious change in foraging behavior supports the idea that predator odors have also extensive effects on behavioral activities not directly associated with defense.

In Part 1 of this doctoral thesis, urine samples of many different predator species (fox, wolf, bobcat, puma, lion) have been used. As previously discussed, each predator urine type evoked innate behavioral fear responses in rats whereby the type of fear behavior (avoidance, risk assessment, freezing, USVs) was particularly dependent on features of the testing environment. If one can assume that predator urine independently of its species origin is clearly fear-inducing in rats, one could also suggest that rats assess predatory threats by detecting common urinary molecules shared by all predators (probably derived from a carnivorous diet). An alternative but more sensory complex detection mechanism would involve the individual detection of specific chemicals derived from different predator species (Papes et al., 2010). On the basis of the first hypothesis, in *Experiment 1*, rats were also exposed to PEA (0.04; 0.4; 4 µmol/l), a biogenic amine identified to be highly concentrated in a predator urine blend. Rats avoided PEA (0.04; 0.4; 4 µmol/l) supporting the finding that PEA has the capacity of an universal danger-signaling kairomone. However, comparing the efficiency of natural predator urine samples to induce avoidance behavior with those of PEA showed that the PEA-induced fear response was only modest. Thus, PEA might be only one, but not an exclusive component of a predator urine blend and suggests that the full expression of fear behavior to predator odors requires the detection of the complete predator odor blend.

For future experimental work, it would be helpful to quantify PEA levels in the natural predator urine samples by means of high performance liquid chromatography assays. This is

especially interesting when rats tested in the same testing apparatus respond differently to urine samples of different predator species. For instance, in *Experiment 1*, we observed that urine from species of the canidae family (fox, wolf) is stronger avoided by rats than urine of members of the felidae family (bobcat, puma). The latter urine samples, however, probably induced also periods of risk assessment. This suggests that urine samples from canids and felids might signal “fear situations” of different intensity thereby recruiting different sets of defensive responses. Such differences in behavioral responses might be caused by differences in the amount of PEA or other key compounds in the urine samples.

4. PART 2: NEURAL BASIS OF PREDATOR ODOR-INDUCED FEAR

4.1. Introduction

In the following chapter, research done to delineate the neural circuit underlying innate fear responses in rodents is summarized. Hereby, the focus is on studies using predator-prey interactions as animal models of innate fear.

Over the last years, the understanding of the neural circuit underlying innate fear to predator odors has been substantially improved (see review articles of Takahashi, 2014; Fendt et al., 2005a; Takahashi et al., 2005; Rosen et al., 2008). The brain structure most commonly discussed in terms of its importance in the processing of fear is the amygdala. The amygdala is an almond-shaped structure, located deep within the anterior temporal lobe and was first characterized by the anatomist Burdach in the early 19th century (Swanson and Petrovich, 1998). Although the amygdala was previously assumed to be a coherent mass of gray matter, nowadays it is known that the amygdala is a highly differentiated region comprising several nuclei and subnuclei. Therefore, it can rather be spoken of the amygdala complex instead of a single structure. As part of the limbic system, the amygdala is probably the brain structure most affiliated with emotions and emotional processing. Traditionally, the amygdala was thought of primarily as a "protection device" (Murray, 2007) in the brain which is essential for both the detection of threatening stimuli and the generation of defensive and autonomic behaviors during fear (LeDoux, 2000; LeDoux, 2012; Wallace and Rosen, 2001; Davis, 1992). The significance of the amygdala for fear processing was first supported by the fact that discrete lesions of the amygdala produce a dramatic loss of emotional reactivity in primates. These amygdalectomized animals were unable to attach the appropriate significance to sensory input (e.g., snakes) assuming that the perceived object can no longer evoke a state of fear (Bechara et al., 2003). In humans, bilateral damage of the amygdala causes impairments in the recognition of fearful facial expressions (Adolphs et al., 2005).

Although most studies of amygdala functioning emphasize its role in fear learning (Fendt and Fanselow, 1999; Davis, 1992), the contribution of single amygdaloid subnuclei in the processing of predator odor-induced innate defensiveness is still poorly understood. Because the basolateral nucleus of the amygdala (BLA) is well known to be crucial for fear conditioning, a role of the BLA in innate fear has been suggested, too. However, the findings

are rather inconsistent. Using TMT as predator odor, Wallace and Rosen (2001) showed that rats with permanently inactivated BLA (caused by excitotoxic fiber-sparing lesions) displayed normal unconditioned freezing responses to TMT, while Müller and Fendt (2006) reported a delayed fear response to TMT after temporary inactivation of the BLA by injecting the *gamma*-aminobutyric acid receptor A ($GABA_A$) agonist muscimol). In contrast to this, Vazdarjanova et al. (2001) reported that pharmacological inactivation of the BLA impairs freezing and avoidance behavior of rats in response to a ball of cat hairs. This is in aggrement with findings of Takahashi et al. (2007) using a cloth containing cat odor. The medial nucleus of the amygdala (MeA) is another target of interest in predator odor studies since it receives both direct and indirect input from the accessory olfactory system (Canteras et al., 1995; Martinez-Marcos, 2009). It was demonstrated that the MeA critically participate in the neural processing of fear in rats exposed to either cat odor (Li et al., 2004; Blanchard et al., 2005) or TMT (Müller and Fendt, 2006; Takahashi et al., 2007; Vazdarjanova et al., 2001) In addition, rats exposed to cat odor showed changes in neuronal plasticity and increased c-fos expression (c-fos is a marker for neural activity) within the MeA (Collins, 2011; Dielenberg et al., 2001b). Unlike the MeA, lesions or pharmacological inactivation of other subnuclei of the amygdala like the lateral and central nucleus did not reliably affect predator odor-induced behavioral changes (Fendt et al., 2003; Li et al., 2004; Wallace and Rosen, 2001).

Other brain structures known to be part of the defensive circuit for unconditioned fear to predator odors are the lateral septum (LS) and the bed nucleus of the stria terminalis (BNST). This was supported by different c-fos studies showing that both structures are strongly activated in rats exposed to TMT (Day et al., 2004), cat odor (Dielenberg et al., 2001b; McGregor et al., 2004) or ferret odor (Masini et al., 2005). In addition, TMT-evoked freezing behavior in rats could be blocked by temporary inactivations of the LS or BNST (Endres and Fendt, 2008; Fendt et al., 2003; Fendt et al., 2005b).

Downstream to all mentioned structures, lies the hypothalamic defensive circuit, an highly interconnected system of three hypothalamic structures which has strongly been implicated in the processing and organization of defensive behaviors in dangers (Canteras, 2002). This circuit consists of the anterior hypothalamic nucleus (AHN), the dorsomedial part of the ventromedial nucleus (VMHdm) and dorsal premammillary nucleus (PMd) whereby the latter one is thought to act as output structure for projections to the periaqueductal grey (PAG), a structure largely associated with the behavioral output of defense (Canteras, 2002; Cezario et al., 2008). The hypothalamic defensive circuit receives major inputs from the MeA

and the BNST (Canteras et al., 1994; Rosen et al., 2008). Exposure of rats to a live cat or cat odor induces a marked increase in c-fos expression in all parts of the hypothalamic defensive circuit, especially in the PMd (Staples et al., 2008; Canteras et al., 1997; Pagani and Rosen, 2009; Dielenberg and McGregor, 2001). To corroborate this, discrete bilateral ibotenic acid lesions of the PMd have been shown to cause a dramatic reduction in freezing and avoidance behavior in rats exposed to cat odor (Markham et al., 2004) or confronted with a live cat (Canteras et al., 1997). Using TMT as predator odor, Pagani and Rosen (2009; Rosen et al., 2008) observed that only fibers passing through the AHN and VMHdm and not neurons within these nuclei are part of the innate fear circuit underlying TMT-induced fear. Whether the same is true for innate fear evoked by exposure to predator odors others than TMT, has not been investigated so far.

The following two experiments were part of a CBBS-funded NeuroNetwork that was aimed at elucidating the neural circuit underlying predator odor-induced fear behavior in rats. Within this cooperation, Dr. Jürgen Goldschmidt and M.Sc. Daniel Vincenz-Zörner (Leibniz Institute of Neurobiology) identified different brain sites activated or inactivated during exposure to fox urine using SPECT-imaging of the rCBF. Specifically, they showed that blood flow of rats exposed to fox urine was significantly increased in the AOC when compared to the activity level of this brain region during exposure to water. Significant changes in rCBF were also evident in the Hb-IPN system, whereby the Hb was clearly activated and the IPN clearly inactivated in rats exposed to fox urine. Part of the present doctoral project was then to further investigate the relevance of these brain regions by means of temporary (*Experiment 1*, microinjections of the $GABA_A$ receptor agonist muscimol) and permanent (*Experiment 2*, electrolytic lesions) inactivation techniques in innate fear processes. At this point, I would like to refer to the abstracts of *Experiment 1* and *2* of Part 2 where a more detailed description of these brain regions is given.

4.2. General Materials and Methods

4.2.1. Subjects

Subjects were 70 male Sprague-Dawley rats (2-3 months old) weighting 220-350 g at the time of the surgery. Rats were bred and reared at the local animal facility (original breeding stock: Taconic, Denmark). Animals were housed in groups of 5-6 animals in standard Macrolon Type IV cages (48 cm x 37.5 cm x 21 cm) in constant temperature- and humidity-controlled rooms (22 ± 2 °C, 50-55 %) and maintained on a 12 h light/dark cycle with lights on at 6:00 am. Tap water and standard laboratory rodent chow (Sniff Spezialitäten GmbH, Soest, Germany) were available *ad libitum*. Experiments were conducted between 8:00 am and 2:00 pm.

All experiments were carried out in accordance with the international ethical guidelines for the care and use of laboratory animals for experiments (2010/63/EU), and were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42505-2-1172 UniMD).

4.2.2. Odor Samples

Urine samples of foxes (*Vulpes vulpes*) were purchased from Maine Outdoor Solutions Inc. (Hermon, ME, USA) and were aliquoted into 1 ml portions and stored at -18 °C until usage. Furthermore, common tap water served as control odor.

4.2.3. Testing Apparatus

Testing took place in one of four identical testing boxes (45 cm x 45 cm x 30 cm) constructed from opaque polyvinyl chloride (see Fig. 1). Each box was located in a sound-attenuating chamber provided with four light sources (illumination: < 10 lux) and an observation camera (CCTV-Camera Miniature-CCD-Camera, RS Components GmbH, Mörfelden-Walldorf, Germany). Four small glass bowls (4 cm outer diameter, 2.5 cm height) were placed and fixed in each corner of the testing box using double-side adhesive tape. Each box was placed inside an animal detection frame (TSE Systems, Bad Homburg, Germany) with infrared light sensors (14 mm distance between two sensors).

For *Experiment 1* the animal detection frame was used to automatically measure the movements of the rats. In *Experiment 2,* the video tracking software EthoVision XT (Version 10, Noldus Information Technologies, Wageningen, The Netherlands) was used.

4.3. Experiment 1: Role of the AOC in predator odor-induced innate fear

Over the last years, our understanding of the neural systems underlying innate fear to predator odors has substantially been improved. As previously mentioned, the amygdala is important for both the detection of threatening stimuli and the generation of defensive behaviors (LeDoux, 2000; LeDoux, 2012; Takahashi, 2014). However, the contribution of single amygdaloid subnuclei in processing predator odor-induced defensiveness is poorly understood. Since the medial nucleus of the amygdala (MeA) receives strong olfactory input (Martinez-Marcos, 2009; Canteras et al., 1995), most of the research was focused on this subnucleus (e.g., Müller and Fendt, 2006; Li et al., 2004; Blanchard et al., 2005).

Using SPECT-imaging of the rCBF, Dr. Jürgen Goldschmidt and M.Sc. Daniel Vincenz-Zörner showed that the AOC but not the MeA was significantly activated during exposure of rats to fox urine (Fig. 11).

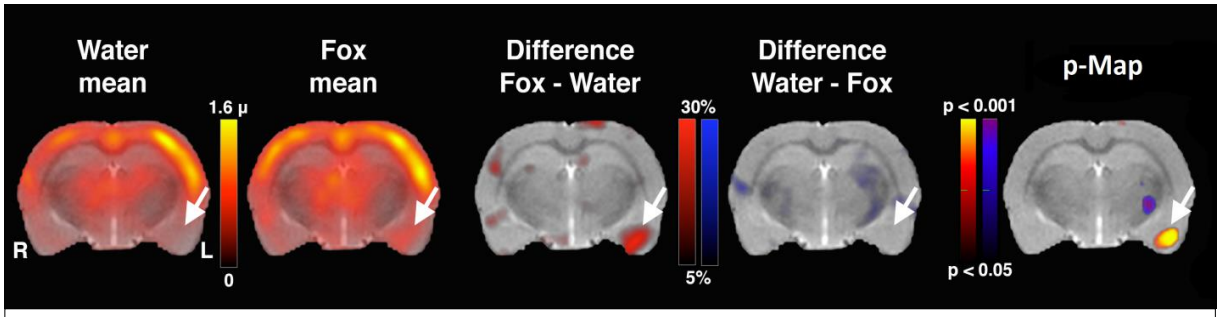

Figure 11 SPECT data indicate increased rCBF in the AOC. Shown from left to right are mean tracer uptake during exposure to water and to fox urine, respectively, mean increases (colored in red) and decreases (colored in blue) in tracer uptake under fox urine exposure versus water exposure. The corresponding probability map (p-map; $0.001 < p < 0.05$) is shown in the right column. Significant increase in rCBF is color coded from red to yellow and significant decrease from blue to violet. All images are overlaid on the same template MR image. White arrows point to the AOC.

In addition to the MeA, the AOC also receives massive projections from both the main and accessory olfactory system (Swanson and Petrovich, 1998; Gutiérrez-Castellanos et al., 2014) – thereby making the AOC a strategic brain region to directly affect odor-guided behaviors. The AOC, as defined by Swanson and Petrovich (1998), includes the cortical nucleus of the amygdala (CoA), the amygdalo-piriform transition zone (APir) and the nucleus of the lateral olfactory tract. Interestingly, the AOC also heavily projects to the medial hypothalamus (Swanson and Petrovich, 1998; Gutiérrez-Castellanos et al., 2014; Mohedano-Moriano et al., 2012) which is critical for the expression of innate defensive behaviors to predators (Canteras, 2002). Although different studies emphasized the importance of the AOC in socio-sexual behaviors e.g., copulatory behavior (Maras and Petrulis, 2008; Meurisse

et al., 2009), a potential role in the processing of innate fear behavior has not been studied yet.

Based on these literature findings and the results of the imaging study, we decided to further investigate the role of the AOC in predator odor-induced fear behavior in rats. Therefore, the AOC was temporary inactivated by local microinjections of the $GABA_A$ receptor agonist muscimol. Then, the effects of AOC inactivation on fear behavior (avoidance behavior, general motor activity) induced by exposing the animals to samples of fox urine were tested.

4.3.1. Materials and Methods

Surgery

Rats were anesthetized with 2.0-2.5 % isoflurane (5 % for induction) in oxygen (2.4 l/min) and fixed in a stereotactic frame with blunt ear bars and with the incisor bar set at -3.3 mm relative to the interaural line to ensure a flat skull position and thereby the exact placement of the cannula. Stainless steel guide cannulas (8.0 mm length; 0.7 mm outer diameter) were implanted bilaterally aiming at the AOC. Using Paxinos and Watson's "Stereotaxic Brain Atlas" (1997), the coordinates for the injection sites in the AOC were as follows: - 4.4 mm rostral, ± 4.8/5.5 mm lateral, - 9.3 mm ventral to bregma). The cannulas were fixed to the skull with three anchoring stainless steel screws and dental acrylic cement (Paladur, Heraeus Kulzer GmbH, Hanau, Germany). Finally, the scalp was sewed. After surgery and between testing, stainless steel stylets (8.0 mm length; 0.3 mm outer diameter) were inserted into the guide cannulas to prevent occlusion. Rats were allowed one week to recover prior to the start of behavioral testing. A more detailed standard operation protocol for cannula implantations is given in the appendix.

Behavioral testing procedure

Rats were given three 10-min habituation sessions without odor to familiarize themselves to the testing boxes (once per day on different days). On the following days, each animal received (in a pseudo-randomized order) bilateral microinjections of either 2.6 nmol muscimol (dissolved in 0.3 µl saline) or 0.3 µl of only saline into the AOC. For this, stylets were carefully removed whilst the rats were gently restrained. Intracranial infusions were given using custom-fabricated stainless steel injection cannulas (0.3 mm outer

diameter), attached via a polyethylene tube to two 10-µl microsyringes (Hamilton, Bonaduz, Switzerland). Solutions were injected at a rate of 0.2 µl/min using a microinfusion pump (CMA Microdialysis, AB, Kista, Sweden). Following injections, the injection cannulas were left in place for an additional min to allow diffusion into the tissue. After the injection, injection cannulas were removed and the stylets replaced (see protocol for microinjection in the appendix).

10-15 min later, the animals were placed into the testing boxes and either exposed to 1 ml water or 1 ml fox urine. For this, the respective odor sample was pipetted into a glass bowl in one corner. Each rat was tested once per day and four times in total in a pseudo-randomized order (saline injection/exposure to water, saline injection/exposure to fox urine, muscimol injection/exposure to water, muscimol injection/exposure to fox urine) with the odor corner also changed in a pseudo-randomized fashion.

The rat's behavior was monitored for 10 min by the infrared detectors (sample rate: 100 Hz). The TSE software automatically calculated the rat's location for predefined time intervals and the general motor activity (distance traveled [cm], number of rearings). Avoidance behavior was measured for each min by calculating the mean percentage of time the rat spent in the quadrant (8 x 8 sensors) containing the test stimulus. The testing boxes were thoroughly cleaned with soapy water after each test and ventilated with clean air.

Histology

After completion of behavioral testing, rats were sacrificed by carbon dioxide. Brains were rapidly removed from the skull and stored in 10 % sucrose-formalin solution (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) followed by 20 % and 30 % sucrose-formalin solution for cryoprotection (see protocol for brain removel in the appendix). Afterwards, serial 40 µm coronal sections were sliced with a cryostat (Leica, Nussloch, Germany) at a temperature of –21 °C. After drying overnight, sections were Nissl-stained with cresyl violet (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany)(see protocol for cryostat sectioning and Nissl staining in the appendix). The injection sites were verified under a light microscope and mapped on schematic coronal sections from employing the "Stereotaxic Brain Atlas" of Paxinos and Watson (1997).

Statistical analyses

Behavioral data were expressed as means ± standard errors of the mean (SEMs). Since data was not normally distributed (D'Agostino and Pearson omnibus test), avoidance behavior (mean percentage time in odor quadrant) was analyzed using a non-parametric analysis of variance (Friedman test) followed by Dunn's multiple comparisons test. For the analysis of the normally distributed motor activity data, we accomplished a two-way repeated measures analysis of variance (ANOVA) using drug treatment (saline/muscimol) and odor (water/fox urine) as within-subject factors. Post-hoc analysis was performed using Sidak's multiple comparisons test. For all statistical evaluations, a $p < 0.05$ was considered statistically significant. All analyses were carried out using GraphPad Prism (Version 6, GraphPad Software Inc., La Jolla, USA).

4.3.2. Results

Histology

Figure 12 A shows a schematic reconstruction of the injection sites of the rats receiving infusions of saline and muscimol into the area of the AOC as verified by histological examination. A photomicrograph of a Nissl-stained section from a representative injection site into the AOC is shown in Figure 12 B. Only animals with bilateral injections localized within or very close to the AOC, i.e. inside the posterior division of the lateral/medial cortical or basolateral nucleus of the amygdala (PLCo, PMCo, BLP), the posterolateral division of the amygdalo-hippocampal area (AHiPL) or the amygdalo-piriform transition zone (APir), were included in the behavioral analysis (n = 15).

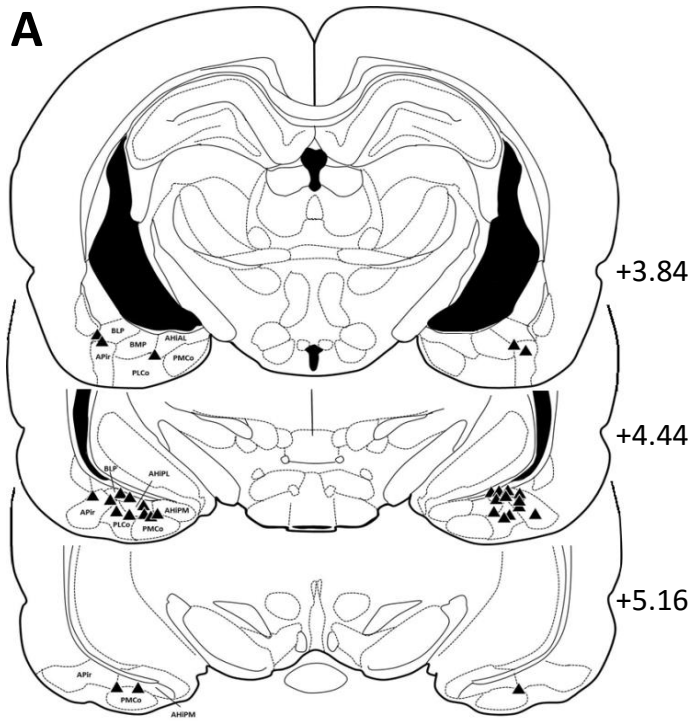

Figure 12 Injection sites into the AOC. (A) Reconstruction of the injection sites (filled triangles) on schematic diagrams of coronal brain sections (Paxinos and Watson, 1997). Numbers indicate the caudal distance (in mm) from Bregma. *Abbreviations*: AHiAL, anterolateral part of the amygdalo-hippocampal area; AHiPL, posterolateral part of the amygdalo-hippocampal area; AHiPM, posteromedial part of the amygdalo-hippocampal area; APir, amygdalo-piriform transition area; BLP, posterior part of the basolateral amygdaloid nucleus; BMP, posterior part of the basomedial amygdaloid nucleus; PLCo, posterolateral cortical amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus. **(B)** Photomicrograph showing a Nissl-stained coronal section with a representative injection site in the AOC (Wernecke et al., 2015).

Avoidance behavior

Two rats were excluded from this analysis since they displayed inappropriate behavioral responses towards fox urine after saline injections (approach instead of avoidance behavior). Figure 13 illustrates the mean percentage of time rats spent in the odor quadrant during the 10-min exposure to water or to fox urine, respectively, for the different treatment conditions. A non-parametric repeated-measure ANOVA showed that the different test conditions (odor and treatment) significantly affected the time spent in the odor quadrant (Friedman test: $Q = 18.28$, $p = 0.0004$). Post-hoc pairwise comparisons revealed that after saline injections into the AOC, rats spent significantly less time in the quadrant with the fox urine sample than in the quadrant with water (Dunn's test: $p < 0.001$), i.e. the quadrant with fox urine was avoided. After muscimol injections, this avoidance response towards fox urine was significantly reduced (Dunn's test: comparison saline-fox urine vs. muscimol-fox urine: $p < 0.001$). Notably, the behavior during exposure to the control odor water was not affected (Dunn's test, comparison saline-water vs. muscimol-water: $p > 0.05$).

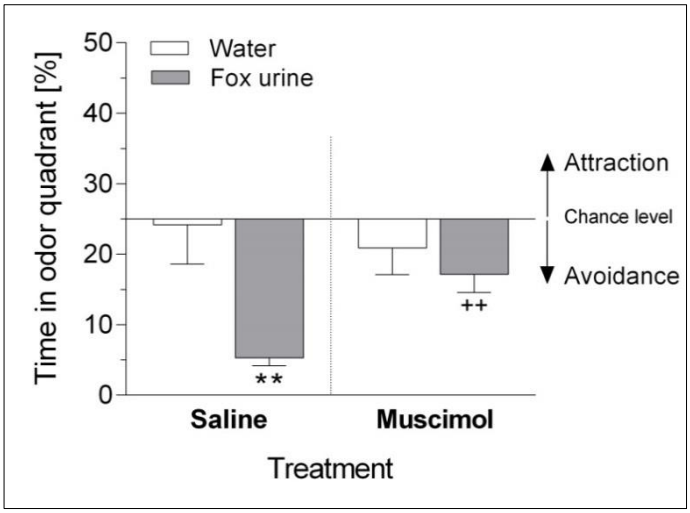

Figure 13 Temporary inactivation of the AOC disrupted fox urine-induced avoidance behavior. Mean percentage of time (- SEM) rats (n = 15) occupied the odor quadrant (chance level = 25 %), containing either water (white bars) or fox urine (grey bars) samples. Rats received bilateral injections of either saline or muscimol into the AOC. ** $p < 0.01$ comparison with the appropriate water control; ++ $p < 0.01$ comparison with the appropriate saline condition (Dunn's multiple comparison tests after significant main effects in a Friedman test)(Wernecke et al., 2015).

General motor activity

The distance traveled (Fig. 14 A) of the rats was not affected by exposure to fox urine (ANOVA, factor odor: $F_{(1,14)} = 1.31$, $p = 0.35$) but by muscimol injections (factor treatment: $F_{(1,14)} = 15.88$, $p = 0.003$). However, the interaction between odor and treatment clearly failed to reach statistical significance ($F_{(1,14)} = 0.72$, $p = 0.30$) indicating that the weak increase of locomotor activity in muscimol-injected animals cannot explain the effects on avoidance behavior described above. Regarding vertical activity (number of rearings), the statistical analysis revealed that neither the treatment factor ($F_{(1,14)} = 0.71$, $p = 0.41$) nor the odor factor ($F_{(1,14)} = 0.0001$, $p = 0.99$) exhibited significant differences (Fig. 14 B).

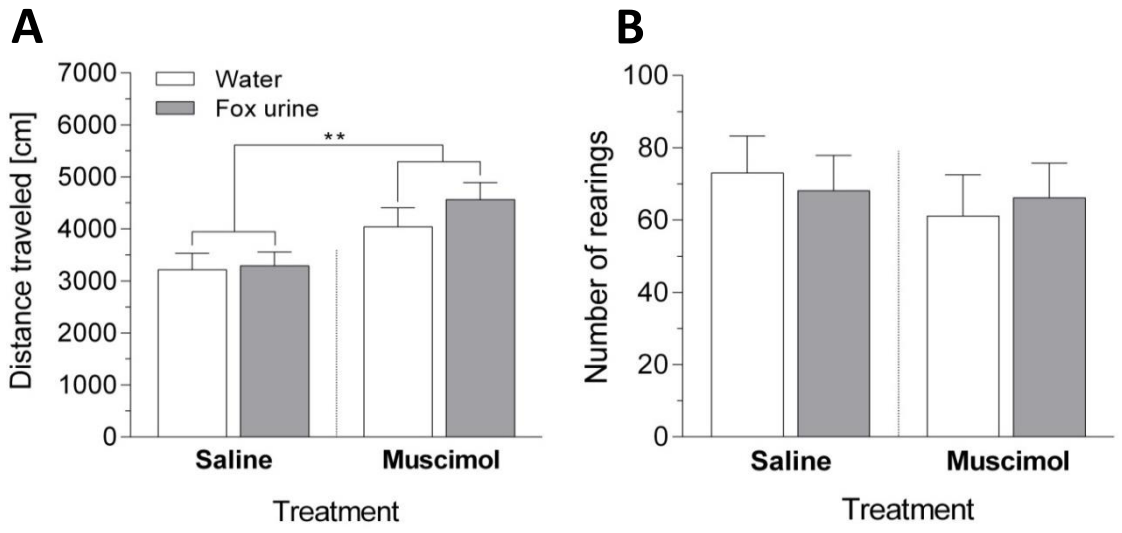

Figure 14 Effects of fox urine exposure on general motor activity. (A) Distance traveled in cm (+ SEM) and **(B)** number of rearings (+ SEM) rats (n = 15) showed during the 10-min exposure test under the indicated treatments (saline/muscimol injections) and exposure conditions (water/fox urine). ** $p < 0.01$ compared with the appropriate saline condition (Sidak's multiple comparison test after significant main effects in an ANOVA)(Wernecke et al., 2015).

4.3.3. Discussion

Based on findings of a SPECT-imaging experiment, the aim of the present experiment was to test whether the AOC is involved in fox urine-induced fear behavior. Specifically, the AOC was temporarily inhibited by local microinjections of the $GABA_A$ receptor agonist muscimol and the effects of fox urine exposure on avoidance behavior and locomotor activity were measured. The results demonstrated that temporary inactivation of the AOC substantially disrupted avoidance behavior of rats in response to fox urine that is strongly avoided under the control condition (saline injection). Also, muscimol treatment slightly increased the distance traveled of rats exposed to both fox urine and water, while leaving horizontal activity unaffected.

In the present experiment, saline-treated rats displayed robust avoidance behavior, i.e. approximately 6 % of their time was spent in the quadrant with fox urine (chance level would be 25 %). Similar fear-evoking properties of fox urine have been reported previously in Part 1 of this thesis. In other studies, fox urine-exposed rats and mice showed also higher levels of freezing, suppressed locomotor activity and elevated c-Fos immunoreactivity in brain regions involved in fear (Liu et al., 2010; Kopec et al., 2007; Farmer-Dougan et al., 2005; Funk and Amir, 2000; Fendt, 2006). This data together with the present results, indicate that fox urine functions as a predatory signal that reliably induces fear behavior in rodents. In contrast to urine samples of larger carnivores (e.g., coyote, bobcat, puma, dingo; see Experiment 1 Part 1,Fendt, 2006; Ferrero et al., 2011; Osada et al., 2013; Xu et al., 2012), urine samples of smaller carnivores (e.g., cat, ferret) often failed to induce fear-like behavioral effects (Blanchard et al., 2003c). As previously reported, Ferrero et al. (2011) identified a biogenic amine, PEA to be the key constituent of carnivore urine blends that triggers innate avoidance behavior in rodents. In comparison to the PEA-enriched urine of larger carnivores, both the urine from cats and ferrets contain relative low levels of PEA which could be a likely explanation for the absence of fear behavior to these odors. In line with this, Fendt (Fendt, 2006) observed that the urine of larger carnivores (e.g., bobcat, cougar) induced even stronger avoidance responses than fox urine.

As mentioned in the Introduction of this thesis, two of the most widely used predator odors in recent research are cat odor from fur/skin and TMT. These odors not only elicit avoidance behavior as observed in the present study with fox urine but also induce freezing and risk assessment behaviors such as flat back approaches or head outs (Munoz-Abellan et al., 2010; McGregor et al., 2002; Wallace and Rosen, 2000; Endres and Fendt, 2009;

Dielenberg and McGregor, 2001; Blanchard and Blanchard, 1989a). In the present experiment, fox urine-exposed rats displayed only infrequent risk assessment (two animals) and no freezing behavior. The different behavioral observations in the present experiment may be caused by differences in the setup. For instance, Dielenberg and McGregor (2001) gave rats the opportunity to retreat into a hide box and the Blanchards (1989a) established a semi-natural visible burrow system. In contrast, here rats were tested in rather simple testing boxes to which they were well habituated (three habituation sessions) and that do not offer any possibility to hide. This suggests that a more complex testing environment may support risk assessment. Freezing behavior, on the other hand, is expressed when the threatening stimulus is very intense (e.g., foot shock, high concentration of TMT) and inescapable (e.g., when tested in a small, confined test chamber) (Wallace and Rosen, 2000; Rosen et al., 2008). Samples of fox urine, as used in the present study, may be considered as relatively weak threatening stimuli. Taken together, the experimental setup and the intensity of odor samples used in the present study seem to specifically induce avoidance behavior and do not necessarily support risk assessment or freezing behavior.

In the present experiment, the $GABA_A$ receptor agonist muscimol was used to reversibly inactivate neurons within the AOC. Local muscimol injections lead to an unselective and rapid suppression of local neuron activity which lasts between one and two hours (Arikan et al., 2002; Martin, 1991; van Duuren et al., 2007). Such temporary inactivations represent an important alternative to chronic lesions because they allow within-subject designs. However, one issue of this method is that it is difficult to determine the spatial spread of the drug around the injection site. Injections of 0.3 µl muscimol, labelled with a dye, have been shown to diffuse 0.5-1 mm away from the injection site (Allen et al., 2008). Therefore, we assumed that muscimol injected very close to the AOC, i.e. into the AHiPL and BLP, will most probably diffuse to the AOC, too. Due to this, we also included rats with injection sites within the AHiPL and BLP into the behavioral analysis of the present experiment. Otherwise, diffusion of muscimol to the ventral hippocampus is unlikely since myelinated fiber tracts (alveus of the hippocampus, deep cerebral white matter) that separate hippocampus and amygdala usually act as effective diffusion barriers.

The main finding of the present study is that, in rats, temporary inactivation of the AOC by local muscimol injections prevents avoidance behavior towards fox urine. To the best of our knowledge, this is the first study showing that the AOC is crucial for predator odor-induced fear behaviors. Nevertheless, these findings are not surprising since the AOC is

strongly interconnected with both olfactory systems, as well as brain areas mediating fear behaviors. Now the question arises how the AOC is implemented into the brain circuit of innate fear? Olfactory information from the main olfactory bulb is transmitted to the anterior (ACoA) and posterior-lateral CoA (PLCo), as well as to the APir. Projections from the accessory olfactory bulb, known to also convey predator-related pheromonal information (Tirindelli et al., 2009), primarily terminate in the posterior-medial CoA (PMCo) (and the MeA) (Swanson and Petrovich, 1998; Gutiérrez-Castellanos et al., 2014; Ottersen, 1982). Therefore, the AOC could be regarded as the first limbic/emotional brain structure after the primary sensory brain regions, where olfactory information is prossessed and integrated. With regard to our injection sites within both main olfactory- and vomeronasal-recipient areas of the amygdala, we cannot say which olfactory system plays a critical role in the processing of fox urine. Until recently, little is known about how predator odor-derived kairomones are detected and processed. Funk and Amir (2000) observed that fox urine induced high Fos expression in main olfactory pathways that is strongly modulated by the circadian cycle. Also, both TMT and lion urine-derived PEA seem to exert their aversion responses by stimulating subsets of olfactory sensory neurons located in the dorsal olfactory epithelium (Ferrero et al., 2011; Kobayakawa et al., 2007). On the other side, Osada et al. (2013) demonstrated that volatile pyrazine analoges that were identified in wolf urine and initiate defensive behaviors in mice, activate the accessory olfactory bulb, whereby the contribution of the main olfactory system could not be excluded. Indeed, the involvement of both olfactory systems is conceivable because there is a complex network of interconnections between subregions within the AOC. Especially, the PMCo and PLCo are good candidate structures for olfactory-vomeronasal convergence, since both subnuclei are strongly bidirectional connected (Gutiérrez-Castellanos et al., 2014; Pitkänen et al., 1997). In addition, the AOC provides via the BLP and BMP strong input to the “medial hypothalamic defense circuit”, a network of distinct medial hypothalamic subnuclei that plays a key role in initiating and regulating fear behaviors (Canteras, 2002; Swanson and Petrovich, 1998). Moreover, different studies using Fos immunohistochemistry and lesion methods have provided evidence that these hypothalamic areas are involved in the control of antipredator defensiveness following exposure to both live predators or their smell (Markham et al., 2004; Blanchard et al., 2005; Dielenberg et al., 2001b). As a consequence, the AOC could modulate fear behavior by directly affecting hypothalamic function which in turn controls other brain areas involved in the expression of fear behavior (e.g., PAG).

Muscimol injections into the AOC further enhanced horizontal locomotor activity (distance traveled) during exposure to both water and fox urine. However, vertical locomotor activity (rearings) was not affected. We think that this increase in horizontal locomotor activity reflects a general effect of AOC inactivation on novelty-induced fearfulness, suggesting that the AOC is also part of an amygdala network that is implicated in neophobic responses towards novel but not frightening stimuli (Misslin and Ropartz, 1981; Wright et al., 2003). Consistent with this view, bilateral excitotoxic lesions of of the BLA enhanced exploratory behavior of rats to both fake and real cat hair (Vazdarjanova et al., 2001) and increased locomotion in a novel environment (Burns et al., 1996).

Taken together, the present study demonstrate that the fox urine-induced avoidance response is diminished by temporary inactivation of the AOC, an amygdala region that receives strong olfactory input. This indicates that the AOC is part of an innate fear circuit mediating fear responses towards predatory odors.

4.4. Experiment 2: Role of the Hb and IPN in predator odor-induced innate fear

Much research on the neurobiology of innate fear has focused on the amygdala, as a central fear region in the vertebrate brain (Li et al., 2004; Müller and Fendt, 2006). In the previous experiment, we revealed an important function of the AOC in predator odor-induced fear. However, parts of the innate fear circuit should be phylogenetically older than the amygdala, since even the most primitive vertebrates (e.g., teleosts, lampreys) without developed amygdala (Pabba, 2013; Maximino et al., 2013) react in response to danger – thereby adapting their behavioral responses in order to survive (Bals and Wagner, 2012).

Interestingly, using SPECT-imaging of the rCBF M.Sc., Daniel Vincenz-Zörner and Dr. Jürgen Goldschmidt from our CBBS NeuroNetwork observed that neural activity of the rat's Hb complex and its direct projection target, the IPN, was significantly changed during exposure to fox urine suggesting that both brain regions might be involved in predator odor-induced fear (Fig. 15).

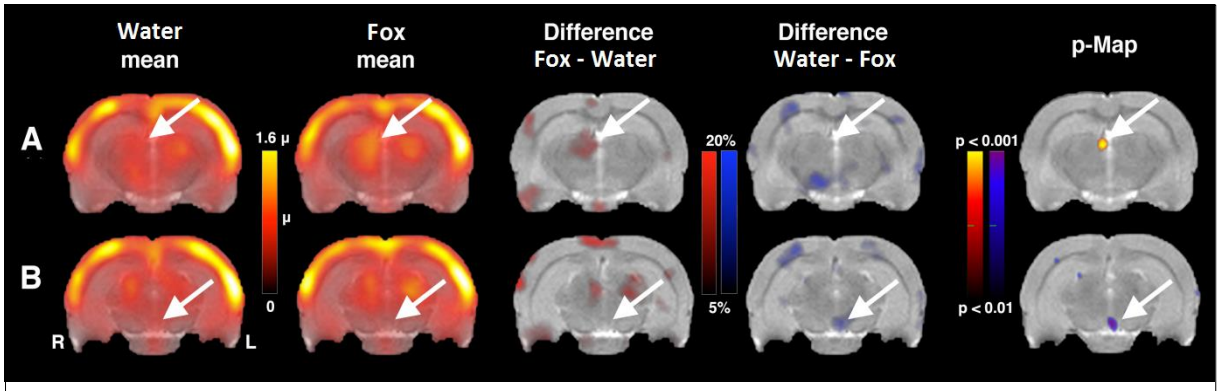

Figure 15 SPECT data indicate rCBF changes in the Hb-IPN axis. Shown from left to right are mean tracer uptake during exposure to water and to fox urine, respectively, mean increases (colored in red) and decreases (colored in blue) in tracer uptake under fox urine exposure versus water exposure. The corresponding probability map (p-map; $0.001 < p < 0.01$) is shown in the right column. Significant increase in rCBF is color coded from red to yellow and significant decrease from blue to violet. All images are overlaid on the same template MR image. Significant increase in rCBF was found in the right Hb (arrows in A) and significant decrease was found in the IPN (arrows in B).

Compared to other forebrain structures (e.g., the amygdala), the Hb-IPN axis is phylogenetically older. In the literature, the Hb-IPN axis has been described as a highly conserved connection in the vertebrate brain that links limbic (e.g., septum, BNST, lateral hypothalamic areas) and basal ganglia structures with key regions in the midbrain and brainstem (Klemm, 2004; Lecourtier and Kelly, 2007; Hikosaka et al., 2008). Many of the afferent and efferent projection structures identified have also been implicated in fear processing making the Hb-IPN axis ideally situated to modulate fear responses. Indeed,

Agetsuma et al. (2010) demonstrated that genetic silencing of the Hb-IPN axis biased zebrafish to a passive strategy of freezing rather than the normal flight response in a classical fear conditioning paradigm. Furthermore, genetic ablation of two neural pathways connecting the posterior septum with the Hb-IPN system has been shown to differentially regulate fear and anxiety responses of mice to immediate threats (Yamaguchi et al., 2013; Okamoto and Aizawa, 2013). That indicates that the Hb-IPN axis is involved in learned fear and anxiety-driven behaviors. However, since the Hb-IPN system is even present in the phylogenetically oldest vertebrates, it can be suggested that its basic function might be to control more fundamental, innate fear processes and that this system may have been adapted during evolution to regulate also higher cognitive processes including fear learning by changing afferent inputs.

The present experiment was conducted to explore the role of the Hb and the IPN in innate defensive fear responses to predator odors. More specifically, the effects of electrolytic lesions of either the Hb or the IPN on avoidance and risk assessment behaviors induced during exposure to fox urine were evaluated. Electrolytic lesions were applied because this lesion technique is often used to receive first information about how a certain brain structure is implemented within the whole brain circuit since lesions were not restricted to a certain type of neurons. In contrast to reversible inactivation techniques (e.g., by injecting neurotransmitter agonists or antagonists) where the spatial spread of the drug around the injection site is difficult to determine, electrolytic lesions offer the advantage that the extent of the lesion can be easily controlled by changing the intensity and duration of the applied current. This is very usefull when investigating brain regions of small size such as the Hb complex.

4.4.1. Materials and Methods

Surgery

As in the previous experiment, rats were anesthetized with 2.0-2.5 % isoflurane (5 % for induction) in oxygen (2.4 l/min) and fixed in a stereotactic frame. The incisor bar was set at 3.3 mm relative to the interaural line and blunted ear bars were used. Electrolytic lesions of the Hb or the IPN were made using stainless steel electrodes (TeWa acupuncture needle, B-type, Asia-med GmbH & Co. KG, Suhl, Germany, 0.30 mm in diameter) insulated with varnish except for the approximately 500 µm long tip. The electrode was lowered to the Hb (- 3.7 mm rostral, ± 0.6 mm lateral, - 5.4 mm ventral to bregma) or to the IPN (- 6.3 mm

rostral, ± 0.0 mm lateral, - 9.4 mm ventral to bregma). Bilateral lesions were then generated by passing an approximately 0.1 mA anodal current through the electrode tip for 10-15 sec, whereby the rat's tail served as cathode. The current was provided by a pulse stimulator (A-M Systems, Model 2100, Sequim, USA) and monitored by a digital multimeter (Metex Corporation, M-3610B, Seoul, Korea). At the end of the surgery, the incision was closed by a suture and the animals were allowed to recover for at least three days. A different group of animals served as sham-operated control group. Here, the electrode was lowered to the same coordinates within the Hb or the IPN, but no current was applied. A more detailed standard operation protocol for electrolytic lesions is given in the appendix.

Behavioral testing procedure

Rats used in the experiment underwent one 10-min habituation session. Then, baseline behavioral responses to 1 ml water and 1 ml fox urine were assessed for 10 min in two pre-surgery test sessions. Only one odor was tested per day. For this, the respective odor sample was pipetted into one of the glass bowls. The order of odor presentation and the corner with the odor was chosen in a pseudo-randomized fashion. Within two days after this pre-surgery testing, rats underwent stereotactic surgeries (see above). The animals were randomly assigned either to a sham-operated control group or to a Hb/IPN-lesioned group. Post-surgery test sessions began earliest three days after surgery. These sessions were identical to the pre-surgery test sessions and were used to control for the effects of the general surgical procedure. The testing boxes were thoroughly cleaned with soapy water after each test and ventilated with clean air.

In the previous experiment, the behavior of the animals became more variable with increasing test duration (data not shown). To decrease this variability, the behavioral data analysis was limited on the first 5 min of testing. Using EthoVision XT software for video tracking (Version 10, Noldus Information Technologies, Wageningen, The Netherlands), the percentage of time an animal spent in the odor quadrant (as a measure of avoidance behavior) was calculated. Numbers of contacts with the glass bowl containing the odor sample were scored manually (as a measure of risk assessment behavior).

Histology

After completion of behavioral testing, sham-operated and Hb/IPN-lesioned animals were sacrificed by carbon dioxide. Brains were identically processed as in the previous experiment. Besides being Nissl-stained with cresyl violet (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany)(see protocol for cryostat sectioning and Nissl staining in the appendix), exemplary brain sections were also stained with gold chloride (see Gold chloride staining protocol in the appendix) to assess potential damage of fibers-of-passage. The extent of complete tissue destruction or gliosis was verified under a light microscope and mapped on schematic coronal sections from the "Stereotaxic Brain Atlas" of Paxinos and Watson (1997).

Statistical analyses

Behavioral data were expressed as means + standard errors of the mean (SEMs). The behavior (avoidance, risk assessment behavior) of the sham-operated and Hb/IPN-lesioned groups was compared separately for the pre- and post-operative test sessions using two-way repeated measures analyses of variance (ANOVA) with group (sham vs. lesion) as a between-subject factor and odor (water vs. fox urine) as a within-subject factor. To assess differences in behavioral responses between testing in pre- and post-surgery test sessions, two-way repeated measures ANOVAs with time (pre-surgery testing vs. post-surgery testing) and odor (water vs. fox urine) as within-subject factors were performed for each experimental group. Significant ANOVAs were followed by Holm-Sidak's multiple comparisons tests. For all statistical evaluations, a $p < 0.05$ was considered statistically significant. All analyses were carried out using GraphPad Prism (Version 6, GraphPad Software Inc., La Jolla, USA).

4.4.2. Results

Histology

After pre-surgery testing, animals received either discrete electrolytic lesions of the Hb (n = 12) or IPN (n = 11) or sham lesions (Hb: n = 18; IPN: n = 14). The Figures 16 A and 17 A show serial schematic reconstructions of the lesion sites within the Hb or IPN. Electrolytic lesions were characterized by the presence of neural loss and gliosis. Rats included in the lesion groups encompasses bilateral lesions of intermediate and posterior parts of either the Hb (bilateral) or the IPN with the most anterior part of these structures as well as neighboring structures e.g., the thalamus being barely damaged. In addition, sparse neural

degenerations were observed in nearby parts of the fasciculus retroflexus for both lesion sites. However, microscopic verification of exemplary gold chloride stained-brain sections showed that electrolytic lesions of the Hb or the IPN were mainly restricted to the target regions and did not strongly affect nearby ascending or descending fiber tracts (Fig. 16 D).

Almost no neuronal loss or gliosis was observed in brains of sham-operated rats. Photomicrographs of Nissl-stained brain sections from a representative sham-operated and Hb/IPN-lesioned animal are shown in Figure 16 B + C and 17 B + C.

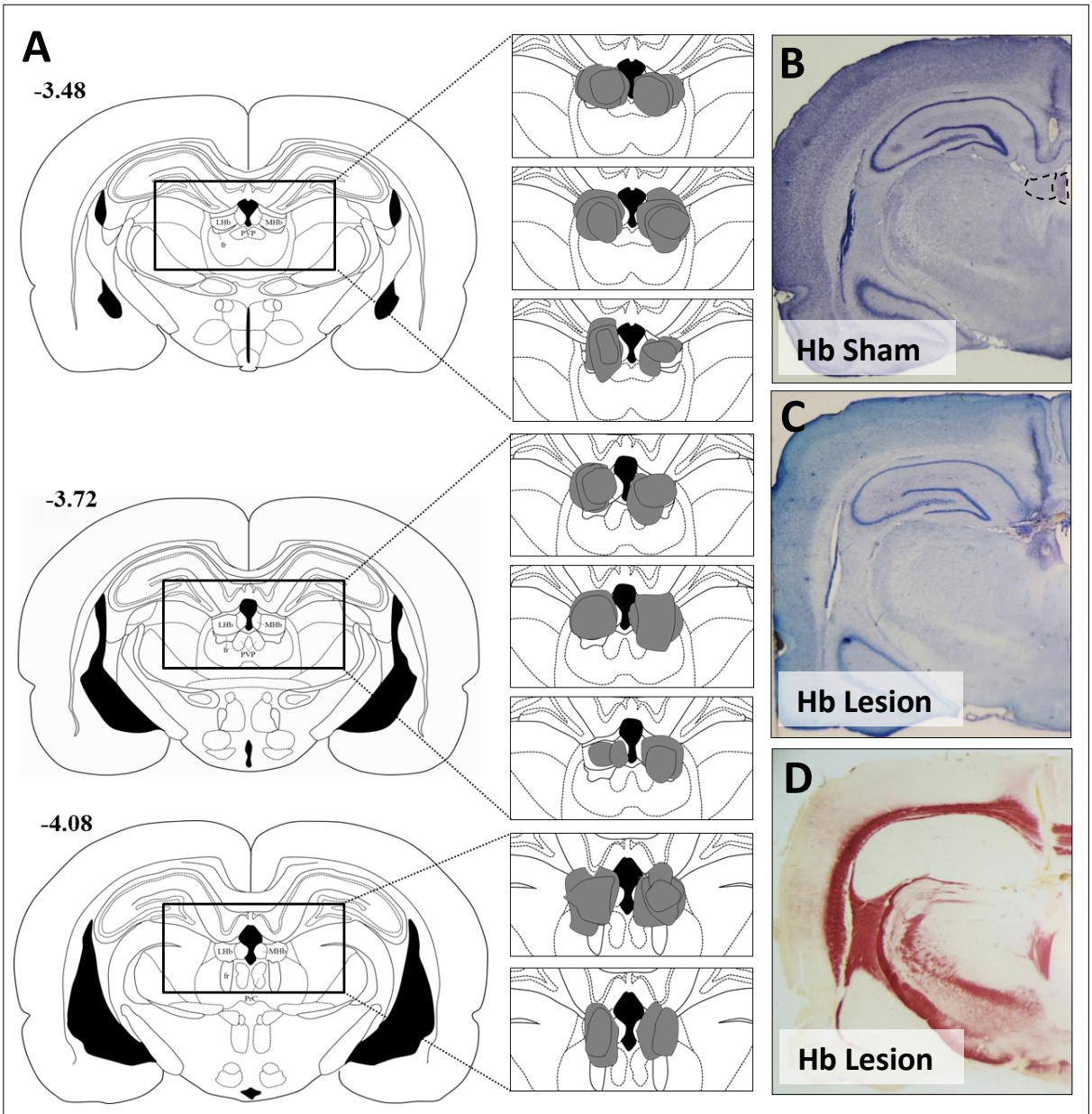

Figure 16 Electrolytic lesions of the Hb (A) Schematic diagrams of coronal brain sections at the level of the Hb showing the distribution of all the electrolytic lesions (n = 12). Lesions were determined by the presence of neuronal loss and glial proliferations (Paxinos and Watson, 1997). Numbers indicate the caudal distance (in mm) from bregma. *Abbreviations*: fr, fasciculus retroflexus; LHb, lateral habenular nucleus; MHb, medial habenular nucleus; PVP, posterior paraventricular thalamic nucleus. **(B)** Representative photomicrographs showing Nissl-stained coronal sections through the Hb of a sham-operated **(C)** and a Hb-lesioned rat. **(D)** Representative photomicrograph showing the destruction of fiber tracts by electrolytic lesions of the Hb. The section was stained with a gold chloride solution for myelin detection.

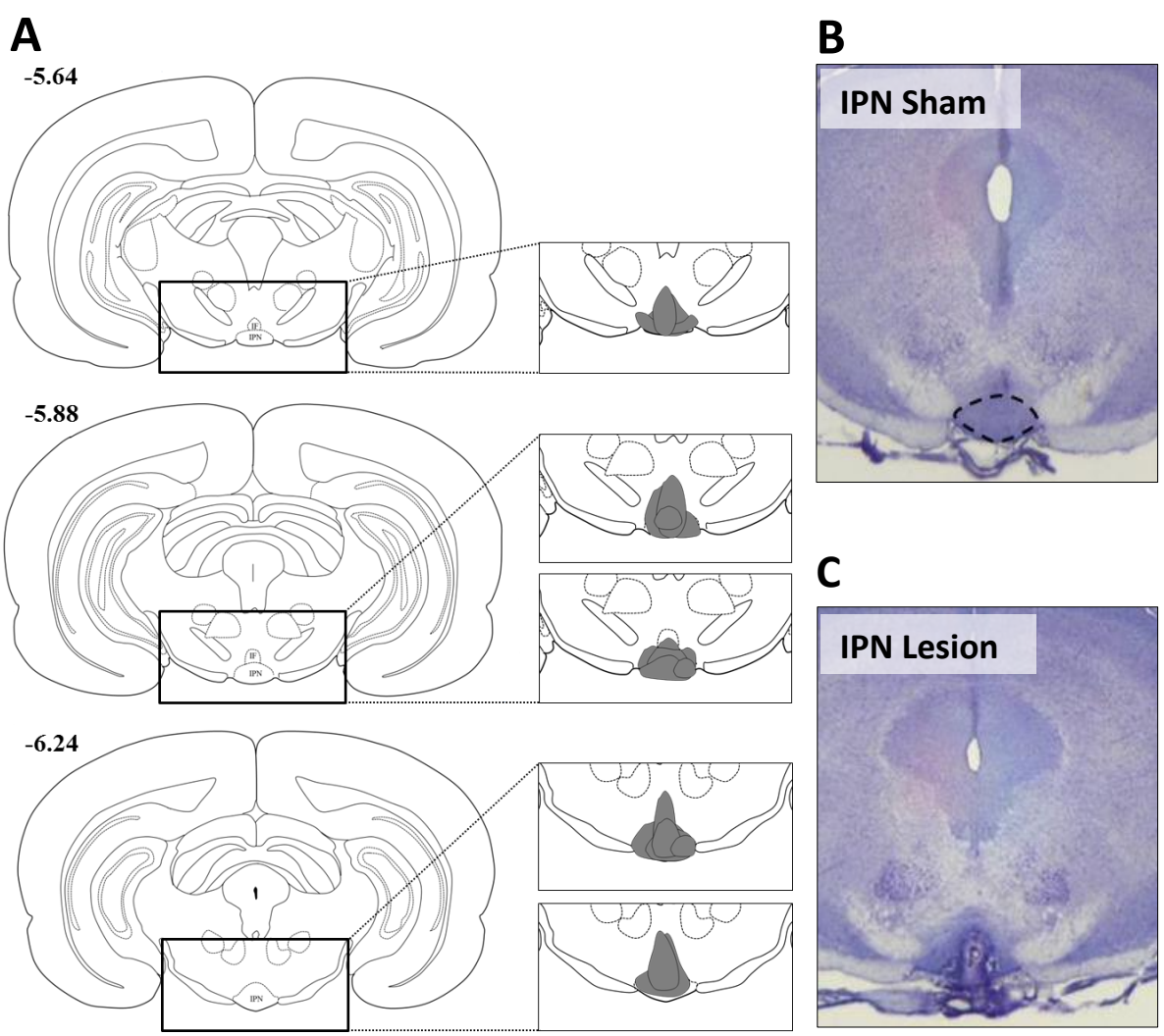

Figure 17 Electrolytic lesions of the IPN (A) Schematic diagrams of coronal brain sections at the level of the IPN showing the distribution of all the electrolytic lesions (n = 14). Lesions were determined by the presence of neuronal loss and glial proliferations (Paxinos and Watson, 1997). Numbers indicate the caudal distance (in mm) from bregma. *Abbreviations*: IP, interpeduncular nucleus; IF, interfascicular nucleus. **(B)** Representative photomicrographs showing Nissl-stained coronal sections through the IPN of a sham-operated and **(C)** IPN-lesioned rat.

Avoidance behavior

The effects of electrolytic lesions of the Hb or of the IPN on avoidance behavior are illustrated in Figure 18. Two-way repeated-measure ANOVAs showed that rats assigned to either the sham-operated or lesion groups spent comparably amounts of times in the odor quadrant during pre-surgery testing (Factor group: $F_{(1,28)} = 0.29$, $p = 0.59$ (Hb); $F_{(1,23)} = 0.15$, $p = 0.70$ (IPN)) and that the odor quadrant occupancy was significantly affected by the factor odor (factor odor: $F_{(1,28)} = 16.76$, $p = 0.0003$ (Hb); $F_{(1,23)} = 11.11$, $p = 0.003$ (IPN)). Post-hoc pairwise comparisons revealed that during exposure to fox urine, all rats spent significantly less time in the quadrant with the fox urine sample compared to the control test sessions with exposure to water (Holm-Sidak's tests: $p = 0.01$ (Hb-Sham), $p = 0.01$ (Hb-Lesion);

p < 0.05 (IPN-Sham, IPN-Lesion)), i.e. the quadrant with fox urine was avoided. After surgery and recovery for a few days, rats that received sham operations of either the Hb or the IPN expressed the same amount of avoidance behavior during exposure to fox urine as before the sham surgeries (factor time: $F_{(1,17)}$ = 1.44, p = 0.25 (Hb); $F_{(1,13)}$ = 0.08, p = 0.78 (IPN); factor odor: $F_{(1,17)}$ = 13.44, p = 0.002 (Hb); $F_{(1,13)}$ = 7.74, p = 0.02 (IPN)). This was very different in Hb- and IPN-lesioned animals as indicated by a significant interaction effect (Time x Odor interaction: $F_{(1,11)}$ = 9.29, p = 0.01 (Hb); $F_{(1,10)}$ = 9.99, p = 0.01 (IPN)). That means that both Hb and IPN lesions significantly blocked avoidance behavior towards fox urine (Holm-Sidak's tests: p = 0.54 (Hb), p = 0.23 (IPN)).

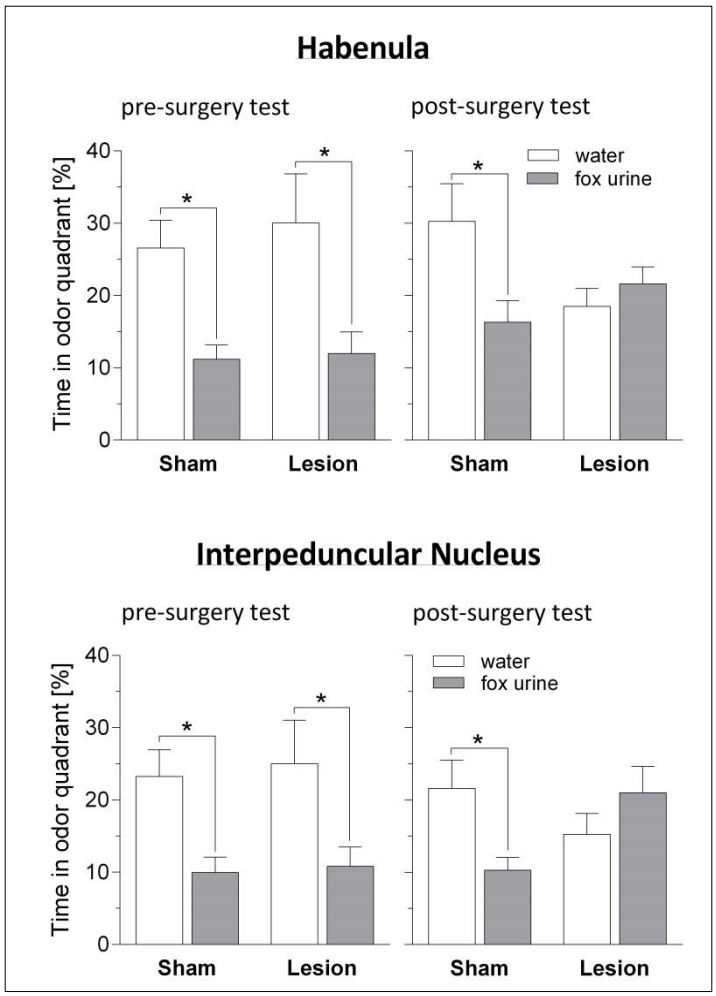

Figure 18 Electrolytic lesions of the Hb or IPN blocked avoidance behavior to fox urine. Results are shown for both sham-operated (Hb: n = 18; IPN: n = 14) and Hb or IPN-lesioned (Hb: n = 12; IPN: n = 11) rats for the first 5 min of pre-surgery and post-surgery testing during exposure to water (white bars) and fox urine (grey bars). Avoidance behavior is expressed as percentage of time (mean + SEM) rats occupied the odor quadrant (chance level = 25 %) containing the respective odor sample. *p < 0.05, Holm-Sidak's multiple comparisons tests after significant effects in two-way repeated measures ANOVAs.

Risk assessment behavior

Figure 19 depicts the effects of electrolytic lesions of the Hb or of the IPN on the number of odor contacts as a measure of risk assessment. Two-way repeated-measure ANOVAs revealed no significant differences in the numbers of odor contacts between the lesion groups and their respective sham-operated control groups (Factor group: $F_{(1,28)} = 1.43$, $p = 0.24$ (Hb); $F_{(1,23)} = 0.01$, $p = 0.91$ (IPN)) during pre-surgery testing. There were also no significant odor effects (Factor odor: $F_{(1,28)} = 0.30$, $p = 0.59$ (Hb); $F_{(1,23)} = 0.34$, $p = 0.56$ (IPN)). As expected, rats that received sham operations of either the Hb or the IPN expressed similar amounts of risk assessment behavior during exposure to water and fox urine during post-surgery testing (Factor time: $F_{(1,17)} = 1.05$, $p = 0.32$ (Hb); $F_{(1,13)} = 0.71$, $p = 0.41$ (IPN)). Interestingly, Hb lesions significantly increased the numbers of odor contacts with the fox urine sample (Factor time: $F_{(1,11)} = 13.01$, $p = 0.004$; Factor odor: $F_{(1,11)} = 12.08$, $p = 0.005$) while risk assessment was not affected by IPN lesions (Factor group: $F_{(1,10)} = 2.40$, $p = 0.15$; Factor odor: $F_{(1,10)} = 0.34$, $p = 0.57$).

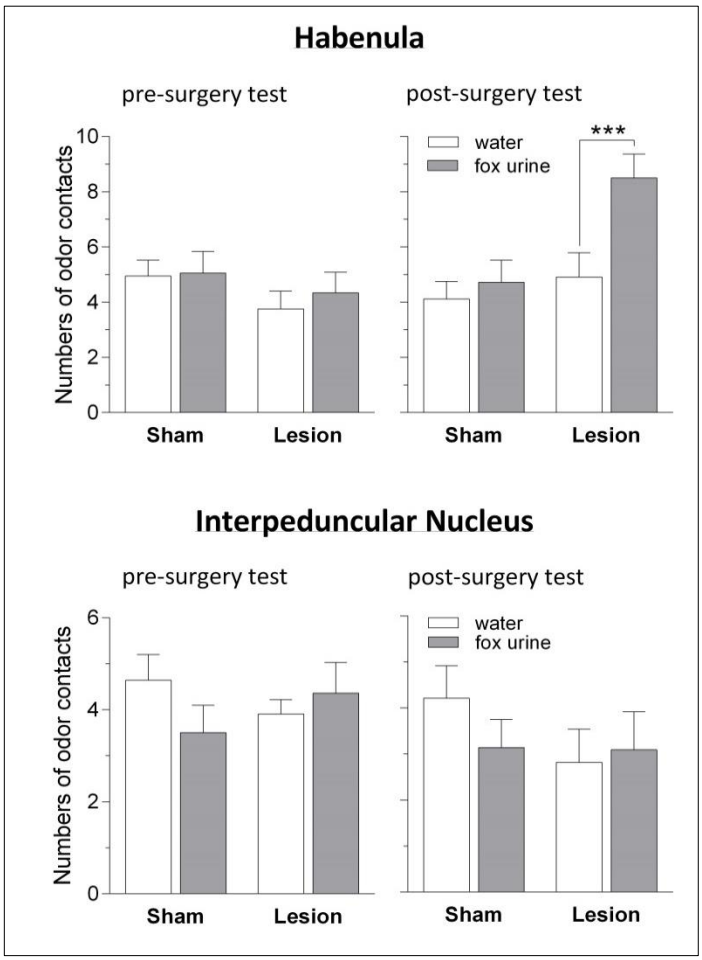

Figure 19 Electrolytic lesions of the Hb increased risk assessment to fox urine. Results are shown for both sham-operated (Hb: 18; IPN: n = 14) and Hb or IPN-lesioned (Hb: n = 12; IPN: n = 11) rats for the first 5 min of pre-surgery and post-surgery testing during exposure to water (white bars) and fox urine (grey bars). Risk assessment behavior is expressed as the number of odor contacts (mean + SEM) with the odor samples. ***p < 0.001, Holm-Sidak's multiple comparisons tests after significant effects in two-way repeated measures ANOVAs.

4.4.3. Discussion

In lower vertebrate species such as teleosts and lampreys, the Hb is considered as a higher olfactory center receiving direct innervations from mitral output cells in the olfactory bulb – thereby modulating odor-driven behaviors (Miyasaka et al., 2009; Krishnan et al., 2014; Okamoto et al., 2012; Stephenson-Jones et al., 2012). In mammals, such a direct olfactory innervation of the Hb is lacking. Whether the rodents' Hb, despite its direct olfactory innervation vanished during evolution, is still important for odor-evoked behaviors has not been investigated so far. On the contrary, at the mammalian level, Hb functioning has often been linked to the emotional control of behaviors and certain forms of emotive decision making mainly by influencing the activity of monoaminergic neurons located in the midbrain (Hikosaka et al., 2008; Hikosaka, 2010; Pobbe and Zangrossi, 2008). Common in all vertebrates is probably the intuitive and thereby innate selection of appropriate defensive responses when facing a threat. Whereas a few studies already investigated the role of the Hb complex in learned fear processes (Winter et al., 2011; Thornton and Bradbury, 1989; Rausch and Long, 1974; Wilcox et al., 1986), its role in innate fear remains largely unknown.

The present experiment was designed to investigate whether the Hb and one of its main projection targets, the IPN, are involved in fox urine-induced fear responses. Specifically, electrolytic lesions were applied to permanently destroy the Hb or the IPN. Effects of electrolytic lesions on avoidance and risk assessment behavior were measured.

The present study clearly demonstrated for the first time that predator odor-induced innate avoidance behavior in rats requires the contribution of both the Hb and the IPN. During pre-surgery testing, all rats spent significantly less time in the odor quadrant with fox urine indicating that fox urine induced robust avoidance behavior. Avoidance behavior in response to fox urine was also observed in other experiments of this doctoral thesis which further supports that predator urine samples can reliably be used as threat stimuli. Interestingly, electrolytic lesions of the Hb blocked avoidance behavior during post-surgery testing while sham-operated rats continued to avoid fox urine. The absence of an effect in animals of the control group is of particular importance since it excludes the possibility that rats habituate to the odor sample upon multiple presentations. The failure to show habituation effects upon repeated predator exposures gave rise to the conclusion that fear responses to predator odors are largely innate (Takahashi et al., 2005; cf. Blanchard et al., 2003b; Berton et al., 1998; Wallace and Rosen, 2000). Apart from reduced avoidance behavior, electrolytic lesions of the Hb also increased the number of contacts with the fox

urine sample. Avoidance behavior and also freezing behavior are usually considered as passive response strategy. On the other hand, an active response strategy includes risk assessment behaviors and the cautious investigation of the threat source (Ebner et al., 2005; Fendt and Endres, 2008; Taghzouti et al., 1999). Applied to the present results, it appears that lesioning the Hb bias rats from the more passive strategy of avoiding the fox urine sample to the more active strategy of risk assessment. Interestingly, electrolytic lesions of the IPN only reduced avoidance behavior without affecting risk assessment behavior presuming that the IPN is more implicated in the expression of predator odor-induced avoidance behavior and that its function may not be related to the control and choice of fear response strategies. A role of the Hb and probably also of its afferent connection with the IPN in controlling fear responses has also been observed in the zebrafish. Agetsuma and colleagues (2010) showed that zebrafish with genetically inactivated lateral subnucleus of the dorsal Hb, which corresponds to the medial nucleus of the Hb (MHb) in mammals, increased freezing behavior to a conditioned fear stimulus (passive defensive strategy) while control zebrafish switched to a normal flight response (active defensive strategy). In rats, deficits in the ability to change and select appropriate behavioral strategies during stress situations were observed following large electrolytic lesions of the Hb. This has been shown by Thornton and Evans (1989) using an one-way active avoidance learning paradigm. In their study, habenular-lesioned rats displayed a failure to escape shocks under stressful testing conditions (increased shock intensity, short intertrial interval) in comparison to control animals, but not under milder testing conditions using shocks of less intensity and a longer intertrial interval. On the basis of these findings, authors hypothesized a role of the Hb in behavioral flexibility, especially under stressful conditions.

In the present experiment, we made use of electrolytic lesions to permanently destroy neurons within the Hb and IPN. This lesion technique is often used for very first investigations to unterstand the function of a certain brain structure within the whole brain circuit since lesions were not restricted to a certain type of neurons. As previously mentioned, in contrast to reversible inactivation techniques (e.g., by injecting neurotransmitter agonists or antagonists) where the spatial spread of the drug around the injection site is difficult to determine, electrolytic lesions offer the advantage that the extent of the lesion can be easily controlled by changing the intensity and duration of the applied current. This is very usefull when investigating brain regions of small size such as the Hb complex.

However, given the small size of the Hb and the extent of tissue damage caused by electrolytic lesions, it is not possible to differentiate between the MHb and lateral nucleus of the Hb (LHb). In fact, different studies reported a prominent role of both subnuclei in the adjustment of behavioral responses to aversive and stressful events mainly by regulating monoaminergic transmission within the raphe nuclei (serotonin) and the ventral tegmental area (VTA, dopamine)(Hikosaka, 2010; Pobbe and Zangrossi, 2008; Mirrione et al., 2014). For instance, the rat's LHb has been shown to be activated in aversive situations (e.g., during exposure to inescapable electric shocks, social defeat) but also in response to stimuli that signal the absence of reward or that indicate behavioral errors (reviewed in Bianco and Wilson, 2009; Hikosaka, 2010). An important role of the MHb in fear behavior of mice and zebrafish has been suggested, too (Yamaguchi et al., 2013; Okamoto et al., 2012; Agetsuma et al., 2010; Lee et al., 2010; Mathuru and Jesuthasan, 2013). For instance, zebrafish with targeted expression of the light chain tetanus toxin (potent inhibitor of neurotransmitter release) within the MHb have been shown to remain for a longer time near the bottom in a fish tank and to display increased episodes of slow swimming and pauses than control zebrafish when using an overhead shadow that is thought to mimic a predator threat (Mathuru and Jesuthasan, 2013).

In all vertebrate species, the MHb projects almost exclusively to the IPN (Lecourtier and Kelly, 2007; Bianco and Wilson, 2009). In the SPECT-imaging study, the neural activity of the IPN was significantly reduced during exposure to fox urine (see Fig. 15). Notably, the IPN represents one of the brain regions containing the highest levels of GABA receptors with $GABA_B$ receptor binding sites being primarily located on presynaptic terminals of the Hb input (Price et al., 1984; Margeta-Mitrovic et al., 1999; Kawaja et al., 1989). Activation of these GABA receptors by excitatory projections arising from the activated MHb should lead to a suppression of neural activity in the IPN as it was also observed in the SPECT-imaging study (reduced rCBF in fox urine-exposed rats). Major projection targets of the IPN are the raphe nuclei and the dorsal tegmental area (Bianco and Wilson, 2009). Moreover, different studies revealed that predominantly glutamatergic projection neurons arising within the LHb preferentially targeted GABAergic neurons of the VTA and raphe nuclei (Kalen et al., 1986; Ferraro et al., 1996; Ji and Shepard, 2007). In line with results from the SPECT-imaging experiment, fox urine-exposed rats that displayed increased neural activity within the Hb showed also reduced rCBF within the VTA and raphe nuclei (see appendix) which further

evidenced the strong inhibitory upstream control of the Hb on dopaminergic and serotonergic brain activity.

In the present experiment, the most robust behavioral response of rats during confrontation with the fox urine sample is to avoid. This assumption is supported by the finding that both Hb lesioned rats and sham control rats avoided the quadrant containing the fox urine sample during pre-surgery testing while latter animals also expressed avoidance behavior during post-surgery testing. Considering that neural activity within monoaminergic midbrain centers obviously needs to be suppressed in order to select the avoidance strategy in response to fox urine (as it was the case in the SPEC-imaging experiment), we suggest that a neural activity change within these midbrain centers due to less inhibitory control by the lesioned Hb might have influenced the decision process in favor of selecting an active behavioral strategy. Interestingly, IPN lesions only blocked fox urine-induced avoidance behavior of rats but did not increase risk assessment. To our knowledge this is the first study showing that the IPN plays an important role in predator odor-induced fear behavior, i.e. avoidance behavior. In contrast, the IPN might not be involved in the processing of risk assessment behaviors. Why IPN lesions did only blocked avoidance behavior in rats while not affecting risk assessment behavior is not clear. However, following the neural suppression hypothesis as previously mentioned, it might be that electrolytic lesions of the IPN could have only partially and not sufficiently affected the “natural inhibition of the midbrain centers” required for switching behavioral fear responses possibly because neural projections from the IPN to GABAergic neurons within the VTA are only modest. In contrast, strong inhibitory control over the VTA and raphe nuclei is predominantly provided by the LHb as stated previously. Alternatively, the IPN could simply play no role in risk assessment.

Based on the results of the SPECT-imaging study and the present lesion experiments, our data suggest that the Hb might function as regulator for fear response strategies by directly affecting neural activity within monoaminergic midbrain centers. The IPN, in contrast, might only play a role in the processing of avoidance behavior.

From where in the brain the olfactory input to the Hb arises, is not clear. In higher vertebrates, the Hb receives a strong innervation from the septal complex (Klemm, 2004; Bianco and Wilson, 2009). This area is homolog to the dorsal-posterior part of the telencephalon in the zebrafish which possess projections from the mitral output cells in the olfactory bulb (Kermen et al., 2013). This suggests that the Hb might receive indirect

olfactory input from the septal complex. How the Hb and the IPN are functionally localized in the neural circuit of predator odor-induced fear is not entirely clear. However, given its anatomical position as pivotal interface in the vertebrate brain that links parts of the limbic and basal ganglia circuits with major midbrain centers and brainstem nuclei implicated in motor responses (Klemm, 2004), both the Hb and IPN are ideally situated to cross-talk with brain structures also implicated in fear processing. Within this circuit, the VTA and raphe nuclei might occupy central positions since both brain regions receive either direct or indirect (via the IPN) projections from the LHb and MHb – thereby exerting strong inhibitory control over dopaminergic and serotonergic activity in the midbrain (Ji and Shepard, 2007; Groenewegen et al., 1986).

Taken together, the present study demonstrates that electrolytic lesions of Hb complex blocked fox urine-induced avoidance behavior and increased risk assessment behavior in rats as measured by the number of odor contacts. Therefore, lesioning the Hb bias rats from a more passive strategy of avoiding to a more active strategy of risk assessment indicating that the Hb functions as a regulator site for fear response strategies possibly by affecting inhibitory upstream control on monoaminergic midbrain centers. Electrolytic lesions of the IPN, one major projection target of the Hb, only reduced avoidance behavior to the fox urine sample without affecting risk assessment. This suggests that the IPN is more implicated in the expression of predator odor-induced avoidance behavior and that its function may not be related to the choice of behavioral fear response strategies.

4.5. Summary and Conclusion Part 2

Exaggerated fear learning has long been accepted as a central feature in the development of anxiety disorders. However, an increasing body of human studies is now also emphasizing the importance of abnormal innate fear responses in fear-and anxiety-related pathologies. A striking argument for this arises from the fact that primeval anxiogenic stimuli such as spiders or situations where an escape is impossible or difficult often play an important role in human phobias as similar to the stimuli inducing defensive behaviors in a variety of mammalian species (Staples et al., 2008; Rosen et al., 2008).

Part 2 of this doctoral thesis was aimed at elucidating the neural circuit underlying predator odor-induced fear behavior in rats. The investigation of the neural substrates required to express innate fear in a rat model, contributes an indispensable step towards a better understanding of fear processing in general. This in turn might help us to better assess pathologies in the human and might be potentially useful for identifying new treatment strategies for patients suffering from such anxiety disorders.

Using SPECT-imaging of the rCBF, the group of M.Sc. Daniel Vincenz-Zörner and Dr. Jürgen Goldschmidt (Leibniz Institute of Neurobiology) identified different rat brain sites either activated or inactivated during fox urine exposure. Of all identified brain regions, the AOC, Hb and IPN were selected to directly validate their relevance in predator odor-induced defensiveness. We showed that both the AOC and IPN are critically involved in fox urine-induced avoidance behavior. The Hb complex seems to differently contribute to the processing of innate fear responses in rats. Hb lesions led to a switch in the defensive strategy from a passive to an active one suggesting that the Hb acts as possible regulator for the choice of fear strategies.

This brings now up the question of how the three brain regions are integrated into the brain circuit of innate fear? Further, how is this circuit connected to primary olfactory brain structures? From all three brain regions, the AOC is the only brain region where a direct innervation from both the main olfactory and accessory olfactory system is certainly known (Swanson and Petrovich, 1998; Gutiérrez-Castellanos et al., 2014). As previously mentioned, in teleosts and lampreys the Hb receives direct innervation from mitral cells of the olfactory bulb implying a specific role of the Hb in olfaction (Miyasaka et al., 2009; Krishnan et al., 2014; Okamoto et al., 2012; Stephenson-Jones et al., 2012). So far as we know, there are no studies showing that such connection of the Hb with the olfactory system also exists in higher vertebrates such as rodents. This implies that the Hb underwent diverse anatomical

and thereby also functional changes during evolution. In addition to its olfactory input, the AOC also strongly projects to distinct medial hypothalamic subnuclei (Canteras, 2002; Swanson and Petrovich, 1998). Together with the PAG, which is densely interconnected with all parts of the medial hypothalamic subnuclei, the medial hypothalamus and the amygdala have traditionally been thought to form an innate fear circuit (Canteras, 2002). This neural fear circuit has been suggested to present a common pathway for the expression and regulation of different defensive strategies in threat situations such as freezing or escape responses. If this fear circuit serve as the basis for innate fear processing, then which role plays the Hb-IPN axis? Both the Hb complex and the IPN are highly conserved structures in the vertebrate brain, even older than the amygdala, that link forebrain basal ganglia and limbic structures with key monoaminergic centers in the midbrain and brainstem (Klemm, 2004; Lecourtier and Kelly, 2007). Surprisingly, a direct neural connectivity of the Hb or the IPN with the ventromedial defence circuit has not been describes so far. Based on literature findings, we figured out that neural activity within the VTA, a midbrain region critically involved in the control of emotions and behavior, is largely modulated by both the amygdala (via the Nucleus accumbens, NAc) and the Hb-IPN system (Russo and Nestler, 2013; Stamatakis et al., 2013; Goncalves et al., 2012). A significant neural activity change within the VTA caused by fox urine exposure was also observed in the neural imaging study (see appendix) which further supports a specific role of the VTA in predator odor-induced fear behavior. Therefore, each of our study regions (AOC, Hb, IPN) might contribute to the processing of fear by excerting direct (mostly inhibitory) control on VTA neurons. To test this hypothesis, future experiments (e.g., microdialysis measurement of transmitter release while electrical stimulation of the Hb, combination with behavioral experiments) should be performed.

5. PART 3: ESTABLISHMENT OF THE OLFACTORY HOLE-BOARD TEST

5.1. Introduction

Challenged by the large diversity of natural odor blends in their environment, most mammalian species have developed highly sensitive olfactory systems to identify and discriminate biologically-relevant odors. In rodents, the detection of some odors is of critical importance because they trigger different basic behaviors essential for their survival. In particular, odors transmitted between individuals of the same species (pheromones) are used to communicate information on the gender, reproductive state, social status and subject identity. Thus, pheromones have been highly associated with the mediation of e.g., mate choice, parental care and territorial behaviors (Fortes-Marco et al., 2013; Brennan and Kendrick, 2006). Predator odors, in contrast, warn prey animals of a potential confrontation with a predator. For example, odors derived from cats or other carnivorous species elicit a range of innate defensive behaviors in rodents including avoidance and hiding behavior, an increase in risk assessment behaviors and the suppression of non-defensive behaviors such as foraging, sexual behavior and overall locomotor activity (Blanchard and Blanchard, 1989a; Endres et al., 2005; Masini et al., 2005; Apfelbach et al., 2005; Wernecke et al., 2015; Fendt, 2006).

Research focused on the effects of odors on rats' behaviors often include multi-trial paradigms where animals experience a sequence of single odor exposures (e.g., Wallace and Rosen, 2000; Fendt, 2006; Ferrero et al., 2011; Rivard et al., 2014). In the current set of experiments, a modified version of the olfactory hole-board test (Moy et al., 2008) have been used to study behavioral effects of different odors within single trials. In this procedure, rats are placed in a standard hole-board apparatus with automated recording of nose pokes, also called head dips. Previous work has shown that the hole-board test offers a simple method for measuring exploratory behavior of animals in an unfamiliar environment (Brown and Nemes, 2008; Takeda et al., 1998). Whether an animal prefers or avoids a hole results from an inner conflict between the natural drive of rodents to explore and the potential aversive properties of the hole. Thus, according to this hypothesis, a general decrease in head dipping behavior is interpreted to reflect increased anxiety in animals, while high levels of head dipping behavior are defined as a decline in anxiety (Crawley, 1985; Lister, 1990; Saitoh et al., 2006).

In part 3 of this doctoral thesis, a series of four experiments has been conducted to investigate if the hole-board test can be used to investigate behavioral responses of rats to different odors within single trials. In *Experiment 1*, rats were tested for hole preference in the classical 16-hole configuration. *Experiment 2* and *3* were conducted to assess whether rats display a shift in hole preference when both aversive and attractive odors were presented in the preferred corner holes. *Experiment 4* tested if avoidance behavior to holes with carnivore urine samples can be reduced by treating the rats with the anxiolytic compound buspirone.

5.2. General Materials and Methods

5.2.1. Subjects

Testing was carried out using 64 experimentally naive male Sprague-Dawley rats (2-3 months old) weighing 200-350 g at the time of testing. Rats were bred and reared at the local animal facility (original breeding stock: Taconic, Denmark). They were housed in groups of 5-6 animals in standard Macrolon Type IV cages (55 cm x 33 cm x 20 cm) with water and food available *ad libitum*. Cages were kept in temperature and humidity-controlled rooms (22 ± 2 °C, 50-55 %) under a 12 h light/dark cycle with lights on at 6:00 am. Behavioral testing was conducted during the light phase between 8:00 am and 3:00 pm.

All experiments were carried out in accordance with international ethical guidelines for the care and use of laboratory animals for experiments (2010/63/EU) and were approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42505-2-1172 UniMD).

5.2.2. Testing Apparatus

All experiments were conducted in a computer-controlled hole-board apparatus (Fig. 20 A, ActiMot2 Hole-Board System, TSE Systems, Bad Homburg, Germany) consisting of three testing boxes constructed from transparent Plexiglas (51.5 cm x 51.5 cm x 41 cm) and a height-adjustable frame with infrared detectors (sample rate: 100 Hz). A removable hole-board with 16 holes (3 cm diameter) in a grid-pattern was placed on the floor of the testing box. Holes were categorized into four corner holes (holes 1, 4, 13, 16; see Figure 21 B Inlay), four back wall holes (holes 2, 3, 5, 8), four front wall holes (holes 9, 12, 14, 15) and four center holes (holes 6, 7, 10, 11). Supplier-specific lids were used to close particular holes meaning that the number and/or the location of the holes could be modified as required for each experiment. The apparatus was located in a small testing room with dimmed illumination (ca. 30 lux).

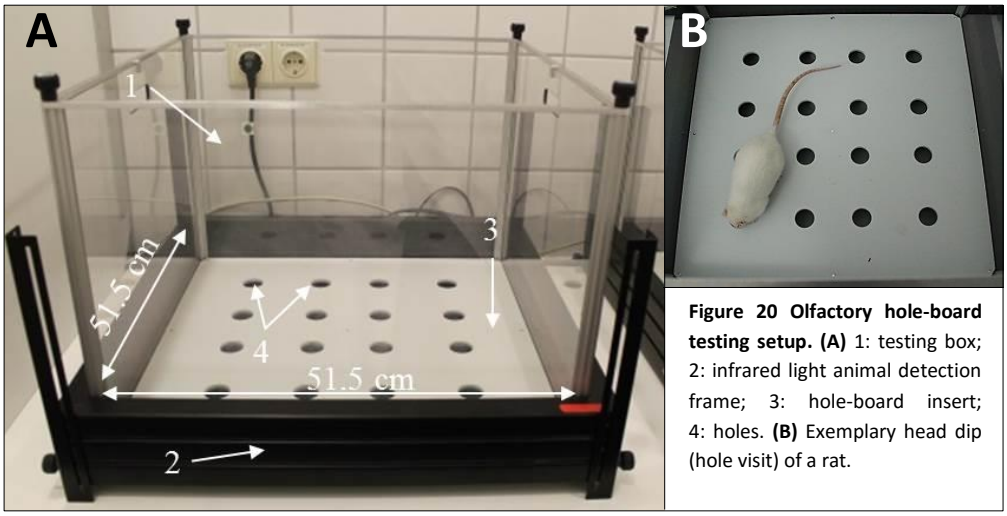

Figure 20 Olfactory hole-board testing setup. (A) 1: testing box; 2: infrared light animal detection frame; 3: hole-board insert; 4: holes. **(B)** Exemplary head dip (hole visit) of a rat.

5.2.3. Testing Procedure and Odor Presentation

For *Experiment 1*, the hole-board was used in its 16-hole configuration. Rats were individually placed into the testing boxes and tested for 20 min. Rats head dipping behavior was monitored by the infrared detectors. The software automatically measured the total number of head dips (hole visits) for each single hole. More specifically, a head dip was counted when the animal placed its head into a hole for at least 300 msec with the ears even with the floor of the hole-board (Fig. 20 B). A minimal time interval of 300 msec had to elapse after a head dip before a new hole visit was counted. For the experiments with odor presentations (*Experiment 2-4*), the holes of the center region were covered (12-hole configuration). Then, animals were individually placed into the testing box and exposed to four odors simultaneously. For this, 1 ml odor samples (described in detail below) were pipetted into small glass bowls (4 cm outer diameter, 2.5 cm height) and placed underneath the hole of each corner prior to testing. The animals were not able to touch the odor samples. For each test session, one corner hole contained only water which served as a control odor. The location of the different types of odor samples was pseudo-randomly changed across individuals (*Experiments 2-4*) and tests (*Experiment 4*). The wall holes were always left empty. Head dips into the wall holes were used to assess baseline levels of exploration behavior and to control for individual differences in the total number of head dips. After each test, the testing boxes were thoroughly cleaned with soapy water and ventilated with clean air, before the next rats were tested.

5.2.4. Odor Samples

Urine samples from foxes (*Vulpes vulpes*), bobcats (*Lynx rufus*), pumas (*Puma concolor*) and coyotes (*Canis Iatrans*) were purchased from Maine Outdoor Solutions Inc. (Hermon, ME, USA). Urine samples from elks (*Cervus elaphus)* and mona monkeys (*Cercopithecus mona*) were obtained from the local zoo (Zoologischer Garten Magdeburg, Magdeburg, Germany). Female rat urine (*Rattus norvegicus*) was self-collected by placing adult female Sprague-Dawley rats (n = 12, 3-6 months old) individually in a metabolic cage (Tecniplast, Hohenpeißenberg, Germany) for ca. 30 min on consecutive days. Female urine samples of individual animals were mixed up to ensure that urine from all estrus cycle phases were present. All urine samples were aliquoted into 1 ml portions and stored at -18 °C until usage.

5.2.5. Descriptive and Statistical Analysis

Hole visits were expressed as percentages of total hole visits. In all figures, behavioral data are shown as box-and-whisker plots. The horizontal line represents the median and the box the lower and upper quartiles. The whiskers were calculated with the Tukey method (GraphPad Prism 6.00, GraphPad Software Inc., La Jolla, USA).

For statistical analysis, data were first tested on normal distribution (D'Agostino and Pearson omnibus test). For normally distributed data, analyses of variance (ANOVA) and post-hoc comparisons by Holm-Sidak's test were used. Non-normally distributed data were analyzed using the Friedman test followed by Dunn's multiple comparisons test. Either hole location (*Experiment 1*) or odor (*Experiments 2-4*) was used as within-subject factors. $p < 0.05$ was considered statistically significant. All analyses were carried out using GraphPad Prism.

Pilot tests revealed that the hole visit behavior of animals that are extremely active or extremely inactive is only marginally modulated by odors (floor/ceiling effects; see also discussion of *Experiment 1*). In *Experiments 2-4*, we therefore excluded animals with more than 65 or less than 15 total hole visits from further analysis.

5.3. Experiment 1

The first experiment was conducted to determine whether rats display a specific exploration pattern in the hole-board test when no odors are present. From other exploration-based tasks (e.g., open-field) it is known that rodents prefer to remain in the periphery of the apparatus (thigmotaxis), whereas the bright and unprotected areas are usually avoided (Lister, 1990; Wallace and Rosen, 2000; Litvin et al., 2008). Therefore, we expected that our rats would show preference (i.e. a high number of visits) to the holes in the corners and along the side walls and avoidance (i.e. a low number of visits) to the four holes in the center of the box. This was also observed in the mouse version of the olfactory hole-board test (Moy et al, 2008).

5.3.1. Materials and Methods

14 male Sprague-Dawley rats were tested. They were put into the middle of the hole-board (all 16 holes open, no odors) and their hole visits were recorded for 20 min.

5.3.2. Results

Figure 21 illustrates the local distribution of hole visits (16 holes, no odor). The total number of hole visits ranged between 15 and 74 head dips (Fig. 21 A) with a mean of 39 head dips. The subsequent analysis revealed that there were clear differences in the percentage number of total hole visits according to the position of the holes (corner vs. center vs. front wall vs. back wall: ANOVA: $F_{(3,39)} = 66.21$; $p < 0.0001$; Fig. 21 B + C). Corner holes were visited significantly more often than holes with other locations (Holm-Sidak's tests: ps < 0.0001). Furthermore, the back wall holes were visited more often than the center holes ($p = 0.001$). There were no significant differences in the hole visits within the different hole categories (corner holes: Friedman test: $Q = 3.24$; $p = 0.36$; wall holes: ANOVA: $F_{(7,91)} = 2.21$; $p = 0.08$; center holes: Friedman test: $Q = 6.49$; $p = 0.09$).

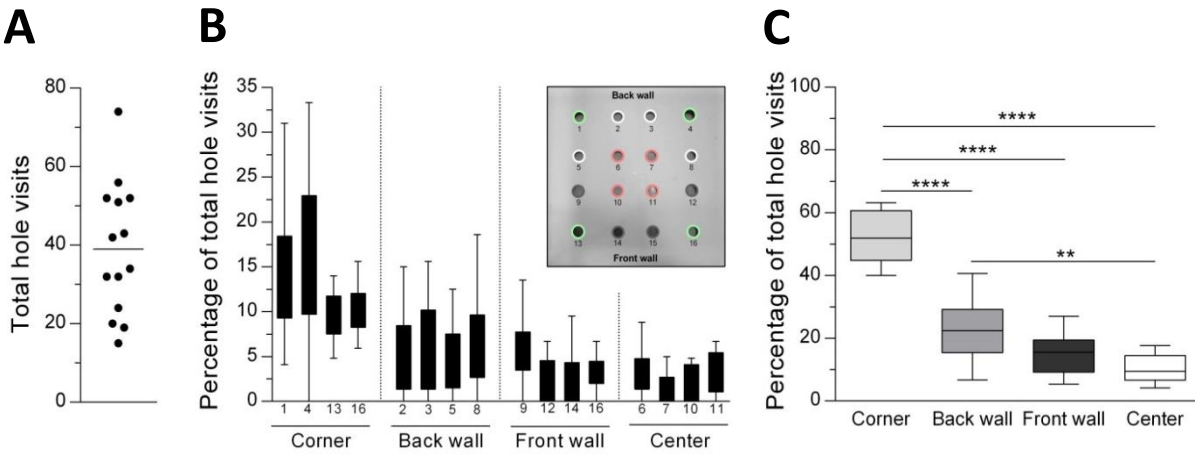

Figure 21 General distribution of hole visits (median) during a hole-board test showing a clear preference for corner holes. **(A)** Total numbers of hole visits for individual animals (n = 14) are displayed as a scatter dot plot with the horizontal line describing the mean. Number of hole visits (% of total) are depicted for **(B)** all 16 holes and **(C)** summed up for each hole category. **p < 0.01; ****p < 0.0001 comparisons as indicated (Holm-Sidak's multiple comparison test after significant main effects in an ANOVA)(Wernecke & Fendt, 2015).

5.3.3. Discussion

The behavior of animals in the hole-board test, originally described by Boissier and Simon (1962; 1964), is determined by a conflict between curiosity-based exploration and fear-based avoidance from novel, unknown locations (Brown and Nemes, 2008; Hughes, 2007). Thus, altered head dipping activity is often interpreted as changes in the anxiety state of the animals (Brown and Nemes, 2008; Takeda et al., 1998). In *Experiment 1*, rats were tested in the 16-hole configuration of the hole-board without any odors. Our results indicate that rats showed the highest rate of head dips for corner holes and the lowest rate of head dips for center holes (Fig. 18 B + C). This finding is in line with the results of previous hole-board and related exploration-based rodent models and can be explained by thigmotaxis (Lister, 1990; Moy et al., 2008; Lamprea et al., 2008).

Interestingly, Moy et al. (2008) also established an olfactory hole-board test, however, to model repetitive behavior, a core symptom of autism, in mice. In their study, different appetitive odor samples (e.g., familiar cage bedding, food items) were presented in the less-preferred center holes and the ability of mice to shift their hole preference was assessed. In contrast to this, we wanted to mainly investigate how hole visits are influenced by aversive odors. Based on previous studies from our laboratory (Wernecke et al., 2015; Fendt, 2006; Ferrero et al., 2011), we expected that holes with aversive odors will be visited less often, i.e. avoided. Such avoidance is much easier to observe when holes are very often visited under control conditions. Therefore, our approach was to place the test odors under the

corner holes. To further increase the number of corner hole visits, we closed the four center holes. Given that the hole visit activity was very different for individual animals, we also decided that this individual variance should be included into the analyses of odor effects on hole visits. Therefore, hole visits were presented as the percentage of total head dips (cf. Moy et al., 2008).

To avoid floor or ceiling effects, we further excluded animals from the behavioral analysis when these rats were either too inactive (i.e. few total hole visits) or too active (i.e. many total hole visits). Based on these thoughts, the testing protocols of the following studies were designed and the exclusion criteria were defined.

5.4. Experiment 2

It is well established that aversive odors, such as predator odors, innately induce a variety of defensive responses including avoidance and escape behavior (Apfelbach et al., 2005; Dielenberg and McGregor, 2001; Masini et al., 2005). On the other hand, attractive odors, such as the odors of female conspecifics, are approached (Liberles, 2014). To investigate whether these behaviors can also be observed in the olfactory hole-board test for rats, we placed urine samples of carnivores, female conspecifics and a water control sample under the corner holes of the hole-board. We expected that holes with aversive odors will be avoided (i.e. less hole visits) and holes with attractive odors will be preferred (i.e. more hole visits).

5.4.1. Materials and Methods

14 male Sprague-Dawley rats were used in this experiment. The following odor samples were presented: fox urine (*Vulpes vulpes*), bobcat urine (*Lynx rufus*), female rat urine (*Rattus norvegicus*) and water. The locations of the odor samples were pseudo-randomized.

5.4.2. Results

The percentages of total hole visits for each corner hole of the present experiment are shown in Figure 22. The different odor samples significantly affected the corner hole visits (ANOVA: $F_{(3,39)} = 54.85$; $p < 0.0001$). Post-hoc pairwise comparisons with the water control indicated a strong increase of visits to the holes with female rat urine (Holm-Sidak's test: $p < 0.0001$), while holes with fox urine ($p = 0.03$) or bobcat urine ($p = 0.02$) were visited less often. The mean number of total hole visits was 39 (data not shown).

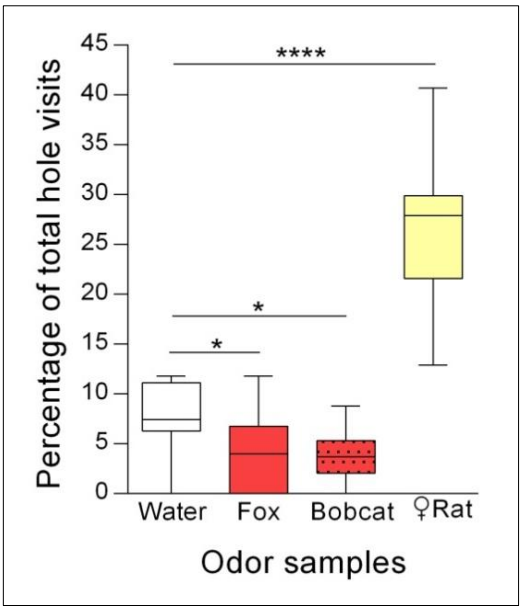

Figure 22 Rats (n = 14) avoided holes with carnivore urine, whereas holes with female rat urine were preferred. Percentages of total hole visits (median) for the different corner holes are shown. *p < 0.05; ****p < 0.0001 comparisons as indicated (Holm-Sidak's multiple comparison test after significant main effects in an ANOVA) (Wernecke & Fendt, 2015).

5.4.3. Discussion

Experiment 2 investigated whether simultaneous presentation of both aversive and attractive odors led to changes in hole visit behavior. We showed that holes with carnivore urine samples were clearly visited less often than the hole with water, i.e. carnivore urine was avoided. These results support findings from previous studies showing avoidance behavior to carnivore urine. For instance, Osada et al. (2013) observed that mice similarly avoided the short arm of a Y-maze when it contained wolf urine. Using an open-field test, we previously showed that rats avoid the quadrant or corner of the testing arena containing carnivore urine (Wernecke et al., 2015; Fendt, 2006; Ferrero et al., 2011). This is confirmed by field studies demonstrating that carnivore urine samples (e.g., dingo, coyote, bobcat, wolf) are effective repellents protecting forestry and agricultural areas from feeding-related damage (Parsons et al., 2007; Bramley and Waas, 2001; Nolte et al., 1994).

Our second observation is that rats were attracted to the hole containing urine from female rats. Sexually naive male mice similarly preferred to investigate female urine over water in a Y-maze test (Pankevich et al., 2006). In the present experiment, the female urine sample was presented simultaneously with aversive carnivore urine samples. Since we were able to measure these appetitive effects of the female urine samples, we suggest that the different odors samples did not strongly diffuse within the hole-board testing apparatus and that avoidance/preference responses were most likely restricted to the holes containing the particular odor sample. Otherwise, an increase in general anxiety due to the recognition of aversive carnivore odors should be detectable. This would most probably reduce sexually

motivated behaviors like approach to female urine samples (cf. Rhees et al., 2001; Retana-Marquez et al., 1996; Kobayashi et al., 2013).

Taken together, the present experiment is in agreement with the rats' natural motivation to approach odors of potential mating partners (Liberles, 2014) and to avoid odors of carnivores (Apfelbach et al., 2005; Masini et al., 2005). Importantly, using the olfactory hole-board test, we are able to study olfactory avoidance and preference behavior to different types of odors presented on the same hole-board in the same test session.

5.5. Experiment 3

The previous experiment showed that rats avoid holes with carnivore urine and preferred holes with female rat urine. However, this phenomenon could also be explained by a simple avoidance of odors from other species, whereas odors from conspecifics are preferred. To exclude this possibility, we exposed rats to urine samples from an herbivorous species (elk), an omnivorous species (mona monkey) and a carnivorous species (puma). Based on previous studies (Ferrero et al., 2011; Fendt, 2006), we would expect that carnivore but not herbivore urine samples will be avoided, whereas omnivore urine may lead to an intermediate response.

5.5.1. Materials and Methods

22 male Sprague-Dawley rats were included in this experiment. Rats were exposed to carnivore urine (Puma, *Puma concolor*), herbivore urine (Elk, *Cervus canadensis*), omnivore urine (Mona monkey, *Cercopithecus mona*) and water as a control odor.

5.5.2. Results

Again, the holes with the different odor samples were differently visited by the rats (Fig. 23) as indicated by a significant odor effect (Friedman test: $Q = 20.98$; $p = 0.0001$). Post-hoc pairwise comparisons of the percentages of corner hole visits with the percentage of water control hole visits showed that the holes with the urine from mona monkeys and pumas were avoided (Dunn's test: $p = 0.009$ (mona monkey urine); $p = 0.0001$ (puma urine)), whereas the holes with elk urine were not differently visited ($p = 0.14$) than the water control hole. Rats displayed on average 31 hole visits during testing (data not shown).

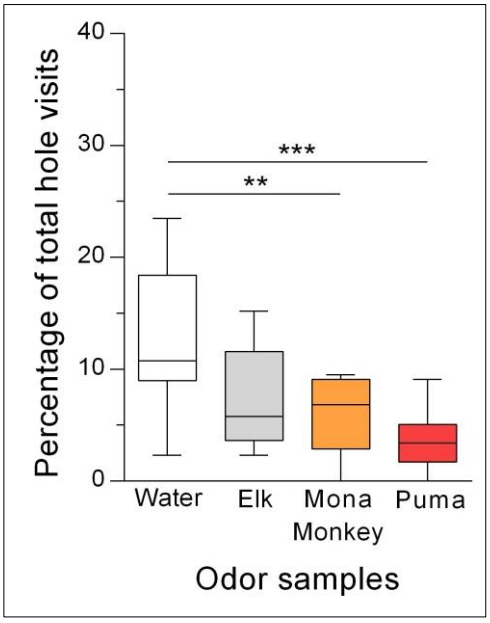

Figure 23 Rats (n = 22) avoid holes with omnivore and carnivore urine, but visited holes with herbivore urine. Percentages of total hole visits (median) for the different corner holes are shown. **p < 0.01; ***p < 0.001 comparisons as indicated (Dunnett's multiple comparison test after significant main effects in the Friedman test)(Wernecke & Fendt, 2015).

5.5.3. Discussion

Rats were exposed to urine samples from an elk, mona monkey and puma, as representatives for herbivore, omnivore and carnivore species, respectively. Rats avoided the holes containing urine from either the puma or the mona monkey, whereas the holes with elk urine appeared to be neutral (Fig. 23). These findings as well as similar findings from literature (Ramp et al., 2005; Du et al., 2012; Fendt, 2006) suggest that prey animals are able to discriminate between urine of harmless herbivore species and urine of omnivore or carnivore species, both being potential predators. This would be an important evolutionary adaption since rats would only invest energy for the defense from potential predators but would not waste energy with defensive responses to odors of herbivore species which are no threat to rats. The question now is by which mechanisms do rats innately recognize urine from potential predators? One possibility is that rats detect predators through common metabolites derived from a carnivorous diet (Berton et al., 1998; Ferrero et al., 2011; Nolte et al., 1994). Such a metabolite could be PEA, a component of most carnivore species' urine and also of some omnivore species' urine (Ferrero et al., 2011). Only moderate concentrations of PEA have been identified in urine samples of omnivores or smaller carnivores (e.g., ferret, fox, cat, human), while higher amounts of PEA are present in urine samples of larger feline carnivores (e.g., tiger, lion, jaguar). These different PEA levels in the urine may be responsible for the intensity of the expressed avoidance behaviors.

5.6. Experiment 4

The aim of the present experiment was to test whether anxiolytic treatments specifically modulate the avoidance of carnivore urine holes without affecting the preference of rats to female rat urine.

Benzodiazepines are highly effective anxiolytic substances in both humans and animals (Gelfuso et al., 2014). When tested in predator odor exposure tests, different benzodiazepines (e.g., midazolam) have been reported to change defensive responsiveness to cat odor leading to decreased hiding behavior and increased approach behavior (Dielenberg et al., 1999; McGregor and Dielenberg, 1999; Blanchard et al., 1998a; Siviy et al., 2010a). However, we observed that treatment of rats with midazolam (0, 0.19, 0.38 mg/kg) had sedative effects and strongly dose-dependently reduced the number of total hole visits (Friedman test: Q = 20.46; p < 0.0001; see Fig. 24). This makes it very difficult to evaluate whether midazolam treatment affects the avoidance response to carnivore urine.

An established anxiolytic compound with only minor sedative properties is the 5-HT_{1A} receptor agonist buspirone (Carli et al., 1989; Kehne et al., 1988; Moser, 1989). Therefore, rats were treated with buspirone and the effects on olfactory hole-board performance were tested.

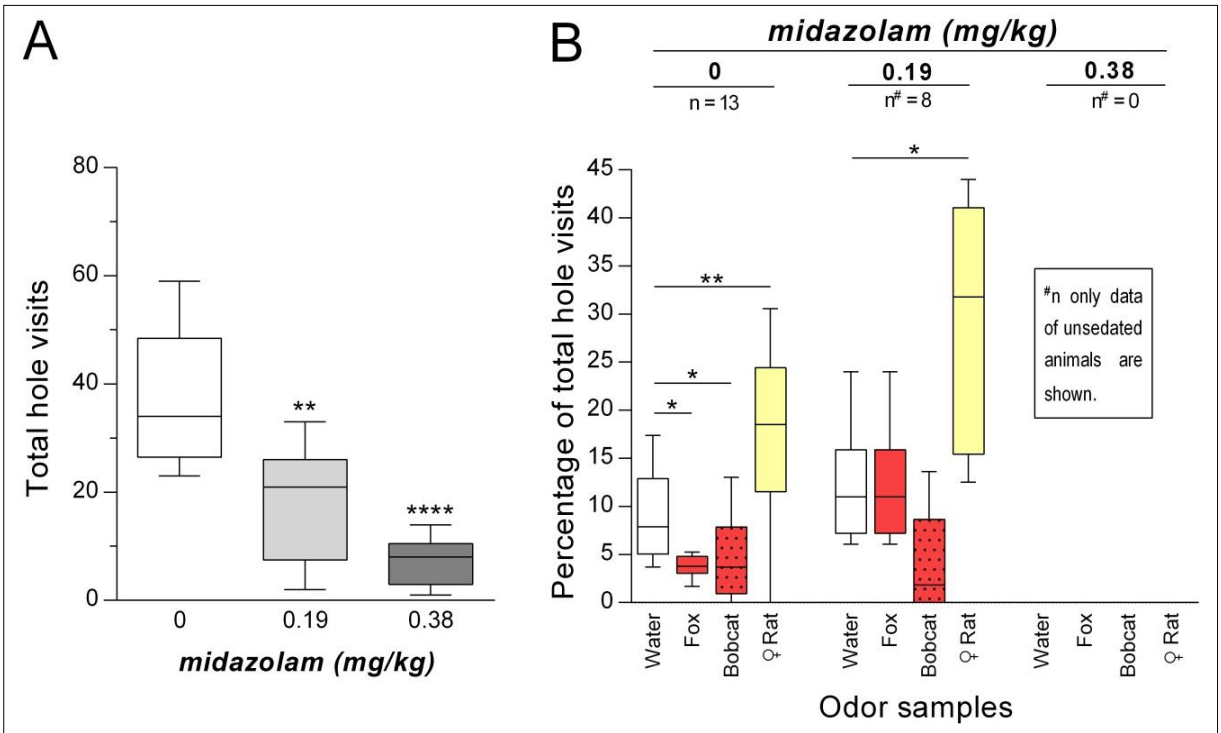

Figure 24 Midazolam treatment dose-dependently sedated rats (A) Total number of hole visits (median) of rats (n = 13) after treatment with midazolam (0; 0.19; 0.38 mg/kg midazolam). **(B)** Percentages of total hole visits (median) for the different corner holes and for each treatment are shown. **p < 0.01; ****p < 0.0001 comparison to saline treatment (0 mg/kg); *p < 0.05; **p < 0.01 comparison as indicated (Holm-Sidak's/Dunn's multiple comparison test after significant main effects in an ANOVA/Friedman test) (Wernecke & Fendt, 2015).

5.6.1. Materials and Methods

Experiment 4 included 14 male Sprague-Dawley rats. 20 min prior to testing, each animal was pretreated with the vehicle (saline) or the 5-HT_{1A} receptor agonist buspirone (0.1, 1 mg/kg). Injections were given intraperitoneal (i.p.) and were administered at a volume of 1 ml/kg. Each rat received each of the three treatment conditions in a pseudo-randomized order with 24 h between each test. Rats were exposed to fox urine (*Vulpes vulpes*), coyote urine (*Canis Iatrans*), female rat urine (*Rattus norvegicus*) and water as control odor.

5.6.2. Results

The analysis of the total number of hole visits confirmed that buspirone has only minor sedative properties and did not significantly affect the total number of hole visits (ANOVA: $F_{(2,26)} = 2.28$; $p = 0.129$, Fig. 25 A).

The percentages of corner hole visits after treatment with saline or buspirone (0.1 mg/kg; 1 mg/kg) are illustrated in Figure 25 B. We performed separate ANOVAs for each treatment. In saline-treated rats, there was a significant main effect of odor (Friedman test: $Q = 29.10$; $p < 0.0001$). Post-hoc comparisons with the water control indicated that rats significantly avoided the holes with fox (Dunn's test: $p = 0.016$) or coyote urine ($p = 0.039$). However, there was no effect of female rat urine ($p = 0.237$). After treatment with 0.1 mg/kg buspirone, there was still a significant main effect of odor (ANOVA: $F_{(3,39)} = 14.81$; $p < 0.0001$) with holes with fox urine (Holm-Sidak's test: $p = 0.005$) or coyote urine ($p = 0.042$) being avoided. The holes with female rat urine were not visited more often by rats than the water hole ($p = 0.105$). Notably, different effects were observed after treatment with 1 mg/kg buspirone. Although there was again a main effect of odor (Friedman test: $Q = 12.05$; $p = 0.007$), the percentages of total hole visits for the different corner holes were not different from water (Dunn's tests: $p > 0.999$ (fox urine); $p = 0.563$ (coyote urine); $p = 0.171$ (female rat urine)).

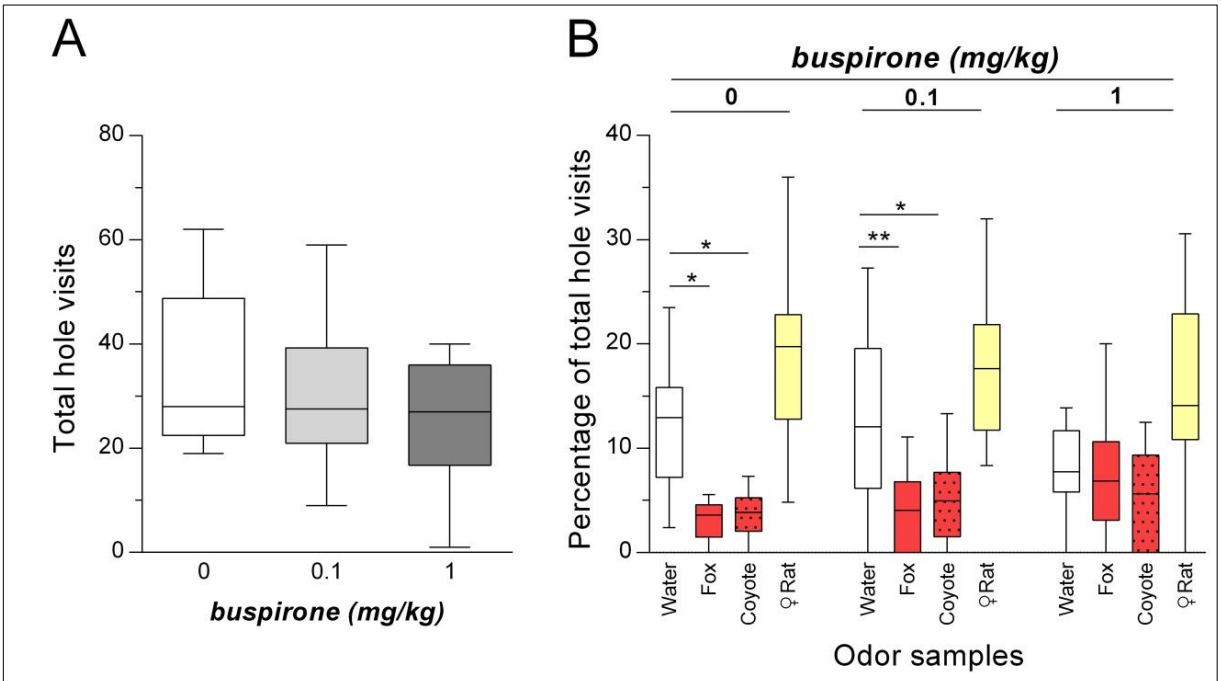

Figure 25 Buspirone treatment specifically blocked the avoidance of holes with carnivore urine without sedating the rats. (A) Total number of hole visits (median) of rats (n = 14) after treatment with buspirone (0; 0.1; 1 mg/kg buspirone). **(B)** Percentages of total hole visits (median) for the different corner holes and for each treatment are shown. *p < 0.05; **p < 0.01 comparison as indicated (Holm-Sidak's/Dunn's multiple comparison test after significant main effects in an ANOVA/Friedman test)(Wernecke & Fendt, 2015).

5.6.3. Discussion

Treatment with the 5-HT_{1A} receptor agonist buspirone dose-dependently blocked the avoidance of holes with carnivore urine in the olfactory hole-board test. Importantly, buspirone did not affect the total hole visits and the visits of holes with attractive female rat urine (Fig. 25) indicating a specific anxiolytic effect on behavioral changes induced by carnivore urine. We further showed that the olfactory hole-board test is inappropriate for testing compounds with strong sedative effects such as midazolam (Fig. 24).

Our finding that buspirone reduced predator odor-induced defensive behavior supports previous findings showing that treatment with 8-OHDPAT ((±)-8-hydroxy-2-(di-n-propylamino) tetralin), another 5-HT_{1A} receptor agonist, decreases freezing and increases approach behavior to TMT (Shields and King, 2008). Similarly, it has been demonstrated that buspirone effectively reduces anxiety in other rodent anxiety models such as the elevated-plus-maze test and the black/white exploration test (Hendrie et al., 1997; Moser, 1989).

Comparison of the experiments with female rat urine presentation (*Experiments 2, 4*) showed that the used female rat urine sample were variably effective to induce hole preference. It has been demonstrated that male rats are more attracted to odors of estrous females than that of non-estrus females (Achiraman and Archunan, 2006; Hosokawa and

Chiba, 2005; Achiraman et al., 2010). Here, urine regardless of the female's estrus cycle stage was collected. Therefore, different amounts of estrus urine in the different odor samples may serve as a likely explanation for the varying effectiveness of female rat urine to attract male rats.

5.7. Summary and Conclusion Part 3

Rodents, as most other mammals, are predominantly olfactory oriented and largely depend on olfactory cues for operating in their environment (Galliot et al., 2012; Sotnikov et al., 2011). Therefore, odors are of considerable significance in guiding nearly every class of animal behavior (Doty, 1986) and their perception and discrimination are believed to be crucial for survival and reproduction. In this sense, the recognition of predator odors and odors of the sexual counterpart is critically important. The former induces defensive behaviors, whereas the latter induces attraction behavior in rats.

The aim of Part 3 of this doctoral thesis was to assess whether the olfactory hole-board test can be used as a behavioral paradigm for investigating olfactory preference and avoidance to biologically-relevant odors, as well as whether such a preference or avoidance can be selectively modulated by pharmacological treatments. We made use of the rats' natural preference for corner holes in the hole-board test and examined whether this pattern of hole preference could be manipulated by placing both appetitive and aversive odors under these holes. Using the innate preference for corner holes allowed us to circumvent a floor effect since odor-induced avoidance responses are more easily detected when the holes are frequently visited under control conditions. The key advantage of the olfactory hole-board test is that it allows testing animals' responses to four odors in a single test session. Moreover, since appetitive odor samples can be presented simultaneously with aversive odor samples under different corner holes, we were further able to test preference and avoidance responses at the same time. This is unique, since most research focused on the effects of biologically-relevant odors on the behaviors of rats used multi-trial paradigms (e.g., olfactory habituation/dishabituation task) with sequential presentations of different odors (Silverman et al., 2010; Lehmkuhl et al., 2014; Mandairon et al., 2009). Moreover, because rodents in nature are not exposed to only one pure odor but to several odors at the same time, the olfactory hole-board may present also a more natural test situation.

As mentioned in the discussion of *Experiment 1,* Moy et al. (2008) had also developed an olfactory version of the hole-board test. In contrast to the present study, they made use of the mice's natural aversion of the center holes and tested whether the placement of novel, appetitive odors in these holes may modify this innate aversion. Consequently, a lack of a hole preference shift to the center holes has been interpreted to reflect the resistance to adapt their behavioral responses in regard to environmental factors (Moy et al., 2008). This suggests that the hole-board test in association with the presentation of odor samples is

versatile allowing the study of multiple research issues. While the mouse olfactory hole-board was only used to test appetitive odor samples, in the present version, rats were exposed to both appetitive and aversive odor samples.

The quality of the olfactory hole-board test in testing aversion and preferences in the same test session was shown by *Experiment 2* and *4*. Rats avoided visiting corner holes with urine of potential predators (fox, bobcat, puma, coyote, mona monkey). Simultaneously, rats visited holes with urine from female rats more often indicating attraction behavior. Importantly, the odor-induced avoidance response was specific to the urine of omnivore and carnivore species. In contrast, the number of visits to holes with urine of an herbivore species was indistinguishable to that of the control holes. This supports the idea that rats are able to discriminate between urine of different species, both predator and non-threatening species (Fendt, 2006; Ramp et al., 2005; Du et al., 2012; Ferrero et al., 2011).

The present study further tested whether the avoidance of carnivore urine holes can be reduced by treating the rats with the anxiolytic compounds midazolam or buspirone (*Experiment 4*). Treatment with buspirone specifically abolished the avoidance response to holes with carnivore urine. Notably, such effects are difficult to detect when the anxiolytic compound has strong sedative effects, as was the case with midazolam since too few hole visits were observed after midazolam treatment. The finding that the olfactory hole-board test provides direct measures of olfactory responses in rats that can be specifically pharmacologically manipulated further makes it possible to use this test to examine, for instance, the specificity of anxiolytic treatment effects.

In conclusion, Part 3 of this doctoral thesis demonstrates that the olfactory hole-board test may provide an appropriate tool for the assessment of olfactory aversion and preferences in rats. In contrast to many other testing paradigms, this paradigm allows testing of up to four odors simultaneously in single trials. Furthermore, the olfactory hole-board test is applicable to test anxiolytic treatments without sedating properties indicating predictive validity.

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7. APPENDIX

7.1. SPECT-Imaging data

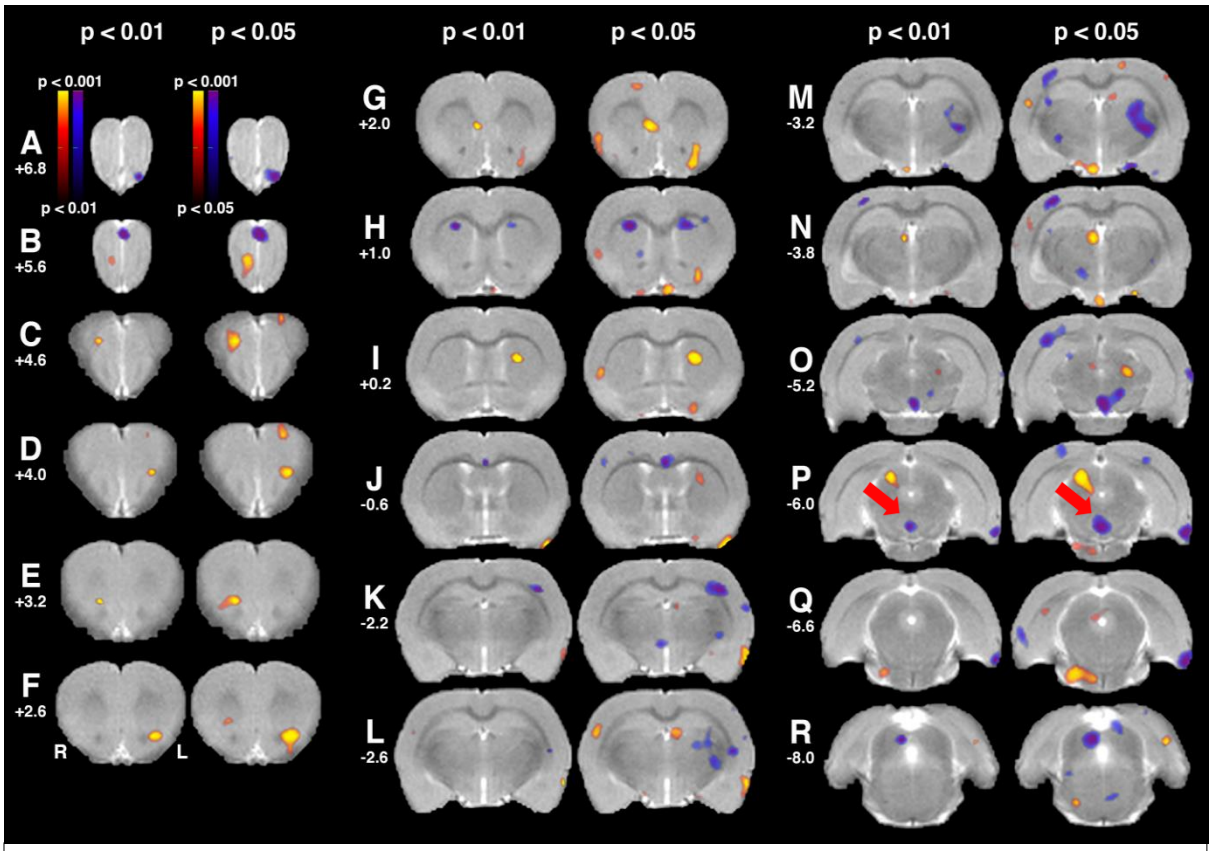


Figure 25 Probability maps indicating significant changes in rCBF during fox urine exposure. Probability maps are shown at two different significance levels (right panels: $p < 0.05$; left panels: $p < 0.01$) in order to illustrate the different spatial extent of significant voxels above threshold. Sections are arranged from rostral (A) to caudal (R). Numbers on the left side of the sections indicate coordinates relative to bregma. Significant increase in rCBF is color coded from red to yellow and significant decrease from blue to violet. All images are overlaid on the same template MR image. The red arrows point to the VTA.

7.2. Protocols

Inhalation anesthesia

Materials

Isoflurane (Baxter Deutschland GmbH, Unterschleißheim, Germany)
Oxygen bottle (Air Liquide Medical GmbH, Düsseldorf, Germany)
Anesthesia setup (Rothacher Medical GmbH, Heitenried, Switzerland):

- Isoflurane vaporizer (Rothacker & Partner, Bern, Switzerland)
- Induction chamber
- Electronic flowmeter (Dräger Medical Schweiz AG, Liebefeld, Swiss)
- Gas anesthesia head holder (David Kopf Instruments, Tujunga, USA)
- F/AIR filter (A.M. Bickford Inc., New York, USA)

Stereotaxic alignment system with blunted ear bars (David Kopf Instruments, Tujunga, USA)

Methods

1. Verify the isoflurane level in the isoflurane vaporizer
2. Check the weight of the F/AIR filter (> 50 g needs replacement)
3. Check the content of the oxygen bottle
4. Open the valve of the oxygen bottle
5. Turn on the electronic flowmeter and adjust the O_2 flow until 2.4 l/min is reached
6. Place the rat into the induction chamber and close the lid
7. Turn the vaporizer control to 5 % (induction of anesthesia)
8. Open the selector for the induction chamber
9. As soon as the animal is deeply anesthetized, close the selector for the induction chamber
10. Open the induction chamber and mount the animal into the stereotactic alignment system
11. Fix the gas anesthesia head holder over the nose of the animal
12. Open the selector for the gas anesthesia head holder
13. Lower the amount of isoflurane (2-2.5 %) depending on animal's breathing and pain reaction (check the pedal and eye reflex)
14. After surgery, turn the vaporizer control to 0 %, close the selector for the gas anesthesia head holder
15. Close the valve of the oxygen bottle, turn the electric flowmeter off

Cannula implantation

Materials

Stereotaxic alignment system with blunted ear bars (David Kopf Instruments, Tujunga, USA)
Shaver (B. Braun Vet Care GmbH, Tuttlingen, Germany)
Scalpel, scissors, forceps, extractors, swabs, cotton sticks
Stainless steel injection cannulas, stainless steel guide cannulas, stainless steel stylets
Screws, screwdriver
Dental drill (Nouvag AG, Goldach, Switzerland)
Eye ointment (e.g., Vidisic®, Bausch + Lomb GmbH, Berlin, Germany)
Tissue glue (e.g., Histoacryl®, B. Braun Melsungen AG, Melsungen, Germany)
Dental acrylic cement (e.g., Paladur, Heraeus Kulzer GmbH, Hanau, Germany)
Saline (0.9 % NaCl, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany)
70 % Ethanol, 10 - 30 % H_2O_2, 1 ml syringe (B. Braun Melsungen AG, Melsungen, Germany)

Methods

1. After securing the rat into the stereotactic alignment system, shave the surgical field
2. Disinfect the skin with 70 % ethanol, cover the eyes with eye ointment
3. Make a midline incision using a scalpel or a scissor, starting at the back of the eyes and extending posteriorly for about 2 cm
4. Attach retractors to the periosteum to keep skin away from the surgical field
5. Remove the blood and tissue from the skull, dab off the skull with H_2O_2 to stop bleedings
6. Fix the injection cannula to the arm holder block of the stereotactic alignment system
7. Determine coordinates of Bregma and Lambda using the tip of the injection cannula
8. Ensure a flat skull position by checking the ventral/dorsal coordinate of Bregma and Lambda (difference > 0.1 mm needs adjustment of the head holder)
9. Calculation of the target coordinates
10. Drill holes at the target coordinate(s), drill 1-2 holes next to the target hole(s) (for screws)
11. Tighten screws slightly
12. Install guide cannula over the injection cannula (will stick together when guide cannulas were wetted with 70 % ethanol)
13. Position the injection cannula (with attached guide cannula) at the determined coordinates
14. Fixate guide cannula with dental acrylic cement, wait 5 min for drying
15. Remove the injection cannula (guide cannulas should now be fixed to the skull)
16. If necessary, repeat the step 13-17 for cannula implantation on the bilateral hemisphere
17. Close the surgical field (e.g., by using tissue glue)
18. Prevent occlusion of guide cannulas by inserting stylets
19. Intraperitoneal or subcutaneous injection of saline (2-3 ml)
20. Let the animal recover from surgery for at least 3 days

Intracranial microinjection

Materials

Stainless steel injection cannulas, stainless steel stylets
Polyethylene tubes
1-µl Hamilton microsyringes (Hamilton, Bonaduz, Switzerland)
Microinfusion pump (CMA Microdialysis AB, Kista, Sweden)
Small cage with bedding material and food pellets
Saline (0.9 % NaCl, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany)
Drug solution to be microinjected

Methods

1. Connect the injection cannula(s) with the polyethylene tube(s)
2. Rinse the tubing system with saline and check the permeability of the injection cannula(s)
3. Install 1-µl Hamilton microsyringes in the microinfusion pump
4. Connect the other end of the tube(s) with the 1-µl Hamilton microsyringe(s)
5. Withdraw the plunger of the microsyringe(s) that air is pulled into the tube
6. Place the tip of the injection cannula into the drug solution
7. Continue to withdraw the plunger of the microsyringe (drug solution is now separated from the saline in the tube system by the air pocket
8. Determine the injection rate (0.2 µl/min) and the volume (0.3 µl) at the microinfusion pump
9. Run the microinfusion pump until a drop of drug solution is observed at the tip of the injection cannula
10. Place the animal into the small cage
11. Remove the stylet
12. Insert the injection cannula(s) into the guide cannula(s) while gently restraining the animal
13. Run the microinfusion pump until the predefined volume of the drug solution is injected (infusion rat: 0.2 µl/min)
14. Leave the injection cannula(s) in place for 1 min to allow diffusion of the drug solution into the tissue
15. Remove the injection cannula(s) from the guide cannula(s) and insert the stylets
16. Check again the permeability of the injection cannula(s) to confirm that the injection tubing system did not become occluded during the injection

Surgery protocol for electrolytic lesions

Materials

Stereotaxic alignment system with blunted ear bars (David Kopf Instruments, Tujunga, USA)
Shaver (B. Braun Vet Care GmbH, Tuttlingen, Germany)
Scalpel, scissors, forceps, extractors, swabs, cotton sticks
Acupuncture needle (insulated except for the tip, asia-med GmbH & Co. KG, Suhl, Germany)
Dental drill (Nouvag AG, Goldach, Switzerland)
Eye ointment (e.g., Vidisic®, Bausch + Lomb GmbH, Berlin, Germany)
Tissue glue (e.g., Histoacryl®, B. Braun Melsungen AG, Melsungen, Germany)
Saline (0.9 % NaCl, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany)
70 % Ethanol, 10 - 30 % H_2O_2
1 ml Syringe (B. Braun Melsungen AG, Melsungen, Germany)
Pulse stimulator (A-M Systems, Sequim, USA)
Digital multimeter (Metex Corporation, Seoul, Korea)

Methods

1. After securing rat into the stereotactic alignment system, shave the surgical field
2. Disinfect the skin with 70 % ethanol, cover the eyes with eye ointment
3. Make a midline incision using a scalpel or a scissor, starting at the back of the eyes and extending posteriorly for about 2 cm
4. Attach retractors to the periosteum to keep skin away from the surgical field
5. Remove the blood and tissue from the skull, dab off the skull with H_2O_2 to stop bleedings
6. Fix the acupuncture needle to the arm holder block of the stereotactic alignment system
7. Determine coordinates of Bregma and Lambda using the tip of the acupuncture needle
8. Ensure a flat skull position by checking the ventral/dorsal coordinate of Bregma and Lambda (difference > 0.1 mm needs adjustment of the head holder)
9. Calculation of the target coordinate(s)
10. Drill holes at the target coordinate(s)
11. Position the acupuncture needle at the determined coordinates
12. Connect the cathode of the pulse simulator to the acupuncture neddle
13. Connect the anode of the pulse simulator to the tail of the animal (earthing)
14. Generate an electrolytic lesion by passing a ~ 0.1 mA anodal current through the electrode tip for 10-15 sec
15. While lesioning, monitor the current by the digital multimeter
16. If necessary, repeat the step 11-15 for lesioning at the bilateral hemisphere
17. Close the surgical field (e.g., by using tissue glue)
18. Intraperitoneal or subcutaneous injection of saline (2-3 ml)
19. Let the animal recover from surgery for at least 3 days

Brain removal

Materials

Small animal guillotine
Scissors, spatula, forceps, rongeurs, glass vial
10 %, 20 %, 30 % sucrose-formalin solution (fixation solution)

Methods

1. Upon completion of behavioral testing, animals were killed e.g., through exposure to CO_2
2. Decapitate the rat directly behind the ears using a guillotine
3. Remove the skin and muscles form the dorsal and posterior part of the skull
4. Use rongeurs to remove small parts of the bone. Start at the upper edge of the foramen magnum
5. Carefully remove the remaining bone along the dorsal and lateral surface of the brain
6. Remove the bone over the posterior part of the nasal cavity
7. Remove the dura mater from the brain using fine forceps
8. Lift the brain using a spatula, cut the optical nerves with the spatula
9. Take out the brain and store it in a glass vial with 10 % sucrose-formalin solution for fixation
10. When brain is sagged, transfer brain into the 20 % sucrose-formalin solution
11. Repeat step 10 using 30 % sucrose-formalin solution

Preparation of gelatin-coated slides

Materials

Microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany)
Glass staining jar, glass staining rack, glass funnel, filter paper, beaker, thermometer
Magnetic stirrer with hot-plate (IKA®-Werke GmbH & CO. KG, Staufen, Germany)
100 ml distilled H_2O
270 mg gelatin + 25 mg chromalaun ($KCr(SO_4)_2 \cdot H_2O$)

Methods

1. Heat 100 ml distilled H_2O up to about 50 °C on the magnetic stirrer with hot-plate
2. Dissolve gelatin, stir gently until it clears
3. Add chromalaun, stir gently until it clears
4. Filter solution using a funnel with filter paper into a glass staining jar
5. Let the solution cool down to room temperature
6. Dip staining rack filled with microscope slides into the staining jar filled with gelatin-solution
7. Dry the microscope slides for 1 h, then redip
8. Air-dry microscope slides over night

Cryostat sectioning

Materials

Gelatin-coated microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany)
Cryostat (Leica Biosystems Nussloch GmbH, Nussloch, Germany)
Microtome blades (Leica Biosystems Nussloch GmbH, Nussloch, Germany)
Specimen disc (Leica Biosystems Nussloch GmbH, Nussloch, Germany)
Tissue freezing medium (Leica Biosystems Nussloch GmbH, Nussloch, Germany)
Scalpel, forceps, small brush
Petri dish
Brain

Methods

1. Place the fixed brain in a petri dish
2. Make a straight cut through the tissue at a position that will not be analyzed (e.g., olfactory bulb, cerebellum) to get a supporting surface
3. Apply a drop of tissue freezing medium on the center of the deeply grooved specimen disc
4. Place the brain's supporting surface on the drop of tissue freezing medium and align the brain straightly
5. Place the specimen disc in a supporting hole of the cryostat to allow the tissue to freeze for at least 30 min (when frozen tissue freezing medium turns white)
6. Attach a new microtome blade into its holder in the cryostat
7. When frozen, place the specimen disc with the brain into the disc holder
8. Set the desired section thickness (40 µm)
9. Take sections turning the hand wheel clockwise
10. Position the desired brain sections on the pressure plate of the cryostat using a fine brush
11. Lower a gelatin-coated microscope slide to the brain section until it just touches it and allow the section to melt onto the microscope slide
12. Air-dry slides before Nissl and Gold chloride staining

Nissl staining protocol

Materials

Microscope slides with brain sections
Glass staining jar, glass staining rack
Funnel, filter paper
Microscope cover slips (Gerhard Menzel GmbH, Braunschweig, Germany)
Magnetic stirrer with hot-plate (IKA®-Werke GmbH & CO. KG, Staufen, Germany)
Stop watch
1 % cresyl violet staining solution
50 %, 70 %, 96 %, 100 % ethanol
Acetic acid
Rot-Histol
DPX / Entellan
Distilled water

Methods

Preparation of cresyl violet staining solution:

- Dissolve 5 g cresyl violet in 100 ml 100 % ethanol
- Stir the solution using a magnetic stirrer with hot-plate at 50 °C for 30 min
- Add 500 ml distilled H_2O, stir gently
- Filter the solution using a funnel with filter paper into a brown glass bottle

Nissl staining:

- Transfer microscope slides with dried brain sections into a glass staining rack
- Then, dip the staining rack in the following solution for the specified time
    1. 70 % ethanol for 2 h
    2. 50 % ethanol for 15 min
    3. Cresyl violet for 1-3 min, control staining
    4. Distilled H_2O, only for a few sec
    5. 70 % ethanol for 2 min
    6. 96 % ethanol for 2 min
    7. 96 % ethanol + 12 drops of acetic acid for 2 min
    8. 100 % ethanol for 2 min
    9. 100 % ethanol for 2 min
    10. Roti-Histol for 10 min
    11. Roti-Histol for 5 min
    12. Coverslip brain sections with DPX/Entellan

Gold chloride staining protocol

Materials

Microscope slides with brain sections
Glass staining jar, glass staining rack
Microscope cover slips (Gerhard Menzel GmbH, Braunschweig, Germany)
Stop watch, magnetic stirrer, autoclave
0.2 % gold chloride staining solution ($AuCl_3$; 0.2 M phosphate buffer)
2.5 % sodium thiosulfate ($Na_2S_2O_3$)
50 %, 70 %, 96 %, 100 % ethanol
Distilled water
Roti-Histol
DPX/Entellan

Methods

Preparation of 0.2 M phosphate buffer:

- Stock solution A (500 ml): dissolve 13.8 g NaH_2PO_4-H_2O in 500 ml distilled water
- Stock solution B (500 ml): dissolve 14.20 g Na_2HPO_4 in 500 ml distilled water
- Mix 11 parts of stock solution A with 39 parts of stock solution B (in total 100 ml, ≙ 0.1 M phosphate buffer)
- Check and adjust if necessary the pH (7.4)
- Prepare a 0.02 M phosphate buffer solution (2 ml 0.1 M phosphate buffer + 98 ml distilled water)
- Stir the solution using a magnetic stirrer

Gold chloride staining:

- Transfer microscope slides with dried brain sections into a glass staining rack
- Incubate microscope slides for 1.5 h in the 0.2 % gold chloride staining solution (0.5 g $AuCl_3$ in 250 ml 0.02 M phosphate buffer + 0.9 % NaCl)
- Rinse microscope slides with distilled H_2O
- Fixate brain sections for 5 min in freshly prepared 2.5 % sodium thiosulfate solution (6.25 g $Na_2S_2O_3$ in 250 ml distilled water)
- Keep microscope slides for 30 min under running tap water
- Keep microscope slides for a few sec in distilled water
- Dehydrate brain sections by keeping microscope slides in an ascending series of alcohol (50 %, 70 %, 96 %, 100 % ethanol) for each 2 min
- Fixate brain sections for 10 min in Roti-Histol
- Coverslip brain sections with DPX/Entellan

PUBLIKATIONEN UND ERKLÄRUNG ZUM EIGENANTEIL

Bis auf die unten erwähnten Ausnahmen wurden alle in dieser Dissertationsarbeit vorgestellten Experimente von mir geplant, durchgeführt und statistisch ausgewertet. Einige Verhaltensexperimente wurden zum Teil innerhalb von LabRotations durchgeführt, die von mir geplant und betreut wurden. Ein Teil der Daten wurde bereits in Form von drei Publikationen veröffentlicht, die von mir selbstständig verfasst wurden. Mein Betreuer Prof. Dr. Markus Fendt übernahm hierbei die klassischen Korrekturarbeiten.

PART 1

Experiment 2:

Judith Brüggemann (Integrative Neuroscience Master Programm, Otto-von-Guericke Universität Magdeburg) erhob im Rahmen ihrer von mir betreuten LabRotation einen Großteil der Daten des Futtereintrage-Experimentes. Ergänzende Experimente wurden von mir eigenständig durchgeführt.

Publikation:

Wernecke KEA, Brüggemann J and Fendt M (2016). Predator odor exposure increases food-carrying behavior in rats. *Physiol Behav* 154:15-19.

Experiment 3:

Im Rahmen seiner Beschäftigung als wissenschaftliche Hilfskraft erhob Marcel Brosch (Integrative Neuroscience Master Programm, Otto-von-Guericke Universität Magdeburg) unter meiner Anleitung die Daten des Experimentes 3.

PART 2

Experiment 1:

Daten für das Manuskript „Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex" wurden vollständig von mir alleine erhoben, analysiert und diskutiert. Die anderen Mitglieder des CBBS NeuroNetzwerkes sind Mitautoren der daraus hervorgegangenen Publikation, da sie an der Konzeption dieser Studie mitbeteiligt waren.

Publikation:

Wernecke KEA, Vincenz D, Storsberg S, D'Hanis W, Goldschmidt, Fendt M (2015). Fox urine exposure induces avoidance behavior in rats and activates the amygdalar olfactory cortex. *Behav Brain Res* 279: 76-81.

PART 3

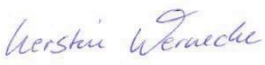
Experiment 3:

Ein kleiner Teil der Verhaltensdaten stammt aus einer von mir betreuten LabRotation von Sinthyia Ahmed (Integrative Neuroscience Master Programm, Otto-von-Guericke Universität Magdeburg).

Publikation:

Wernecke KEA, Fendt M (2015). The olfactory hole-board test in rats: a new paradigm to study aversion and preferences to odors. *Front Behav Neurosci* 9:223.

Magdeburg, den 18.12.2015



DANKSAGUNG

Wenn drei Jahre wie im Flug vergehen und man die Zeit noch einmal Revue passieren lässt, stellt man fest, dass es viele Menschen gibt, denen man **Danke** sagen möchte:

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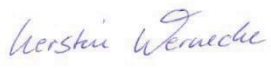
SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit versichere ich an Eides statt, die vorliegende Dissertation mit dem Titel „Predator odor-induced fear in rats: a behavioral characterization and neural substrate analysis“ selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Wörtliche oder sinngemäße Zitate habe ich als solche gekennzeichnet.

Weiterhin erkläre ich, dass ich bisher keine Promotionsversuche unternommen habe. Diese Dissertation wurde weder in der jetzigen noch in einer ähnlichen Form oder auszugsweise bei einer anderen Prüfungsbehörde zu Promotionszwecken eingereicht.



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