How predator odors affect avoidance behavior of laboratory and wild rat strains

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„Wenn die Menschen nur über das sprächen, was sie begreifen,
dann würde es sehr still auf der Welt sein.“

Albert Einstein, 1879 - 1955

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"Weißt du was passiert, wenn man sich immer alle Türen offen hält?
Dann zieht's mein Freund! Dann wird man krank!"
Das Känguru — Marc-Uwe Kling

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Abbreviations

Abbreviation	Standing
%	percent
°C	degree Celsius
µg	microgram
µL	microliter
µm	micrometer
AB-Complex	avidin-biotin-complex
ANOVA	analysis of variance
AOB	accessory olfactory bulb
AOC	amygdalar olfactory cortex
AOS	accessory olfactory sytem
APir	amygdalo-piriform transition zone
BLA	basolateral amygdala
BLP	basolateral amygdaloid nucleus, posterior part
BSA	bovine serum albumin
BNST	bed nucleus of stria terminalis
CeA	central amygdaloid nucleus
CENIT	Centre for Neuroscientific Innovation and Technology
cm	centimeter
DAB	Diaminobenzidine
dest.	distilled
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPl	external plexiform layer of the olfactory bulb
EPlA	external plexiform layer of the accessory olfactory bulb
EtOH	ethanol
g	gram
GABA	gamma-amino-butyric acid
GAD	general anxiety disorder
h	hour

HPA	hypothalamic–pituitary–adrenal axis
i.p.	intraperitoneally
IEG	immediate early gene
L	liter
LH	Lister Hooded
LHbM	lateral habenular nucleus, medial part
M	molar
m	meter
max	maximum
MeA	medial amygdaloidal nucleus
MeAV	medial amygdaloidal nucleus, anteroventral part
mg	milligram
MiA	mitral cell layer of the accessory olfactory bulb
min.	minute
mL	milliliter
MOB	main olfactory bulb
MOS	main olfactory system
mRNA	messengerRNA
NaCl	sodium chloride
NGS	normal goat serum
nm	nanometer
ns	not significant
OR	olfactory receptors
ORN	olfactory receptor neurons
OvGU	Otto-von-Guericke University
PB	phosphate buffer
PB+T	phosphate buffer with 0.3 % Triton X-100
PFA	paraformaldehyde
pH	potential of hydrogen
Pir	piriform cortex
PLCo	posterolateral cortical amygdaloidal nucleus

PTSD	posttraumatic stress disorder
PV	paraventricular thalamic nucleus
RIA	radioimmunoassay
RNA	ribonucleic acid
ROI	regions of interest
Rpm	rounds per minute
RT	room temperature
SAD	social anxiety disorder
s.c.	subcutaneously
SO	supraoptic nucleus
SPECT	Single Photon Emission Computed Tomography
t	tendency
TMT	2,5-dihydro-2,4,5-trimethylthiazoline
TSA	Tyramide Signal Amplification
USA	United States of America
VNO	vomeronasal organ
WWCPS	Warsaw Wild Captured Pisula Stryjek

1 Introduction

1.1 Fear, anxiety and anxiety disorders

The Oxford dictionary defines fear as an "unpleasant emotion caused by the threat of danger, pain, or harm" and anxiety as "a feeling of worry, nervousness, or unease about something" {Waite 2012}. In addition to this linguistic differentiation there is also a psychological one. Harriet Lerner, a US American clinical psychologist, describes this differentiation in an article published in *Psychology Today* with the following words: "The sudden re-arrangement of your guts when an intruder holds a knife to your back (fear), is different from the mild nausea, dizziness and butterflies in your stomach as you're about to make a difficult phone call (anxiety)" {Lerner 2009}. The authors Sadock and colleagues describe anxiety as “a diffuse, unpleasant, vague sense of apprehension…” and reaction to an unspecific, more general threat. Fear, in contrast, is an emotional response to an explicit, specific threat or immediate danger {Sadock et al. 2007}. Mice or rats share this vital “emotion” and show different behavioral escalation levels which distinguish between fear and anxiety {e.g. Ohl et al. 2008; Sotnikov et al. 2011}. However, anxiety and fear are important protective and survival mechanisms especially for prey animals {Spielberger 1972}. The physical reactions interrelate or merge, and both, morbid and exuberant anxiety or fear are symptoms of several human mental disorders such as phobias, anxiety disorders (e.g. posttraumatic stress disorder (PTSD)) or panic attacks {Kessler 1995; NHS 2015; NIMH 2016}.

In the European Union the prevalence for PTSD is estimated between 1 % to 9 % {Paris 2000; Siegmund & Wotjak 2006 & 2007; Mackenzie et al. 2010}. In the United States of America an estimated 8 % of inhabitants suffer from PTSD and similar numbers are published for general anxiety disorder (GAD) {PTSD United 2013}. These data clearly indicate that anxiety disorders are a serious problem among mankind {WHO 2017a}. However, mental disorders are as manifold as their triggers. These triggers can be traumatic experiences, often characterized by a potential threat to life. Examples of traumatizing events are wars, terrorist attacks, accidents, distress during emergencies, witnessing the death of others, injuries or threatening natural events {Frommberger et al. 1998; Reddemann et al. 2008; NIH 2013; WHO 2017a & 2017b}. However, for mice or rats other situations are potentially traumatic like experiencing heights, brightly illuminated places or encountering a predator. Whether such experiences cause mental diseases is modulated by predisposition and conditions, both psychologically and genetically {Lang et al. 2000; Campos et al. 2013}. Nonetheless, fear and anxiety are important emotions as well {LeDoux 2012}. Innate fear is what saves us from walking straight into a grizzly bear cave. It is also what keeps us alarmed when we walk alone in a

park at night and a villain jumps at us from behind a bush to run away fast; or to freeze in shock. Both of these reactions, freezing and flight are well described innate reactions towards a predator threat {Dielenberg & McGregor 2001}. In contrast to conditioned or learned fear, innate fear appears even though one has never encountered a similar traumatic situation before and induces defensive behavior {Blanchard & Blanchard 1989}.

In animal research scientists model traumatic experiences in order to understand altered innate fear circuits and predispositions in patients which suffer from anxiety disorders {Lissek et al. 2005; Cohen et al. 2009; Toth & Neumann 2013} but the underlying neuronal circuitries are currently not fully understood. Animal models are essential to investigate these complex connections among genetics, physiology, psychology and behavior in anxiety and anxiety disorders. The possibilities to test animal behavior in the laboratory or even in the field are as numerous as behavioral variability {Schöner et al. 2017}. As a reaction to a stressor there are primary and secondary defense strategies in prey animals {Apfelbach et al. 2005}. Primary responses help to avoid a conflict or discovery. Secondary responses include flight and hide responses, active and passive avoidance, freezing, burrowing, grooming or several risk assessment behaviors such as rearing, leaning or approach {Blanchard et al. 2011} with various characteristics. To test behaviors, manifold paradigms have been established such as Y- or T-maze, elevated plus maze, 3 chamber box or open field, to name a few. In general, one can distinguish conditioned and innate fear paradigms {Buccafusco 2009}. In typical conditioned fear paradigms, neutral stimuli are paired with aversive events to elicit fear and investigate principal learning and memory {Cushman & Fanselow 2010, Tovote et al. 2015}. In addition, pairing with rewarding events is also possible {e.g. Robinson et al. 2014; Morris & Rottenberg 2015; Simon & Moghaddam 2015}, but not used to investigate fear or anxiety. To test the innate fear reaction of an animal, unconditioned fear paradigms are used {Ennaceur 2014}. These and other tests can be arranged to analyze olfactory capabilities as well. In simple habituation or preference tests the animal is confronted with one or two known and unknown odors to test for discrimination abilities, preference, avoidance or odor memory {Schellinck et al. 1995; Staples et al. 2010; Frederick et al. 2011; Arbuckle et al. 2015}. Such olfactory tests are quite frequently performed with appetitive or aversive odors to investigate conditioned fear, aversive learning tasks and innate behavior {e.g. Staples 2010; Wernecke & Fendt 2015}. Another behavioral reaction, which is frequently analyzed, is the reaction to novelty as an indicator for fear, coping mechanisms, visual discrimination, learning or even schizophrenia {Saayman et al. 1964; Steimer & Driscoll 2003; Cole et al. 2017; Martinelli et al. 2017}. Rats show high

levels of natural exploration behavior. This is not only due to curiosity and rather to the fact that these animals depend on constant information about their habitat and surrounding. Some behavioral studies have been performed to analyze the benefit of an enriched environment on development, the functional maturation of brain connectivity and stress level {Alvarez et al. 2014; Xu et al. 2014 Illa et al. 2017}. Behavioral responses to fear are also used as readout to test anxiolytic medications and therefor are very important in treatment and drug development {Dielenberg & McGregor 2001; Ganella & Kim 2014}.

How are these emotions processed in the brain? A brain region with central role in processing emotions, such as anxiety and fear, is the amygdala and it is apparent that in anxiety disorders its function might be affected {Mitra & Sapolsky 2010; Andero et al. 2013; Janak & Tye 2015; Tsvetkov et al. 2015; Lee et al. 2017; Neumeister et al. 2017; Sah 2017}. In 2005 Adolphs and colleagues reported about bilateral amygdala defects to cause impairments in fear recognition {Adolphs et al. 2005} although Feinstein and colleagues published that the amygdala is not obligatory to evoke panic reactions {Feinstein et al. 2013}. Especially the medial amygdaloid nucleus (MeA) seems to process not only behavioral but also endocrine responses to stressful events {Feldman et al. 1994; Dayas et al. 1999; Masini et al. 2009}. Another interesting publication from 2014 describes the role of this nucleus in innate fear response. Toxoplasmose gondii infected rats lose their innate aversion towards predators due to DNA methylation in the MeA {Hari Dass & Vyas 2014) which clearly shows the important role of this nucleus in processing innate fear. However, the MeA receives direct input from both, the main and accessory olfactory system {Petrovich et al. 2001; Meredith & Westberry 2004; Pro-Sistiaga et al. 2007} and is involved in mediating freezing responses after TMT exposure {Müller & Fendt 2006} as well as the basolateral complex of the amygdala (BLA). This nucleus of the amygdala processes sensory information depending on fear memory {Ribeiro et al. 2011} which helps to evaluate the quality of the sensed threat {Etkin et al. 2009}. The BLA then activates the central nucleus of the amygdala, which is responsible for defensive responses and modulation of species-specific behavior by modulating further projections to the hypothalamus or brainstem {Scalia & Winans 1975; Pitkänen et al. 1997; Jolkkonen & Pitkänen 1998; LeDoux 2000; Davis & Whalen 2001; Aggleton et al. 2003; Sah et al. 2003}. However, literature indicates even a more complex role. A very recent publication from Lee and colleagues describes an involvement of the BLA in fear and anxiety discrimination, with activation of different neuronal subpopulations to discriminate these two emotions {Lee et al. 2017}.

1.2 Sense of olfaction

The importance of the human sense of olfaction is not highly appreciated, only when it is absent, for example when we are caught with a cold. Humans become aware of the loss of olfaction because the sense of taste, which is tightly linked to olfaction, is impaired as well and we can no longer experience flavor. Since smell and taste are not essential for the human´s survival, humans are considered to be microsmatic animals. On the other hand, there are reports about people losing their sense of smell and taste, e.g. after an accident, developed depression and committed suicide. The same holds true for elderly people in whom these senses deteriorate over time. Some publications even describe connections between anosmia and dementias {McShane 2001; Doty 2012; Roberts et al. 2016} and olfactory tests as sensors or models in Parkinson's disease {Chen et al. 2017; Maremmani et al 2017; Zapiec et al. 2017}. In contrast, mice, rats, and other rodents rely on olfaction as it is crucial for survival, as guidance to food, mates or pups, and as detector of danger or predators {Kats 1998; Stowers & Kuo 2016}. These animals are considered macrosmatic. The perceivable molecules belong to the group of semiochemicals {Omkar 2016} which are substances with a certain function in communication {The Society of American Foresters 2008}. These "communication signals" are classified by emitter and receiver into allelochemicals (interspecific communication) and pheromones (intraspecific communication) {Sbarbati & Osculati 2006; Wyatt 2008}. Allelochemicals are further divided into kairomones, allomones, and synomones {Brown et al. 1970; Sonenshine 1985; Ruther et al. 2002}. Synonomes are beneficial for both, emitter and receiver. Allomones carry messages which are only beneficial for the emitter and kairomones are only beneficial for the receiver. This makes especially kairomones an important cue for prey animals to recognize a predator early.

Some working groups have identified and isolated single repellent components of predator odors and found out that the diet has special influence, besides age and gender {Jorgenson et al. 1978; Nolte et al. 1994; Berton et al. 1998; Hendriks et al. 2008; Apfelbach et al. 2015}. Typical metabolites of meat digestion are sulfur and nitrogen, compounds which seem to be responsible for the repellent effect. Experiments with coyote urine showed a lack of this effect after vegetarian diet. The same holds true after chemical removal of the compounds containing sulfur {Nolte et al. 1994; Apfelbach et al. 2005}. Pyrazines und pyridines, which contain sulfur, are described to induce freezing behavior in mice comparable to wolf urine {Osada et al. 2013}. Other well-known isolated components of predator odors, are 2-amino-7-hydroxy-5-dimetyl-4-thiaheptanoic acid (L-felinine), 2,5-dihydro-2,4,5-trimethylthiazoline (TMT), and 2-phenylethylamine (PEA) {e.g.

Voznessenskaya & Malanina 2013; Voznessenskaya 2014; Apfelbach et al. 2015; Pérez-Gómez et al. 2015}. The latter was isolated from different predator urines and characterized by Ferrero and colleagues {Ferrero et al. 2011}. This research group discovered that PEA causes a robust avoidance response in rats similar to predator urine. Additional findings were that the repellant probabilities of predator urine were lost after chemical removal of PEA, which was restored after its addition {Ferrero et al. 2011}. These findings characterize PEA as important fear evoking predator urine compound. L-felinine, a cat pheromone precursor, which was well described by Hendriks and colleagues {Hendriks et al. 1995, 2001 & 2008}, is known to prevent pregnancy in female mice {Voznessenskaya 2014}. And TMT, a well investigated compound of red fox feces, induces avoidance and fear responses in several prey species like mice, rats, voles or lagomorphs {Sullivan et al. 1988; Wallace & Rosen 2000; Perrot-Sinal et al. 2000; Fendt et al. 2005 & 2008; Staples et al. 2006; Endres & Fendt 2009}.

Hence, receiver animals are able to recognize chemical compounds to discriminate olfactory stimuli. In 2014 Bushdid and colleagues stated the human main olfactory system (MOS) can discriminate more than a trillion volatile olfactory stimuli. This number was the result of an extrapolation after discrimination experiments with healthy subjects {Bushdid et al. 2014}. In contrast, it is known that dogs or rats are able to perceive and discriminate smallest amounts of odors (e.g. tracking and police dogs, mine rats) which humans are incapable of detecting {Thesen et al. 1993; Settle et al. 1994; Hart et al. 2000; Furton & Myers 2001; Uchida & Mainen 2003; Wells & Hepper 2003; Pinc et al. 2011; apopo.org 2017}. On the neuronal and connectivity level olfactory systems in mammals are very similar. The first part of the MOS is the olfactory epithelium, which is located in the roof of the nasal cavity. It consists of thousands of different olfactory receptor neurons (ORN) and supporting cells. The olfactory receptors (OR) are located at the basal cilia of the ORN. Different odor molecules activate a different combination of these receptors which makes it possible to discriminate way more odors than receptors are present {Mombaerts et al. 1996; Serizawa et al. 2004; Masurkar & Chen 2009; Masini et al. 2010}. Once activated, the axon of the ORN sends the information through the *lamina cribrosa* to the main olfactory bulb (MOB). The MOB is a layered structure and consists of a glomerular layer, an external plexiform layer, a mitral cell layer and a granule cell layer. The axons from the ORN expressing the same receptors, convergent activate a specific set of glomeruli. In these glomeruli the ORN form synapses with periglomerular interneurons, tufted cells and centrifugal cells. With these cells the olfactory information is transported via the

lateral olfactory tract to higher brain areas {Scalia & Winans 1975; Masurkar & Chen 2009; Nagayama et al. 2014; Simpson & Sweazey 2015}.

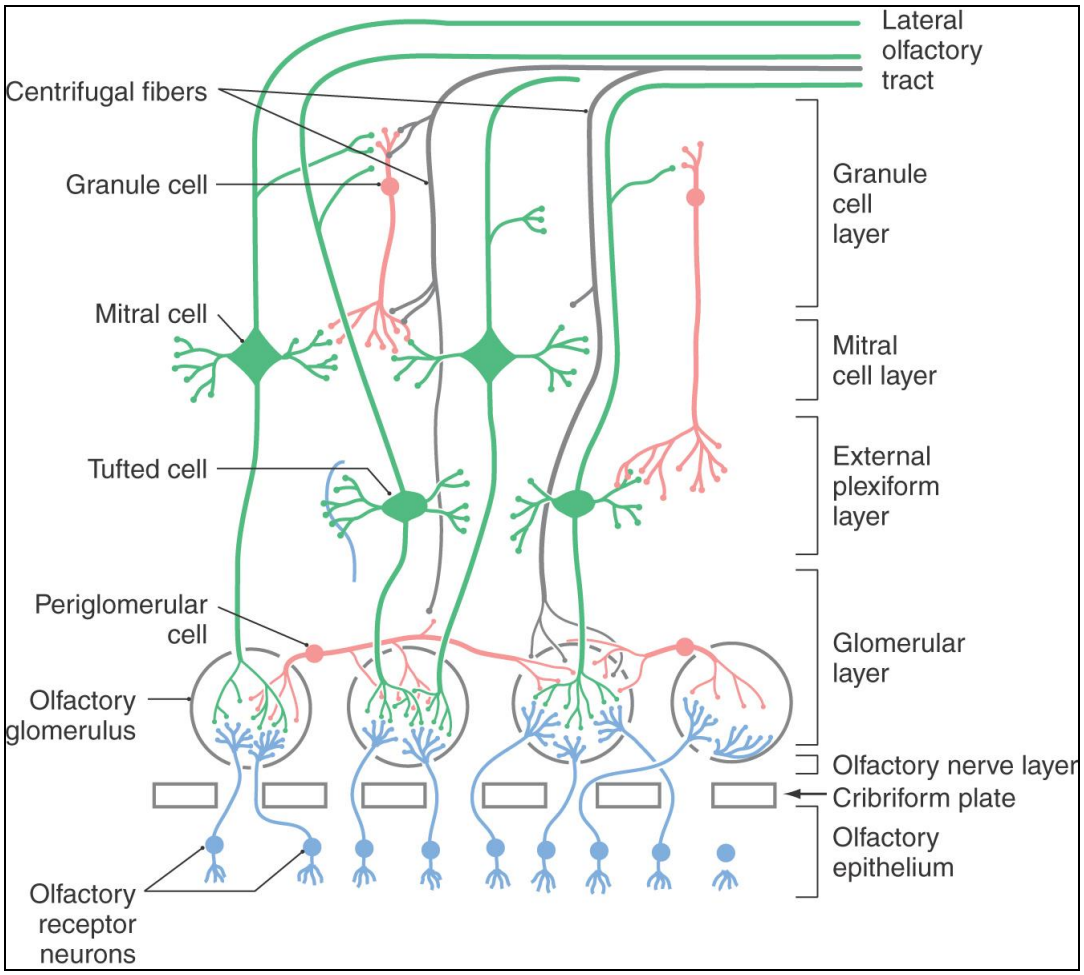

Fig. 1: Schematic sketch of the olfactory bulb with the layered structure. Blue: olfactory receptor neurons, red: periglomerular interneurons, green: mitral and tufted cells, black: centrifugal cells {Simpson & Sweazey 2015}.

A variety of animals have an additional olfactory system, the vomeronasal organ (VNO) to perceive non-volatile olfactory cues like pheromones {Scalia & Winans 1975; Kobayakawa et al. 2007}. Typically, this organ is located in a pit next to the vomer in the nasal cavity and is capable of detecting pheromones, allomones or kairomones {Fortes-Marco et al. 2013; Kiyokawa et al. 2013}. The VNO is part of the accessory olfactory system (AOS) and is activated through receptors on basal microvilli. The axons of the ORN project to a posterior part of the olfactory bulb, the accessory olfactory bulb (AOB), and from there to higher brain areas {Keverne 1999; Hagino-Yamagishi 2008}. The existence of a human VNO is controversially discussed {Meredith 2001, Trotier 2011}. Currently scientists state an existence during gestation but a lack of typical structures in adulthood {Monti-Bloch et al. 1998}. Nevertheless, humans are capable of processing pheromone-like molecules, although a typical VNO could not be found recently {Witt & Hummel 2006; Schulz 2008}.

1.3 Rats and strains

In order to investigate certain issues and disorders, experiments on animals are of crucial importance. Only in animal models it is possible to investigate certain disease triggers, genetic preconditions, and develop intervention strategies and drug treatment strategies. Rats show biological, genetic, and behavioral characteristics which closely resemble those of humans and therefore rats are common subjects in scientific research since about 100 years. Until today there are more than 400 laboratory rat strains available {GV Solas 2004}. These strains can be roughly divided in two groups: inbred and outbred strains. An inbred strain is produced by at least 20 generations of brother-sister pairing which is traceable to the founding pair. The resulting animals are genetically relatively stable, should show unique phenotypes and therefore should decrease experimental variations. Outbred strains, in contrast, show an inter-individual diversity due to regular introduction of unrelated mating partners, which increases genetic and experimental variation as well {Charles River 2015}.

Since the late 19th century rats have been used in scientific research as model organisms. Every laboratory rat resulted from the wild Norway rat, but ever since the albino forms were preferentially bred. The Wistar Institute for Anatomy and Biology in Pennsylvania (USA) is of particular importance and already in the 70s of the 20th century about 100 inbred lines were established here {The Wistar Institute of Anatomy and Biology 2017}. This breeding caused several changes in the animals. Most of the internal organs and the cerebral volume of laboratory rats are smaller compared to wild rats. Cerebral regions important for locomotion (corpus striatum and cerebellum) and olfaction are reduced or less developed whilst the pituitary gland is enlarged {Niethammer & Krapp 1978}. Additionally, there are clear differences in behavior. Laboratory rats show less locomotor activity, aggressiveness or neophobia {Stryjek et al. 2012}. Barnett and colleagues already describe a form of wariness in wild rats during exploratory behavior, which is absent in laboratory strains {Barnett et al. 1975}. Wild rat strains also tend to avoid novel objects under natural conditions {Barnett 1958}.

These are all clear signs of domestication which might influence research conclusions when behavioral results are transferred into humans {Hughes & Boice 1973; Hughes 1975}. To understand and cure processing of anxieties one has to understand healthy processing of these emotions. But this can be altered by domestication, which has to be taken into account.

1.3.1 Sprague Dawley® (SD) rats

SD rats are an outbred albino strain established in the 1920s {Janvier-labs 2017} presumable with participation of Wistar rats. SD rats are suitable models in many research fields such as oncology, pharmacology, toxicology or physiology, just to name a few. These animals are also often used in behavioral tests due to their tameness and easy handling {Charles River 2017a; Janvier-labs 2017}.

1.3.2 Lister hooded (LH) rat

The LH strain is an outbred strain and was established at the Lister Institute in the 1920s {Velaz 2013a}. These rats have white and black fur; usually the body is white and the so called hood is black. The eyesight is superior due to pigmentation and the animals are easy to handle because of their tame and docile nature {Charles River 2017b}. The LH rats are often used in behavioral studies and can easily be trained {e.g. Harpur & D'Arcy 1975; Clemens et al. 2014}.

1.3.3 Warsaw Wild Captured Pisula Stryjek (WWCPS) rat

The WWCPS strain originates from breeding pairs of wild Norway rat colonies in Warsaw and was established in 2006 in the animal facility of the Institute of Psychology of the Polish Academy of Sciences in Warsaw by Rafał Stryjek and Wojciech Pisula {Stryjek & Pisula 2008}. Wild rats are systematically captured and introduced to the colony to maintain the population free of domestication effects. These rats show a more aggressive behavior, they move faster and behave “jumpy” when confronted with an experimenter. The anatomical appearance at the juvenile state is more slender and lightweight. The animals display agouti colored fur on dorsum and extremities and grey on the abdomen.

1.4 Objectives and conceptual formulation

The main conceptual idea of this thesis was part of the NeuroNetzwerk 8, funded by the Center for Behavioral Brain Sciences (CBBS) Magdeburg. The overarching aim was to investigate neuronal circuitries underlying predator odor innate fear processing in rats. In this collaborative project methodological and expert experience were combined.

Dr. Jürgen Goldschmidt with his former PhD student M.Sc. Daniel Vincenz-Zörner at the Leibniz Institute for Neurobiology used SPECT analyses to visualize the cerebral blood flow during odor exposures {Vincenz et al. 2017}. The results indicated participation of brain areas via visualized activation and deactivation. Prof. Markus Fendt and his former PhD student Dr. Kerstin Wernecke at the Institute for Pharmacology and Toxicology used these brain areas as target regions for local $GABA_A$-receptor agonist muscimol microinjections to analyze changes in behavior during following

odor exposure test {Wernecke et al. 2015}. Dr. Wolfgang D´Hanis and myself decided for target regions for the tracer injections on basis of the results of these former analyses. The purpose was to further investigate the (inter)connections of these brain areas and their activation during odor exposure to support or confirm previous findings of our collaboration partners.

Specific objectives of this thesis were

- to analyze neuronal and behavioral responses of innate fear towards predator odor and the possibilities of multiple odor test paradigms by exposing rats consecutively to predator odor and water, in different order.
- to evaluate differences in behavioral and neuronal effects of a physiological predator odor (urine) and a single predator odor compound (TMT).
- to compare differences in behavior response toward predator odor between laboratory and semi-wild rats.

In **Experiment 1** iontophoretically tracer-injected SD rats were tested two times in an opaque square shaped box paradigm. The animals were exposed once to a water sample and once to a fox urine sample in randomized order. The objectives were to analyze whether fox urine has avoidance inducing capabilities in the square shaped box, if a double-trial paradigm is possible and whether the animals habituate or sensitize. Neuronal connections and their activation were investigated by subsequent c-Fos/retrograde tracer colocalization study.

In **Experiment 2** SD rats were exposed to either water samples, TMT samples or puma urine samples. Puma urine was selected as powerful feline predator odor in contrast to the canine derived TMT. The composition of the predator urines we purchased was unknown and the smell differed between batches, presumably depending on the predator's diet. Therefore the question was examined whether TMT, with a defined potency, has an avoidance-inducing effect similar to predator urines. This was investigated by means of a behavioral paradigm with subsequent c-Fos immunoactivity study and serum corticosterone analysis.

In **Experiment 3** the aim was to investigate behavioral and physiological differences between laboratory and wild rats when exposed to predator odor. Based on the results from the experiments in domesticated animals, we tested a wild rat strain (WWCPS) and laboratory strain (LH) with TMT as predator odor in a behavioral paradigm with subsequent serum corticosterone analysis.

2 Material and Methods

2.1 Animals

Sprague Dawley (SD) and Wistar rats were compared for their response to predator odors, since different avoidance responses to TMT in these two rat strains were published earlier {Rosen 2006}. The pilot study was performed by our collaborating Dr. Kerstin Wernecke at the Institute of Pharmacology and Toxicology. 11 male Wistar and 12 male SD rats were tested in the later used square shaped box odor paradigm. On basis of the pilot study results (see Results 3.1) the experiments in this thesis were performed by taking advantage of 79 Sprague Dawley (SD), 16 Lister Hooded (LH) and 16 Warsaw Wild Captive Pisula Stryjek (WWCPS) rats (111 rats in total, see attachments). All rats were 8 - 12 weeks old, male and naive.

2.1.1 Housing of Sprague Dawley rats

The SD rats were either bred and reared in the animal facility of the Institute for Pharmacology and Toxicology at the Medical Faculty of the Otto-von-Guericke University (origin: Taconic, Denmark) or ordered from Charles River Laboratories (Sulzfeld, Germany) and housed in the central animal facility in the Centre for Neuroscientific Innovation and Technology (CENIT) of the aforesaid faculty. All rats were kept in Macrolon Type IV cages in groups of 2-6 animals with *ad libitum* access to food and water. A 12 hour light/12 hour dark cycle was provided in a temperature and humidity controlled environment (22°C ± 2°C and 50–55 %)). All experiments were performed according to international ethical guidelines for the care and use of laboratory animals for experiments (2010/63/EU) and the German law on the protection of animals. All experiments were approved by local authorities (State Administration Office Saxony-Anhalt, file reference IANA/G/04-1178/12).

2.1.2 Housing of Warsaw Wild Captive Pisula Stryjek and Lister Hooded rats

The WWCPS rats were bred and reared in the animal facility of the Institute of Psychology of the Polish Academy of Sciences in Warsaw by Rafał Stryjek. The LH rats were obtained 2 weeks before the test (Charles River Laboratories, Sulzfeld, Germany) and as well kept in the aforesaid animal facility. All rats were housed in Eurostandard Type IV cages in groups of 4 animals with *ad libitum* access to food and water. A 12 hour light/12 hour dark cycle was provided in a temperature and humidity controlled environment (22°C ± 2°C and 50–55 %). All procedures described were approved by the local Ethics Commission on Animal Experimentation, Warsaw, Poland, and were in accordance with the Regulation of the Polish Minister of Science and Higher Education of January 15th, 2015 on the protection of animals used for scientific and educational purposes.

2.2 Laboratory equipment and materials

Described chemicals, proteins and antibodies were prepared and used as declared by the manufacturers. Solutions and buffers were freshly prepared in our laboratory. The manufacturers, recipes and compositions are described in detail in the attachments (5.3 & 5.4). Miscellaneous materials and equipment are listed with model name and manufacturer or vendor in the attachments (5.5) as well.

2.3 Methods

2.3.1 Stereotactic surgery and tracer injection

Prior to stereotactic surgery each animal was intraperitoneal (i.p.) injected with a combination of 6 - 8 mg/kg xylazine and 90 - 120 mg/kg ketamine. As soon as the animal was deeply anesthetized (loss of righting reflexes, lid closure reflex, flexor reflex and exophthalmos) the scalp was shaved with an electric shaver and the eyes were coated with artificial tear fluid. The animal was fixated into the stereotactic frame with surface anesthetic lidocaine gel coated ear bars. Next, the incisor bar was set to assure a flat skull position by adjusting bregma and lambda on the same level. The scalp was disinfected with surface disinfection spray and sagittal incised from the center of the frontal bone (approximately eye level) to lambdoid suture (approximately ear level). The skin incision was dilated with one or two sharp iris hooks and the pericranium was scraped off of the skull in this area (see Fig. 2). The skull was cleaned with aseptic 0.9 % NaCl and bleeding was stopped with 0.3 % H_2O_2 applied with cotton buds.

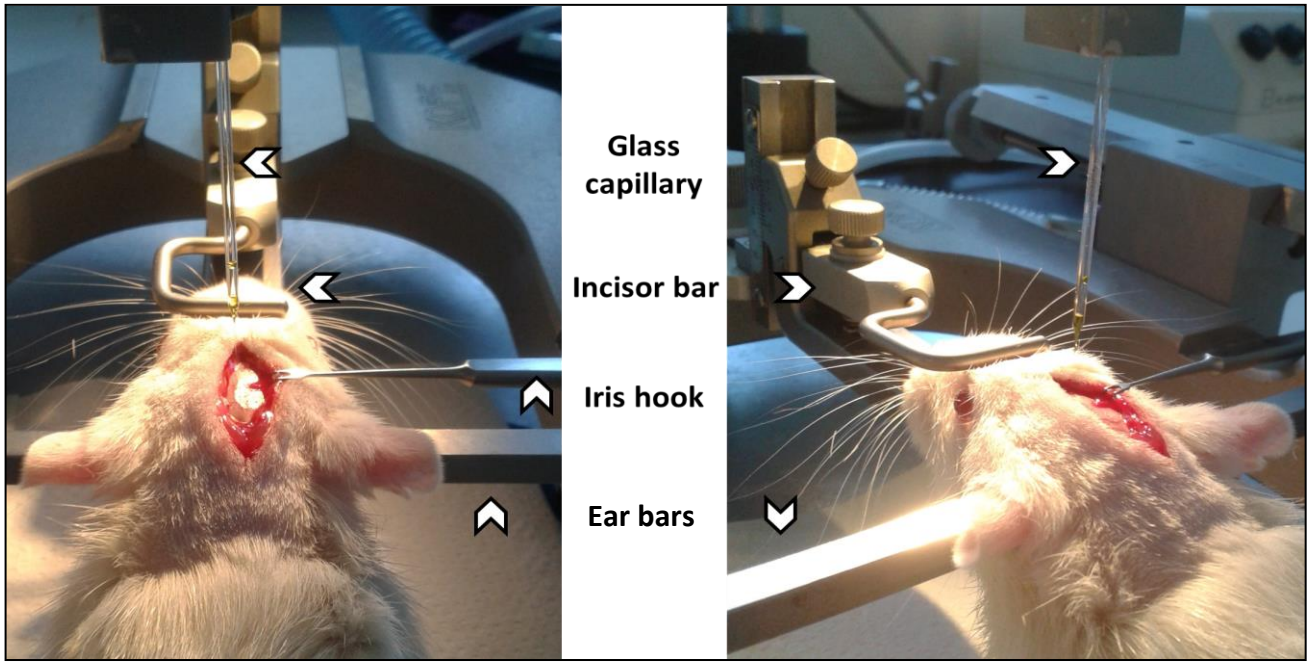

Fig. 2: Operation field for stereotactic surgery. The head of the rat is fixed with ear pins and incisor bar, the glass capillary is filled with FluoroGold™ and the incision is dilated with an iris hook.

Custom pulled borosilicate glass capillaries were used (see Attachment 5.5.1) with average outer tip diameter of about 40 µm to iontophoretically dispense the retrograde tracer FluoroGold™ {Schmued & Fallon 1986}. The capillary, filled with FluoroGold™, was fixated on the stereotactic arm and adjusted at bregma. At this anatomical point on the skull the coronal and sagittal suture intersect. The target coordinates for the tracer injections sites (regions of interest, ROI) were determined with help of the coordinates of bregma and the stereotaxic rat atlas {Paxinos & Watson 2007}. The skull was tagged at the specific point defined by the anterior-posterior and medial-lateral coordinates. A hole, wide enough for the capillary, was vertically drilled with a dental drill into the skull, the dura mater was cut in and the capillary was lowered to the dorsal-ventral coordinate.

For the iontophoretic application a current source (custom build) was used with 5 s on/off pulse and + 5 µA for 10 min {Pieribone & Aston-Jones 1988}. After the injection the glass capillary remained with no current at the injection site and was removed after 3 min. The wound was clamped and covered with lidocaine gel and 1 mL aseptic 0.9 % NaCl were subcutaneously (s.c.) injected. After 10 - 12 days of recovery all animals were able to perform the behavioral test sessions without any impairment. The surgical wounds were completely closed and healed. The time span of 10 - 12 days is particular needed to ensure a complete tracer migration and labeling of distal structures.

In general, the regions of interest were the bed nucleus of stria terminalis, the amygdala, and the habenula. On the basis of the previous findings in our CBBS NeuroNetwork, i.e. the BNST and PLCo are of crucial importance for predator odor-induced fear behaviors {Wernecke 2015, Wernecke & Fendt 2015} and that the habenula shows significant induced cerebral blood flow in the SPECT during predator odor exposure {Vincenz et al. 2017}, we decided for the following target injection sites. The detailed coordinates are given in Table 1.

General region	Detailed region	rostral-caudal	medial-lateral	dorsal-ventral
BNST	STMPM	- 0.8 mm	1.1 mm	- 6.0 mm
Amygdala	PLCo	- 4.4 mm	- 4.8 mm	- 9.3 mm
Habenula	LHbM	- 3.8 mm	- 0.5 mm	- 5.0 mm

Table 1: Target coordinates for tracer injection. The coordinates were chosen according to the rat brain atlas from Paxinos and Watson {Paxinos & Watson 2007}.

2.3.2 Behavioral test paradigm

2.3.2.1 Opaque square shaped box

The behavior in the experiments 1 and 2 was tested in a non-transparent, square-shaped polyvinyl chloride box (Fig. 3) with the dimensions 45 cm x 45 cm x 30 cm (width x depth x height). This arena was either placed inside a sound-attenuating chamber with a light-beam frame for movement detection and a camera in the roof or inside a closeable laboratory fume hood with a camera installed above. Each corner was equipped with a glass bowl (Ø 4 cm, 2.5 cm height), fixed with hook-and-loop tape. Illumination was approximately 10 lux.

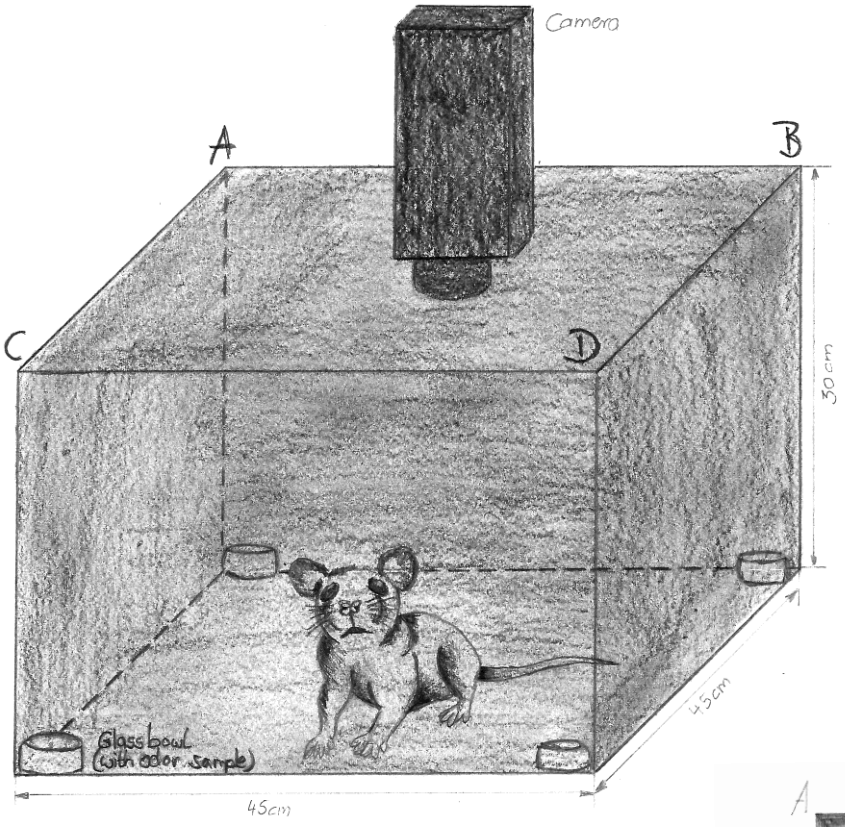

Fig. 3: Sketch of the opaque square shaped box

The corners were labeled with A, B, C and D in reading direction and the quadrants will be referred to as quadrant A, B, C and D (Fig. 4).

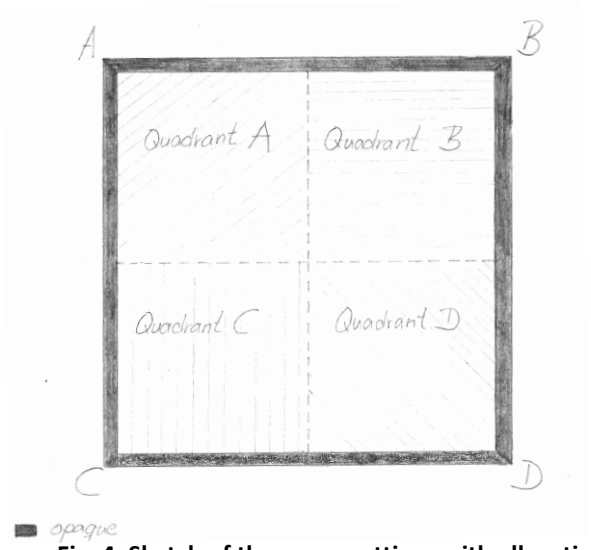

Fig. 4: Sketch of the arena settings with allocations

2.3.2.2 Square shaped box with one transparent wall

The behavioral tests of experiment 3 in Warsaw were performed in a square-shaped acrylic glass box with the dimensions 47 cm x 47 cm x 50 cm (width x depth x height). Due to the aggressiveness of the WWCPS the box was built with three opaque and one transparent side for observation purposes. Each corner was equipped with a glass bowl (Ø 4 cm, 2.5 cm height), fixed with play dough (Fig. 5). Illumination was approximately 60 lux. The arena was placed inside a closeable fume hood (custom build). For the behavioral tests with the WWCPS rats a 30 cm acrylic glass buildup was added.

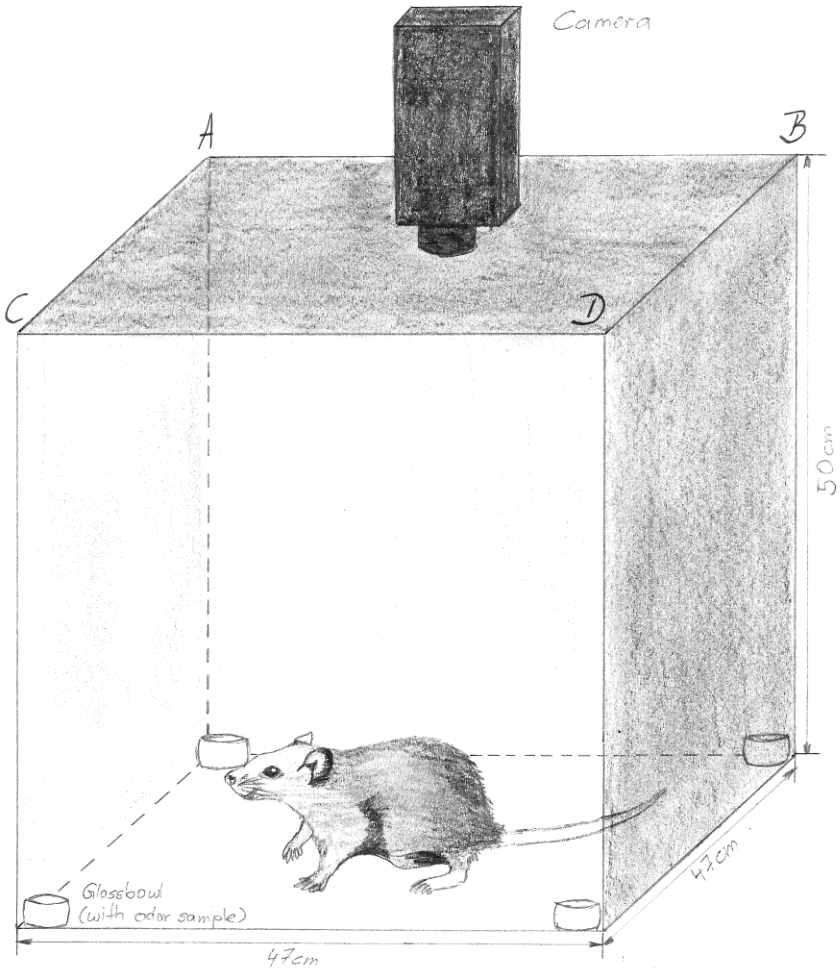

Fig. 5: Sketch of the square shaped box with transparent front

The corners were labeled with A, B, C and D in reading direction. The corners A and B were bilateral bordered with opaque walls, the corners C and D were unilateral bordered with a transparent wall. In the further analysis the quadrants A and B will be referred to as “area AB” and the quadrants C and D as “area CD” (Fig. 6).

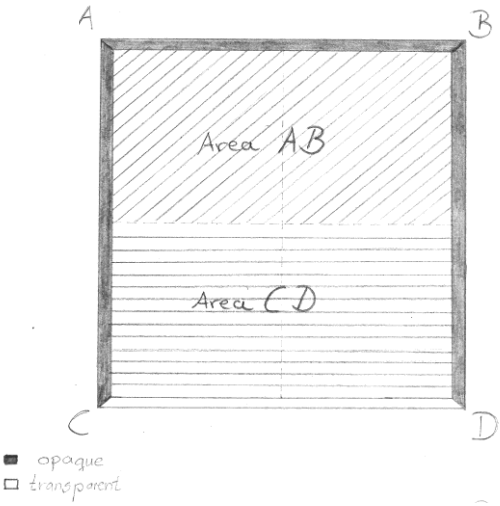

Fig. 6: Sketch of the arena setting with allocations

2.3.2.3 Odor test

The experimenter had to use the same drugstore products (i.e. shower gel, shampoo, deodorant) through the whole testing phase and was not allowed to use perfume at all. It was also important to keep the animals prior to testing in a separate room from the animals which returned from the test to avoid exposure to odor left-overs in the fur and the emission of fear related pheromones or vocalization. Therefore separate rooms were used. Each rat experienced three habituation sessions of 10 min without odor exposure, one session per day during the light phase on three consecutive days. On the fourth day the odor test took place during the light phase. Each animal was exposed to either control odor (1 mL or 5 µL tap water) or predator odor (1 mL urine (puma / fox) or 5 µL TMT) for 10 min. The test odor was presented in one of the four glass bowls in a randomized corner. After each habituation and test session the arena was thoroughly cleaned with soapsuds, rinsed with tap water and dried. Due to the aggressiveness of the WWCPS it was necessary to use special bite proof cloves and a small cardboard transportation box in experiment 3 to carry the animals in and out of the testing arena {Stryjek 2008 & 2010}. Immediate after the odor test the animals were kept in fresh home cages and separate rooms assorted by particular test odor until perfusion or decapitation. In the experiment 1 each rat was exposed to both test odors on two consecutive days and this procedure is illustrated in Fig. 7.

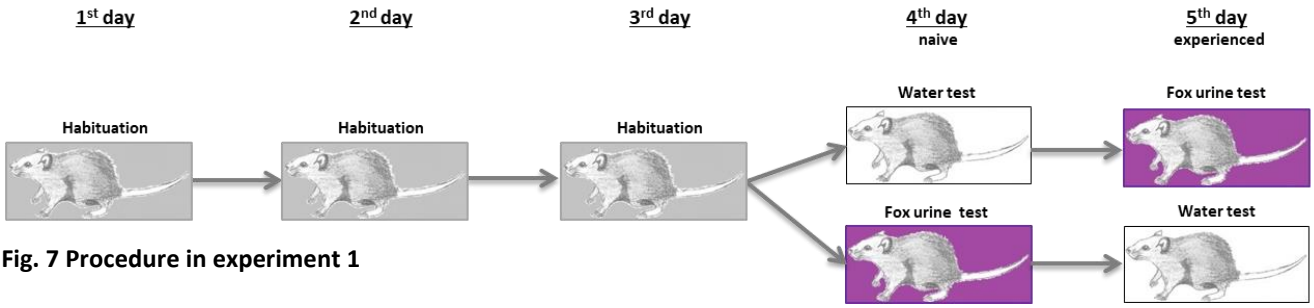

Fig. 7 Procedure in experiment 1

In the experiments 2 and 3 each rat performed only one test. The experimental procedure is illustrated in Fig. 8.

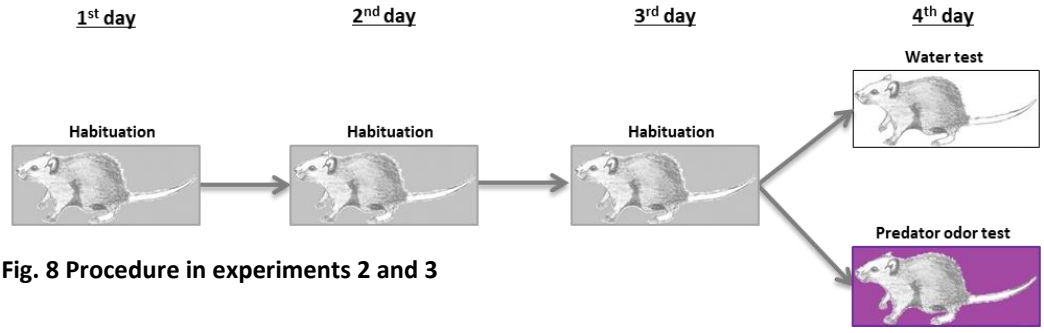

Fig. 8 Procedure in experiments 2 and 3

2.3.2.4 Behavioral analysis

The behavior of the rats was video recorded and off-line analyzed with the video tracking software EthoVision XT 10 (Noldus). Nose- and center-point detection was used. In addition, grooming and freezing behavior were manually scored. Freezing was defined as a minimum of 3 seconds without moving. In the experiment 1 the movement of the animals was recorded with infrared light beam detectors (TSE).

2.3.3 Sampling

2.3.3.1 Perfusion

Exactly 90 min after the onset of the odor tests of experiments 1 and 2 the animals were narcotized with an i.p. injection of 1-2 mL 6 % pentobarbital-sodium and the animals were transcardially perfused via a pericyclic pump. To wash out the blood 150 - 200 mL 0.9 % NaCl were used and afterwards about 400 mL 4 % paraformaldehyde solution with picric acid (“Immunfix”) to fix the three dimensional structure of the proteins in the tissue. The brains were extracted and kept overnight in Immunfix on a circulatory shaker. In experiment 2 blood samples were additionally taken before perfusion with a cannula and 1 mL Eppendorf pipette from the heart. The samples were filled in 1.5 mL Eppendorf safe lock tubes and centrifuged for 10 min with 14.000 U/min (MiniSpinPlus). The upper phase (serum) was collected and the probes were stored at – 80°C for corticosterone level analysis via Radioimmunoassay (RIA).

2.3.3.2 Decapitation

Exactly 90 min after the onset of odor tests of experiment 3 the rats were anesthetized with isoflurane and decapitated with a guillotine. Blood samples were taken immediately from the trunk with a 1 mL Eppendorf pipette, filled in 1.5 mL Eppendorf safe lock tubes and centrifuged for 10 min with 14.000 U/min (MiniSpinPlus). The upper phase (serum) was collected and stored at – 80°C for enzyme-linked immunoabsorbent assay (ELISA) analysis. The brains were removed expeditiously and bisected at the medial longitudinal fissure. The right hemisphere was directly fixed in paraformaldehyde for about 2 weeks; the left hemisphere was further dissected as described from Janitzky and colleagues {Janitzky et al. 2009} and cerebellum, amygdala, BNST, PV, Hippocampus and olfactory bulbus were shock frozen and stored at – 80°C. These brain samples were preserved for upcoming analyses and are currently worked on.

2.3.4 Immunohistochemistry

2.3.4.1 Brain sample preparation

Perfused brains were, depending on fixation status, left in Immunfix on a circulatory shaker for 12-24 hours. Native removed brains were fixated in 4 % paraformaldehyde solution for about 2 weeks. After fixation the brains were incubated in 20 % sucrose in phosphate buffer (PB) until subsidence. The subsided brains were frozen with 2-methylbutane and stored at -80 °C. In the following 40 µm thick frontal cryosections were prepared in four series. The first series of brain sections was placed on object slides and Nissl-stained. The fourth series was stained for the immediate early gene protein c-Fos. The second and third series were stored at –20°C in cryoprotective solution.

2.3.4.2 Nissl-staining

The object slides with the brain sections of the first series were incubated 1 - 4 min in the buffered 0.5 % cresyl violet staining solution. Next, the object slides were rinsed in freshly prepared acetate buffer. Afterwards the staining was differentiated in 100 % EtOH and rinsed in another cuvette of 100 % EtOH. Next, the brain sections were two times dehydrated in xylol and mounted on xylol basis (Entellan®). The alkaline dye cresyl violet recognizes the nucleic acids and the Nissl bodies of the rough endoplasmic reticulum. These appear dark violet while cytoplasm appears light violet.

2.3.4.3 C-Fos immunostaining

2.3.4.3.1 Free floating c-Fos immunostaining

The brain sections of the fourth series were stained for c-Fos utilizing the free floating method. First the endogenous peroxidase was blocked with POX-Block solution for 10 min. After washing three times for 10 min with PB+T, blocking solution was added and incubated for 30 min. After the removal of the blocking solution the rabbit c-Fos antibody was applied without washing steps. A 1 : 10.000 dilution in PB+T with 1 % NGS was used and incubated for 2 days at 4°C on a horizontal shaker. Next, after three 10 minute washing steps the secondary antibodies, biotinylated anti rabbit, were applied in a 1 : 200 dilution in PB+T and incubated for 2 h at room temperature (20°C) and on a horizontal shaker. Three washing steps followed and next the AB-complex was applied and incubated for 1 h at room temperature on a horizontal shaker. After another three washing steps 5 % DAB solution was used as substrate for the color reaction or TSA-Rhodamine {Hopman et al. 1998} for fluorescence staining. After activation with 0.3 % H_2O_2 the color reaction was stopped after microscopic evaluation and the fluorescence reaction was stopped after 15 min.

2.3.4.3.2 Substrate comparison

For the later analysis of c-Fos expression and scanning of c-Fos and tracer co-localization it was necessary to compare the staining with the substrates Diaminobenzidine (DAB) and Tyramide Signal Amplification (TSA)-Rhodamine.

In this methodological test 2 rats were i.p. injected each either with 2 mL 0.9 % NaCl solution or 2 M NaCl solution {Berghorn et al. 1994}. The brains were taken as described under 2.3.3.1 and preceded as described in 2.3.4.1 with 2 series. Both series were stained as described in 2.3.4.3.1, one with DAB as substrate and the other with TSA-Rhodamine.

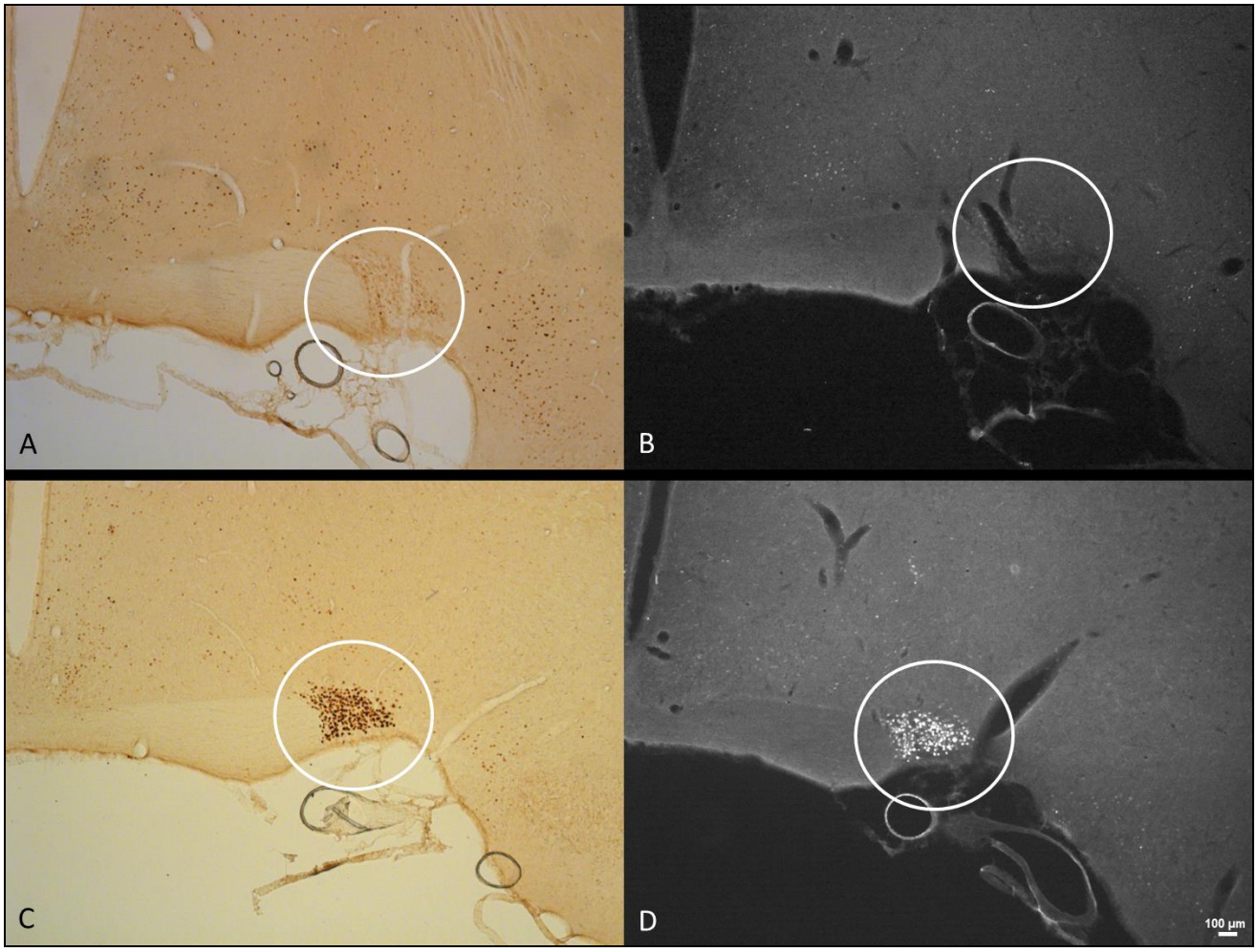

Fig. 9 C-Fos immunopositive cells in the supraoptic nucleus (SO) stained with DAB-substrate (A & C) or TSA-Rhodamine substrate (B & D). No c-Fos immunopositive cells in the SO after i.p. injection of 0.9 % NaCl (white circle in A and B). Numerous c-Fos immunopositive cells in the SO are found after i.p. injection of 2 M NaCl solution (white circle in C & D).

Both staining techniques with DAB-substrate and TSA-Rhodamine substrate showed optically comparably results in the light microscope (Fig. 9A & C) and fluorescence microscope (Fig. 9B & D). Therefore the results of these two staining techniques are comparable used in the following.

2.3.4.4 Regions of interest (ROI) and cell counting

On basis of the ROI for the tracer injections, discussions in the CBBS NeuroNetwork 8 and countability the brain regions in which the c-Fos immunopositive cells were counted were selected. For counting the AxioVision Imaging software 4.8 with AxioCam (Carl Zeiss) was used. The region to count was selected in the Nissl-series slices, photographed and framed in the image to transfer the contours on the image of appropriate slices of the DAB- or TSA-Rhodamine-series. The c-Fos immunopositive cells were manually marked and counted. In each animal the same number of slices in the same range of figures (as described in the rat brain atlas from Paxinos & Watson, 2007) for each region was counted on rostro-caudal level. The region "anteroventral part of the medial amygdaloid nucleus" (MeAv) for example extends from figure 47 to 55. C-Fos immunopositive cells were counted in 5 slices between these figures. For the statistical analysis the mean number of cells in the right and left hemisphere per animal was taken.

2.3.4.5 Tracer analysis

For the tracer analysis the injection sites were first controlled for tracer halos (Fig. 16). Next, the whole series was manually scanned for cells which had taken up FluoroGold™ and checked for co-localization with c-Fos by changing the fluorescence channel between fluorescence and ultraviolet light (FluoroGold™ excitation emission max: 408 nm, excitation max: 323 nm). Whilst c-Fos will be exclusively visible in the nucleus, this one will be devoid of tracer. FluoroGold™ will be visible in the soma, dendrites and axon.

2.3.5 Corticosterone analysis

Serum was taken as described under 2.3.3. Corticosterone levels were quantified via enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). All samples of the experiment were tested as duplicates within the same assay.

2.3.5.1 Radioimmunoassay (RIA)

The RIA was performed in the laboratory of Prof. Markus Fendt, Institute for Pharmacology and Toxicology at the Otto-von-Guericke-University in Magdeburg, Germany (see 5.1) as described by Pryce et al. {Pryce et al. 2001}.

2.3.5.2 Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed using the corticosterone ELISA kit ab108821 from abcam. First, the microplate strips and reagents had to equilibrate to room temperature (20°C). 25 µL corticosterone

standard or sample were pipetted per well and 25 µL 1 x biotinylated corticosterone were added immediately. The wells were covered with sealing tape and incubated for 2 h at room temperature. Next, it was washed 5 times with 200 µL 1x wash buffer, added 50 µL 1x SP conjugate to each well and incubated for 30 min. Again it was washed 5 times with 200 µL 1x wash buffer, added 50 µL chromogen substrate and incubated for about 12 min until a blue color developed. Finally, 50 µL stop solution were added and the color changed to yellow. The absorbance was read immediately at a wavelength of 450 nm.

2.3.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 6.03. After Shapiro-Wilk normality test normally distributed data were analyzed either with one-sample t-test, unpaired t-test or ANOVA followed by Holm-Sidak´s multi comparison test. Not normally distributed parameters were analyzed using either Mann-Whitney test or Kruskal-Wallis test with Dunn´s correction. Identified outliers (ROUT test, Q = 1) were excluded from the particular analysis and quoted in the results.

The duration of the behavior responses was measured in sec and in parts converted to % with the duration of one individual behavior test session taken as 100 %. The probability of a rat staying in one of the four quadrants was 25 % and defined as chance level (100 % / 4 quadrants = 25 %). Significant differences to chance level were considered as avoidance (significantly less than 25 %) or preference (significantly more than 25 %).

3 Results

3.1 Pilot study: Behavioral comparison between Sprague Dawley and Wistar

Kerstin Wernecke found that SD rats express more robust avoidance behavior to predator odor than Wistar rats (Fig. 10).

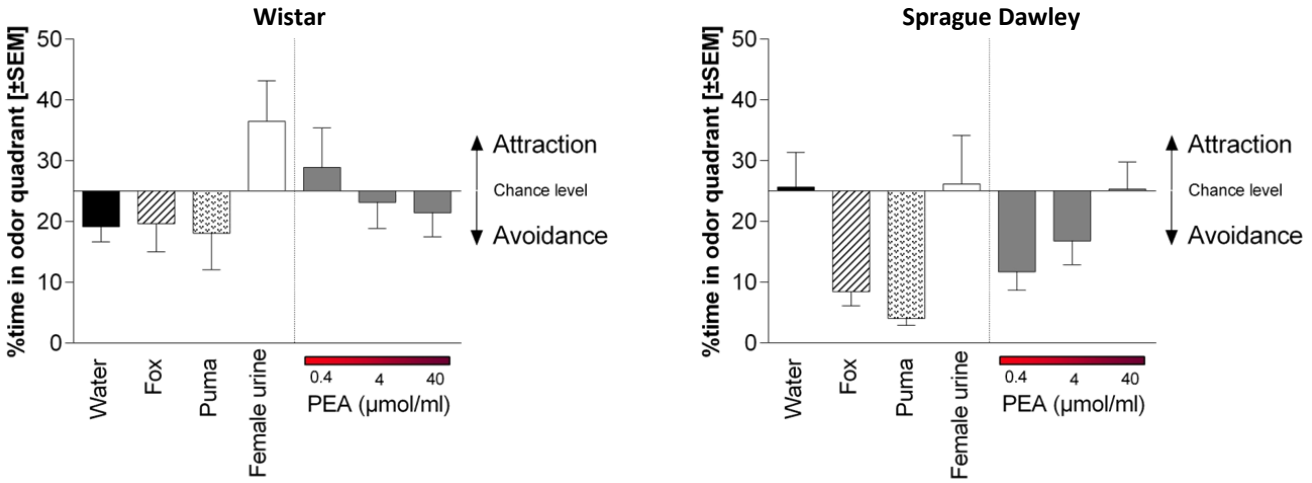

Fig. 10: Predator odor- induced avoidance behavior in two outbred rat strains. Illustrated is the mean percentage of time (± SEM) Wistar (n = 11) and Sprague Dawley (n = 12) rats occupied the odor quadrant during 10-min exposure test. Unpublished data, taken from a corporate presentation at the SFB779 PhD student retreat 2014 in Wernigerode, Germany {Wernecke & Storsberg 2014}.

In contrast to SD rats, Wistar rats avoided the neutral water control sample in the same magnitude as the predator odor samples. Although, Wistar rats showed attraction to the female urine samples whilst SD did not, our paradigm focused on avoidance behavior. However, SD showed robust avoidance towards predator odor samples such as fox and puma urine and also to phenylethylamine (PEA), a component of predator urine in general {Ferrero et al. 2011}. So it was decided to take advantage of SD rats in the experiments 1 and 2.

3.2 Experiment 1: Effects of fox urine exposure on naive and experienced laboratory rats

In these behavioral experiments iontophoretically tracer-injected Sprague Dawley® rats (SD) were tested in the opaque square shaped box paradigm as described in 2.3.2.1. Each animal experienced two trials with opposed samples to analyze whether innate avoidance is shown and affected (see Fig. 7). The position of the animals was detected automatically as well as contacts to the odor glass and covered distance, additionally grooming was manually scored. Following a c-Fos/tracer colocalization study was performed to visualize activated neuronal connections.

3.2.1 Avoidance behavior

The animals which experienced water in the first trial were confronted with a fox urine sample in the second trial and vice versa. The time an animal stayed in the odor quadrant was measured. The result of the first trial is illustrated in Fig. 11 as the mean residence time in percent rats spent in the odor quadrants with the probability to stay in one of the four quadrants (25 %) given as chance level. Here naive rats were confronted with either water or fox urine samples for the first time. The same animals were tested in a second trial with the opposite odor, respectively. The combined results are shown in Fig. 12.

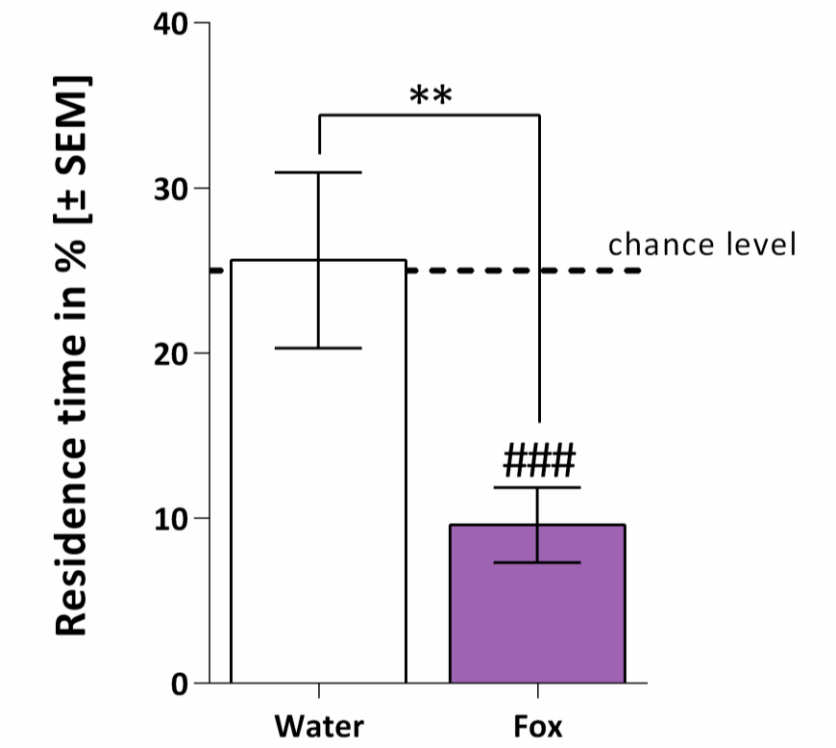

Fig. 11: Mean residence time in % of naive rats in odor quadrant (± SEM) containing water (n = 12) or fox urine sample (n = 14) during trial 1. The rats significantly avoided the odor quadrant during fox urine sample presentation compared to water control experiment and to chance level (25 %). ** $p < 0.001$ compared to water control experiment (Mann-Whitney test); ### $p < 0.0001$ compared to chance level (25 %) (one sample t-test).

The residence time of naive SD rats in the odor quadrant containing the water control sample did not differ significantly from chance level (one sample t-test, p = 0.91), whereas residence time in the quadrant containing the fox urine sample differed significantly (one sample t-test, p < 0.0001). Mann-Whitney test revealed a significant effect of the odor samples on residence time in the odor quadrants (p = 0.002; Fig. 11). However, the second trial with experienced rats revealed a less distinct effect (see Fig. 12).

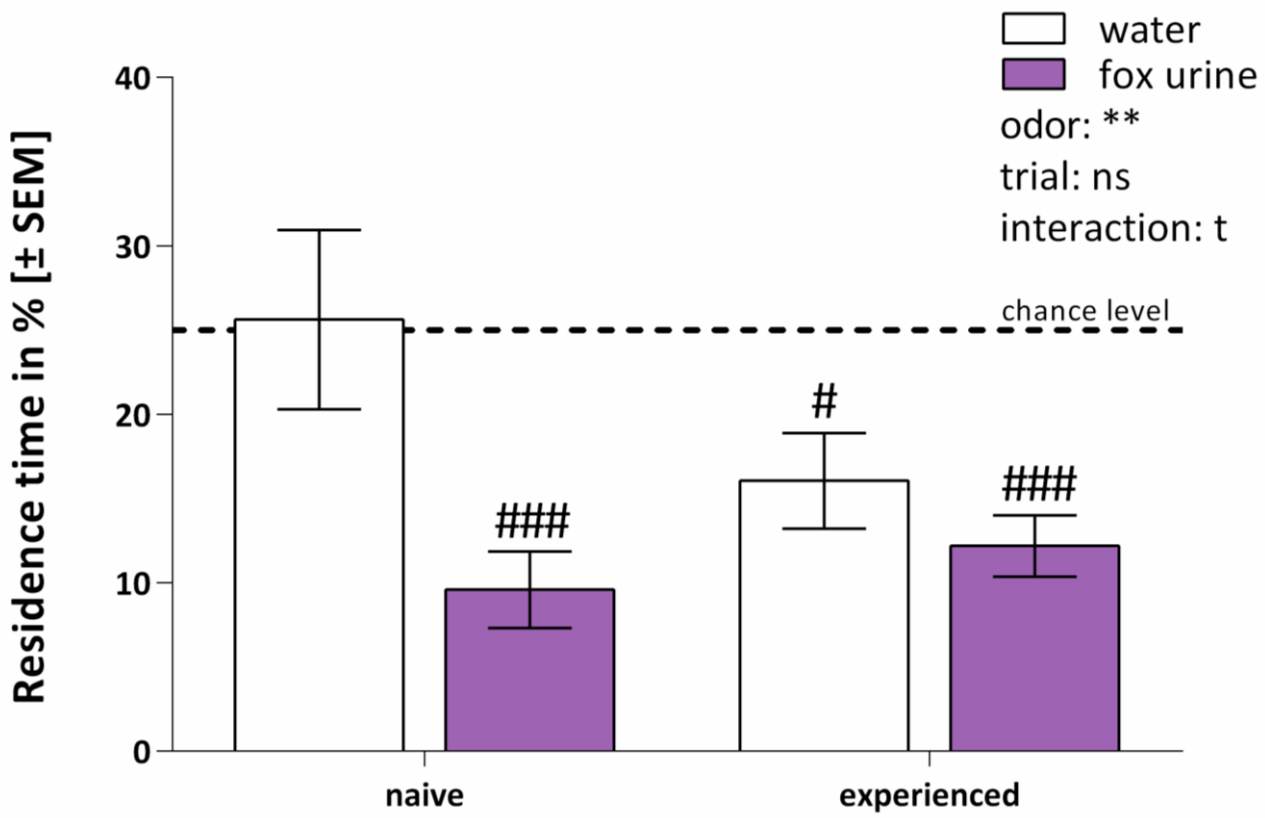

Fig. 12: Mean residence time in % in odor quadrant (± SEM) containing water or fox urine sample during first (naive) and second trial (experienced). The rats significantly avoided the quadrant containing fox urine odor in the first but not in the second trial. ** p < 0.001 compared to water control experiment (two-way ANOVA); ### p < 0.0001; # p < 0.01 compared to chance level (25 %) (one sample t-test, water first n = 12, fox urine first n = 14).

The two-way ANOVA (here and in the following pictured in the graphics legend) showed a significant odor effect ($F_{[1, 48]}$ = 9.4; p = 0.004), a tendency of interaction but no trial effect (interaction: $F_{[1, 48]}$ = 3.5; p = 0.068; trial: $F_{[1, 48]}$ = 1.1; p = 0.29). Whilst fox urine was only avoided during the first trial, as described above, during the second trial the experienced animals also avoided the water samples. Compared to chance level both odor samples were significantly avoided (one sample t-test, p < 0.0001, p < 0.001) and there was no longer a significant different avoidance between the odors. The animals which were confronted with water sample in the first trial avoided the fox urine sample in the second trial comparable to the animals which were confronted with the fox urine sample in the

first trial. The animals which encountered the fox urine sample in the first trial now avoided water in the second trial, too. Although the residence time in the quadrant with the water sample differed significantly from chance level in the second trial ($p = 0.008$), there was no significant difference of residence time in the first and second trial (Mann-Whitney test, $p = 0.12$).

Next, the number of glass contacts were measured with the nose point detection and a circular area (Ø = 6 cm) defined around the glasses. The presence of the nose point inside this area was counted as contact with the odor sample. The result of this analysis is illustrated as the mean number of contacts to the odor glass in Fig. 13.

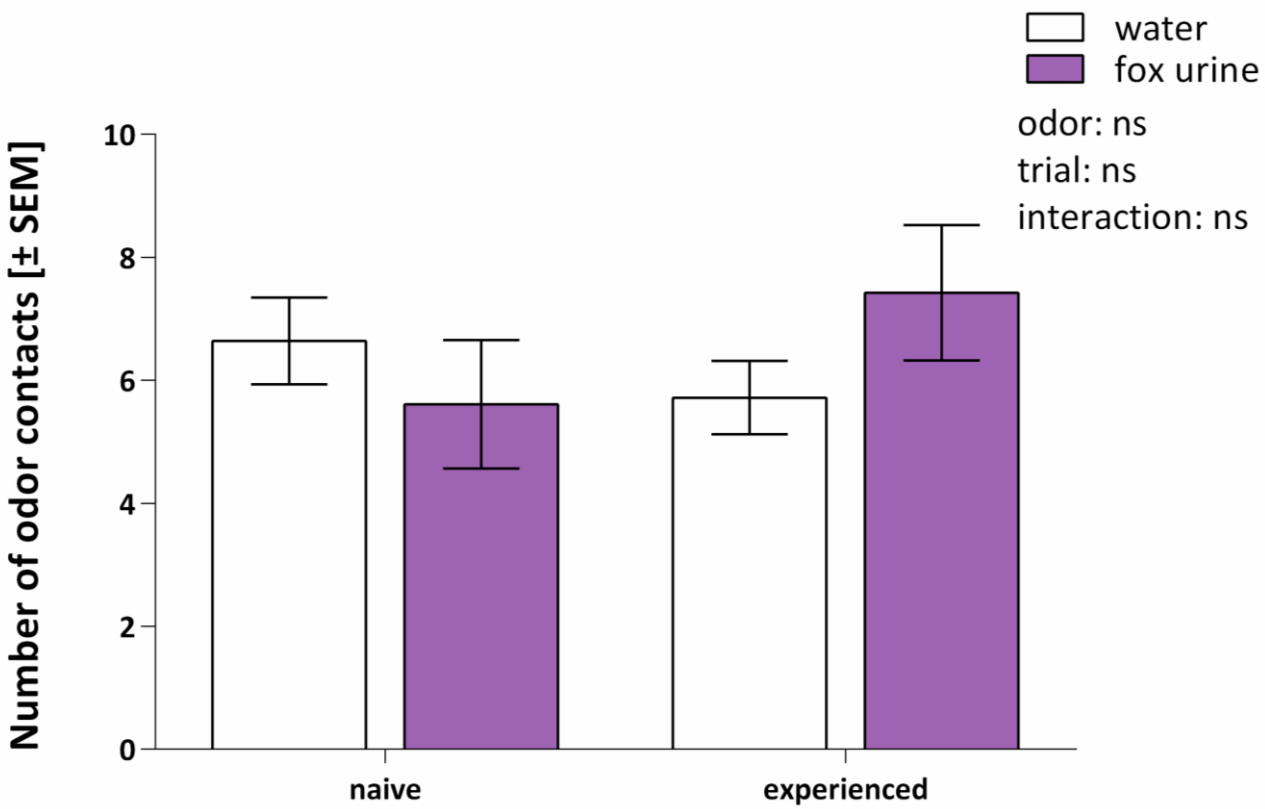

Fig. 13: Mean number of contacts to the odor source (± SEM) during first (naive) and second trial (experienced) with either water or fox urine sample. No significant difference was detectable (two-way ANOVA; water first n = 11, fox first n = 9).

Neither odor samples nor number of trials had any significant effects on the frequency of nose contacts to the odor source (two-way ANOVA, odor: $F_{[1, 45]} = 0.14$, $p = 0.7$; trial: $F_{[1, 45]} = 0.25$, $p = 0.62$) and there was no interaction ($F_{[1, 45]} = 2.4$; $p = 0.13$).

3.2.2 Locomotor activity

To evaluate potential odor effects on motor activity of the animals, moved distance in cm was measured and analyzed. Fig. 14 illustrates the average moved distance during the two trials with the different odors.

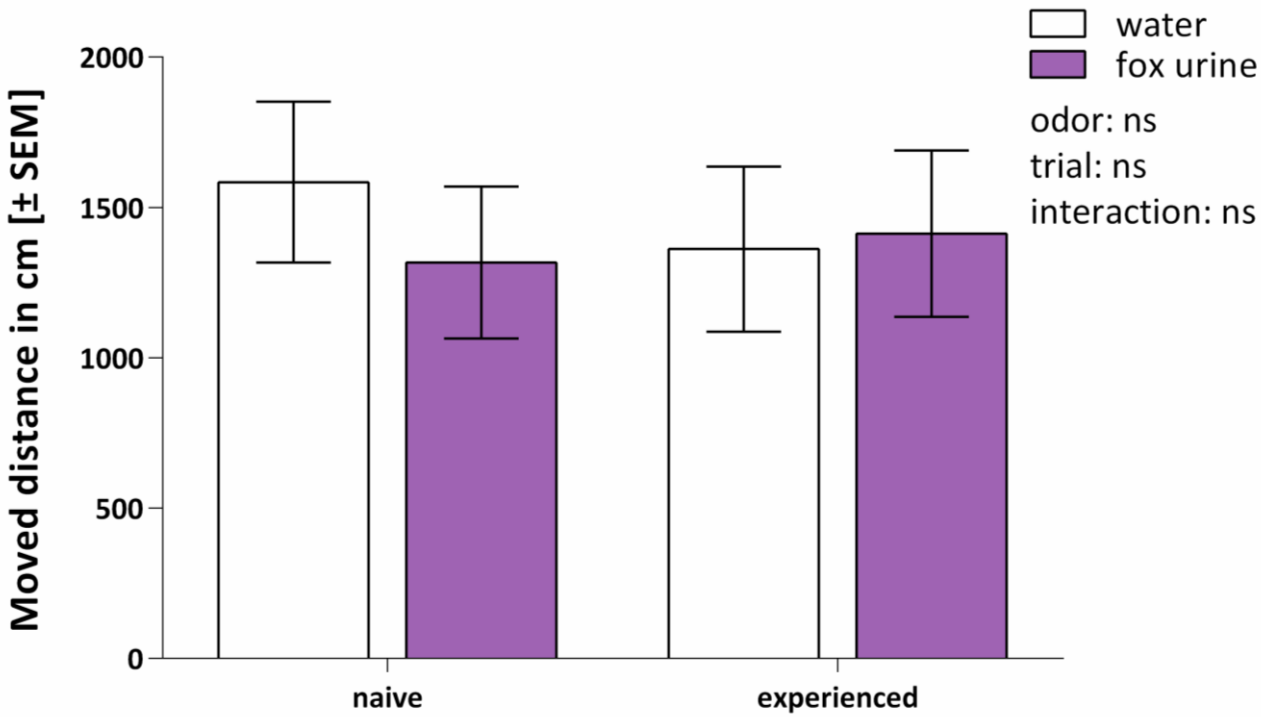

Fig. 14: Mean distance in cm rats moved (± SEM) during first (naive) and second trial (experienced) with either water or fox urine sample. No significant difference was detectable (two-way ANOVA; water first n = 11, fox first n = 9).

The results show that the different samples did not affect the motor activity of the animals (two-way ANOVA, interaction: $F_{[1, 36]} = 0.35$, $p = 0.56$; trial: $F_{[1, 36]} = 0.56$, $p = 0.81$; odor: $F_{[1, 36]} = 0.16$, $p = 0.69$).

In addition, grooming behavior was analyzed as another indicator of fear and stress. The mean time in sec rats showed grooming behavior is illustrated in Fig. 15.

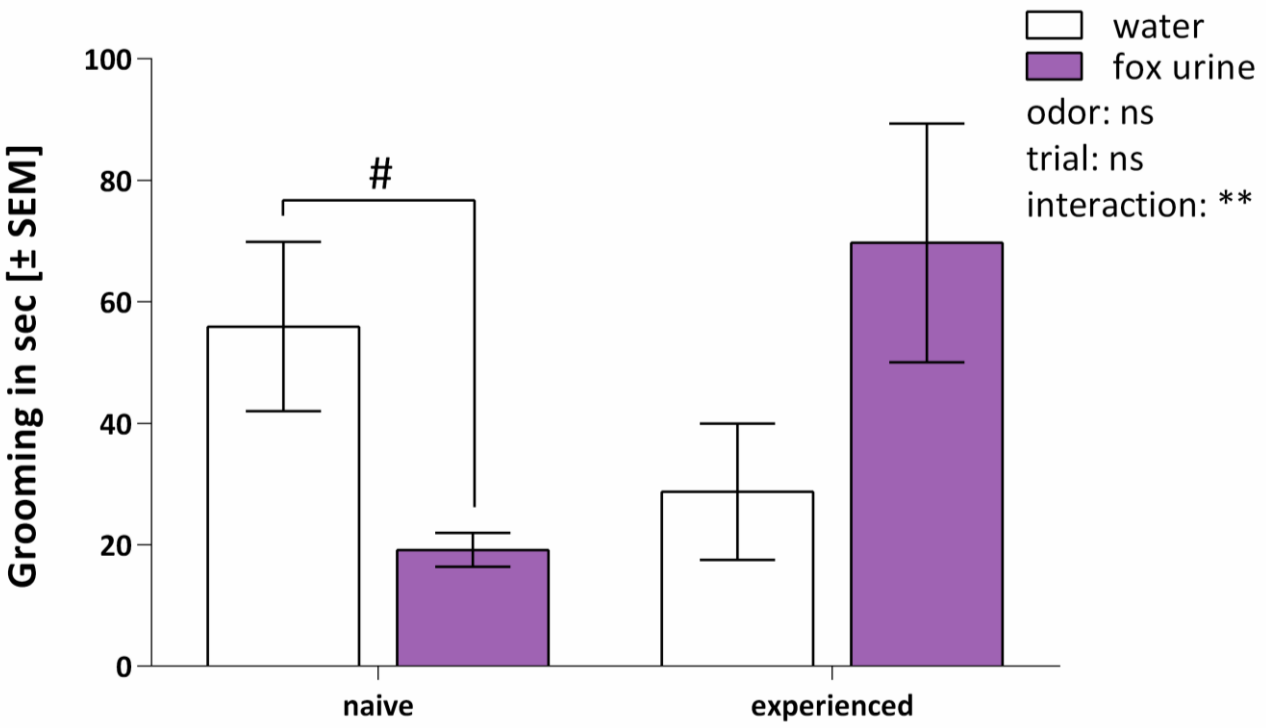

Fig. 15: Mean time in sec rats spent grooming (± SEM) during first (naive) and second trial (experienced). Naive: During water sample exposure significant more grooming behavior was detected. # $p < 0.05$ (unpaired t-test; 2 outliers); **Experienced**: Grooming behavior did not differ significantly. ** $p < 0.01$, compared with the appropriate control experiment (two-way ANOVA with Holm-Sidak´s multiple comparison test, water first n = 9, 2 outliers, fox first n = 9).

During the first trial naive SD rats showed significantly more grooming behavior during exposure to water sample compared to fox urine sample exposure (unpaired t-test, $p = 0.02$), this difference was no longer evident during the second trial in experienced rats. This is shown by a two-way ANOVA which revealed an interaction ($F_{[1, 32]} = 8.50$; $p = 0.007$), but no main odor or trial effect (odor: $F_{[1, 32]} = 0.76$; $p = 0.39$; trial: $F_{[1, 32]} = 0.025$; $p = 0.88$).

3.2.3 Tracer and colocalization

The injections sites were clearly identifiable by a tracer halo labelled with a central gliotic scar. Fig. 16 shows an exemplary picture of a gliotic scar (A) with tracer halo (B) in the APir.

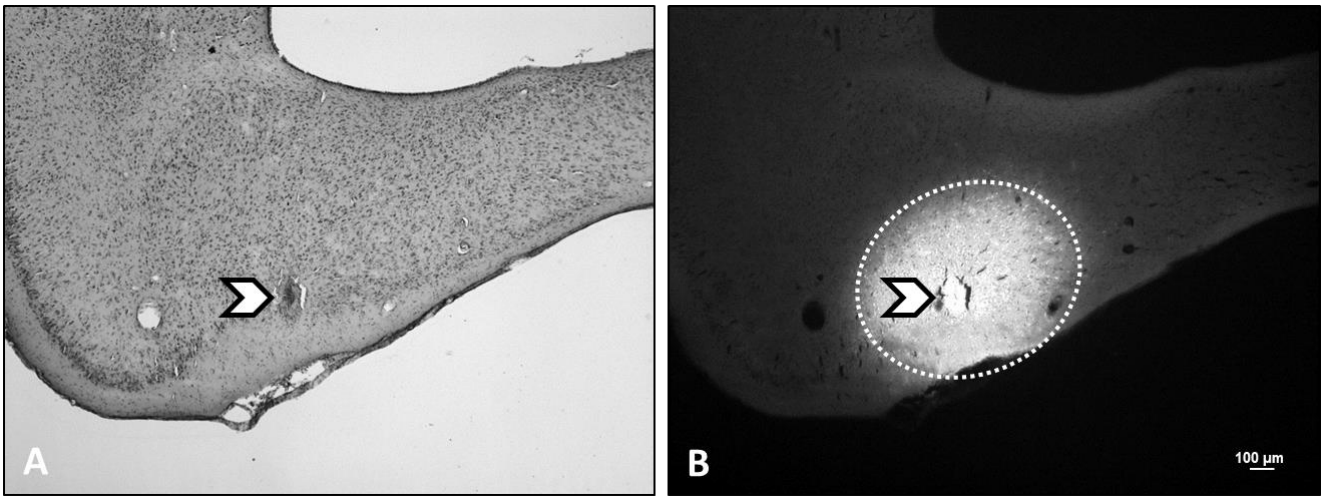

Fig. 16: Exemplary gliotic scar (white arrow) after FluoroGold™ injection into the APir in transmitted light (A) and ultraviolet light (B). The area of the halo is indicated by the white dotted line.

Five animals were injected with tracer at the target coordinates of the BNST. In two animals, the tracer injection area was consistent with the target region. No connectivity was detectable and no colocalization with c-Fos immunopositive cells was observed.

20 animals were injected with amygdalar target coordinates. In five of these animals, the tracer injection area was consistent with the target region amygdalopiriform transition area (APir) and in five animals with the basolateral amygdaloid nucleus, posterior part (BLP). In both cases retrograde connections to the MeAv were visible and are shown in Fig. 17.

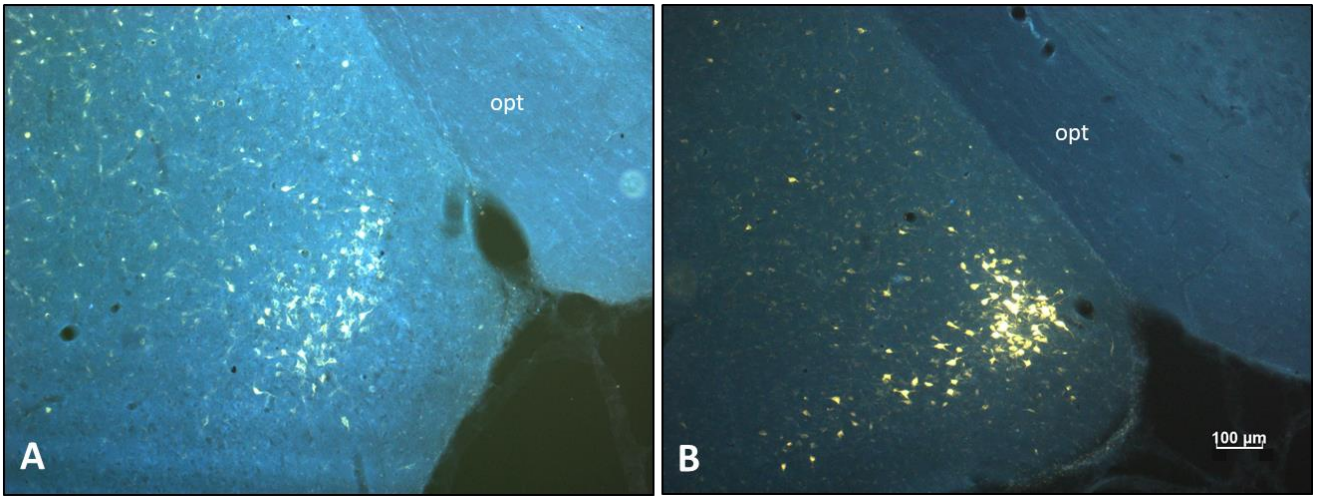

Fig. 17: FluoroGold™ positive neurons in the right MeAv after tracer injection into the BLP (A) and APir (B). Opt = optic tract

In these animals colocalizations of tracer and c-Fos immunoactivity were found in neurons of the MeAv after exposure to fox urine (Fig. 18) and in the paraventricular thalamic nucleus (PV) after exposure to both samples (Fig. 19 & Fig. 20).

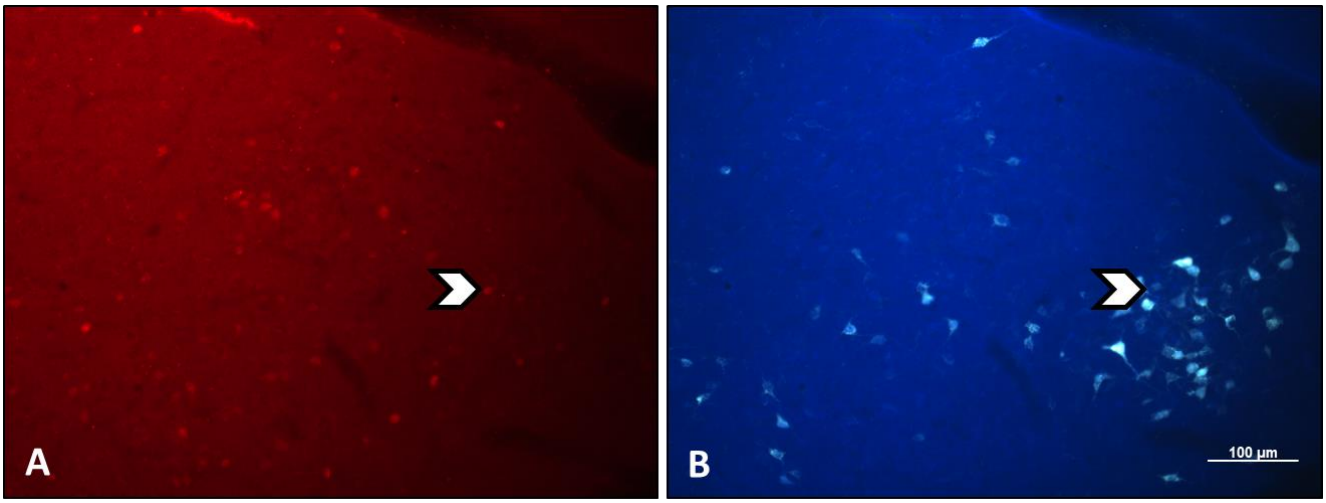

Fig. 18: Colocalization of c-Fos (A) and FluoroGold™ (B) in neurons of the MeAv after tracer injection into the APir and exposure to fox urine. The white arrow highlights a cell which is positive for tracer and c-Fos.

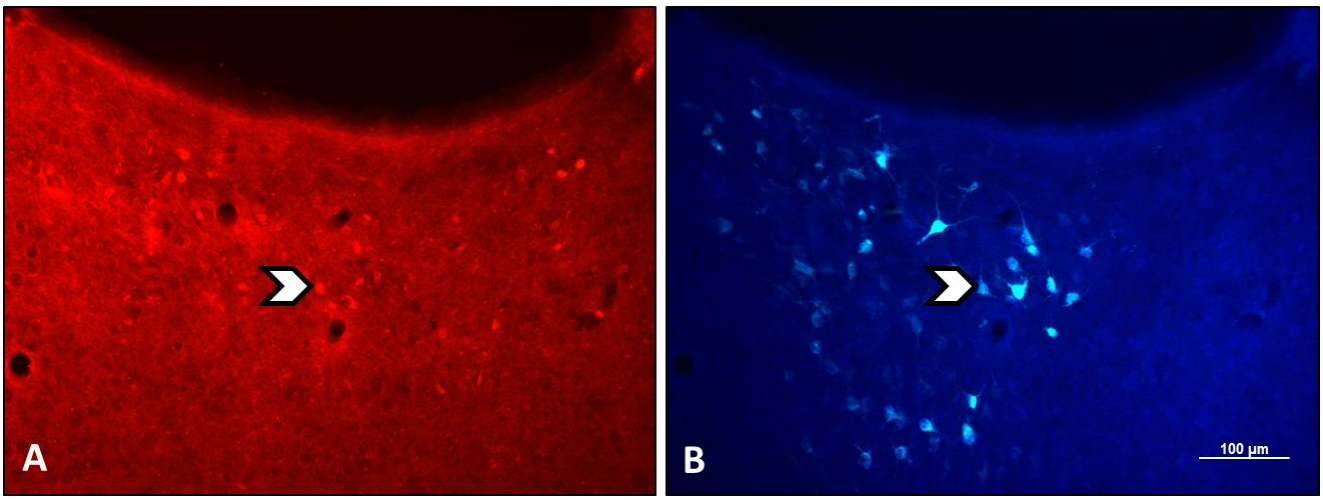

Fig. 19: Colocalization of c-Fos (A) and FluoroGold™ (B) in the PV after tracer injection in the BLP and exposure to fox urine. The white arrow highlights a cell which is positive for tracer and c-Fos.

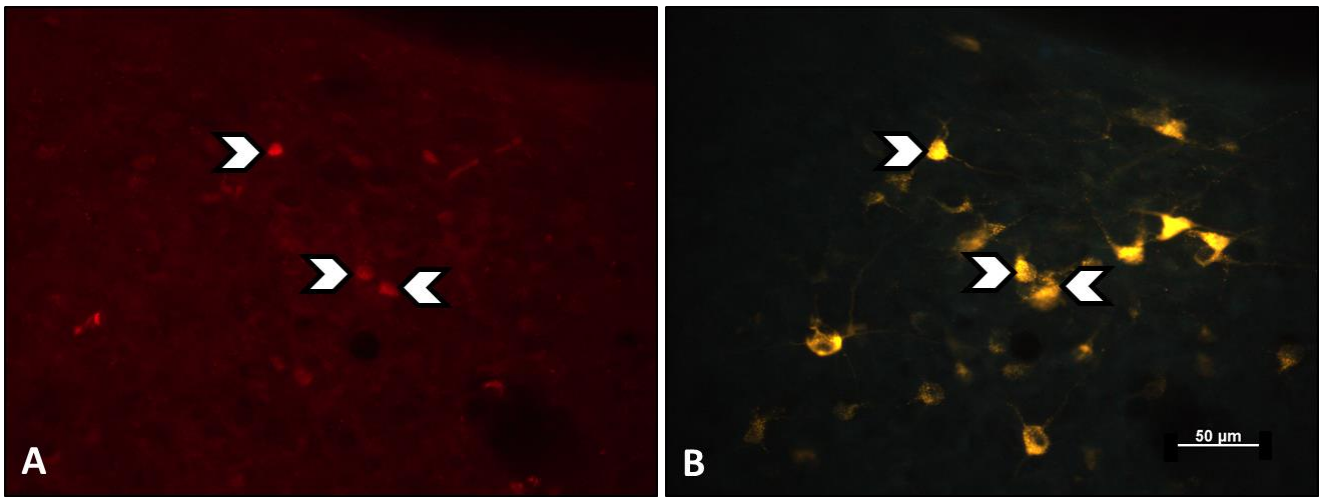

Fig. 20: Colocalization of c-Fos (A) and FluoroGold™ (B) in the PV after tracer injection in the BLP and exposure to water urine. The white arrows highlight cells which are positive for tracer and c-Fos.

Six animals were injected with the target coordinates of the lateral habenula (LHb). In one animal the tracer injection area was consistent with the target area and connections were visible in the triangular septal nucleus (TS) and septofimbrial nucleus (SFi) (Fig. 21).

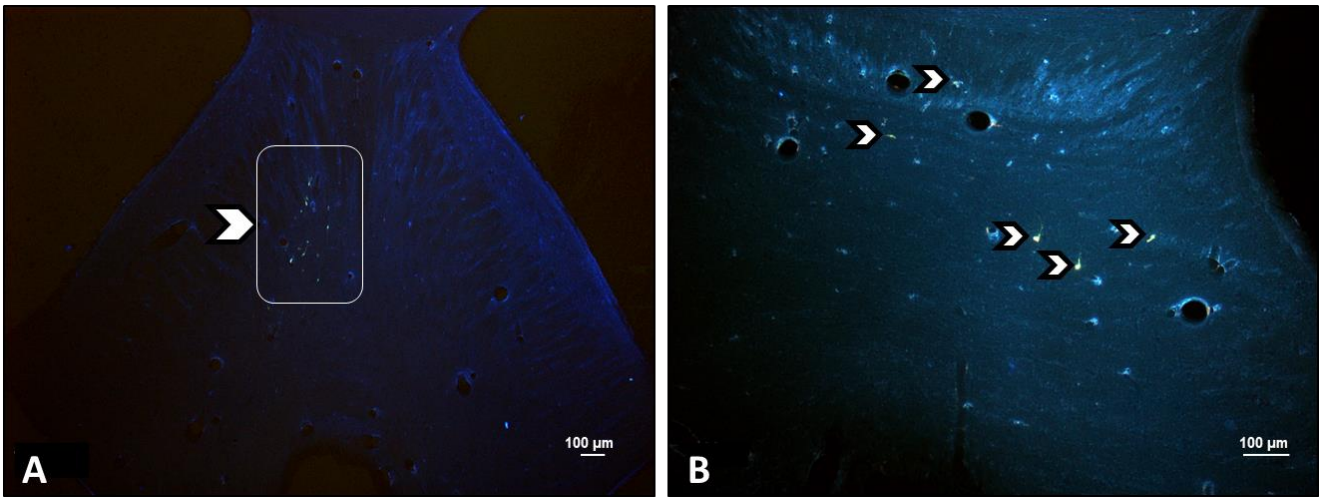

Fig. 21: FluoroGold™ in the TS (A) and SFi (B) after tracer injection in the LHb. The white arrows indicate cells which are positive for tracer.

3.3 Experiment 2: Effects of puma urine and TMT exposure on laboratory rats

In these experiments SD rats were confronted once with one odor sample, either water, TMT or puma urine (see Fig. 8). In the opaque square shaped box paradigm, previously described in 2.3.2.1, the position of the animal´s center point and nose point were detected automatically via EthoVision software. The time spent in the odor quadrant, contacts to the odor sample, moved distance and velocity were measured. Following a c-Fos immunoactivity study was performed to analyze differences in predator odor derived neuronal activation. In addition, serum corticosterone levels of the blood samples were analyzed.

3.3.1 Avoidance behavior

Fig. 22 illustrates the mean percentage of time rats spent in the odor quadrant during the test session with the probability to stay in one of the four quadrants given as chance level (25 %). The analysis of residence time in the odor quadrant showed no significant difference from chance level during water sample presentation (one sample t-test, $p = 0.98$). However, exposure to TMT and puma urine significantly reduced the time rats spent in the odor quadrat below chance level (one sample t-test, TMT: $p < 0.0001$, Puma: $p < 0.0001$). These results indicate avoidance of the TMT and puma urine samples but not of water samples.

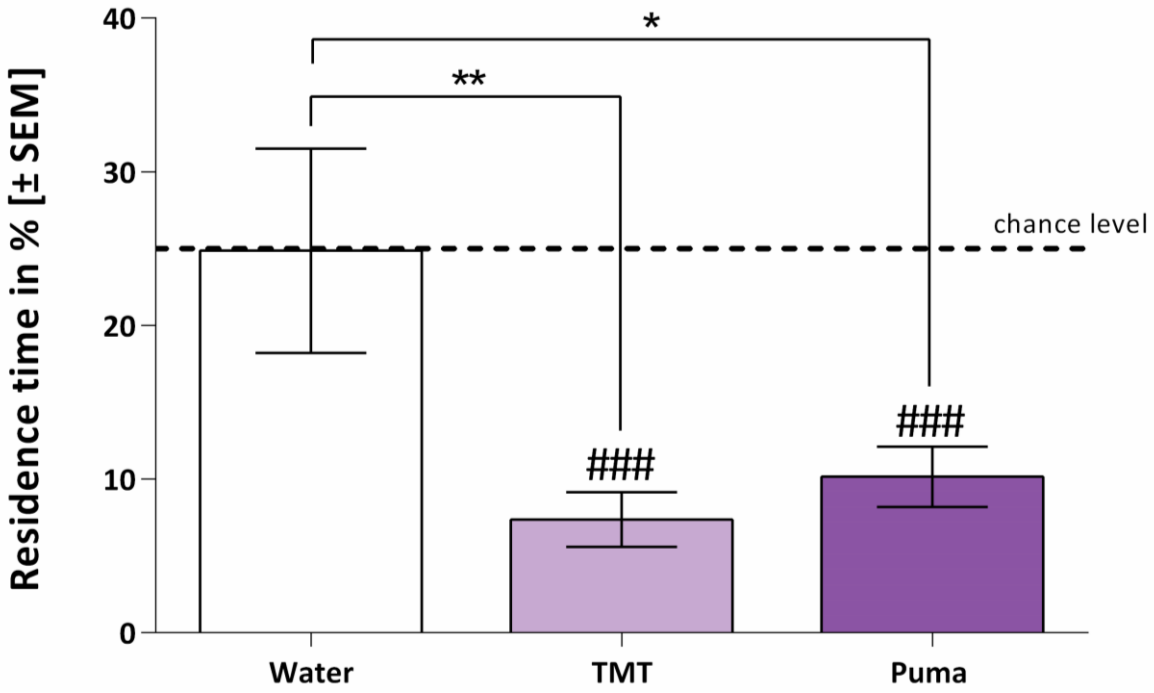

Fig. 22: Mean residence time (%) of rats in odor quadrant (± SEM) containing water, TMT or puma urine sample. The rats significantly avoided the odor quadrant during TMT and puma urine presentation (n = 10 each) compared to water control test (n = 8) and to chance level (25 %). ** $p < 0.01$; * $p < 0.05$ compared to water control experiment (one-way ANOVA with Holm-Sidak´s multiple comparison test); ### $p < 0.001$ compared to chance level (25 %) (one sample t-test).

This is supported by an ANOVA showing a significant effect of the odor samples ($F_{[2,\ 25]}$:= 6.11, p = 0.007). Post-hoc pairwise comparison (Holm-Sidak´s multiple comparison test to water) revealed a significant reduction of residence time when TMT samples (p = 0.009; Fig. 22) and puma urine samples (p = 0.02; Fig. 22) were presented. Thus, there was no significant difference between exposure to TMT and puma urine samples detectable (p = 0.58).

To measure glass contacts the nose point detection was used and a circular area (Ø = 6 cm) was defined around the glass bowl with the samples. The presence of the nose point inside this area was counted as odor sample contact. The results of the analysis are illustrated in Fig. 23 as the mean time in sec (A), the mean number of contacts to the odor glass (B) and the mean duration of one contact event in % (C) of testing time.

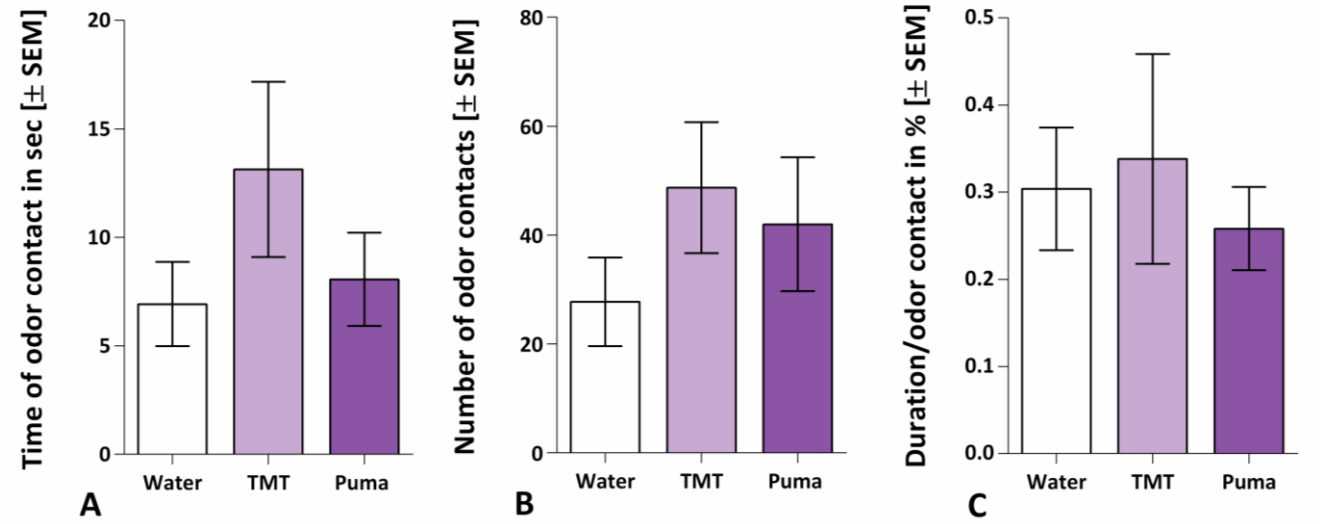

Fig. 23 A: Mean time in sec rats contacted the odor glass (± SEM) containing either water, TMT or puma urine. No significant difference was detected (Kruskal-Wallis test, water n = 8, TMT n = 10, puma urine n = 10). **B: Mean number of contacts to the odor glass (± SEM)** containing either water, TMT or puma urine. No significant difference was detected (Kruskal-Wallis test, water n = 8, TMT n = 10, puma urine n = 10). **C: Mean duration per odor contact event in % (± SEM).** No significant difference was detectable (Kruskal-Wallis test, water n = 8, TMT n = 10, puma urine n = 10).

Kruskal-Wallis test revealed that the mean time rats spent contacting the odor source was not affected by the odor. The same applies to the number of contacts to the odor source and to the mean time one contact event lasted.

3.3.2 Locomotor activity

In addition it was analyzed whether general locomotor activity is influenced by the odor samples. First, the distance the animals moved during the odor test was measured. An ANOVA revealed that the distance moved was not affected by the different odor samples ($F_{[2, 25]}$:= 0.43, p = 0.65, Fig. 24A). Furthermore the velocity of the animals was analyzed. The odor samples had no significant influence on the mean velocity while in mobile state, shown by Kruskal-Wallis test (H = 3.42, p = 0.18, Fig. 24B).

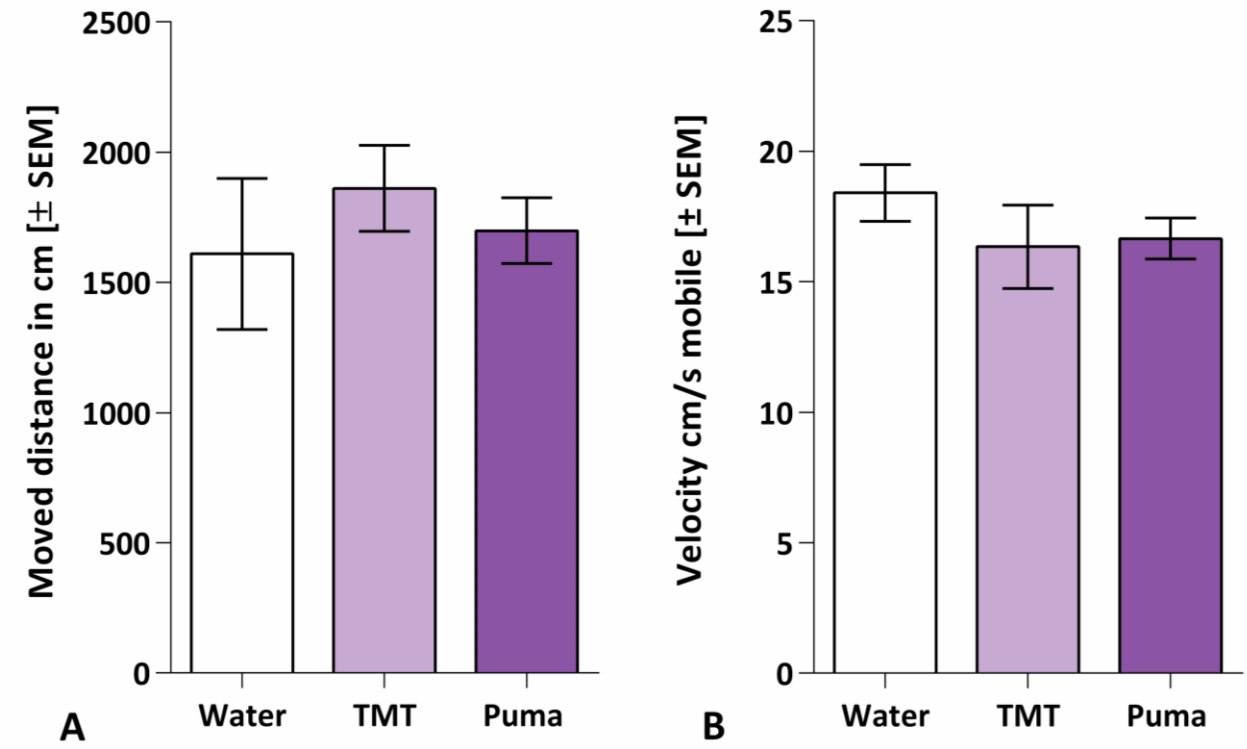

Fig. 24: A: Mean distance in cm rats moved during odor test (± SEM) with either water, TMT or puma urine. No significant difference was detected (one-way ANOVA; water n = 8, TMT n = 10, puma urine n = 10). **B: Mean velocity when mobile (cm/s) (±SEM)** during odor test with either water, TMT or puma urine. No significant difference was detected (Kruskal-Wallis test, water n = 8, TMT n = 10, puma urine n = 10).

In the test conditions the animals were moving freely in the behavior test box and moved a comparable distance with a comparable velocity. The odor samples did not affect these behaviors.

3.3.3 Serum corticosterone levels

The level of serum corticosterone was measured via RIA. The results are illustrated in Fig. 25.

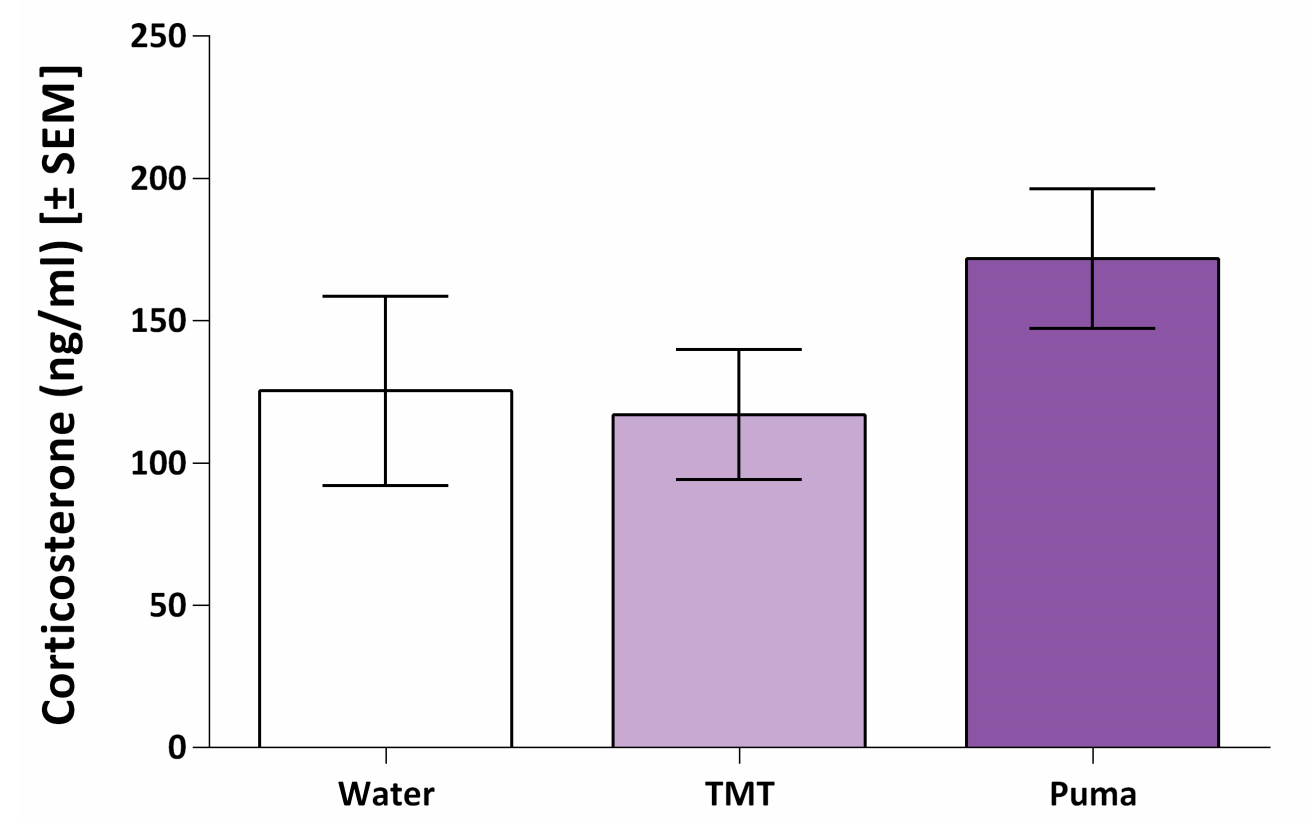

Fig. 25: Serum corticosterone levels after odor test with water, TMT and puma urine. The different odor samples caused no significant differences in serum corticosterone levels (one-way ANOVA; water n = 8, TMT n = 10, puma urine n = 8).

In comparison to the water test the odors TMT and puma urine had no significant effects on the serum corticosterone levels in SD rats (ANOVA $F_{[2, 25]} = 1.31$, $p = 0.29$).

3.3.4 C-Fos activation

C-Fos immunopositive cells were counted in different nuclei of the olfactory bulb and the amygdalar olfactory cortex (AOC), as defined by Wernecke and colleagues {Wernecke et al. 2015}. Statistical analyses showed significant effects of the odor samples on the number of c-Fos immunopositive cells in the external plexiform layers of the olfactory bulb (EPl) and accessory olfactory bulb (EPlA) as well as in the mitral cell layer of the accessory olfactory bulb (MiA), in the anteroventral part of the medial amygdaloid nucleus (MeAv) and in the posterolateral cortical amygdaloid nucleus (PLCo). The particular differences are illustrated in the following (Fig. 26 and Fig. 27).

The TMT odor sample caused a significant decrease in the cell number of c-Fos immunopositive neurons in the three nuclei of the olfactory bulb, EPl, EPlA, and MiA. In contrast, the puma urine sample had no significant influence on the cell number, although the effect of these two odor

samples was significantly different in the EPl (EPl: H = 9.77; p < 0.002; Dunn´s multiple comparisons: Water vs. TMT: p < 0.04; TMT vs. Puma: p = 0.013; Fig. 26A; EPlA: H = 5.46; p < 0.06; Dunn´s multiple comparisons: Water vs. TMT: p = 0.06; Fig. 26B; MiA: H = 7.25; p < 0.02; Dunn´s multiple comparisons: Water vs. TMT: p = 0.04; Fig. 26C).

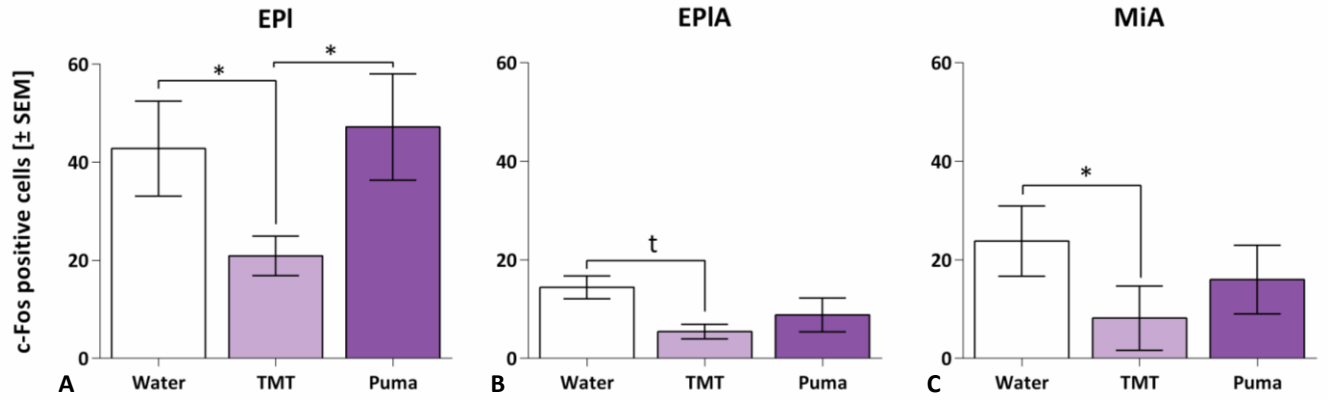

Fig. 26A: Mean number of c-Fos immunopositive cells (± SEM) in the EPl after odor test with water, TMT and puma urine. * p < 0.05 compared to appropriate control experiment (Kruskal-Wallis test with Dunn´s multiple comparisons; water n = 5, TMT n = 6, 1 outlier, puma urine n = 6).**B: Mean number c-Fos immunopositive cells (± SEM) in the EPlA after odor test** with water, TMT and puma urine. t p < 0.07 compared to water control experiment (Kruskal-Wallis test with Dunn´s multiple comparisons, water n = 5, TMT n = 7, puma urine n = 6). **C: Mean Number of c-Fos immunopositive cells (± SEM) in the MiA after odor test** with water, TMT and puma urine. * p < 0.05 compared to water control experiment (Kruskal-Wallis test with Dunn´s multiple comparisons, water n = 5, TMT n = 6; 1 outlier, puma urine n = 6.

In both accessory olfactory bulb areas (EPlA and MiA) the number of c-Fos immunopositive cells was in general lower compared to the olfactory bulb area (EPl). All three areas showed significantly fewer c-Fos immunopositive cells after TMT sample exposure compared to water level. In addition, in the EPl a significant difference in cell numbers after the exposure to TMT and puma urine was detectable. After the puma urine exposure the cell number was comparable to the cell number after water exposure.

Kruskal-Wallis test of the counted c-Fos immunopositive cells in the MeAv revealed a significant decrease in c-Fos immunopositive cells after exposure to the TMT and puma urine samples and a tendency in between the predator odors (H = 50.96; p = 0.0001; Dunn´s multiple comparisons: Water vs. TMT: p = 0.0001; Water vs. Puma: p = 0.0001; TMT vs. Puma: p = 0.05, Fig. 27A). A similar result was found in the PLCo (H = 76.85; p < 0.0001). Here the analysis also showed a significant decrease in the number of c-Fos immunopositive cells after exposure to the TMT and puma urine samples and a significant difference in between the odor samples (Dunn´s multiple comparisons: Water vs. TMT: p = 0.0001; Water vs. Puma: p = 0.0001; TMT vs. Puma: p = 0.02, Fig. 27B).

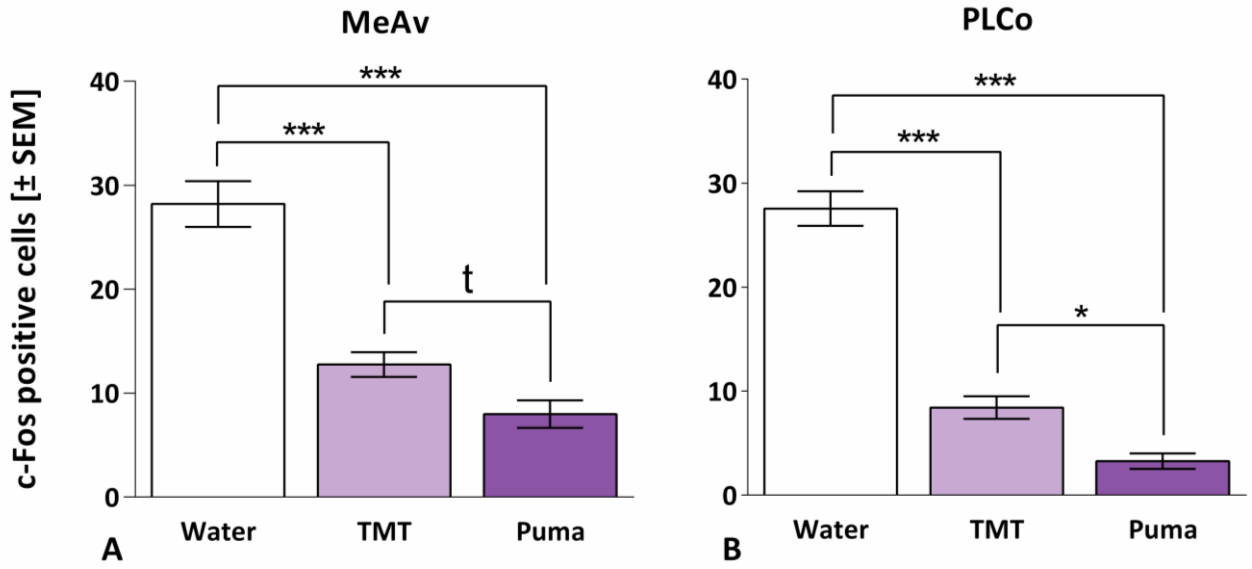

Fig. 27: A: Mean number of c-Fos immunopositive cells (± SEM) in the MeAv after odor test with water, TMT and puma urine. Both test odors caused significant differences in c-Fos immunopositive cells. *** $p < 0.0001$; t $p < 0.07$ compared to appropriate control experiment (Kruskal-Wallis test with Dunn´s multiple comparison, water n = 8, TMT n = 10, puma urine n = 10. **B: Mean number of c-Fos positive cells (± SEM) in the PLCo** after odor test with water, TMT and puma urine. Both test odors caused significant differences in c-Fos immunopositive cells. *** $p < 0.0001$; * $p < 0.05$ compared to appropriate control experiment (Kruskal-Wallis test with Dunn´s multiple comparison, water n = 8, TMT n= 10, puma urine n = 10).

In both amygdaloid nuclei the number of c-Fos immunopositive neurons decreased significantly after exposure to the TMT sample and even more after exposure to the puma urine sample. However, the difference in between the predator odors was only significant in the PLCo but not in the MeAv, though a tendency was found.

3.4 Experiment 3: Different effects of TMT exposure in laboratory and wild rats

In this experiment the semi-wild rat strain Warsaw Wild Captured Pisula Stryjek (WWCPS) was used and the recorded behavior was compared to a laboratory rat strain, Lister Hooded (LH). Each animal was confronted with only one odor; either water or TMT (see Fig. 8) and the square shaped box with one transparent wall was used, which is described in 2.3.2.2. The mean covered distance, time spent mobile and velocity, odor glass contacts, freezing and grooming behavior were analyzed. In addition, the serum corticosterone levels were measured.

3.4.1 Habituation and baseline calculation

A two-way ANOVA of the residence time of the animals in the different quadrants of the box during the three habituation sessions revealed a significant interaction between quadrants and strains ($F_{[3, 90]}$ = 5.20; p = 0.002) but no main effects (strain: $F_{[1,30]}$ = 3.05; p > 0.99; quadrant: $F_{[3,90]}$ = 2.44; p = 0.07) (see Fig. 28).

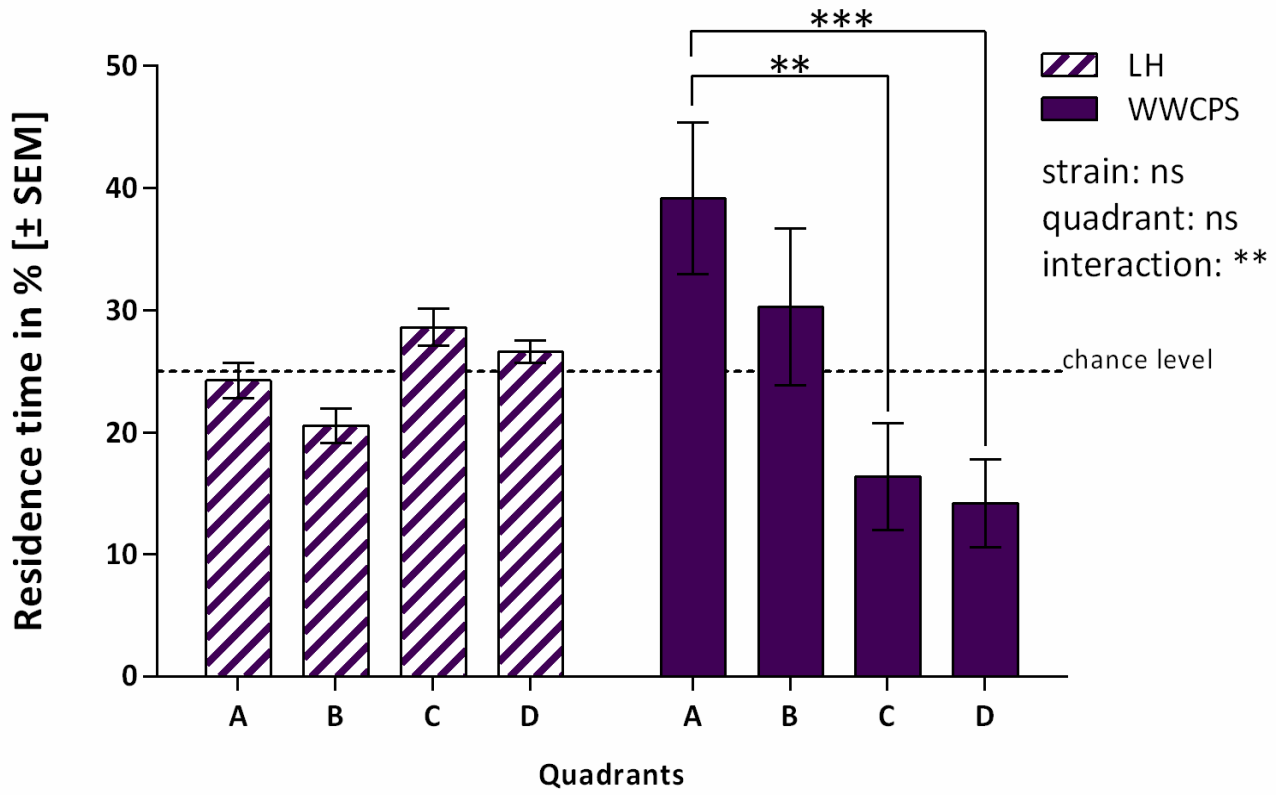

Fig. 28: Mean residence time in % in each quadrant during all habituation sessions (± SEM) of LH (hatched bars; n = 16) and WWCPS rats (dark bars; n = 16). WWCPS prefer quadrant A but avoid the quadrants C and D. LH rats showed no preferences or avoidances. ** p < 0.01; *** p < 0.001, post-hoc Holm-Sidak's multiple comparisons after significant two-way ANOVA.

As previously described, quadrants A and B were bilaterally bordered with opaque walls, quadrants C and D were unilaterally bordered with a transparent wall. Sidak´s multiple comparison tests showed

no differences of residence time between the quadrants in LH rats. In contrast, WWCPS rats stayed the longest time in quadrant A and least in quadrants C and D (see Fig. 28) whereas, LH rats did not show significant preference behavior, except a slight tendency to avoid quadrant B. Accordingly, the area of quadrant A and B were defined as “area AB” and the area of quadrants C and D as “area CD”. Rats in area AB are considered to be unaffected by influences caused by the transparent wall, whereas rats in area CD are considered to be affected by the transparent wall. The analysis of the area preference (see Fig. 29) revealed a significant avoidance of area AB in LH rats and area CD in WWCPS rats (one sample t-test compared to chance level (50 %): LH area AB p = 0.001; WWCPS area CD p = 0.01) and an opposite preference (one sample t-test compared to chance level (50 %): LH area CD p = 0.001; WWCPS area AB p = 0.01). These data are supported by a two-way ANOVA which revealed significant interaction and dependence on area (interaction: $F_{[1, 30]} = 13$; p = 0.001; area: $F_{[1, 30]} = 4.4$; p = 0.04; strain: $F_{[1, 30]} = 0.58$; p = 0.45).

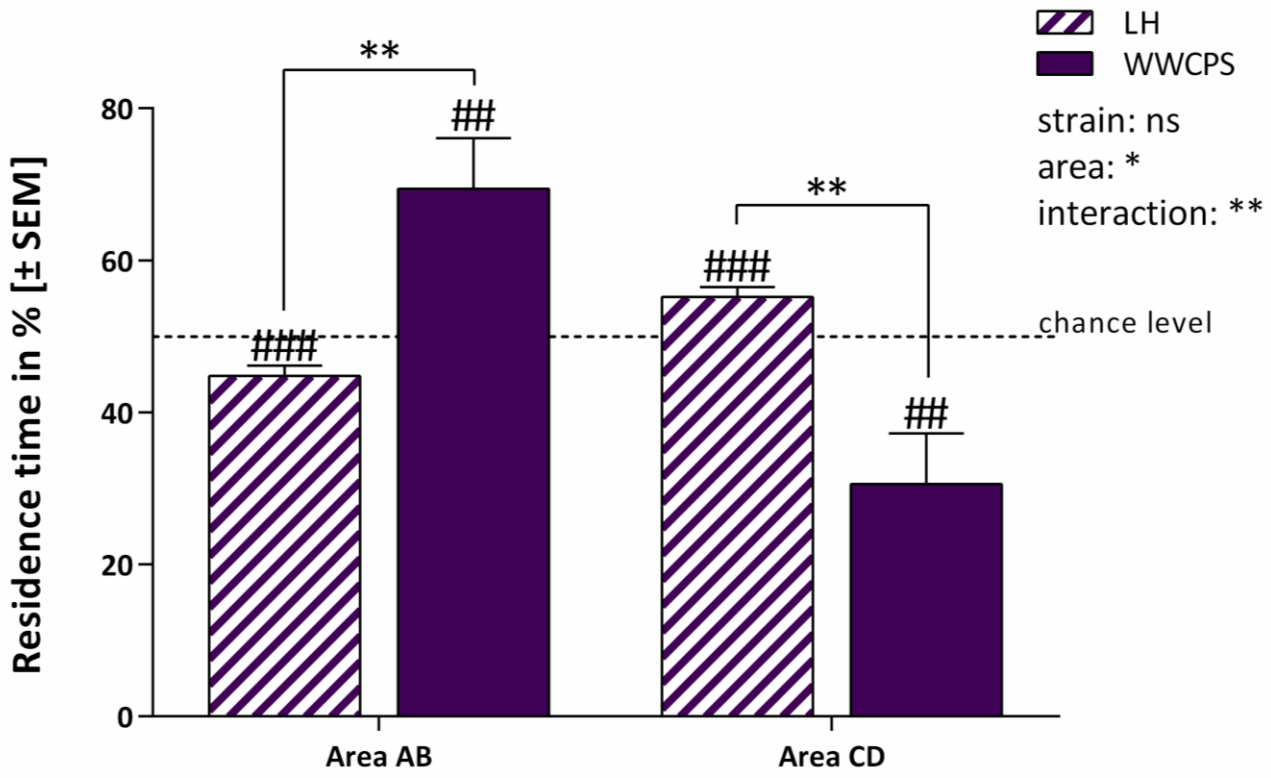

Fig. 29: Mean residence time in % in area AB and CD during all three habituation sessions (± SEM) of LH (hatched bars; n = 16) and WWCPS rats (dark bars; n = 16). WWCPS preferred area AB but avoided area CD. LH avoided area AB and preferred area CD. ## p < 0.01, ### p < 0.001, one sample t-test compared to chance level; ** p < 0.01, post-hoc Holm-Sidak’s multiple comparisons after significant ANOVA.

Post-hoc comparisons showed that the residence time of LH rats in area AB is significantly shorter than those of WWCPS rats and vice versa in area CD (Holm-Sidak´s multi comparisons test: LH Area AB vs. WWCPS Area AB: p = 0.0012; LH Area CD vs. WWCPS Area CD: p = 0.0012).

3.4.2 Avoidance behavior

The mean percentage of time rats spent in the odor quadrant during the odor test session is shown in Fig. 30. LH and WWCPS rats both highly significant avoided the odor quadrant during TMT sample presentation compared to chance level (one sample t-test, p = 0.0001) but WWCPS additionally avoided the water sample (p = 0.0006). A two-way ANOVA showed significant effects of strain ($F_{[1,26]}$:= 26.99, p < 0.0001), odor ($F_{[1,26]}$:= 15.69, p = 0.0005) and interaction ($F_{[1,26]}$:= 4.64, p = 0.04).

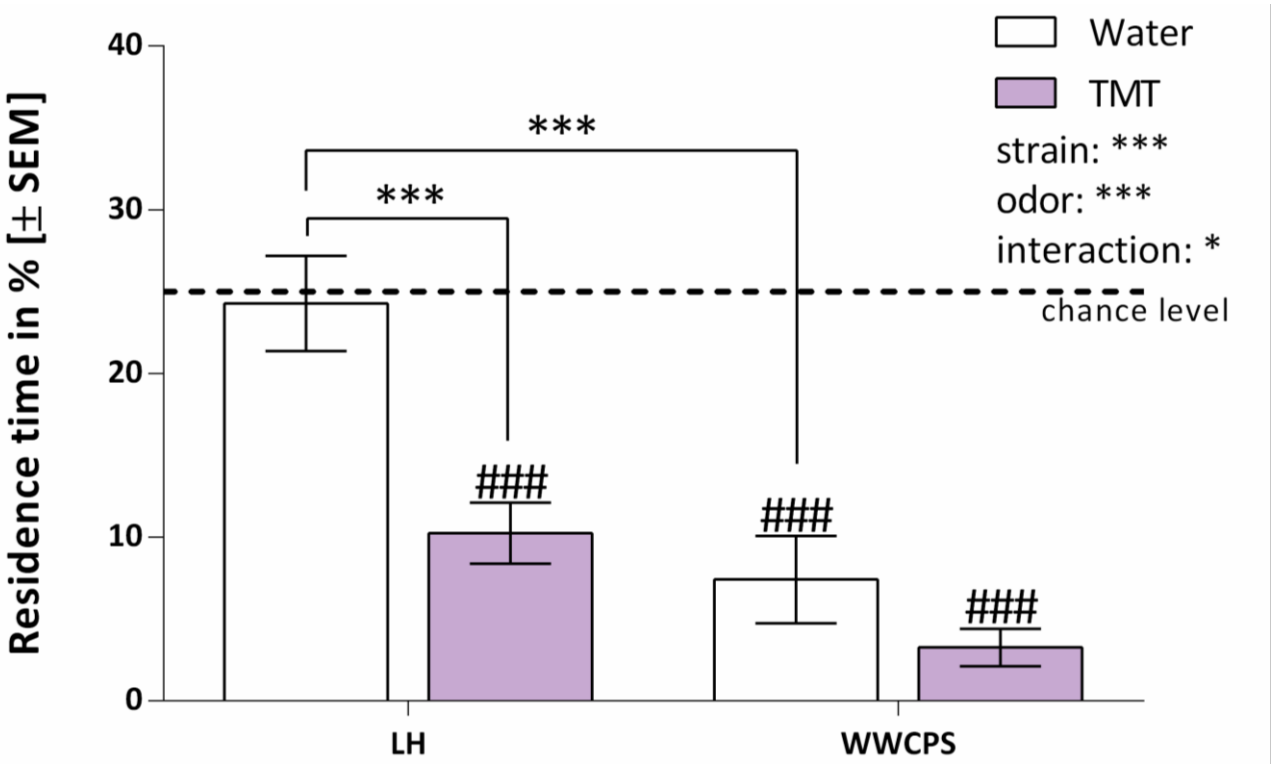

Fig. 30: Mean time in % rats spent in odor quadrant (± SEM) containing either water (white bar) or TMT (lilac bar). LH rats (n = 16) significantly avoided the odor quadrant in the TMT test compared to water control test and to chance level. Compared to chance level WWCPS rats (n = 16) significantly avoided the odor quadrant in the TMT test but also in the water control test. ### p < 0.001 compared to chance level (25 %) (one sample t-test), * p < 0.05; *** p < 0.001 compared with the appropriate control experiment (two-way ANOVA with Holm-Sidak´s multiple comparison).

Post-hoc pairwise comparisons revealed significant avoidance of the TMT sample in LH rats (Holm-Sidak´s multiple comparison test: Water vs. TMT: p = 0.0005) and a significant difference between the water control test sessions (LH Water vs. WWCPS Water: p < 0.0001). Thus, in WWCPS there was no significant difference between the avoidance reactions to the water and TMT samples (Holm-Sidak´s multiple comparison test: WWCPS Water vs. WWCPS TMT: p = 0.40).

However, after correcting for habituation ratios (Fig. 28) LH and WWCPS rats avoided the odor quadrant containing the TMT sample significantly compared to chance level (one sample t-test, LH p < 0.0001, WWCPS p = 0.01) but WWCPS showed no longer avoidance of the water sample (one

sample t-test, p = 0.30). This was supported by a two-way ANOVA with a distinct main odor effect ($F_{[1, 28]}$ = 13.18; p = 0.001), but no strain effect ($F_{[1, 28]}$ = 1.06; p = 0.31; Fig. 31) or interaction. Post-hoc test additionally showed that LH as well as WWCPS rats avoided the odor quadrant containing the TMT sample compared to water control test (Holm-Sidak´s multiple comparison test: LH p = 0.04, WWCPS p = 0.01).

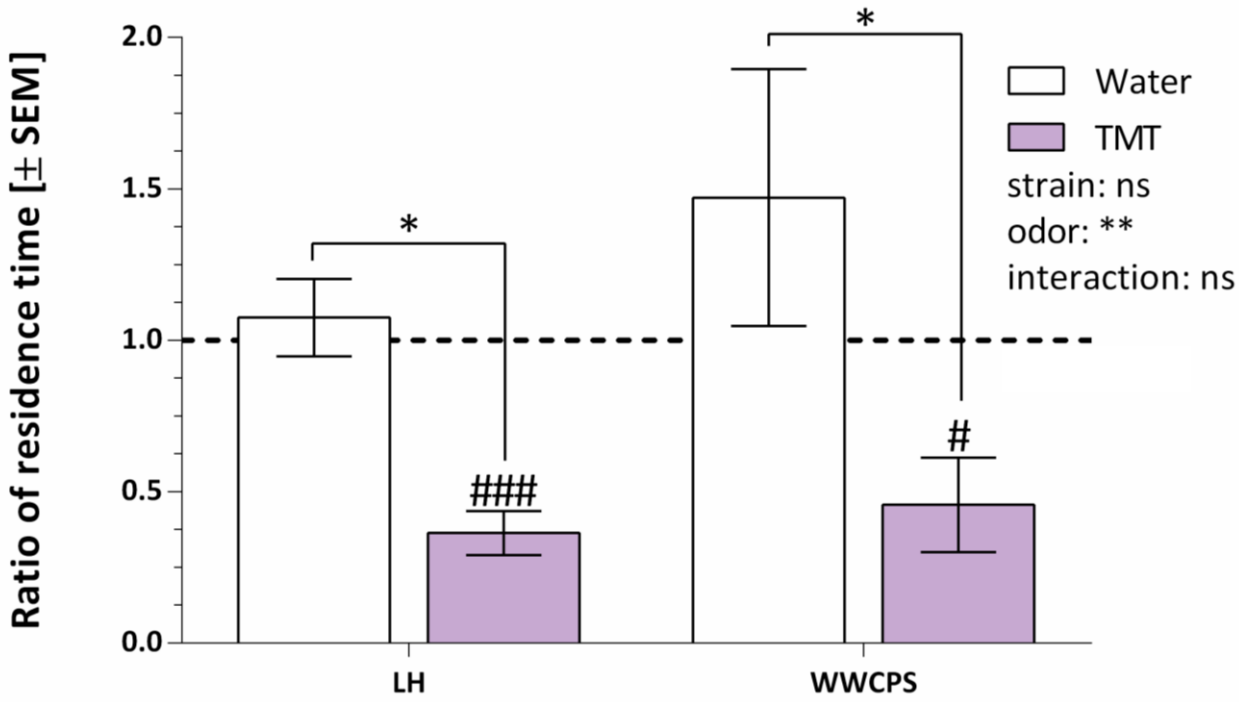

Fig. 31: Ratio of mean residence time rats spent in the odor quadrant (± SEM) during test session with either water (white bar) or TMT (lilac bar). Habituation ratio = time in odor quadrant during test session / mean time in corresponding quadrant over all habituation sessions. LH rats (n = 16) significant avoided the odor quadrant containing TMT compared to chance level and to water control test. WWCPS rats (n = 16) significantly avoided the odor quadrant containing TMT compared to water control and also to chance level. # p < 0.05; ### p < 0.001 compared to habituation (1.0) (one sample t-test), * p < 0.05; ** p < 0.01 compared with the appropriate control experiment (two-way ANOVA with Holm-Sidak´s multiple comparison).

After habituation ratio calculation WWCPS rats showed significant avoidance of the TMT sample compared to water control test (Holm-Sidak´s multiple comparison test: p = 0.027) and also to chance level (one sample t-test: p = 0.01). Only this habituation ratio calculation reveals the significant avoidance of the TMT sample in both rat strains (compare Fig. 30 & Fig. 31).

To take these quadrant preferences into account, the mean time spent in the odor quadrants during habituation sessions (Fig. 28) was taken as a baseline for the following analyses. For each individual animal, the ratio of residence time in the odor quadrant during the particular test session and the residence in this quadrant during habituation was calculated and used for further analyses.

A predator odor sample presentation in one quadrant may "push" the animals to stay in the quadrant which is on the opposite ("vis-à-vis") side, i.e. the farthest away from the possible danger. That kind of reaction could be more a sign of avoidance behavior rather than an approach to the odor source could be a kind of risk assessment behavior. Fig. 32 shows the habituation ratio of residence time in the different quadrants. The odor was presented in the quadrant "odor", the quadrant "vis-à-vis" was directly opposite of the odor quadrant and "left + right" were the quadrants left and right from the odor source summated. Fig. 32A shows the ratio of the mean time LH and WWCPS rats spent in the different quadrants during water sample presentation.

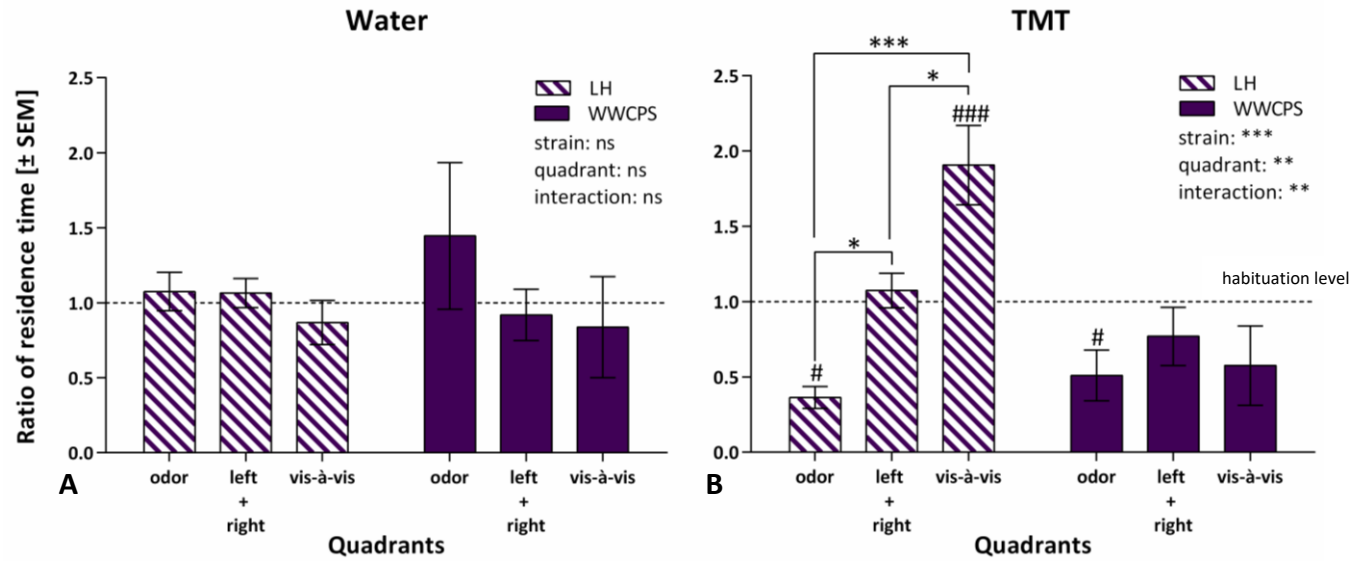

Fig. 32A: Ratio of mean time LH (hatched bars) and WWCPS (dark bars) rats (n = 8 each) spent in quadrants during water sample presentation (± SEM). Shown is the time spent in the odor quadrant, the quadrants left and right from the odor source summed up and the quadrant vis-à-vis. No significant differences were detectable. **B: Ratio of residence time of LH (hatched bars) and WWCPS (dark bars) rats (n = 8 each) in quadrants during TMT sample presentation (± SEM).** Shown is the time spend in the odor quadrant, the quadrants left and right from the odor source summed up and the quadrant vis-à-vis. Both rat strains showed significant avoidance of the odor quadrant and LH rats showed significant preference of the quadrant vis-à-vis. WWCPS showed no such preference. # $p < 0.05$, ### $p < 0.001$ compared to habituation (1.0) (one sample t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the appropriate control experiment (two-way ANOVA with Holm-Sidak´s multiple comparison test).

None of the strains showed significant differences in quadrant residence time during the water control test session, neither compared to habituation (one sample t-test, LH odor $p = 0.58$, LH vis-à-vis $p = 0.40$, LH left + right $p = 0.62$, WWCPS odor $p = 0.30$, WWCPS vis-à-vis $p = 0.65$, WWCPS left + right $p = 0.50$) nor to each other (two-way ANOVA: $F_{[5, 57]}$: = 0.97; $p = 0.44$).

Fig. 32B illustrates the ratio of time spent in the different quadrants during the TMT sample presentation. LH rats showed significant avoidance of the odor quadrant on the one hand and

significant preference of the quadrant vis-à-vis on the other hand (one sample t-test compared to habituation level (1.0): # p = 0.01; ### p = 0.0001). However, WWCPS rats significantly avoided the odor quadrant (one sample t-test compared to ratio of chance level (1.0): p = 0.01) but did not prefer the quadrant vis-à-vis (p = 0.16). The two-way ANOVA confirmed this difference in residence time with major effects of strain ($F_{[1, 13]}$: = 20.70; p = 0.0005), quadrant ($F_{[2, 26]}$: = 7.17; p = 0.003) and interaction ($F_{[2, 26]}$: = 6.27; p = 0.006). The post-hoc analysis showed significant strain differences in preference of the quadrant vis-à-vis (Holm-Sidak´s multiple comparison test: LH odor corner vs. LH vis-á-vis: p < 0.0001; WWCPS vis-á-vis vs. LH vis-á-vis: p < 0.0001).

3.4.3 Locomotor activity

ANOVA followed by post-hoc test revealed that the mean distance covered by the rats during the test sessions is not affected by the odor (two-way ANOVA, Odor: $F_{[1, 28]}$ = 2.27, p < 0.14). However, a clear strain effect showed that LH rats covered a highly significant longer distance than WWCPS rats (Strain: $F_{[1, 28]}$ = 69.64, p < 0.0001; Holm-Sidak´s multi comparison test: LH Water vs. WWCPS Water p < 0.0001., LH TMT vs. WWCPS TMT p < 0.0001; Fig. 33).

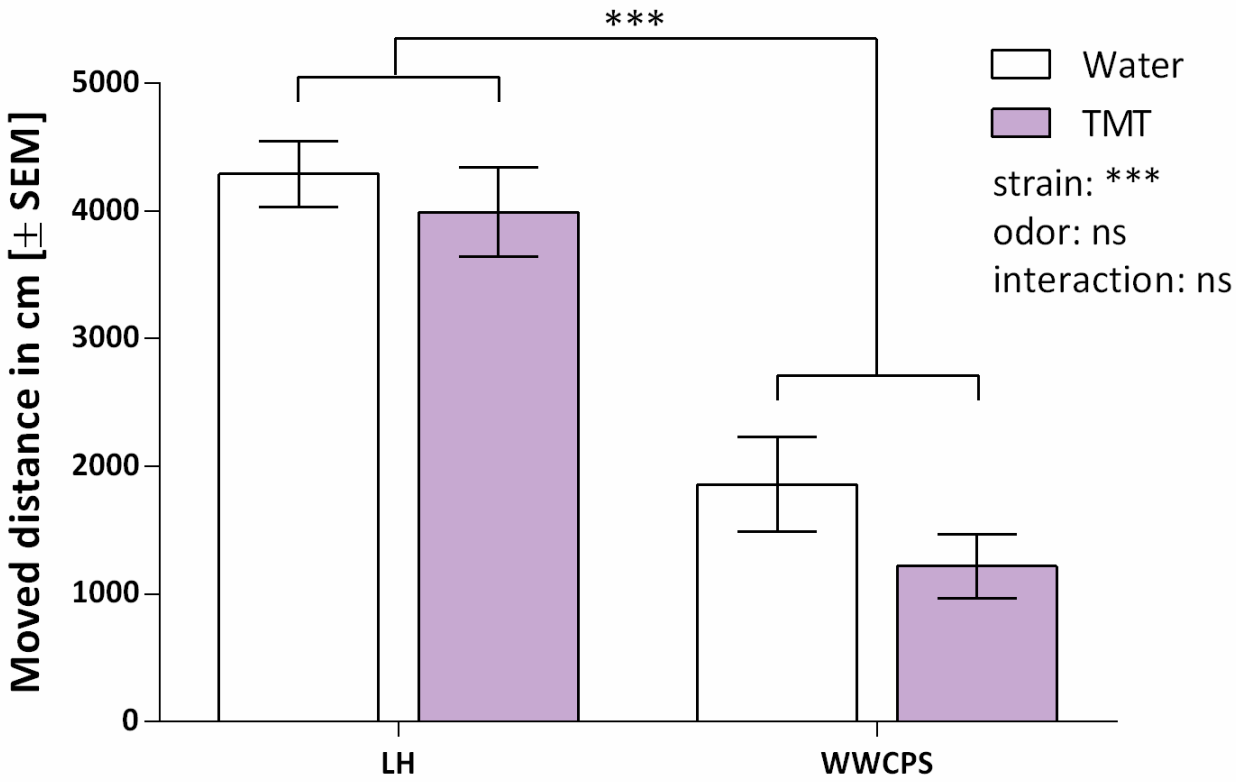

Fig. 33: Mean distance moved in cm during test sessions (± SEM). There is no significant difference in covered distance between water control test (n = 16) and TMT odor test (n = 16). But there is a highly significant effect of strain. *** p < 0.001 (two-way ANOVA).

For further interpretation of this difference, the time rats were mobile and in motion was analyzed (see Fig. 34).

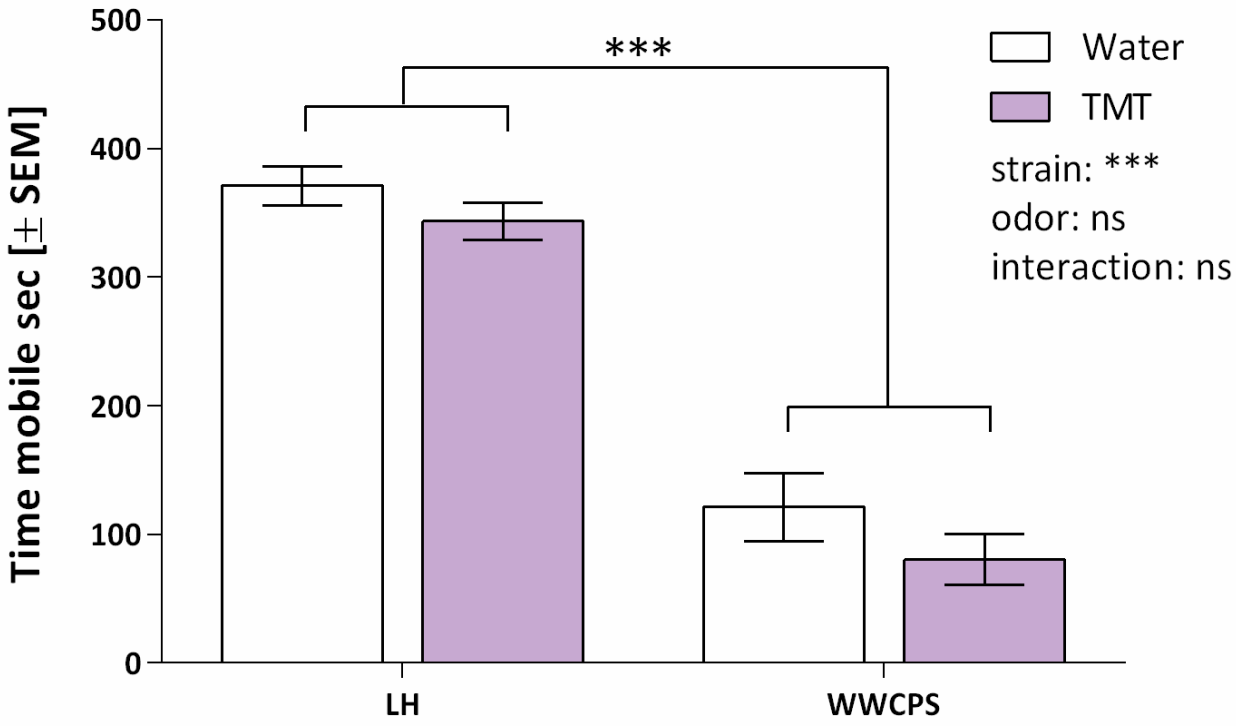

Fig. 34: Mean time rats showed mobility behavior in sec (± SEM) during water control test (n = 16) and TMT sample presentation (n = 16). No odor effect is detectable. However, LH rats spend significantly more time mobile compared to WWCPS. *** $p < 0.001$ (two-way ANOVA).

The ANOVA with post-hoc test revealed that the mean time rats were mobile during the test sessions was not affected by the odor sample (two-way ANOVA, Odor: $F_{[1, 28]} = 3.03$, $p = 0.09$;). However, there was a main effect of strain ($F_{[1, 28]} = 170.80$, $p < 0.0001$; Holm-Sidak´s multi comparison test: LH Water vs. WWCPS Water $p < 0.0001$., LH TMT vs. WWCPS TMT $p < 0.0001$) but no interaction ($F_{[1, 28]} = 0.11$, $p = 0.74$; Fig. 34).

The time rats spent in mobile state was used as basis to analyze the velocity of the animals during the odor test sessions. A two-way ANOVA revealed no odor effect but a significant strain effect which is illustrated in Fig. 35 (strain: $F_{[1, 28]} = 13.36$; ** $p = 0.001$; odor: $F_{[1, 28]} = 0.18$; ** $p = 0.68$).

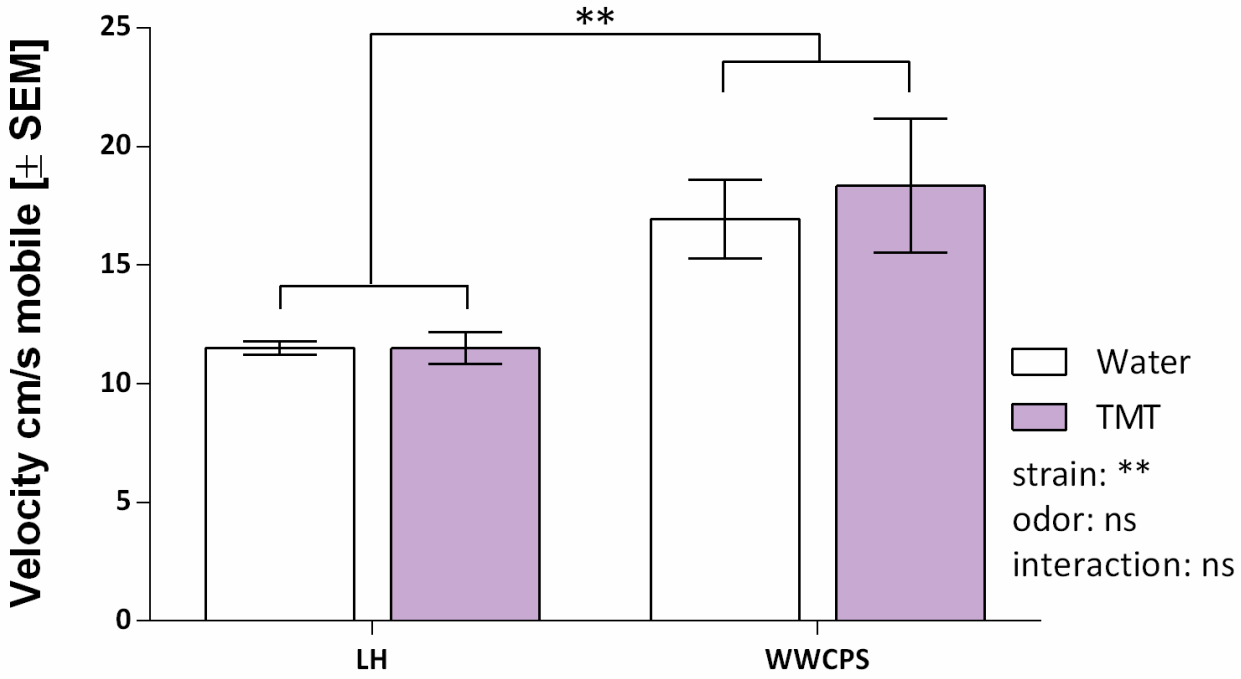

Fig. 35: Mean velocity when mobile (cm/s) (±SEM) during water control test (n = 16) and TMT odor test (n = 16). Testing odor does not affect velocity during test sessions. However, LH rats show a significant lower velocity compared to WWCPS. * $p < 0.01$ (two-way ANOVA).

LH rats showed mean velocity of 12 cm/s during water and 11 cm/s during TMT sample presentation. WWCPS rats showed a higher mean velocity of 17 cm/s and 18 cm/s during water and TMT sample exposure. There are no differences in between the odor presentations. Additionally to the facts that WWCPS covered a significant shorter distance and showed significantly less mobility in the test sessions, these rats showed significantly higher velocity (Fig. 35). WWCPS rats move less but faster.

Last, the freezing behavior of both rat strains was analyzed. A significant odor effect and also strain effect was found (two-way ANOVA: strain: $F_{[1, 28]} = 4.91$, $p = 0.04$; odor: $F_{[1, 28]} = 4.89$, $p = 0.04$) which is illustrated in Fig. 36. The overall time rats spent freezing was significantly influenced by the test odor and rat strain, although Holm-Sidak´s multiple comparison test showed no significant differences (LH Water vs. TMT $p = 0.28$, WWCPS Water vs. TMT $p = 0.10$).

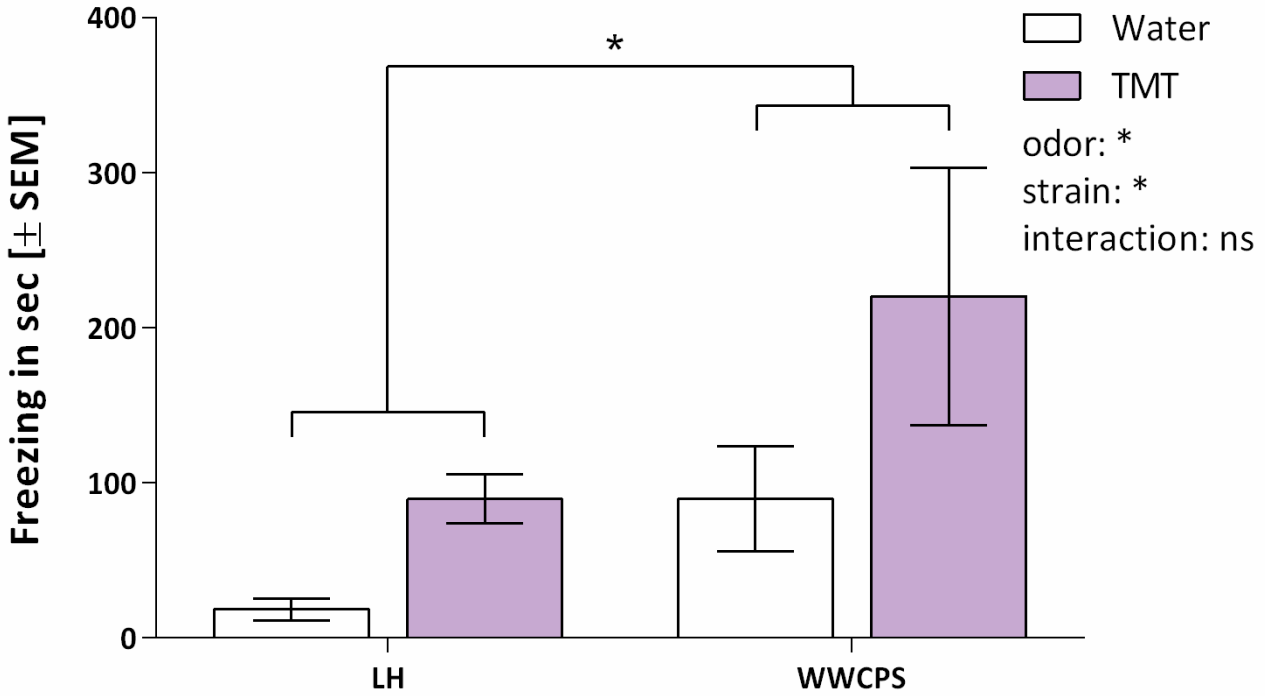

Fig. 36: Mean time rats showed freezing behavior in sec (± SEM) during water control test (n = 16) and TMT odor test (n = 16). * $p < 0.05$ (two-way ANOVA).

The number of freezing events and the mean time of one individual freezing event were measured simultaneously. The ANOVA resulted in a highly significant odor and strain effect and also in significant interaction (two-way ANOVA; strain: $F_{[1, 28]} = 23.44$, $p < 0.0001$; odor: $F_{[1, 28]} = 21.39$, $p = 0.92$; interaction: $F_{[1, 28]} = 21.39$, $p < 0.0001$, Fig. 37).

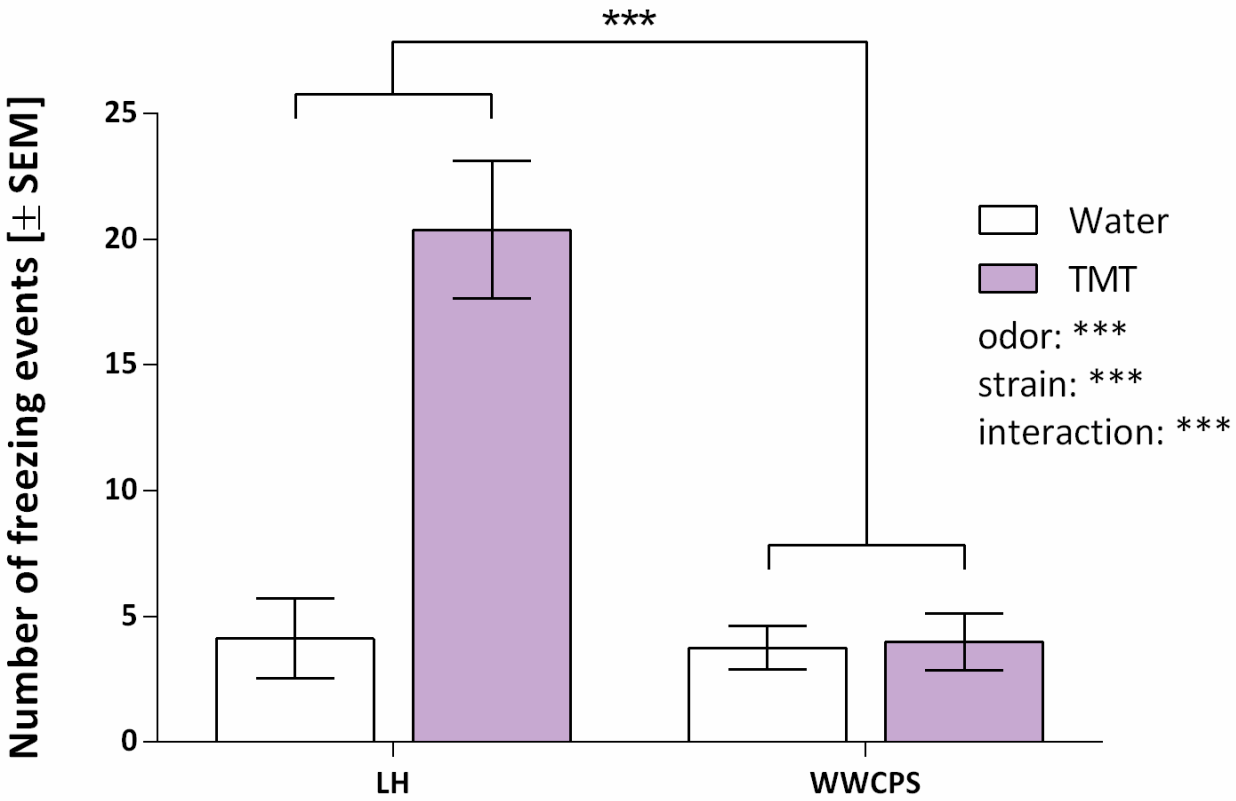

Fig. 37: Mean number of freezing events (± SEM) during water control test (n = 16) and TMT odor test (n = 16). The freezing behavior of LH but not WWCPS rats was affected by the test odor. *** $p < 0.001$ (two-way ANOVA).

Only LH rats showed significantly increased number of freezing events during TMT sample presentation (Holm-Sidak´s multiple comparison test: LH water vs. LH TMT: $p < 0.0001$, WWCPS water vs. WWCPS TMT: $p = 1$) whilst the number of freezing events in the TMT test sessions also differs significantly between the strains (Holm-Sidak´s multiple comparison test: LH TMT vs. WWCPS TMT: $p < 0.0001$)

However, the ANOVA of the mean duration per freezing event revealed a main strain effect but no odor effect or interaction (two-way ANOVA: strain: $F_{[1, 27]} = 7.56$, $p = 0.01$; odor: $F_{[1, 27]} = 2.92$, $p = 0.10$; interaction: $F_{[1, 27]} = 2.60$, $p = 0.12$; Fig. 38).

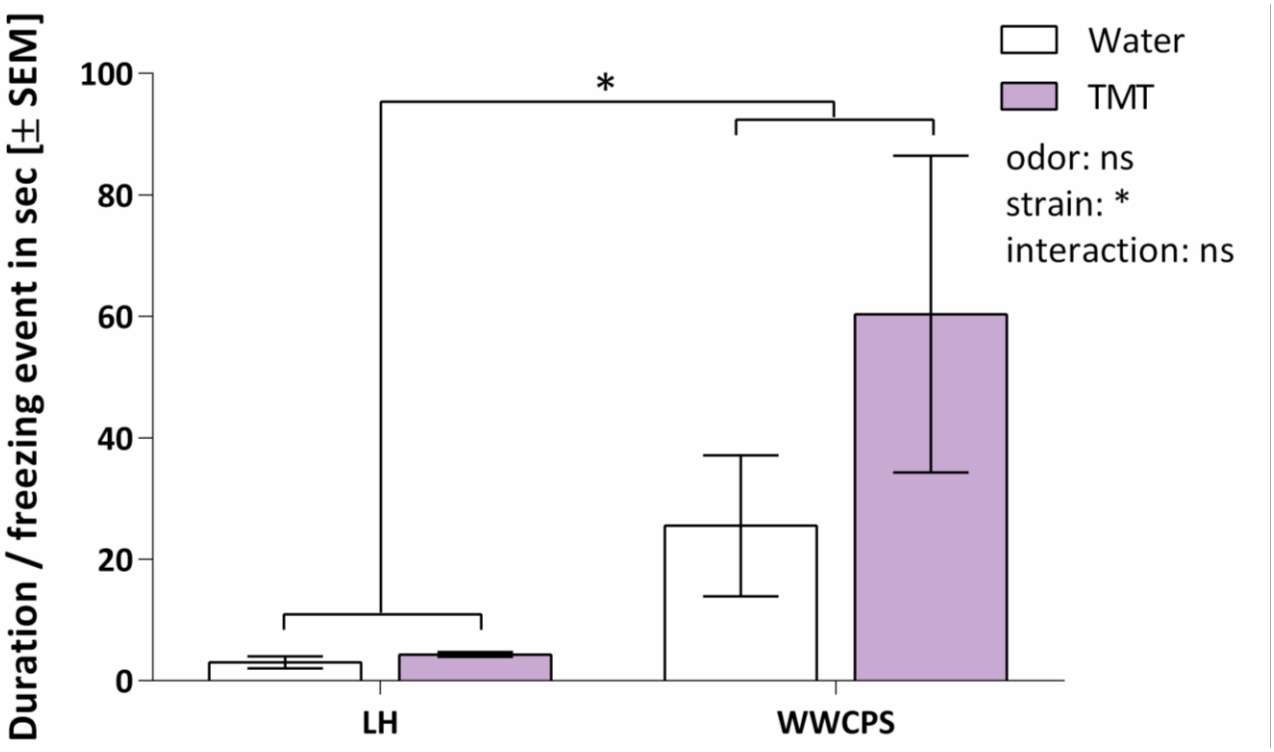

Fig. 38: Mean duration per freezing event in sec (± SEM) during odor tests with water or TMT sample. The freezing events of LH rats (n = 16) were significantly shorter compared to WWCPS rats (n = 16). * $p < 0.05$ (two-way ANOVA).

In general, when WWCPS rats showed freezing, one single event lasts longer compared to LH rats.

The analysis of grooming behavior revealed similar results (see Fig. 39). The two-way ANOVA shows a significant strain effect but no odor effect or interaction on the mean time rats showed grooming behavior during the odor tests (strain: $F_{[1,\ 28]} = 54.75$, $p < 0.0001$; odor: $F_{[1,\ 28]} = 0.33$, $p = 0.59$; interaction: $F_{[1,\ 28]} = 0.46$, $p = 0.50$).

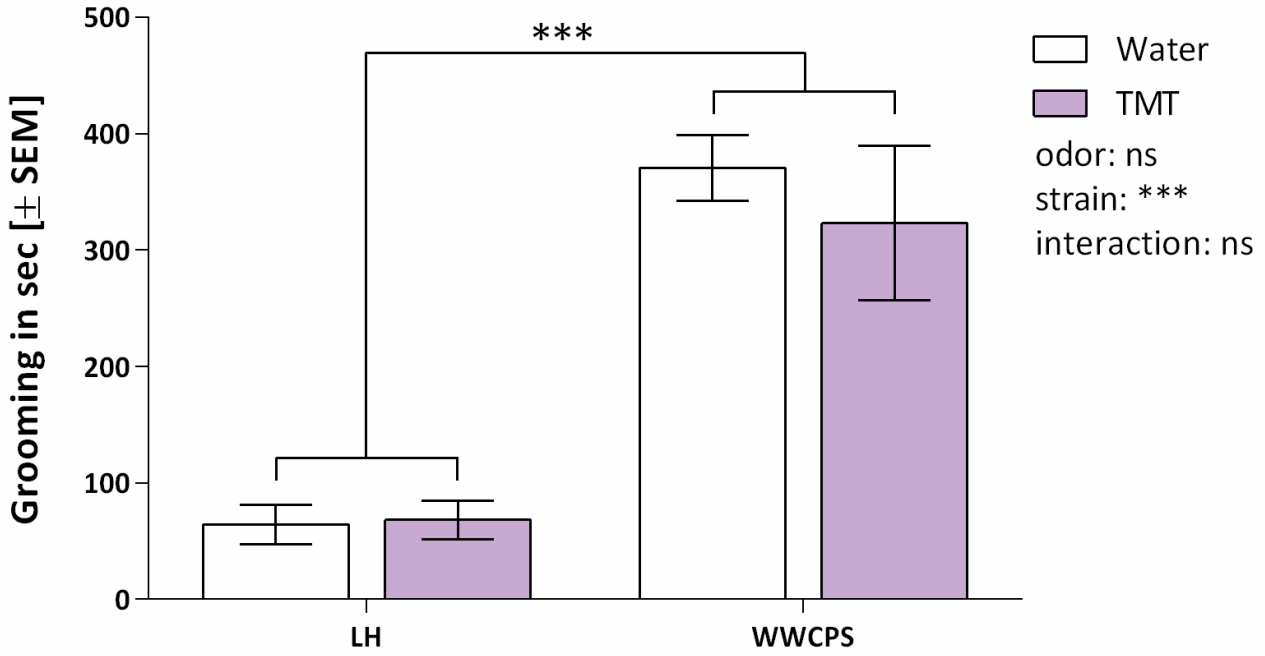

Fig. 39: Mean time rats showed grooming behavior in sec (± SEM) during odor tests with water and TMT sample. LH rats (n = 16) showed significantly less grooming behavior compared to WWCPS (n = 16) in general. *** $p < 0.001$ (two-way ANOVA).

Further analysis and a two-way ANOVA of duration of a single grooming event showed similar results such as a significant strain effect and no effects of odor or interaction (strain: $F_{[1,\ 28]} = 20.43$, $p < 0.0001$; odor: $F_{[1,\ 28]} = 0.41$, $p = 0.53$; interaction: $F_{[1,\ 28]} = 0.02$, $p = 0.88$; Fig. 40). These strain differences were supported by the post-hoc pairwise analysis (Holm-Sidak´s multiple comparison test: LH water vs. WWCPS water $p = 0.02$; LH TMT vs. WWCPS TMT $p = 0.01$).

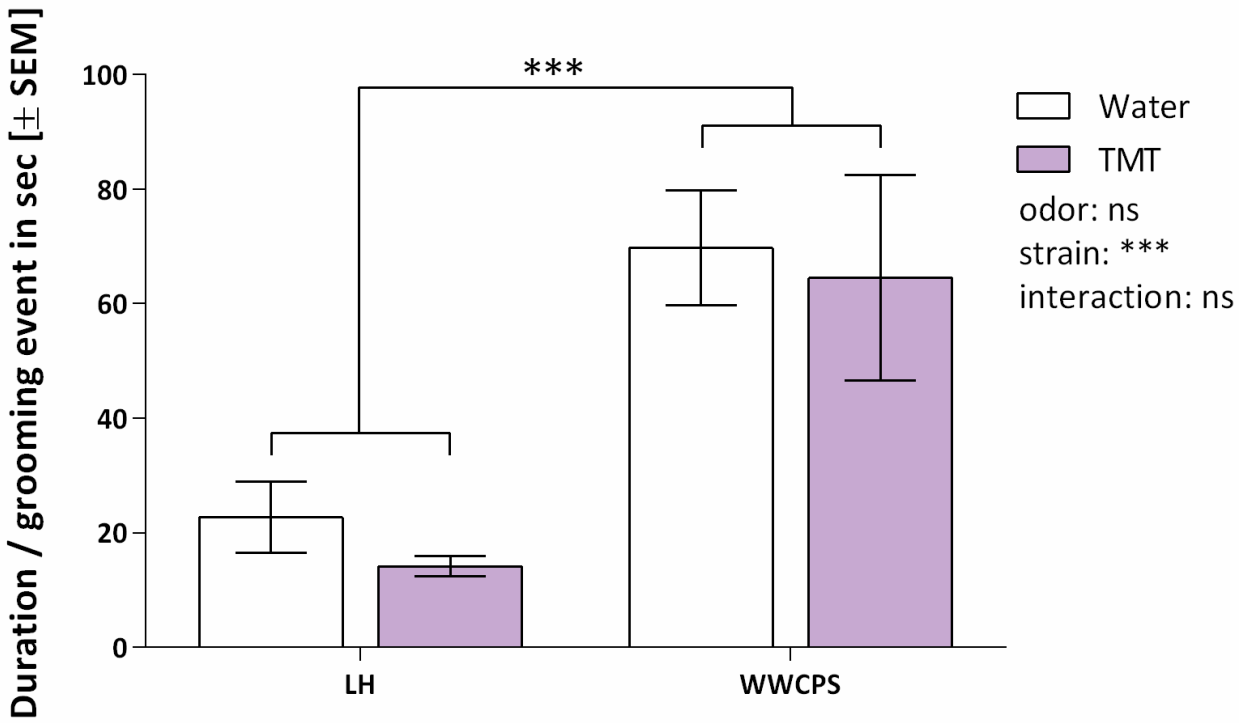

Fig. 40: Mean duration per grooming event in sec (± SEM) during odor tests with water and TMT sample. LH rats (n = 16) showed significant shorter grooming events compared to WWCPS rats (n = 16). *** $p < 0.0001$ (two-way ANOVA).

The average grooming event of WWCPS rats lasted significantly longer compared to the LH, independent of the presented odor sample.

3.4.4 Serum corticosterone levels

To measure endocrine consequences of the exposure to the TMT sample the serum levels of corticosterone were analyzed via ELISA. A two-way ANOVA revealed a significant main effect of strain ($F_{[1, 20]} = 8.04$, $p = 0.01$) but not of TMT exposure or interaction between strain and odor (interaction: $F_{[1, 20]} = 3.61$, $p = 0.07$; odor: $F_{[1, 20]} = 3.72$, $p = 0.07$). In addition, post-hoc comparisons showed a corticosterone increase by TMT in WWCPS rats ($p = 0.03$) which lacks in LH rats ($p = 0.98$; Fig. 41).

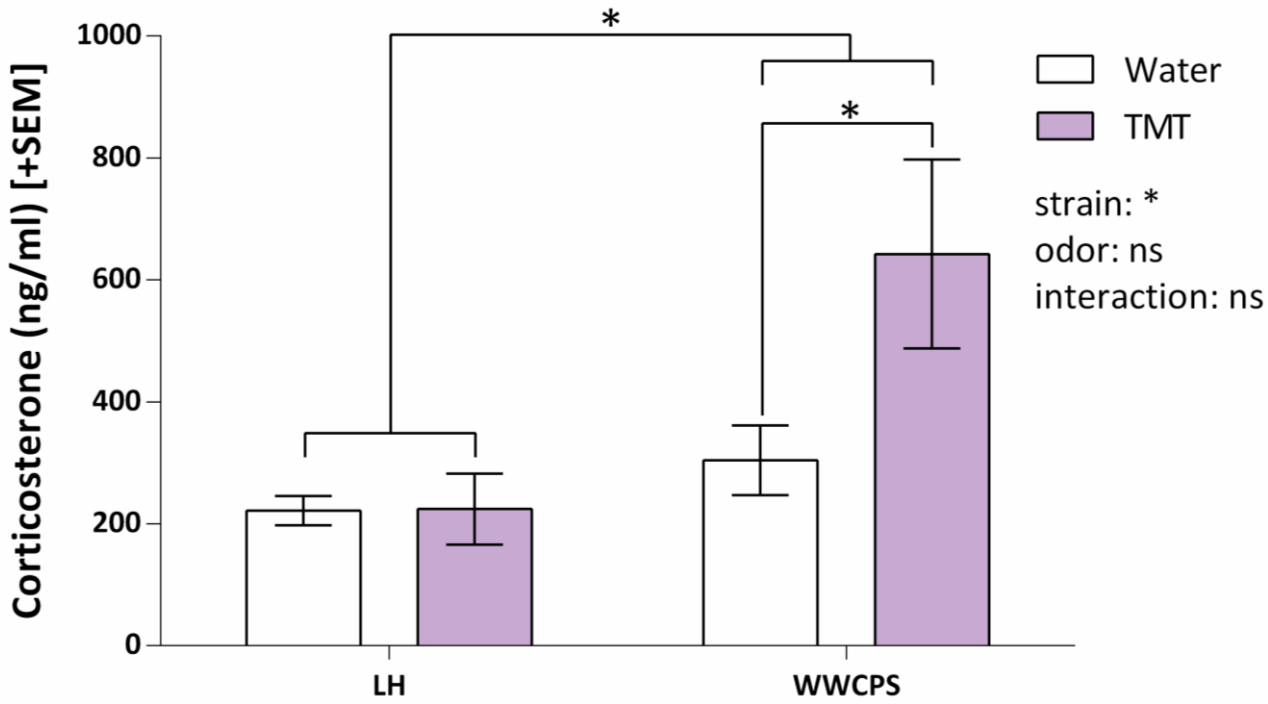

Fig. 41: Serum corticosterone levels in ng / mL (± SEM) after exposure to water (n = 16) or TMT (n = 16) Exposure to TMT increased corticosterone level in WWCPS rats but not in LH rats. * $p < 0.05$ (two-way ANOVA with Holm-Sidak´s multiple comparison test).

The exposure to the TMT sample only significantly affected the corticosterone levels of the WWCPS. Nevertheless, the corticosterone levels of the two strains differed significantly in general.

4 Discussion

4.1 Summary

In this thesis I examined three objective questions on basis of behavioral tests with predator odor exposed rats in an open field paradigm.

In the first experiment the aim was to assess, if repeated odor testing influences behavioral responses. This question was analyzed in two trials with different odors on two consecutive days (see Fig. 7). First, it was confirmed that predator odor naive rats show significant innate avoidance towards fox urine samples in the first trial. In contrast, predator odor experienced rats (i.e. during the second trial) avoided both, water and fox urine samples. A significant interaction of odor and trial was found after analysis of the residence time in the odor quadrants. The mean number of contacts to the odor source was not influence by the odor sample or the trial, the same holds true for the moved distance. However, experienced rats showed significantly less grooming during exposure to fox urine sample. The aim of the tracer injection study was to visualize neuronal connectivity and to show activation of this by means of c-Fos immunostaining. Successful retrograde tracer injections were performed in BLP and APir. These nuclei have both, according to analysis used here, active axonal connections to the MeAv; the BLP has also connections to the PV. In addition, colocalization of tracer and c-Fos immunoactivity was demonstrated there. After injection into the habenula, tracer particles were detected in the septum as well.

The objective of the second experiment was to compare the avoidance inducing qualities of TMT and puma urine. For this purpose the behavior responses, corticosterone levels, and c-Fos immunoactivity in the olfactory bulb and amygdala were analyzed. Both odors induced significant avoidance behavior in SD rats in the behavioral experiments and this avoidance behavior did not differ significantly. Furthermore, the corticosterone serum levels were not affected by exposure to the predator odors. However, the results of the c-Fos analysis were somewhat different. After exposure to predator odor sample, significantly less c-Fos immunopositive cells were found in the amygdaloid nuclei MeAv and PLCo compared to water sample exposure. Additionally, the amount of c-Fos immunopositive cells after puma urine sample presentation was significantly less compared to TMT in the PLCo and showed at least the same tendency in the MeAv. Interestingly, different results were found in the olfactory bulb nuclei EPl, EPlA and MiA. Here the number of c-Fos immunopositive cells after TMT sample presentation was significantly less than after water presentation. However, in the EPlA and MiA there was no such difference after exposure to puma urine sample. Though, in the EPl the

number of c-Fos immunopositive cells after exposure to puma urine was nearly as high as after water control test.

In the third part the behavioral and corticosterone responses of semi-wild WWCPS rats towards predator odor were examined and compared to laboratory Lister Hooded rats. Here we already found differences in behavioral response during habituation session. WWCPS were significantly affected by the transparent front of the testing box. Further behavioral differences were found in locomotor response, grooming and freezing behavior and lead to the hypothesis of different avoidance strategies in the two rat strains LH and WWCPS. In addition, exposure to TMT lead in the WWCPS to a serum corticosterone increase which was absent in LH.

4.2 Methodological consideration

In order to be able to use the same behavioral paradigm test, a well-established and simple behavioral test was chosen {Fendt 2006; Ferrero et al. 2011} which suited the different methodological demands of the participating workgroups of the CBBS NeuroNetwork 8 {see Wernecke et al. 2015 & Vincenz et al. 2017}. In our open fields with a side length of 45 cm & 47 cm, the animals were exposed to the test odors but also able to move freely enough to show active avoidance behavior {Ögren & Stiedl 2010}. A major advantage of the chosen innate fear paradigm was that the animals had not to be trained to perform any tasks. This fact excluded possible learning ability artefacts. However, a disadvantage of the odor paradigm used here was the insufficient controllability of the odor distribution in the testing box. Although the boxes were used under fume hoods, there was no visible distribution control.

In experiment 3 it was necessary to use a square shaped box with one transparent wall due to the aggressiveness of the timid WWCPS rats. On the one hand it was possible to observe the animal´s location when approaching the box after the odor test was finished. On the other hand it was also possible for the animal to recognize an approaching experimenter early. In a completely opaque box the animals startle when the experimenter appears suddenly above the walls to lift them out. This results in aggressive biting attacks and jumping behavior. To reduce these behaviors it was decided by the collaboration partner Rafał Stryjek to use one transparent wall which lead to the interesting results in 3.4.1. and revealed significant strain differences.

One limitation of this study here is that only male animals were analyzed in all the experiments. One reason was that the behavior of male animals is not influenced by the estrous cycle and the use of female rat urine as attractive control was possible. Another reason to confine this study on males

only was that investigating the influence of sex on predator odor induced avoidance behavior, would have at least doubled the total number of animals. In addition, the majority of studies in the literature report results for male rats, thus we wanted to compare published results with ours.

For the behavioral tests in experiment 3 LH rats were used as pigmented comparison rat stock to the WWCPS. In the literature there are reports about possible anosmia in albino rats {Keeler 1942; Sachs 1996; Hanson 2015} which might be dependent on some pigment complexes. Though, chromatographic analyses resulted in no differences between olfactory epithelia of pigmented and albino rats {Amoore et al. 1971}. Since the results of the experiments 1 and 2 show a robust predator odor avoidance response of the testes albino rats (SD) and c-Fos immunoactive neurons in the olfactory bulb, it is reliable that these rats were capable of a functioning olfactory system. However, albino rats were compared to albino rats. In order to test animals with comparable sensory performance in experiment 3, a pigmented laboratory rat stock was chosen. The Brown Norway strain, although these animals originate from a wild trapped rat colony in 1930 {Robinson 1965; Velaz 2013b; Charles River 2017c}, was no option as this rat strain is an inbred strain. In contrast, the LH and SD stocks are both outbred, established in the 1920s {Velaz 2013a; Janvier-labs 2017}, as well as the WWCPS. In order to be able to classify our results, LH stock was chosen as it is a pigmented strain, domesticated for a similar time period as SD.

In 1987 Dragunow and colleagues reported initially about c-Fos distribution in the brain of adult rats {Dragunow 1987} and since this time the immediate early gene (IEG) c-Fos is used as one indicator for neuronal activation {Hunt 1987; Morgan 1987; Sagar 1988; Hoffman 1993; Zhang 2002} and is widely used in olfactory stimulation experiments {e.g. Sallaz 1996; Dardou 2006; Weinberg 2009}. Former experiments in our laboratory with subsequent successful c-Fos visualization {e.g. Janitzky 2015; Lübkemann 2015} and the mass of publications were the reasons for us to use this IEG in combination with the retrograde tracer FluoroGold™. Since it was searched for colocalization of c-Fos immunoactivity and FluoroGold™, it was important to assess whether these two substances interact. A publication by Franklin and Druhan indicates that FluoroGold™ application constrains the expression of c-Fos at the injection site {Franklin & Druhan 2000}. However, in this thesis here the regions c-Fos immunopositive cells were counted were not identical to the injection sites.

The upregulation of the IEG protein c-Fos is triggered by calcium influx after neuronal activation {Morgan & Curran 1986; Morgan et al. 1987}. The protein expression has a peak after 90 min and is still upregulated after 240 min {Staples et al. 2008; Barr et al. 2011; da Silva et al. 2014} up to several hours {Menétrey et al. 1989; Mugnaini et al. 1989}. Hence, to interpret changes in the c-Fos protein

expression it was decided to sacrifice the tested rats 90 min after the odor exposure. This time interval was also appropriate for the analysis of the serum corticosterone level, even though the peak of stress-induced corticosterone elevation is around 60 min {Fuller & Snoddy 1980; Chandralekha et al. 2005}, this increase lasts up to 3 h which makes the chosen time point here, sacrificing the rats 90 min after test onset, reasonable. It was chosen against the possibility of analyzing c-Fos mRNA due to upregulation maximum after 30 min. This would have too much interfered with the rising corticosterone levels.

In this thesis here it was possible to combine c-Fos expression and tracer colocalization analysis with the serum corticosterone level analysis in a best possible way.

4.3 Experiment 1: Effects of fox urine exposure on naive and experienced laboratory rats

The repellant properties of fox urine have been shown before in several prey animals such as hares, rats or mice {e.g. Sullivan & Crump 1986, Farmer-Dougan et al. 2005; Fendt 2006}. Here it was possible to replicate these findings in our behavioral test paradigm (see Fig. 11). The innate avoidance response caused by odor of a predator (displayed in the first trial) is evolutionary beneficial to ensure survival. Fox urine, which belongs to the kairomones, reveals the possible presence of a fox, which is important information for any kind of prey. This beneficial information has impact on the animal and modulates the behavioral reaction. But the perception of the urine of a predator is only evidence for a former presence, which gives no reason for a flight reaction. Therefore avoidance of the particular area seems to be appropriate.

– *Behavior response is affected by the order of exposures to either predator odor or control stimulus*

In some studies, which focus on effects of predator odors, rats undergo multiple odor exposure trials {Ley et al. 1985; Wallace & Rosen 2000; Fendt 2006; Ferrero et al. 2011}. Previous studies in our collaboration within the CBBS also showed the functionality of fox urine as a predator cue in a four trial paradigm {Wernecke et al. 2015}. These paradigm designs have the disadvantage that experience might change innate behavioral reaction of the rats in general due to associative learning effects or contextual fear conditioning. Indeed, most of the animals in the wild are normally confronted more than once with predator odors and habituation to that would not be beneficial for survival. Indeed, predator odor naive rats show an innate fear response when exposed to predator odor {e.g. Vernet-Maury 1968; Funk & Amir 2000; Apfelbach et al. 2005; Wernecke 2015, Wernecke & Fendt 2015; Wernecke et al. 2015} which is also confirmed in this thesis. The change in behavioral response between the first and the second trial (see Fig. 12) indicates that experience with an aversive odor cue changes the behavioral reaction due to associative learning effects or contextual fear conditioning {Hubbard et al. 2004; Akers et al. 2012}, i.e. a generalized fear response during the second exposure in the test situation, which can occur after trauma {Siegmund & Wotjak 2006 & 2007; Perusini et al. 2016}. Long lasting behavioral sensitization has been shown after aversive odor exposure in rats like higher level of anxiety in terms of hyperarousal, stronger startle response etc. {Hebb et al. 2003a; Cohen et al. 2003, 2004a 2004b, 2006a, 2006b, 2008 & 2009; Lewitus et al. 2008} and that might have been the case in the experiments here. The response to the water sample was affected which might be a sign for conditioning. Though, conditioning to the vial should have resulted in an equal avoidance of every corner; conditioning to

the previous odor quadrant only to this particular quadrant and corner. However, since the odor quadrants were randomized, the odor was not presented in the same quadrant twice. To test whether context conditioning took place, a third test with an appetitive odor or fluid could be integrated. In addition, a test design as described in {Dielenberg et al. 2001a} could be examined. Here the authors show conditioned fear after exposure to cat odor to a cue and also to a context. Nonetheless, the results of the experiments here show that general locomotor activity was not affected (see Fig. 14) which is in line with other studies utilizing TMT exposure {Perrot-Sinal et al. 1999; Morrow et al. 2000}. An interesting next step would be to investigate whether there are physiological changes (e.g. change of heart rate or changes in corticosterone level) which may indicate conditioned fear as well {Dielenberg et al. 2001a}.

A significant decrease in grooming behavior was observed when predator odor naive rats were confronted with the fox urine sample, as expected. This effect was no longer observed in the second trial with water (see Fig. 15). Grooming and self-grooming have also been defined as anxiety like behaviors {e.g. Dielenberg & McGregor 2001; Homberg et al. 2002; McGregor et al. 2005}, i.e. in the second trial with water, grooming may indicate the contextual fear conditioning effect. A recent review by Kalueff and colleagues discusses the role of grooming and describes the importance of the cephalocaudal sequence of the grooming behavior to differentiate safety related and anxiety related grooming {Kalueff et al. 2016}. To interpret the relevance of this behavior it would be necessary to further analyze the progression pattern of the grooming sequences and whether this is changed by the predator odor exposure. In contrast to our findings, Dielenberg and colleagues - using TMT as predator odor - showed a significant decrease of grooming in an predator odor free safe environment in predator odor experienced rats {Dielenberg et al. 2001b}. These discrepant findings may be explained by a stronger effect of TMT as a concentrated odor compared to the effect of natural fox urine. In other words, the stronger odor may facilitate context learning, indicated by reduced grooming as sign for conditioned fear response. These findings lead to experiment 2 were the abilities of TMT as physiological predator stressor where further investigated.

- *Activated neuronal connections between BLP and APir to MeA are visualized after odor exposure*

Various studies discovered that conditioned fear is processed with the participation of the basolateral (BLA), central (CeA) and lateral amygdala {Muller et al. 1997; Cousens & Otto 1998; Nader et al. 2001; Gale et al. 2004} but it seems to be still vague how innate fear is processed {Vazdarjanova et al. 2001; Wallace & Rosen 2001; Power & McGaugh 2002; Choi & Brown 2003; Fendt et al. 2003;

Li et al. 2004}. Several publications showed that the BLA is an important structure for assessing sensory input in general {McDonald 1998; LeDoux 2000; Sah et al. 2003; Lanuza et al. 2004; Chen et al. 2006; Silva et al. 2016}. Furthermore, the BLA is involved in generating odor-induced freezing which occurs delayed after temporal BLA inactivation {Müller & Fendt 2005}. Thus, the aim of the tracer experiments was to visualize neuronal (inter)connectivities of these amygdaloid nuclei and to analyze neuronal activation during odor exposure by using c-Fos immunostaining. Successful tracer injections were performed in BLP, APir and habenula. The retrograde tracer injection described in this thesis visualized neuronal connections from BLP and APir to the MeAv (see Fig. 17 & Fig. 18). These connections were found to be activated after exposure to the water sample as well as to the fox urine sample which was visualized by c-Fos immunostaining. After tracer injection into the APir, colocalization of FluoroGold™ and c-Fos immunostaining was found in cells of the MeAv (see Fig. 18). But these were found only after exposure to fox urine and not after water sample exposure. These findings support our hypothesis that the neuronal connection between MeAv and APir plays a role in processing predator odors like fox urine. In contrast, we detected neuronal connections between the BLP and PV colocalized with c-Fos immunostaining expression in both rat groups, exposed to urine and water sample (see Fig. 19 & Fig. 20). These results validate a neuronal connection between BLP and PV and may indicate a role of the double-labelled neurons in signal processing beyond olfactory processing, e. g. signals from the unfamiliar test box, which per se can evoke avoidance responses. A summarizing sketch of the neuronal connections is shown in Fig. 42.

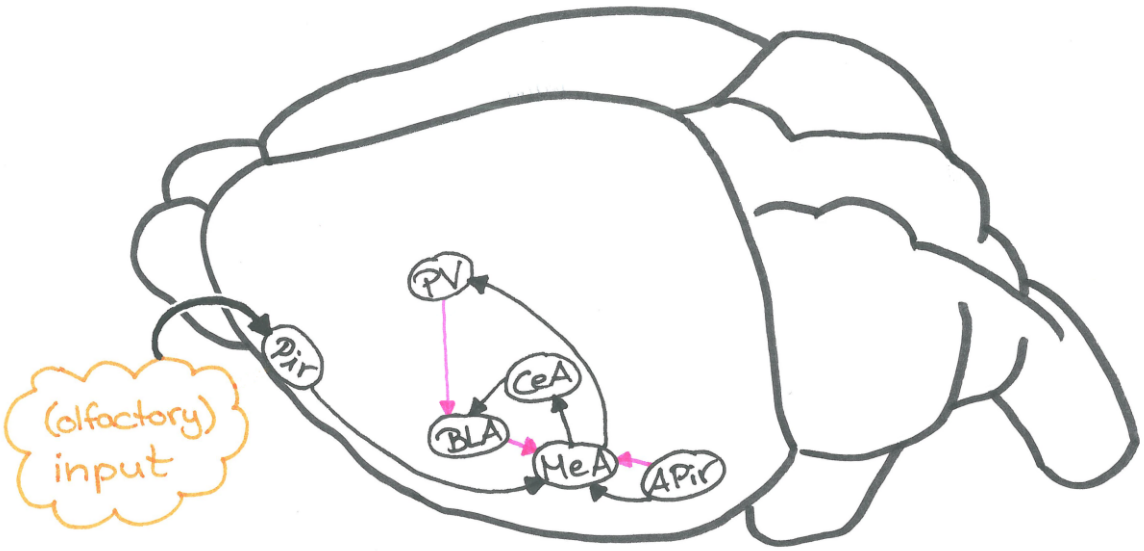

Fig. 42: Sketch of a rat brain with the analyzed nuclei with neuronal connections in their network. The black arrows indicate already described neuronal connections; the pink arrows indicate the visualized and described connections in this thesis. APir: amygdalo-piriform transition zone; BLA: basolateral amygdala; CeA: central amygdala; MeA: medial amygdala; Pir: piriform cortex; PV: paraventricular thalamic nucleus

Olfactory information is detected by the receptor cells of the olfactory epithelium, transferred via the olfactory bulb and olfactory tract to the piriform cortex (Pir), to the medial amygdala (MeA) and from there to the lateral and central nuclei of the amygdala (CeA) {Scalia & Winans 1975; McDonald 1998; Sah et al. 2003; Sosulski et al. 2011}. This is in line with our combined tracing and c-Fos immunostaining results. Previous studies in our collaborative CBBS project revealed a major role of the amygdalar olfactory cortex (AOC), which consists of the PLCo and BLP as well as the APir, in generating fox urine-induced innate fear {Wernecke et al. 2015}. After injections with the $GABA_A$-agonist muscimol into the AOC, to temporarily inactivate this region, avoidance behavior towards fox urine decreased significantly. The present results support these findings by showing that exposure to fox urine sample activates the pathways from APir and BLP both to the MeAv (see Fig. 18) and the PV (see Fig. 19). The amygdaloid nuclei BLP and MeA are known to show increase of c-Fos expression after TMT exposure and to be involved in the processing of this predator cue as well as the APir {Illig & Haberly 2003; Hebb et al. 2004; Sosulski et al. 2011} which is a nucleus also involved in emotion and memory processes {Jolkkonen et al. 2001}. The results of experiment 1 show that this seems to be true for fox urine as well.

Finally, neuronal connections from the habenula to the TS and SFi were visualized, but only in animals exposed to the water sample and no colocalization with c-Fos immunoactive cells was detectable, indicating that these connections are not involved in processing predator odor information.

Taken together the behavioral results showed an innate fear response to fox urine exposure in naive SD rats (first trial). The changed behavioral response in the second trial indicated associate learning or a conditioning of fear to the context. The results of the c-Fos/FluoroGold™ study visualized neuronal connections between MeAv and APir, which seem to play a role in processing predator odors, and neuronal connections between BLP and PV, with an indicated role of the double-labelled neurons in context processing.

4.4 Experiment 2: Effects of puma urine and TMT exposure on laboratory rats

It has intensively been discussed whether TMT is a rather repugnant than fear- or avoidance-inducing predator odor {Dielenberg et al. 2001; McGregor et al. 2002; Blanchard et. al. 2003; Fendt et al. 2008}. Hence, the aim in this experiment was to investigate and compare effects of TMT as synthetic predator odor with puma urine as a very intense smelling natural predator odor. The two odors are from predators of the same order (*Carnivorae*) but from different families. The provided puma urine was collected from the mountain lion (*Puma concolor*, {Linné 1771; Miotto et al. 2007}) which belongs to the family of *Felidae.* TMT on the other hand origins from the red fox` (*Vulpes vulpes,* {Linné 1758; Heltai & Markov 2012}) anal gland {Vernet-Maury 1980} and this animal is biologically classified as a species of the family *Canidae*. However, these two predators and the Norway rat (*Rattus norvegicus*, {Berkenhout 1769}) show a diverse distribution. Whilst the red fox and the Norway rat share the habitats Northern America and Eurasia {Kurtén et al. 1980; Aubry et al. 2009; Statham et al. 2014; Ruedas 2016}, the puma exists mostly in Southern America and today only reduced in Northern America {Nielsen et al. 2015}. Rats are favorite prey animals to red foxes whilst pumas normally prefer larger prey such as rabbits or even foxes. Nevertheless, in the experiment here both predator odors evoked equally aversive responses in the laboratory rats.

– *TMT and puma urine show equivalent abilities as avoidance inducing predator odors*

The behavioral data of experiment 2 show avoidance of both predator odor samples (see Fig. 22) which is a reported reliable behavior {Sullivan et al. 1988; Nolte et al. 1994; Farmer-Dougan et al. 2005; Fendt 2006; Ferrero et al. 2011; Xu et al. 2012} and clearly demonstrates an equivalent ability of TMT and puma urine as avoidance inducing predator odors. This stands in contrast to publications that estimate TMT rather a repugnant and noxious than fear-inducing substance {McGregor et al. 2002; Fendt & Endres 2008} or even ineffective as predator cue to provoke avoidance behavior {Dias Soares et al. 2003}. Avoidance behavior is best detectable in freely moving animals which display locomotor activity. The results here show that the different test odors did not affect the locomotor activity in terms of covered distance or velocity (see Fig. 24). Admittedly, literature describes a decrease in locomotion during odor exposure to TMT {Dielenberg & McGregor 2001} as a sign for fear. However, there is also evidence to interpret the unaffected exploratory behavior as characteristic of active avoidance because the animal actively choses its residence.

The contacts with the glass in which the odor sample was presented, as parameter of the risk assessment behavior {Dielenberg et al. 2001b}, did not show any significant differences as well (see Fig. 23). In this experiment only the contacts per se were evaluated, not how this was achieved. It is quite possible that the animals show a flat back approach during the exposure to predator odor and approach hesitant and more slowly with many stops compared to the water sample exposure. However, it is interesting that the animals approach the predator odor sample as often as the water sample, although the predator odor quadrants are avoided. Nevertheless, the data here clearly demonstrate that TMT functions as well as puma urine as avoidance inducing predator odor.

– *No significant effects on serum corticosterone levels after odor exposure were detectable*

Predator odors are cues for danger and generate stress responses in prey animal. The hypothalamic paraventricular nucleus (PVN) regulates hypothalamic-pituitary-adrenal axis (HPA) hormone secretion via neuronal projections to the median eminence of the hypothalamus {Sawchenko et al. 1996; Herman et al. 2002} and receives itself information from the medial amygdala (MeA) {Petrovich et al. 2001; Meredith & Westberry 2004; Pro-Sistiaga et al. 2007}. This activation of the HPA axis due to predator odor exposure is a well described response in rodents {Perrot-Sinal et al. 1999; Tanapat et al. 2001}. The increase in the corticosterone level leads to an augmented availability of glucose to promote a fight or flight reaction. At the same time metabolic processes are reduced {Tsigos & Chrousos 2002}. The serum corticosterone level rising is a frequently analyzed physiological consequence to predator odor {Vernet-Maury et al. 1984; Blanchard et al. 1998; Morrow et al. 2000; Day et al. 2004} and is also described as an indicator for (innate) fear {File et al. 1993; Whitaker & Gilpin 2015}. The results discussed here show a lack of serum corticosterone level increase in naive SD rats 90 min after exposure to predator urine samples (see Fig. 25), although the animals showed a significant avoidance response. Blanchard and colleagues took reliable blood samples 60 min after a stressful event {Blanchard et al. 1998}; Tinnikov 45 - 50 min after {Tinnikov 1999} and Thomas and colleagues even 2 h after the stressful event {Thomas et al. 2006}. Fuller and Snoddy describe a peak of serum corticosterone after 30 min which was transient after 4 h {Fuller & Snoddy 1980}. Thus, the selected time point for the blood sampling in the experiments in this thesis is reasonable. In line with the results of our analysis, File and colleagues describe non-rising corticosterone levels 30 min after the last of multiple exposures to predator odors but continuing avoidance behavior {File et al. 1993}. The authors suggest a less promptly habituation of the avoidance responses to a predator compared to response caused by

disturbances, which is an ethologically reasonable procedure. In contrast to the results of the experiment 2 in this thesis, Dias Soares and colleagues describe a contrary effect after exposure to TMT for 1 h. The tested animals showed an increase of serum corticosterone levels without displaying avoidance reactions in the following open field test {Dias Soares et al. 2003}. Notably, the behavioral reaction was not tested during TMT exposure, only afterwards. Thus, this experimental setup is only limited comparable to the experimental setup used in this thesis. It would as well be conceivable that our paradigm process itself provokes a corticosterone stress response to such an extent that the exposure to the TMT samples no longer raises this level. However, the animals experience a three-day habituation phase in order to minimize this stress experience. But since there were no blood samples taken and analyzed before the first habituation, it is only possible to speculate about this. Nevertheless, another reason for the lack of serum corticosterone level differences in the results here might as well be a premature decline of serum corticosterone in SD rats.

- *Different significant effects on the number of c-Fos immunopositive cells in amygdala and olfactory bulb after exposure to TMT and puma urine indicate odor depending activation patterns*

It was analyzed whether odor exposure induced changes in c-Fos immunoactivity between TMT and puma urine. In contrast to our working hypothesis we found significantly less c-Fos immunopositive cells in the amygdaloid nuclei MeAv and PLCo after exposure to predator odor compared to water sample presentation. In addition, the amount of c-Fos immunopositive cells after puma urine presentation was significantly less compared to TMT in the PLCo and the same non-significant trend was observed in the MeAv. Interestingly, results in the external plexiform layers of the olfactory (EPl) and accessory bulb (EPlA) and mitral cell layer of the accessory bulb (MIA) were different. Here the number of c-Fos immunopositive cells after TMT sample presentation was significantly lower than after water presentation. However, in the EPlA and MiA there were no such differences after exposure to puma urine sample. Additionally, in the EPl the number of c-Fos immunopositive cells after exposure to puma urine was almost as high as after water control test. Day and colleagues described a TMT specific pattern of c-Fos mRNA {Day et al. 2004}. Though, c-Fos protein expression was analyzed in different regions in this study. Another study from 2003 described specific c-Fos activation patterns after TMT exposure in the glomerular and granular layers of the olfactory bulb {Illig & Haberly 2003}. In our own experiments here we also observed changes in the olfactory bulb, but in the external plexiform layers (EPl) after exposure to TMT sample. The same changes in c-Fos

immunoactivity were found in the EPlA as well as in the MiA (see Fig. 26). Here the c-Fos immunoactivity pattern differed for the two odors: while TMT exposure decreased c-Fos immunoactivity compared to the water control test, c-Fos immunoactivity after puma urine exposure did not differ significantly from the water sample pattern. This indicates that the two odors activate a different set of neurons and therefore form a different activation pattern.

Though, the medial amygdala (MeA) seems to play a central role in freezing generation when it comes to intruders {Kalin et al. 2001; Etkin et al. 2004} and after TMT exposure. Temporal inactivation of this structure results in the complete absence of freezing {Müller & Fendt 2005}. Our findings suggest something different as contrary result was observed in the amygdaloid nuclei MeAv and PLCo (see Fig. 27). After exposure to puma urine c-Fos immunoactivity decreased significantly compared to TMT and water sample exposure, which indicates deactivation of both nuclei. This is in accordance to findings by Hebb and colleagues who found no difference in c-Fos expression after TMT exposure in the BLA and CeA, and also in the MeA, but in mice {Hebb et al. 2003}. In contrast, Asok and colleagues found increased c-Fos mRNA in the BNST, PVN and CeA after TMT exposure, but *in-situ* {Asok et al. 2013}. These data show different activation patterns for TMT and puma urine, which differ comparably to the pattern after water sample exposure. In contrast to the single compound TMT, puma urine contains a mixture of compounds, which highly depend on the predator´s diet. It is likely that these compounds show overlapping or even reversing effects of neuronal activation. Two sketches of the findings of experiment 2 are shown in Fig. 43A &B on the next page.

Taken together, both predator odor samples evoked avoidance behavior and the results confirm TMT as similar predator odor like puma urine. However, there was a lack of serum corticosterone level increase which indicates a lack of HPA activation. The data of the c-Fos/FluoroGold™ study suggest different activation patterns for TMT and puma urine which differ comparably to the pattern of the water sample exposure. The same was described before for TMT and cat odor {Staples & McGregor 2006; Staples et al. 2008}. Here TMT decreased the number of c-Fos immunoactive cells in the EPlA; puma urine increased the number of cells in this area. Contrary was found in the MeA and PLCo which means puma urine activated parts of the accessory olfactory system (AOS), whilst TMT did not.

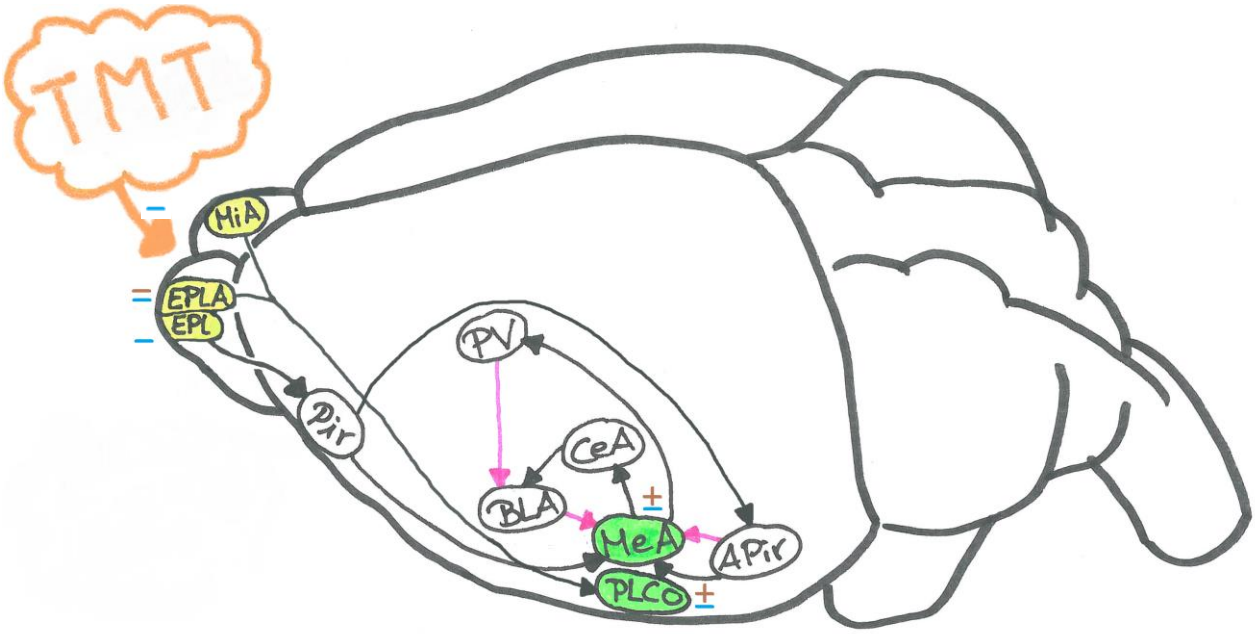

Fig. 43A: Sketch of a rat brain with the analyzed nuclei in their network after TMT exposure. The black arrows indicate already described neuronal connections; the pink arrows indicate the visualized and described connections in experiment 1 of this thesis. Plus and minus signs indicate the difference in number of c-Fos immunopositive cells compared to puma urine (brown) and water (blue).

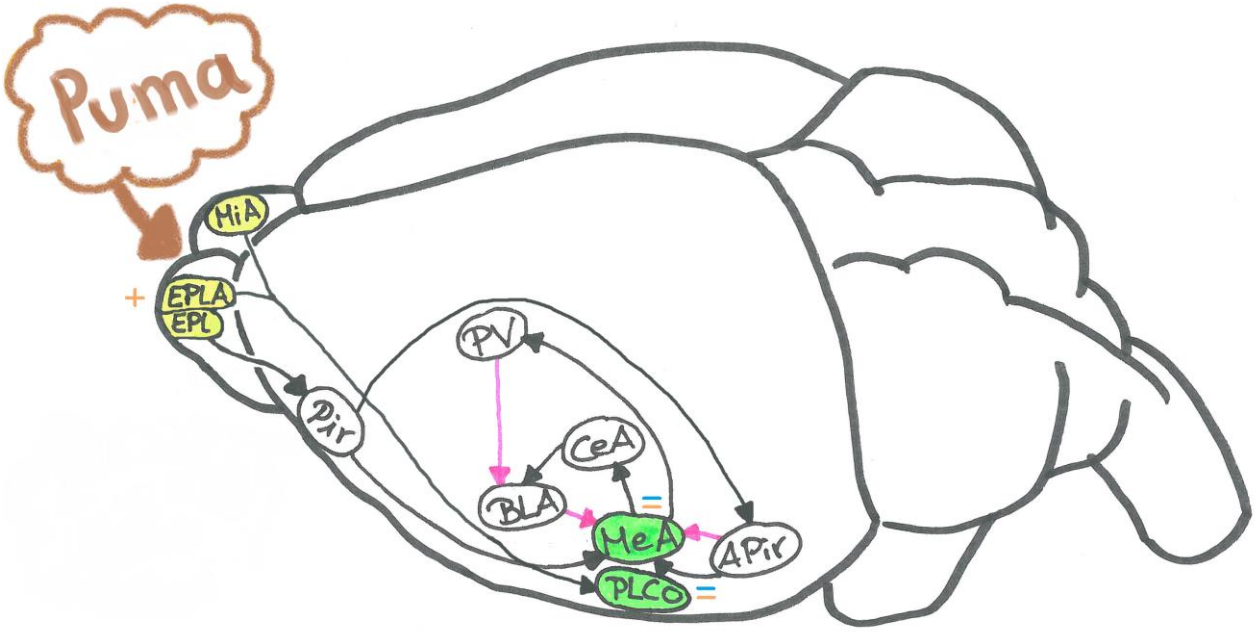

Fig. 43B: Sketch of a rat brain with the analyzed nuclei in their network after puma urine exposure. The black arrows indicate already described neuronal connections; the pink arrows indicate the visualized and described connections in experiment 1 of this thesis. Plus and minus signs indicate the difference in number of c-Fos immunopositive cells compared to TMT (orange) and water (blue). APir: amygdalo-piriform transition zone; BLA: basolateral amygdala; CeA: central amygdala; EPl: external plexiform layer of the olfactory bulb; EPlA: external plexiform layer of the accessory olfactory bulb; MeA: medial amygdala; MiA: mitral cell layer of the accessory olfactory bulb; Pir: piriform cortex; PLCo: posteriolateral cortical amygdala; PV: paraventricular thalamic nucleus.

4.5 Experiment 3: Different effects of TMT exposure in laboratory and wild rats

Plyusnina and colleagues reported about experiments on domestication effects in selected wild Norway rats and other groups took advantage of wild rats as well {Naumenko et al. 1989; Albert et al. 2008; Plyusnina et al. 2011}. These groups selected the animals on basis of their aggressiveness and established tame and aggressive lines over more than 70 generations. In contrast to the tame rats, the aggressive ones showed increased anxiety and decreased exploratory behavior. Thus, the aim of experiment 3 was to examine differences in behavioral and physiological reactions toward TMT in laboratory and wild rats to test the hypothesis that there are domestication effects with respect to innate fear responses. For this, Lister Hooded (LH) rats, a partially pigmented laboratory rat strain, and Warsaw Wild Captured Pisula Stryjek (WWCPS) rats, representing a wild-like rat strain, were compared. Domestication effects are mostly investigated by means of comparisons between laboratory and wild rats caught in the field {Price 1978; Takahashi & Blanchard 1982; Price 1984; Blanchard et al. 1986; Vernet-Maury et al. 1992; Bramley & Waas 2001; Boer et al. 2003}. This bears a methodological problem, i.e. that wild rats are most likely not predator naive and, due to their pre-experience, cannot be compared to laboratory rats, who have never been confronted with predators. The WWCPS experience no predator cues in the laboratory and the utilized F2 and F3 generations are animals which never encountered a predator cue before and behave in an innate way when exposed to predator odors.

– *Significant differences during habituation session between WWCPS and LH*

The first main differences between the two rat strains became clear during the habituation session. WWCPS showed a strong preference of area AB and avoidance of area CD whilst LH showed the opposite (see Fig. 29). LH rats live in transparent cages since the 1920s {Velaz 2013a}. In the habituation sessions the transparent wall seemed not to be aversive for this rat strain and maybe even attractive due to positive linkage to food, water or clean bedding. In contrast, the WWCPS avoided the transparent wall and seemed to seek for shelter just like they would in the wilderness. This might be a reaction to the movement of the leaving experimenter who is identified as potential predator. It could also be a reaction to reflection of the mirror image on the testing box wall, which WWCPS might sense better compared to LH. This would fit to observations of several groups describing obvious differences to laboratory rat strains in terms of anatomy, agility, sensory capabilities, and behavior {Castle 1947; Robinson 1965; Lockard 1968}. These publications indicate that the environment in laboratories works as selector of behavioral repertoire, which might also be

true for the response to transparency and reflections. But the avoidance of the transparent wall might as well be a reaction to novelty.

– *Significant different behavioral responses of WWCPS and LH to TMT exposure*

Further analysis showed a robust avoidance of the TMT samples in both rat strains (see Fig. 31). A more detailed analysis of the quadrant preference during sample exposure revealed different avoidance strategies of the two rat strains. Whilst WWCPS showed no clear preference of one or more non-odor quadrants, LH rats significantly stayed in the quadrants farthermost away from the predator odor source (see Fig. 32). This strain effect can also be seen in the locomotor activity parameters. The WWCPS covered only about half the distance which was covered by LH rats and spend only 1/3 of the time in mobile state (see Fig. 33). However, when moving, the velocity of the WWCPS was 1/3 higher (see Fig. 35). Takahashi and Blanchard described a different behavioral spectrum in wild rats when it comes to intruders and fights among opponents of the same pack {Takahashi & Blanchard 1982}. Appropriately, Blanchard and colleagues observed a fixed behavioral attack pattern in albino rats {Blanchard et al. 1977}. Stryjek and colleagues found significant differences in swimming behavior in WWCPS compared to three different laboratory strains {Stryjek et al. 2012}. Additionally, only WWCPS showed diving attempts which could be a sign of stronger curiosity and high risk tolerance {Stryjek et al. 2012}. Some studies were published on neophobia in laboratory rats which showed that these rats approach to a novel stimulus and explore {Barnett 1958; Barnett et al. 1975; Pisula 2003; Whishaw et al. 2004; Cavigelli et al. 2011}, at least in unstressed situations. In contrast to that, no such intense approach was found in WWCPS rats {Tanaś & Pisula 2011}. This clearly reveals opposite strategies to cope with novelty or potential predation and is supported by the results of the habituation session (see Fig. 30). Our findings on locomotor behavior support this theory. Though, TMT does not affect the distance the rats cover during the odor test, nor the time the animals spent mobile or the velocity. But every analyzed locomotor behavior is influenced by significant strain differences. More precisely, WWCPS walk approximately half of the distance LH rats walk and spent less time in mobile state. However, WWCPS move noticeably faster than LH. Taken together, these data (see Fig. 33, Fig. 34 & Fig. 35) confirm a more strategic and multilayered exploratory behavior of the WWCPS in general as indicated earlier {Stryjek et al. 2012; Tanaś & Pisula 2012}.

– *Significant differences in freezing response: WWCPS freeze less often but overall longer*

To obtain more information about possible differences in intensity of fear we analyzed freezing as well as grooming behavior. The analysis of freezing behavior revealed significant differences between the two strains: a significant difference in the total freezing time was found. In addition, only LH showed an increased number of freezing events when exposed to the TMT sample, whereas the WWCPS showed a base level of freezing during water sample presentation almost as high as displayed by LH rats during TMT sample exposure. LH rats showed freezing behavior to the water sample for about 20 sec and to the TMT sample about 90 sec (see Fig. 36). Asok and colleagues reported on freezing behavior in SD rats during 10 min TMT sample exposure {Asok et al. 2013}. These rats showed freezing for about 260 sec during the TMT exposure test which indicates a quite weak freezing response from the LH in the experiments described here. Nevertheless, total freezing duration of WWCPS during exposure to TMT sample was 2.5 times longer compared to the LH rats, meaning about 220 sec. During water sample exposure the WWCPS showed freezing behavior for as long as the LH during TMT exposure which is represented by the general strain effect without interaction. Interestingly, LH rats showed 5 times more freezing events during exposure to TMT compared to the water control exposure (see Fig. 37). Finally, the analysis of the duration of one average freezing event revealed that WWCPS froze for about 25 sec during water sample exposure and for about 60 sec when exposed to the TMT sample. The LH, in contrast, barely froze more than 5 sec, independent of the sample, which is 8 times shorter than in WWCPS (see Fig. 38). These rats showed freezing during TMT exposure as well as during water sample exposure. But here differences in the duration of one single freezing event were found which supports the hypothesis of different anti-predator strategies in LH and WWCPS rats. Apparently, WWCPS rats display a freezing response to novelty in general but a different freezing response to a predator threat, which suggests an additional role for freezing behavior in these rats. Freezing, defined as the absence of all movement except breathing, is a secondary defense mechanism of prey animals {Apfelbach et al. 2005}. The objective of this behavior is to be worse detectable by predators, which are usually very sensitive to moving stimuli. A possible explanation might be that in WWCPS rats freezing behavior seems to facilitate precise observation of the environment to evaluate the situation and consider next steps. Taken together these findings support a theory of a multilayered exploratory behavior in the WWCPS which seems to have changed in laboratory rat strains by domestication progresses.

The analysis of grooming showed similar results. The WWCPS show strikingly more grooming events and the average grooming event lasted about 3 times longer than in the LH (see Fig. 39 & Fig. 40).

Though, an odor effect and interaction were only evident in the analysis of freezing behavior. Although, the absence of grooming can be interpreted as indicator of fear {Dielenberg et al. 2001b}, but the presence might not necessarily indicate fearlessness. Exposure to TMT samples did not change the grooming behavior compared to water sample exposure; neither in LH, nor in WWCPS rats. Though, a significant strain effect was found (see Fig. 39 & Fig. 40). The significant longer grooming behavior in WWCPS rats is likely to be a displacement activity {Cohen et al. 1979; Pleskacheva 1996} due to extreme stressful experience, which indicates a very high stress level in the WWCPS, during water sample as well as during TMT sample exposure. Although, it is also reported that WWCPS show no increase in grooming during the appearance of novel food which causes food neophobia {Modlinska et al. 2015}. Nevertheless, it would be very interesting to analyze the microstructure of the grooming behavior {Kalueff et al. 2016}. Maybe rat strains differ in cephalocaudal sequence.

- *Serum corticosterone level increase after TMT exposure only in WWCPS*

Another main effect of strain could be found after analysis of serum corticosterone levels. Certainly the analysis of serum corticosterone levels revealed quite surprising results. TMT sample exposure lead to an increase of serum corticosterone, as expected. But this was the case in WWCPS rats only (see Fig. 41). The serum corticosterone levels of WWCPS rats were doubled after TMT exposure whilst the serum corticosterone levels of LH rats did not change, although both strains showed avoidance reactions in the behavioral analyses. Here it seems that the duration of an average freezing event correlates with the corticosterone levels. Accordingly, Plyusnina and colleagues reported of increased corticosterone plasma levels in wild rats after stress {Plyusnina et al. 2011}. Also rising corticosterone levels after predator exposure were described beforehand {e.g. File et al. 1993; Whitaker & Gilpin 2015} but no change in corticosterone levels was found in the experiments with SD rats in experiment 2 of this thesis (see Fig. 25). What strikes the eye here is the allegedly difference in corticosterone levels after water sample exposure tests. LH rats had a serum corticosterone level of about 230 ng/mL and WWCPS about 300 ng/mL. Although these levels are not significantly different, it might be a hint that the testing procedure itself leads to an increase of basis corticosterone levels in WWCPS. To prevent this and to minimize the stress, habituation sessions were performed. It is conceivable, that three sessions were not enough to habituate the WWCPS rats. Either way, a corticosterone level increase during TMT exposure was observed in WWCPS, but not in LH. As described before, the HPA axis is activated by predator odors to promote potential lifesaving

responses {Vernet-Maury et al. 1984; Blanchard et al. 1998; Perrot-Sinal et al. 1999; Morrow et al. 2000; Tanapat et al. 2001; Day et al. 2004}. The findings here show a pronounced defensive behavior in WWCPS and support the theory of a stronger preparedness for a fight or flight reaction.

Taken together these data show clear differences between the two rat strains WWCPS and LH, not only on a behavioral level but also on a physiological one. The results indicate that innate defensive strategies, mostly freezing, and physiological responses to predator cues are affected by domestication effects.

4.6 Conclusion and prospects

The results of experiment 1 confirmed the avoidance inducing property of fox urine in the square shaped box paradigm. In addition, it was examined whether the order of exposures to either predator odor or control stimulus influence the behavior response. Indeed, the neutrality of the control stimulus is affected. Furthermore, the tracer and c-Fos colocalization study confirmed axonal connections from BLP and APir to the MeAv, from BLP to the PV and from the habenula to the septum.

The results of experiment 2 validated the avoidance inducing property of TMT as similar to the avoidance inducing capability of puma urine. Though, the corticosterone levels showed no increase; neither after TMT, nor after puma urine exposure. These findings support the similarity of TMT to predator urine. Accordingly, the c-Fos activity study showed similar amounts of c-Fos immunopositive cells in the EPlA after exposure to TMT and puma urine. Though, different amounts in the EPl, MeAv and PLCo were found. This supports the theory of odor depending neuronal activity patterns as claimed by several studies {e.g. Guthrie & Gall 1995}.

The experiments of experiment 3 were performed in the laboratories of our collaboration partner in Warsaw, Poland. The results showed differences of the behavioral and corticosterone responses towards TMT between semi-wild and laboratory rats. The semi-wild WWCPS rats displayed a different defensive strategy during TMT exposure and habituation session as well. These rats showed, in contrast to LH, striking freezing responses and increased levels of corticosterone.

Taken together, the results in this thesis confirm the property of predator odors as avoidance inducing stressor. The results show similar innate avoidance behavior caused by exposure to fox urine, puma urine and TMT. However, slight differences in the c-Fos expression and tracer study indicate a neuronal discrimination of the different aversive odors which results in the same behavioral output. As stated before, Ferrero and colleagues described the chemical PEA to be present in predator odors {Ferrero et al. 2011}. However, experiments in our NeuroNetwork with the single compound PEA did not show striking behavioral responses {Wernecke 2015}. Taken this into account, the results of this thesis indicate the importance of a defined mixture of compounds to evoke robust avoidance reactions in laboratory rats as well as the particularity of TMT as single predator odor compound. This is supported by the results of experiment 3. The behavioral responses displayed by LH and WWCPS rats during TMT exposure and the serum corticosterone analysis reveals considerable differences between the two rat strains. It might be possible that the sensibility to predator odors slowly vanishes the longer it is not of use or inbreeding is practiced. Though, one should not neglect

that laboratory rats are bred for purposes other than to survive in the wild. The results described in this thesis indicate domestication effects which affect the sensitivity of laboratory rats to predator odors and the neuronal circuits processing this sensory information. The further analysis of the collected brain samples will give more information about the underlying neuronal circuitries and how these differ among the rat strains. The c-Fos immunostaining analysis is ongoing as well as protein isolation and the results described here are currently prepared for publication.

In general it might be insightful to analyze correlations of c-Fos expression patterns and behavioral expressions. Certainly there are responding and non-responding rats among the WWCPS as well as LH or SD. An expanding c-Fos immunoactivity analysis and comparison to further IEG (e.g. Arc, Egr1) would be possible by using the stored brain slice series two and three. Furthermore, it would be interesting to analyze the participating neurons more detailed with regard to neuronal transmitters and receptors. This kind of analysis would characterize the underlying neuronal connections and circuitries in a detailed way regarding excitation, inhibition, convergence or divergence, just to name a few.

To obtain more detailed information about temporal shifts of physiological reactions towards predator odors, an exact time course analysis of the serum corticosterone levels would be necessary. In particular this includes the collection of basal level serum samples. This procedure might be possible in tame laboratory rats without any form of anesthesia. In WWCPS this would not be manageable and the animals need to be anaesthetized which would surely affect the results.

However, a more detailed analysis of the behavioral responses will be conducted in the future. Earlier, unpublished analyses from Dr. Kerstin Wernecke and myself indicated a time depending strategy change during predator odor exposure. It seems that the rats first approach toward the predator odor source and then start to avoid this area. Thus, it will be necessary to analyze time blocks of behavior. Such analyses are currently ongoing and might support the theory of different anti predator strategies among the rat strains.

A concrete connection to the experiments of the first part could be an expansion of the experimental setup with one more test day. At this third test day either the first presented sample or an appetitive stimulus could be presented. It would be interesting to see to which extend previous exposures to odors have an impact on the response to a positive stimulus. It is conceivable to see depression like behaviors which could be analyzed in further adjusted paradigms. In this context a precise analysis of the microstructure of the grooming behavior as described by Kalueff and colleagues would be

interesting {Kalueff et al. 2016}. Maybe rat strains differ in sequences and express stress in different typed of grooming.

Fear and anxiety manifest in many forms and have an important role in maintaining survival. But whenever anxiety becomes pathologic it is important to provide effective help to those affected. The possibilities are manifold and range from drug administration to behavioral therapy. A fairly recent publication from Koek and colleagues describes a Phase I pilot study of a deep brain stimulation therapy for post-traumatic stress disorder {Koek et al. 2014}. In this study patients receive deep brain stimulation of the basolateral amygdala, a nucleus which was also analyzed in this thesis. Basic research, like the one described here, provides important insights and fundamental knowledge for these studies and therapies.

5 Attachments

5.1 Corticosterone in-house radioimmunassay

from Prof. Markus Fendt, Institute for Pharmacology and Toxicology, Otto-von-Guericke-University, Magdeburg, Germany

Preparation of Reagents

Steroid Assay Buffer (SAB):			**2 Liter**	**4 Liter**
Sodium phosphate, dibasic, anhydrous	Na_2HPO_4	MW: 142	17.4g	34.8 g
Sodium phosphate, monobasic, monohydrate	$NaH_2PO_4.H_20$	MW: 138	10.8g	21.6 g
Sodium azide	NaN_3	MW: 65	2.0g	4.0 g
Sodim chloride	NaCl	MW: 58	18.0g	36.0 g
Gelatine (medium Gel strength, 180 g Bloom)			2.0g	4.0 g

Warm 1L deionized water to 50°C.
Dissolve gelatine.
Add salts and dissolve.
Fill up to 2 Liter/ 4 Liter with destilled water.
Once buffer has reduced to room temperature, add NaN3.
Adjust pH to 7.0±0.1.
Store at 4°C.

Corticosterone Standard

Sigma C-2505 MW: 346.5 Standart Verdünnung 29.01.03/emp Std 1, 2, 3

*Stock **1***: Weigh out 10.4mg (0.0104g)
Add to 30mL ethanol abs. = **10^{-3}M**
Store at -20°C
(10^{-3}M = 0.346g/L = 346mg/L = 346,000µg/L = 346µg/mL = 346,000ng/mL)

*Stock **2**:* **Standard 7**: 11.6 µL from Stock **1** + 1000.00 mL SAB= 11.6*10^{-9}M = **1000 pg/250 µL**

*Stock **3**:*

Standard 6:	50.0 mL Stock **2** +	50.0 mL SAB	= **500 pg /250 µL**
Standard 5:	10.0 mL Stock **3**+	10.0 mL SAB	= **250 pg/250 µL**
Standard 4:	5.0 mL Stock **3** +	15.0 mL SAB	= **125 pg/250 µL**
Standard 3:	2.0 mL Stock **3** +	18.0 mL SAB	= **50 pg/250 µL**
Standard 2:	1.0 mL Stock **3** +	19.0 mL SAB	= **25 pg/250 µL**
Standard 1:	0.5 mL Stock **3** +	19.5 mL SAB	= **12.5 pg/250 µL**

Corticosterone Tracer

[1, 2, 6, 7-3H] Corticosterone
Amersham TRK 406 B82: 2.59 Tbq/mmol, 70.0 Ci/mmol
Stored in ethanol:toluene at -20°C
Pipette 20µL into a Schott bottle and stand in fume hood to evaporate solvent
Add 100mL SAB: 100µL in 4mL scintillation fluid = 12 400cpm
Store at 4°C

Corticosterone Antibody

Rabbit anti-corticosterone-3-carboxymethyloxime: BSA
ICN 07120016
Lyophilised antibody: Store at -20°C
Add 1mL distilled or millipore-filtend water (not SAB) and stand for 60min at room temperature
Pipette 100µL aliquots into 10mL plastic tubes
Store at -20°C
(Prior to use add 7mL SAB: final titre = 1: 6 720)

Dextran-coated charcoal (1%)

Activated Charcoal	1.0g
Dextran	0.1g
SAB	100mL

Store at 4°C (3 months maximum)

Preparation of Plasma Samples and RIA Incubation

Day 1

1. Prepare protocols, "RIA Protocol Sheet" and "RIA Protocol Sheet I"
2. Anti-corticosterone:
    a. Remove 1 aliquot from -20°C freezer, write date on tube, allow to thaw, add 9mL Steroid Assay Buffer, secure to rotor mixer and allow to reconstitute for 30-60min
3. Samples and Quality control
    a. Remove samples from -20°C or -80°C freezer and allow to thaw at room temperature
    b. Remove quality control (QC) from -20°C or -80°C freezer and allow to thaw at room temperature
4. Vortex on IKA Multi-tube vortexer for 20 sec.
5. Centrifuge at 2000rpm for 20sec
6. Dilution step:
    a. Using Eppendorf tubes add assay buffer and then sample:
        - Rat: Basal samples = 1 in 100, i.e. 10µL sample + 990µL SAB
        - Stress samples = 1:400, i.e. 2.5µL sample + 1000µL SAB
        - Mouse: Basal samples = 1 in 100, i.e. 10µL sample + 990µL SAB
        - Stress samples = 1:400, i.e. 2.5µL sample + 1000µL SAB
    b. Using Eppendorf tubes add assay buffer and then QC:
        Low QC = 1:400, i.e. 3µL sample + 1200µL SAB
        High QC = 1:100, i.e. 12µL sample + 1200µL SAB
7. Vortex on IKA Multi-tube vortexer for 60sec
8. Binding protein denaturation:
    a. Switch on water bath, fill to required level (with "vollentsalztes" water), set thermostat at 90°C and cover with polystyrene to retain heat during heating.
9. Number glass assay tubes in polypropylene rack:
    a. NB. Standard curve and samples in duplicate = 1-20
    b. Maximum 50 samples, plus QCs at beginning and end of sample run = 21-128
10. Add buffer: Totals = 400µL SAB, NSB = 300µL SAB, B0 = 250µL SAB
11. Add standard and samples: 250µL
12. Binding protein denaturation:
    b. Add tubes in polypropylene rack to water bath and heat for 10min using timer
    c. At end of 10min remove rack, and allow 5 min for cooling or centrifuge for 2 min. at 4^0C
13. 3H-corticosterone: Add 100µL to all tubes using Eppendorf multipipette.
14. Anti-corticosterone:
    b. Add 50µL to all tubes except Totals and NSB's using Eppendorf multipipette
15. Incubation I = competitive steroid-antibody binding:
    a. Quick-vortex all tubes, cover with parafilm and alu-foil and place in refrigerator at 4°C
    b. Incubate for 18-24h, minimum-maximum

Day 2

16. Incubation II = charcoal separation of bound and nonbound hot/cold corticosterone:

a. Remove dextran-coated charcoal (DCC 1.0%) from refrigerator, place in ice on magnetic stirrer and cool and mix for 10min

b. Remove first rack of assay tubes from refrigerator and place on ice

c. Add 100µL DCC 1.0 % all tubes except totals (tubes 1-2) using Eppendorf multipipette

d. Start timer to count 20min

e. Quick-vortex all tubes and incubate on ice in refrigerator

f. Pre-cool centrifuge to 4°C

g. Centrifuge all tubes at 2500rpm for 15min at 4°C

h. Remove tubes back into rack on ice

i. Repeat steps b-h with second rack during centrifugation of tubes from first rack

17. Counting radioactivity:

a. Number scintillation vials (5mL) with numbers corresponding to assay tubes

b. Using a pipette, remove 400µL of the supernatant and pipette on to scintillation vials

c. Add 4mL scintillation cocktail to all vials

d. Cap vials & Shake capped-vials vigorously

e. Place tubes in counter, allow 1h minimum for equilibration, and count for 3min/tube

Quick check for Corticosterone

Corticosterone-antibody: Aliquot [100µl (1:12)] + 7 mL SAB and to equilibrate on stir-table 30-60 min.
Predilution of QC's and Sample:
QCL Neu ab 4. Juli 2002: QCL Plasma (Pool1) 70% B/BO = 46 pg/tube = 74 ng/mL plasma
QCH Neu ab 4. Juli 2002:QCH Plasma (Pool5) 34% B/BO = 245 pg/tube = 392 ng/mL plasma
QCA 6.0 µl +1.2 mL SAB ⇒ 250 µl (1:200)
QCL 2.0 µl + 1.2 mL SAB ⇒ 250 µl (1:600)
QCH 3.0 µl +1.2 mL SAB ⇒ 250 µl (1:400)
& Plasma Sample 2.5 µl + 1.0 mL SAB ⇒ 250 µl (1:400)

Incubation: waterbath 90 ^{0}C for 10 min., 2 min. centrifuge (Programm 1) and Incubation 16-24 h at 4^0 C

Grammalogue	**In duplicat**	**1. SAB-buffer**	**2. Standart/ Sample**	**WATER-BATH 90^{0}C**	**4. Corticoste.-Tracer**	**5. Anti-Coticoste.**	**1% DCC**	Volumen abpipettieren
Valide Date					1.3.2002	6.3.2002		
T	Totals	400µl		10 Min.	100µl			400µl
N	NSB	300µl		10 Min.	100µl		100µl	400µl
B	Standard 0	250µl		10 Min.	100µl	50µl	100µl	400µl
S1 - S7	Standard 1-7		250µl	10 Min.	100µl	50µl	100µl	400µl
A	QCA		250µl	10 Min.	100µl	50µl	100µl	400µl
L	QCL		250µl	10 Min.	100µl	50µl	100µl	400µl
H	QCH		250µl	10 Min.	100µl	50µl	100µl	400µl
1-…	Samples		250µl	10 Min.	100µl	50µl	100µl	400µl

5.2 Rat list

B = Behavior recorded; C = Corticosterone level; I = Immunohistochemistry; T = Tracer injection

Experiment	Ratnumber	Strain	Test substance	Involvement
Experiment 1	R1	SD	Fox urine	T + I
Experiment 1	R2	SD	Water	T + I
Experiment 1	R3	SD	Fox urine	T + I
Experiment 1	R4	SD	Water	T + I
Experiment 1	R5	SD	Water	I
Experiment 1	R6	SD	Fox urine	B + T + I
Experiment 1	R8	SD	Fox urine	B + T + I
Experiment 1	R9	SD	Water	B + T + I
Experiment 1	R10	SD	Water	B + T + I
Experiment 1	R11	SD	Water	B + T + I
Experiment 1	R12	SD	Fox urine	B + T + I
Experiment 1	R13	SD	Water	B + T + I
Experiment 1	R14	SD	Fox urine	B + T + I
Experiment 1	R15	SD	Water	B + T + I
Experiment 1	R16	SD	Fox urine	B + T + I
Experiment 1	R17	SD	Water	B + T + I
Experiment 1	R18	SD	Water	B + T + I
Experiment 1	R19	SD	Fox urine	B + T + I
Experiment 1	R20	SD	Fox urine	B + T + I
Experiment 1	R21	SD	Fox urine	B + T + I
Experiment 1	R22	SD	Fox urine	B + T + I
Experiment 1	R23	SD	Water	B + T + I
Experiment 1	R24	SD	Water	B + T + I
Experiment 1	R25	SD	Water	B + T + I
Experiment 1	R26	SD	Water	B + T + I
Experiment 1	R31	SD	Fox urine	B + T + I
Experiment 1	R32	SD	Water	B + T + I
Experiment 1	R33	SD	Fox urine	B + T + I
Experiment 1	R34	SD	Water	B + T + I
Experiment 1	R35	SD	Fox urine	B + T + I
Experiment 1	R36	SD	Water	B + T + I
total	**31 Rats**			

Experiment	Ratnumber	Strain	Test substance	Behavior recorded
Experiment 2	R37	SD	TMT	I
Experiment 2	R38	SD	TMT	I
Experiment 2	R39	SD	TMT	I
Experiment 2	R40	SD	Water	I
Experiment 2	R41	SD	Puma urine	I
Experiment 2	R42	SD	Puma urine	I
Experiment 2	R43	SD	Puma urine	I
Experiment 2	R44	SD	Water	I
Experiment 2	R53	SD	Water	B + C
Experiment 2	R54	SD	Water	B + C
Experiment 2	R55	SD	Puma urine	B + C
Experiment 2	R56	SD	Puma urine	B + C
Experiment 2	R57	SD	TMT	B + C
Experiment 2	R58	SD	TMT	B + C
Experiment 2	R59	SD	Water	B + C
Experiment 2	R60	SD	Water	B + C
Experiment 2	R61	SD	Puma urine	B + C
Experiment 2	R62	SD	Puma urine	B + C
Experiment 2	R63	SD	Puma urine	B + C
Experiment 2	R64	SD	TMT	B + C
Experiment 2	R65	SD	TMT	B + C
Experiment 2	R66	SD	TMT	B + C
Experiment 2	R67	SD	Water	B + C
Experiment 2	R68	SD	Puma urine	B + C
Experiment 2	R69	SD	Puma urine	B + C
Experiment 2	R70	SD	TMT	B + C
Experiment 2	R71	SD	TMT	B + C
Experiment 2	R72	SD	TMT	B + C
Experiment 2	R73	SD	TMT	B + C
Experiment 2	R74	SD	Water	B + C
Experiment 2	R75	SD	Water	B + C
Experiment 2	R76	SD	Water	B + C
Experiment 2	R77	SD	Puma urine	B + C
Experiment 2	R78	SD	Puma urine	B
Experiment 2	R79	SD	Puma urine	B
Experiment 2	R80	SD	TMT	B + C

Experiment	Ratnumber	Strain	Test substance	Behavior recorded
Experiment 2	R81	SD	Water	I
Experiment 2	R82	SD	Water	I
Experiment 2	R83	SD	Puma urine	I
Experiment 2	R84	SD	Puma urine	I
Experiment 2	R85	SD	Puma urine	I
Experiment 2	R86	SD	TMT	I
Experiment 2	R87	SD	TMT	I
Experiment 2	R88	SD	TMT	I
total	**44 Rats**			
Experiment 3	P1	LH	Water	B + C
Experiment 3	P2	LH	Water	B + C
Experiment 3	P3	LH	Water	B + C
Experiment 3	P4	LH	Water	B + C
Experiment 3	P5	LH	Water	B + C
Experiment 3	P6	LH	Water	B + C
Experiment 3	P7	LH	Water	B + C
Experiment 3	P8	LH	Water	B + C
Experiment 3	P9	WWCPS	Water	B + C
Experiment 3	P10	WWCPS	Water	B + C
Experiment 3	P11	WWCPS	Water	B + C
Experiment 3	P12	WWCPS	Water	B + C
Experiment 3	P13	WWCPS	Water	B + C
Experiment 3	P14	WWCPS	Water	B + C
Experiment 3	P15	WWCPS	Water	B + C
Experiment 3	P16	WWCPS	Water	B + C
Experiment 3	P17	LH	TMT	B + C
Experiment 3	P18	LH	TMT	B + C
Experiment 3	P19	LH	TMT	B + C
Experiment 3	P20	LH	TMT	B + C
Experiment 3	P21	LH	TMT	B + C
Experiment 3	P22	LH	TMT	B + C
Experiment 3	P23	LH	TMT	B + C
Experiment 3	P24	LH	TMT	B + C
Experiment 3	P25	WWCPS	TMT	B + C
Experiment 3	P26	WWCPS	TMT	B + C

Experiment	Ratnumber	Strain	Test substance	Behavior recorded
Experiment 3	P27	WWCPS	TMT	B + C
Experiment 3	P28	WWCPS	TMT	B + C
Experiment 3	P29	WWCPS	TMT	B + C
Experiment 3	P30	WWCPS	TMT	B + C
Experiment 3	P31	WWCPS	TMT	B + C
Experiment 3	P32	WWCPS	TMT	B + C
total	**32 Rats**			
NaCl-Test	R45 / NaCl #1	SD	0,9 % NaCl	I
NaCl-Test	R46 / NaCl #2	SD	0,9 % NaCl	I
NaCl-Test	R47 / NaCl #3	SD	2M NaCl	I
NaCl-Test	R48/ NaCl #4	SD	2M NaCl	I
total	**4 Rats**			
TOTAL	**111 Rats**			

5.3 Chemicals, Proteins and Antibodies

Chemicals, Proteins and Antibodies	Manufacturer
2-methylbutane	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
3,3-Diaminobenzidine (DAB)	Sigma-Aldrich Chemie GmbH, Munich, Germany
Anti-C-Fos Antibody (Ab-5) (4-17) Rabbit pAB (Cat.No. PC38)	Calbiochem / Merck KGaA, Darmstadt, Germany
Artificial tear fluid (Vidisic® Gel)	Bausch & Lomb GmbH, Berlin, Germany
Aseptic 0,9 % NaCl	Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany
Biotinylated Anti-rabbit IgG	Vector Laboratories, Burlingame, California, USA
Bovine serum albumin (BSA)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
C-Fos ELISA kit ab108821	Abcam, Cambridge, United Kingdom
di-Sodiumhydrogenphosphate-Dihydrat	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Elite ABC Kit (ABC)	Biozol Diagnostica Vertrieb GmbH, Eching, Germany
Ethanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethylenglycole	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
FluoroGold™	Fluorochrome, Denver, Colorado, USA
Fox urine (*Vulpes vulpes*) and puma urine (*Puma concolor*)	Maine Outdoor Solutions Inc., Hermon, Maine, USA

Glycerine	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
H_2O_2	Sigma-Aldrich Chemie GmbH, Munich, Germany
Isoflurane	Baxter Deutschland GmbH, Unterschleißheim, Germany
Ketamine (Ketavet® 100 mg / mL)	Zoetis Deutschland GmbH, Berlin, Germany
Kresyl violet	Sigma-Aldrich Chemie GmbH, Munich, Germany
Lidocaine (Xylocain® Gel 2 %)	AstraZeneca GmbH, Wedel, Germany
Methanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Normal goat serum (NGS)	Biozol Diagnostica Vertrieb GmbH, Eching, Germany
Paraformaldehyde	Sigma-Aldrich Chemie GmbH, Munich, Germany
Pentobarbital-Natrium (Narcoren®)	Merial GmbH, Hallbergmoos, Germany
Picric acid 1.2 % (saturated)	Dr. K. Hollborn & Söhne GmbH & Co. KG, Leipzig, Germany
Roti®-Histofix 4 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodiumdihydrogenphosphate-Monohydrat	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sucrose / D(+) Saccharose	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
surface disinfection spray (Octenisept®)	Schülke & Mayr GmbH, Norderstedt, Germany
TMT (2,5-dihydro-2,4,5-trimethylthiazoline)	Contech Enterprises Inc., Victoria, Canada / Pherotech International Inc., British Columbia, Canada
Triton X-100	Merck KGaA, Darmstadt, Germany,
TSA-Rhodamine	Custom made {Hopman et al. 1998}
Xylazin (Rompun 2 %)	Ceva Tiergesundheit GmbH, Düsseldorf, Germany

5.4 Solutions and buffers

Solutions and buffers	**Compounding**
Acetate buffer	1 part 2.72 % Sodium acetat trihydrate 4 parts 1.2 % Acetic acid
Avidin-biotin-complex (AB-Complex)	per 1 mL PB+T 8.8 µL solution A 8.8 µL solution B
Blocking solution	10 % BSA in PB+T 10 % NGS

Cryoprotective solution	30 % Ethylenglycole 25 % Glycerine 0.05 M PB
DAB solution	5 % DAB in PB+T Activation with 1.1 % H_2O_2 (0.3 %)
Immunfix	500 mL 0,2 M PB 40 g 4 % Paraformaldehyde heated up (75 °C) 350 mL Aqua dest. cool down 150 mL 15 % saturated Picric acid
Kresyl violet staining solution	0.5 g Kresyl violet 100 mL Acetat buffer (pH 3.8-4.0)
PB+T 0.1 M	PB 0.3 % Triton X-100
Phosphate buffer 0.2 M (PB)	PB 1 filled up with PB 2 until pH 7.35- 7.4
Phosphate buffer solution 1 (PB 1)	35,6 g di-Sodium hydrogenphosphate dihydrate 1000 mL Aqua dest.
Phosphate-Buffer Solution 2 (PB 2)	8,28 g Sodium dihydrogenphosphate monohydrate 300mL Aqua dest.
POX-Block solution	5 % Methanol 1 % H_2O_2 (30 %) in PB+T

5.5 Material, machines & equipment

Material / Machines / Equipment	**Manufacturer**
acA1300-30gc GigE-Camera (fume hood)	Basler AG, Ahrensburg, Germany
AxioVision Imaging software 4.8 with AxioCam	Carl Zeiss, Jena, Germany
Borosilicate glass capillaries (outer Ø 1.6mm)	Hilgenberg GmbH, Malsfeld, Germany
CCTV-Camera Miniature-CCD-Camera (roof)	RS Components GmbH, Mörfelden-Walldorf, Germany
Circulatory shaker 3005	GFL GmbH, Burgwedel, Germany.
Coverslips Menzel 24 x 60 mm	Thermo Fisher Scientific, Waltham, Maine, USA
Cryostat CM 3050 S	Leica Biosystems Nussloch GmbH, Nussloch, Germany
Dental drill	Nouvag AG, Goldach, Switzerland

Electric shaver	B. Braun Vet Care GmbH, Tuttlingen, Germany
Entellan®	Merck KGaA, Darmstadt, Germany
Eppendorf pipettes	Eppendorf AG, Hamburg, Germany
Eppendorf safe lock tubes	Eppendorf AG, Hamburg, Germany
EthoVision Version XT 10	Noldus, Wageningen, The Netherlands
Glass cuvette	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
GraphPad Prism Version 6.03	GraphPad Software, Inc., La Jolla, California, USA.
Guillotine DCAP-M	World Precision Instruments, Sarasota, Florida, USA
Axioplan 2 Fluorescence Microscope	Carl Zeiss, Jena, Germany
Micropipette puller Model P-97	Sutter instruments Co., Novato, California, USA
MiniSpinPlus centrifuge	Eppendorf AG, Hamburg, Germany
Sound-attenuating chamber with a light beam frame for movement detection (TSE-Multi Conditioning System)	TSE Systems, Bad Homburg, Germany
ELISA - Reader	Tecan Group Ltd., Männedorf, Switzerland
Pericyclic pump Cyclo I	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Standard laboratory feed for WWCPS	Labofeed H, WP Morawski, Kcynia, Poland
Stereotaxic frame	David Kopf Instruments, Tujunga, California, USA
SuperFrost™ Plus object slides	Thermo Fisher Scientific, Waltham, Maine, USA
Surgical instruments	Aesculap, Tuttlingen, Germany
Freezer Hera freeze, -80°C, -20°C	Thermo Fisher Scientific, Waltham, Maine, USA
Tissue freezing medium	Leica Biosystems Nussloch GmbH, Nussloch, Germany
Vortex Reax2000	Heidolph, Schwabach, Germany
Heating cabinet T6060	Heraeus, Hanau, Germany

5.5.1 Borosilicate glass capillaries

Borosilicate glass capillaries with a length of 150 mm, an outer Ø of 1.6 mm and an inner Ø of 0.93 mm were used. The capillary was fixed on the holding of the micropipette puller and the heating coil heated the glass up to the melting point. At this point the glass capillary was pulled apart rejuvenated and narrowed and because of the pulling tension.

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cbbs
center for behavioral
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7 Statement of contribution and originality & declaration of criminal convictions

This PhD thesis has been conducted autonomously with the guidance and advice of my supervisors. To the best of my knowledge and belief, this work contains no material previously written or published by another person except as cited and referenced in the text. The material presented in this thesis has not been previously submitted for a degree at this or any other university.

I hereby also declare that I have not been found guilty of scientific and/or academic misconduct

X

Silke D. Storsberg

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„Nichts wahrhaft Wertvolles erwächst aus Ehrgeiz oder bloßem Pflichtgefühl,
sondern vielmehr aus Liebe und Treue zu Menschen und Dingen…“

Albert Einstein, 1879 - 1955