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(Direktor: Prof. Dr. Roland M. Schmid)

A study of environmental context-dependent learning in C57BL6 mice using rewarding and painful stimuli in a spatial orientation task

Marcel Hess

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Prüfer der Dissertation:

1. Prof. Dr. Roland M. Schmid
2. Prof. Dr. Radu Roland Rad

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Index

1 Introduction

Pain causes strong emotional reactions besides its sensory discriminative component. According to the International Association for the study of Pain (IASP) pain is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Carr et al., 2011). This definition emphasizes the subjective component of pain by mentioning the “emotional experience”. Such a pain state lasting longer than three months is defined as chronic pain. A chronic pain survey in 15 European countries and Israel revealed that about 20 % of the adult participants are suffering of chronic pain (Breivik et al., 2006).

Experimental investigations of the underlying mechanisms of nociception circuits brought detailed knowledge about nociceptors (for example the Transient Receptor Potential Vanilloid TRPV1**),** the conduction via A-delta and C-fibers in peripheral nerves, via the spinocerebral tractus and processing in the brain. But it remains difficult to establish an integral model for investigating the background of the affective component. Basically, the negative affective component is essential in humans and animals as it leads to an avoidance of noxious environments. These mechanisms of self-protection are turning into a serious problem in chronic pain patients and impose a big clinical challenge: about 40 % of the patients suffering of chronic pain are receiving inadequate pain management (Breivik et al., 2006). The costs and the consequences for these patients and the society reflect the huge impact of pain for the modern healthcare systems.

The pain-avoidance concept that explains the origin of chronic pain as a learning process gained importance in pain research in the last 20 years (Vlaeyen & Linton, 2000). According to this concept fear of pain causes avoidance-behaviour to reduce fear and unpleasant feelings in a potentially threatening situation. However such avoidance reinforces fear of pain and can be paraphrased as a “phobia of pain”. The resulting physical distress, poor behavioural performance and hypervigilance to pain stimuli lead to a vicious self-reinforcing circle of chronic pain. This concept emphasizes the role of cognition and learning especially in chronic pain. In other words the affective and cognitive component of pain highly influences the future outcome in somatic and visceral nociception: Enhanced brain responses to expectation of visceral pain, increased attention to threat, and cognition about pain that

overestimate the likelihood of worst possible outcomes, have been implicated as an important mediator of symptom severity in persistent abdominal pain conditions (Mayer & Tillisch, 2011).

Classical Pavlovian conditioning with pain as an unconditioned stimulus is described as an established animal model in pain research. Experimentally inducing pain in a laboratory animal (for example by an electric foot shock) in a conditioning chamber leads to a defensive response with the animal spending more time in a neutral chamber than in the conditioned chamber, a behaviour described as conditioned reaction. In terms of conditioned place avoidance (CPA), fear of pain or punishment is used as strong motivational system and an evolutionarily stable strategy to protect the animal from danger. Besides the advantages of such simple tests practised as animal models to examine the emotional component of pain, it is noteworthy that compared to the cognitive mechanisms in pain in humans these models systems reflect only single aspects of emotional and cognitive nociception; therefore, the transfer of experimental results of pain conditioning in laboratory animals towards humans is limited.

Since pain research mainly focuses on somatic nociception, visceral pain has been regarded as a variant of the somatic pain system and the theoretical concepts behind visceral nociception came from the point of view that both pain systems follow the same principles. In the last decades this simplified view changed and the current status in visceral pain research allows us to name the typical characteristics of the visceral nociception besides similarities. Therefore five highly relevant characteristics for visceral pain can be noted as described by Cervero and Laird:

> *Visceral pain "(1) is not evoked from all viscera (organs such as liver, kidney, most solid viscera, and lung parenchyma are not sensitive to pain); (2) it is not always linked to visceral injury (cutting the intestine causes no pain and is an example of visceral injury with no attendant pain, whereas stretching the bladder is painful and is an example of pain with no injury); (3) it is diffuse and poorly localized; (4) it is referred to other locations; and (5) it is accompanied with motor and autonomic reflexes, such as the nausea, vomiting, and lower-back muscle tension that occurs in renal colic."*(Cervero & Laird, 1999)

In more detail, it was hypothesized that there are high-threshold receptors responding to stimuli within the noxious range (this model could explain that pain is the dominant sensation in visceral organs like the heart or the ureter). Häbler and colleagues showed that pelvic nerves contain unmyelinated afferents with a high-threshold response to noxious mechanical stimulation (distension) of the cat's bladder (Häbler, Jänig, & Koltzenburg, 1990). Sengupta and colleagues were able to demonstrate the existence of high-threshold receptors in the opossum's oesophagus, but also described low-threshold receptors responding to the intensity of a stimulus by increasing their firing rate. Such low-threshold receptors are also able to transmit innocuous stimuli (Sengupta, Saha, & Goyal, 1990). These two classes of nociceptors differ in their representation in the visceral organs. According to Cervero the high-threshold nociceptors have been identified in the heart, lungs and airways, oesophagus, biliary system, small intestine, colon, ureter, urinary bladder, and uterus and the low-threshold intensity-encoding receptors have been described in the heart, oesophagus, colon, urinary bladder, and testes (Cervero, 1994). Facing the special characteristics in visceral pain transmission from the periphery to the spinal cord, the differences in the perception of visceral pain and the paradigmatic change in pain research of the last two decades points out that it is important to regard somatic and visceral pain separately.

Sensory and nociceptive inputs from the gastrointestinal (GI) tract have to be integrated in the central nervous system with other interoceptive inputs and with contextual information from the environment. Therefore the brain's integrated response to various target cells within the GI tract consists of a complex flow of external and internal input. A majority of the interoceptive information reaching the brain is not consciously perceived and serves as input to autonomic reflex pathways. Therefore the brain-gut interaction assures homeostasis and adapts GI function to the overall state of the organism. Findings about these permanent, mostly not consciously perceived, bidirectional brain-gut interaction can be summarized under the model of the brain-gut-axis. It is an important approach to understand the complexity of the underlying pathophysiological processes in functional GI syndromes (e.g. irritable bowel syndrome or functional dyspepsia) and chronic visceral pain:

> *There is an emerging consensus that the various clinical manifestations of chronic abdominal pain can best be viewed as a dysregulation in the complex interplay between events occurring in the gut lumen (including enteric microbiota), the gut mucosa, the enteric nervous system (ENS), and the central nervous system (CNS),*

> *leading to alterations in sensation, motility, mood and affect, and in some circumstances immune function.* (Emeran A. Mayer, 2000)

E. A. Mayer & Tillisch (2011) propose a special model of the pain-gut axis to address these facts to provide new approaches in treatment of visceral pain:

> *The brain-gut axis consists of a hierarchy of reflex loops that assure homeostatic control of GI function. Conscious perception of activity within these reflexes is minimal in health, with the exception of situations that require a specific action (food intake, defecation). Alterations in the gain of these homeostatic reflexes can be associated with alterations in intestinal secretions, motility, blood flow, and afferent sensitivity. Such changes can lead to symptoms of chronic abdominal pain and discomfort and alterations in bowel habits.*(E. A. Mayer & Tillisch, 2011)

Another challenging task for research is to elucidate the influence of cognition and emotion on visceral nociception. On one hand pain leads to spinal reflexive response to protect the organism from injury, but on the other hand pain perception evokes memory and emotions by brain processing. The afferent signals from the viscera are modulated by the CNS, and the complex endogenous pain modulation system is influenced by environmental context, mood or affect which leads to the assumption of a connection between cognition and nociception. The increased attention to threat and cognition about pain leads to greater brain responses to a visceral stimulus (Phillips et al., 2003). Certain types of psychosocial stressors, and acute laboratory stressors have been shown to be associated with enhanced visceral perception (E A Mayer et al., 2008). An upregulation of central stress and arousal circuits has been implicated as an important mediator of symptom severity in persistent abdominal pain conditions. Increased grey matter density in brain regions involved in the stress and arousal circuit can be observed in patients suffering from chronic visceral pain disorders (Schweinhardt, Kuchinad, Pukall, & Bushnell, 2008; Seminowicz et al., 2010). However the underlying mechanisms for these findings are still unexplained. It was hypothesized that enhanced glutamate signalling leading to excitotoxicity or apoptosis as a result of increased cytokine release due to aberrant activation of glial cells might be one reason for the structural changes in the CNS (Mayer & Tillisch, 2011). Besides these hints it still has to be determined what kind of profound structural deficits in the CNS of patients suffering of chronic visceral pain conditions, which possibly cause the persistence of the

symptoms in absence of an adequate stimulus, can be found. In addition, modern functional magnetic resonance (fMRI) of the brain allows to detect active CNS regions in visceral nociception in vivo: A local distension stimulus (e.g. in the oesophagus or colon) is used while the activated brain regions can be detected through functional mapping. Data deducted from such studies show the particular activation of cortical and limbic regions (Binkofski et al., 1998).

Concerning the cognitive process involved in the processing of chronic pain, it is very important to know more about the role of particular regions of the brain in this system. There is evidence indicating that the aversive association is located in the amygdala and the context representation is located in the hippocampus (HPC). For instance, amygdala inhibition of rats by AP5-injections that are exposed to context training showed CPA, which was not seen when the HPC was inhibited. These results indicate that the HPC is involved in context representation of experienced pain (Roesler et al., 2003). Research in this field is lacking a consistent animal paradigms to examine the role of context as a cognitive component of pain and the function of the HPC, which plays a central role in memory and spatial orientation (Burgess, Maguire, & O'Keefe, 2002). Thus, it is necessary to learn more about the processing of pain in this brain region. Although rodents are able to perform complex behavioural tasks and show cognitive flexibility, like finding an invisible platform in a water cross maze and relearning a new strategy after changing the position of the platform using brain regions analogous to humans (Kleinknecht et al., 2012), there are only a few behavioural paradigms testing cognitive processing of pain in mice. Numerous studies about reflexive responses to noxious stimuli in mice can be found. These brainstem-mediated reflex responses are less likely to capture cortical inputs compared with operant behavioural pain models (Tammpere et al., 2005). So far, tests involving higher brain centres like the escape-avoidance paradigm are mostly performed with rats.

The complexity of visceral pain requires to replace symptom related visceral pain models like the colorectal distension model with operant behavioural pain models. Such operant behavioural tests in visceral nociception used as a trigger for analogous structural changes in rodents CNS could open new approaches to examine those. Therefore this work aims to establish a conditioned place preference / avoidance paradigm in visceral pain with C57BL6 mice. The animals should be aware of multiple stimulus elements going beyond a simple cue followed by a single outcome setting to achieve a model of complex memory representation.

Hypotheses of the study:

First, it was hypothesized that the escape-avoidance paradigm can be performed with C57BL6 mice experiencing visceral pain. In detail, mice experiencing visceral pain in a particular context are supposed to show avoidance behaviour in a three chamber box model.

Second, we want to show that mice display spatial orientation in a Tolman maze using a conditioned place preference (CPP) protocol.

Third, the present work should provide a protocol to examine conditioned place avoidance (CPA) in mice after experiencing visceral pain in a spatial orientation task using a modified version of the Tolman maze.

2 Materials and Methods

2.1 Subjects

Male C57BL6 mice delivered from the MPI breeding facility in Martinsried, Germany, at 8 – 12 weeks of age weighing between 22 – 28 g were used in the present work. All mice were housed in individual cages with unlimited access to water and food (standard pellets). The animals were hold on a reversed 12 h light-dark cycle (lights off at 09:00 p.m.) at a temperature of 19–23°C. The experiments were performed in the dark cycle. The animals were kept in a separate holding room next door to the experimental room. The experimental procedures were approved by the local animal welfare authority (Regierung von Oberbayern) and were performed in accordance with the guidelines for the care and use of laboratory animals set by the European Community Council.

2.2 Three chamber box

The three chamber box used for classical place conditioning has three equal sized compartments (A, B, C; L x W x H: 30 cm x 30 cm x 30 cm) with plastic walls and floors

Figure 1: Photo of the three chamber box

(Figure 1: Photo of the three chamber box). The central chamber is open to the other compartments and can be used as start box. In compartment A the floor was textured and the walls squared black and white. There was a plastic made cuboid located in one corner of this compartment. In compartment B the floor was smooth and the walls were covered with horizontal stripes. In this compartment was a conical flask located in one corner. The third chamber (compartment C) was completely neutral (white walls and smooth floor see Figure 2). Such two- or three-compartment conditioning boxes are widely used in various place conditioning experiments with mice or rats in behavioural neuroscience (Cunningham, 2014; Karimi et al. 2014; Prus, James, & Rosecrans, 2009; Sora et al., 1998; Tzschentke, 1998).

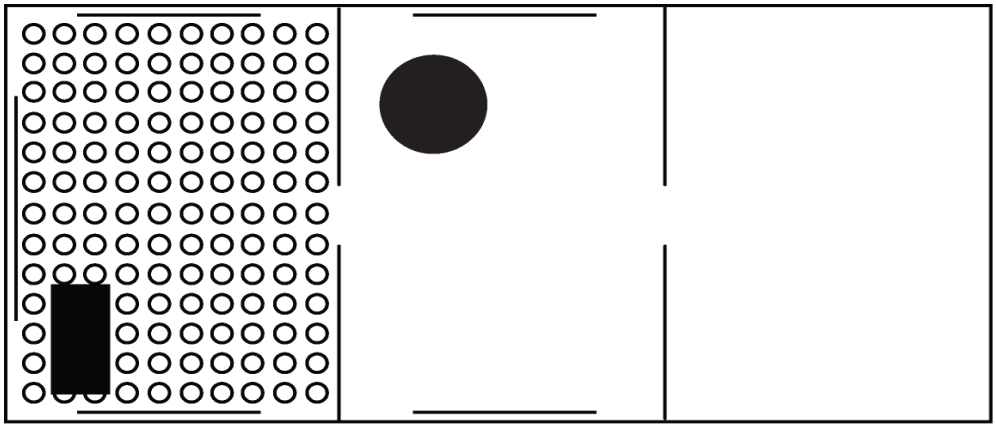


Figure 2: Sketch of three chamber box

2.3 Tolman maze

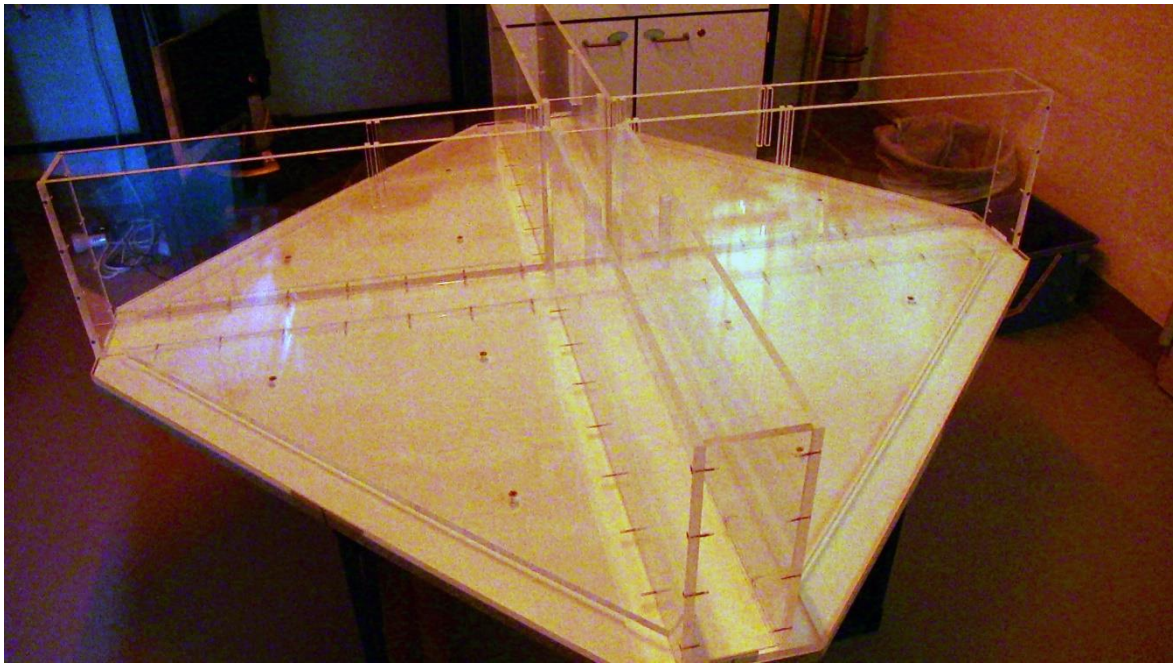


Figure 3: Photo of the Tolman maze

The clear acrylic class Tolman maze used for the spatial learning tasks has four arms forming a cross mounted on a plain white bench (Figure 3). This completely transparent construction allows spatial orientation via distal extra maze cues in the experimental setting. As described by E.C. Tolman and C.H. Honzik (Tolman & Honzik, 1930) the maze can be configured as a T-maze and the start arms can be varied.

Each arm is 10 cm wide, 75 cm long and the clear glass walls are 30 cm high. Every arm can be closed completely or shortened to 25 cm by two clear glass guillotine doors. The arms are clockwise labelled North, East, South and West.

The room was indirectly lit by two 40 W lamps at two opposite corners of the room directed at the wall to avoid averseness and shadows generated by the light. There were cues like two grey doors, a sink, a small blue cabinet, a grey bench, a black waste bin and an empty cage rack in the experimental room (Figure 4). The experimenter wearing a green lab coat was sitting behind the start arm while the experiment was running.

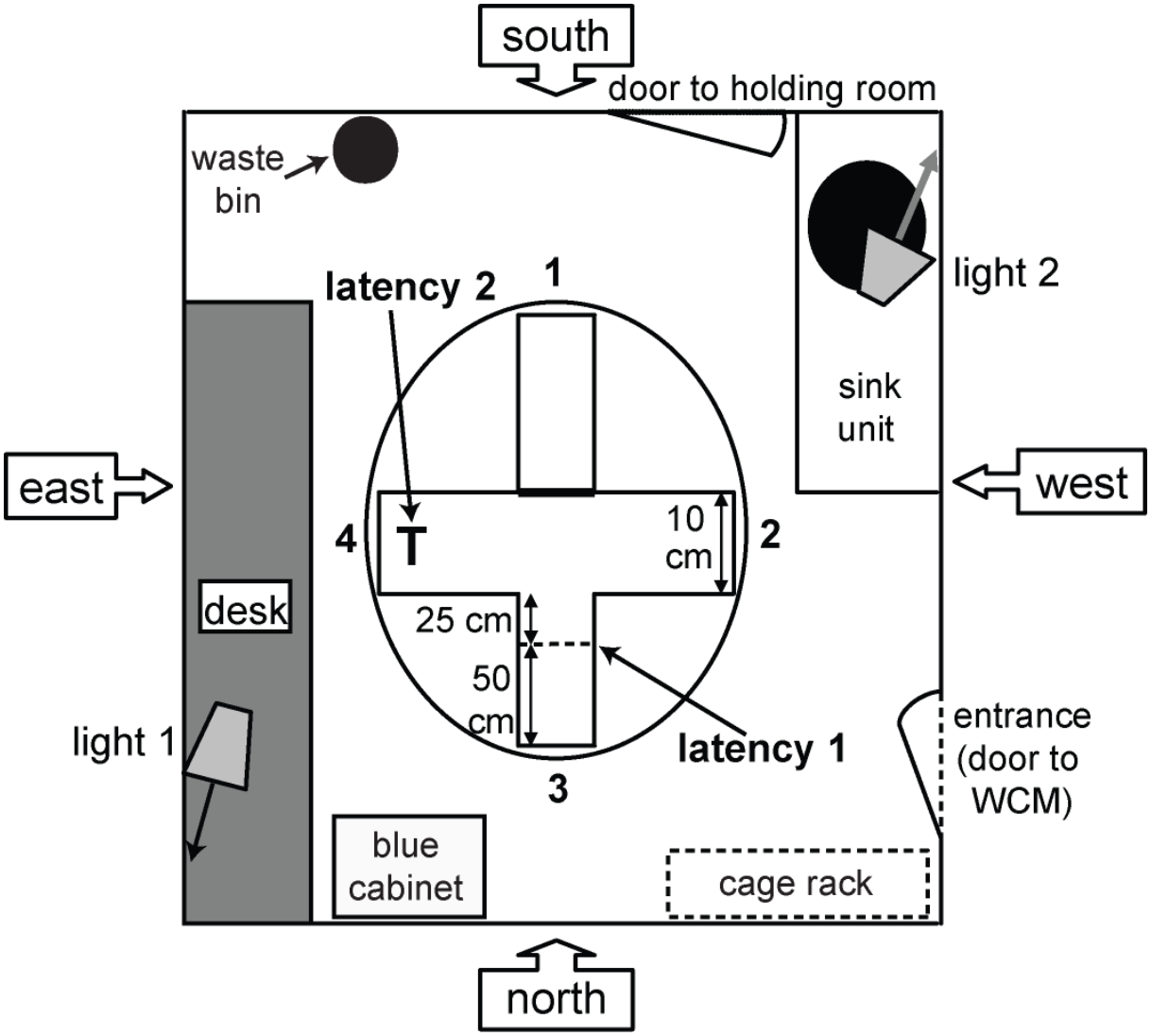


Figure 4: Sketch of experimental room

2.4 Video tracking

For video tracking and automated behavioural analysis the software ANY-maze by Stoelting Co. (IL; U.S.A.) was used. The automatically generated data from the ANY-maze software was compared to manually generated data to guarantee the correct evaluation of data and to minimize errors.

2.5 Animal transport and cleaning the maze

Each animal was transported from the holding room to the testing room in its home cage and transferred into the maze carefully. After finishing each run the animal was gently removed from the maze, placed back into the home cage and transferred to the holding room again. The maze was cleaned after every run in two steps: At first dirt and urine was removed with a paper towel and afterwards the floor and the walls were wiped with a wet towel and rubbed try.

2.6 Injection techniques and agents

2.6.1 Intra-peritoneal injections (i.p.)

Awake mice were carefully picked up by the tail and located on the top of their cage where they could grab the grid and then tightly caught at a skin at the neck using a paper towel and fixing the tail. After having the animal fixed safely in one hand the injection was done in the lowest third of the animal's abdominal wall in a flat angle.

In one experiment (see below) anaesthetised animals were placed on their back and the skin was elevated for injection.

2.6.2 Subcutaneous injections (s.c.)

The subcutaneous injections were performed with anaesthetised animals in a skin fold caught caudal of the animal's shoulder.

2.6.3 Acetic acid i.p. injections

Acetic acid (AA) i.p. injections are commonly used to induce experimental visceral pain. The animals show a typical, short-lasting writhing behaviour (Koster, Anderson, & De-Beer, 1959; Stevenson, Bilsky, & Negus, 2006) after injection. As described by T.J. Ness (Ness, 1999) the animals display body contortions, contractions of abdominal muscles, and extension of the hind limbs as acute signs of visceral nociception in both concentrations, AA 0,5 % or AA 0,9 % dissolved in a standard saline solution 0,9 %.

2.6.4 Cocaine i.p. injections

Intraperitoneal applications of cocaine can induce CPP in a wide range of dosing (from 2 to 30 mg / kg) in mice (Grotewold et al. 2014; Munoz-Cuevas et al. 2013). Based on data by Itzhak and Martin (Itzhak, 2002) and dela Cruz (dela Cruz et al. 2009) showing a significant CPP in mice with doses of 10, 15 and 20 mg / kg, we used 15 mg cocaine / kg body weight in this study.

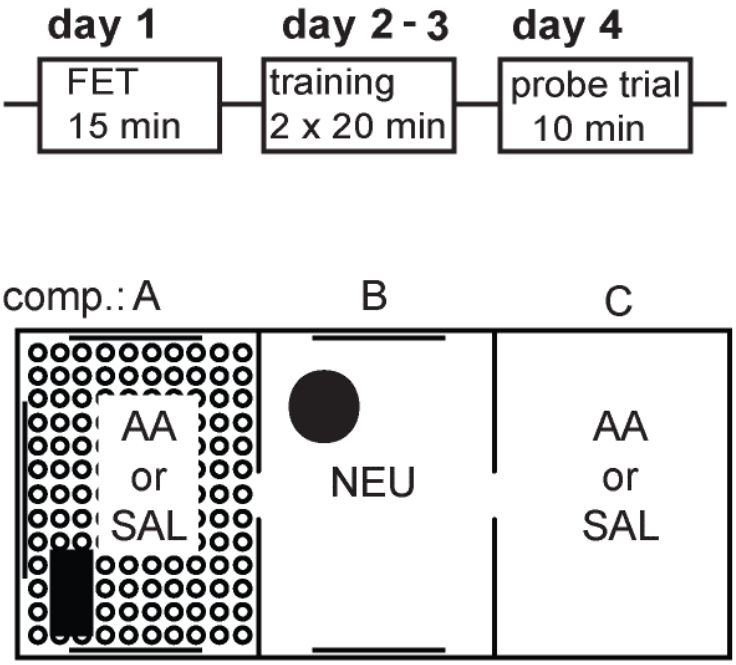
2.7 Isoflurane narcosis

A glass box (10 x 6 x 7,5 cm) was used for the narcosis. A paper-towel located in the box has been pipetted with 1 ml of isoflurane half a minute before the animal has been put into the box. After waking up from narcosis the animal were allowed to recover in their home-cage for 2 minutes.

2.8 Behavioural protocols

2.8.1 Experiment 1: CPA protocol in the three chamber box

This experiment was run for 4 days. During the free exploration trial (FET at day 1) the animals (n = 12) started in compartment B (see Figure 2 and Figure 5) and were allowed to explore the boxes freely for 15 minutes. The compartment, where the animal spent most of the time during FET was later the assigned compartment where the animal received AA during the training trials (compartment A or C). 6 animals were i. p. injected with AA 0,5 % and the remaining 5 were injected with AA 0,9 % at day two and three. The volume administered per injection was 0.01 ml per mg body weight. The animals were also injected with saline in the other compartment at the same day (A or C). They had to remain in one compartment (either comp. A or C) for 20 minutes after the injection. At day 4 the animals were allowed to move freely in the three chambers for 10 minutes and the time spend in each



chamber was counted.

Figure 5: 5 animals were injected i.p. with AA 0,5 % and 4 with AA 0,9 % at day two and three. At the same day the animals were injected with saline in the other compartment. They were placed in the closed compartment (either comp. A or C) for 20 minutes after injection.

2.8.2 Experiment 2: CPP by sucrose solution

On day 1 a FET was performed which was followed by a training lasting 6 days. A cohort of 6 animals was sequentially tested in one run (see Figure 6).

The FET for habituation, exclusion of side preference and equalizing olfactory cuing lasted 5 minutes per animal. The animals (n = 6) started from the centre with all 4 arms open.

During the training the animals had to perform 6 trials per day starting in the north or in the south in a randomized manner. The access to water was limited from 6:00 pm till the next day at 3:00 pm during the training days in order to increase the motivation of the mice to search for liquids during the test. The animals were allowed to drink ad libitum between 3:00 pm and 6:00 pm daily after the sixth run was completed. During training, the animals had to reach a lid of an eppendorf cup filled with a sucrose solution (10 %) at the end of the eastern arm while an empty lid was located at the end of the opposite arm. A visit at the lid (= target) was evaluated when the animal started to drink the solution. If the animal missed to reach the target in the east, the trial was stopped after 5 minutes. The measures taken were, latency 1 (time needed to travel 50 cm from start), latency 2 (time needed to reach target: maximum 301 seconds if the animal did not reach target after five minutes), number of arms visited and wrong target visits.

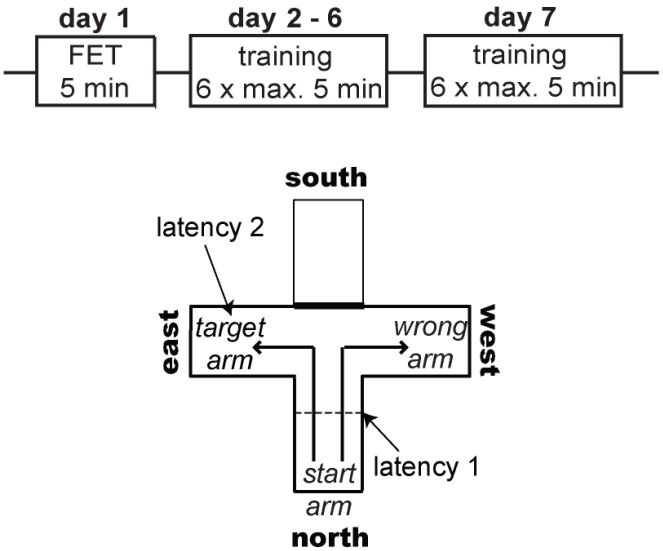


Figure 6: Animals (n = 6) were sequentially tested in one run performing 6 trials per day starting in the north or in the south arm (randomized). A lid of an eppendorf cup filled with sucrose solution was placed at the end of the eastern arm, while an empty lid was located at the end of the wrong arm. If the animal did not drink from the solution within 5 minutes, the run was stopped by the experimenter.

2.8.3 Experiment 3: CPP by hazelnut-spread

In the second protocol (n = 7) based on the protocol described above (see 2.8.2), hazelnut spread was used as target instead of sucrose solution. A similar lid of an eppendorf cup was filled with hazelnut spread (Nutoka® Aldi Sued – amount of hazelnuts: 13 %), and the animals were allowed to enter a cardboard tube while eating from the hazelnut spread (= target) in the east. Another cardboard tube was closed and the cup remained empty in the west. In a pretest session the animals where habituated to both cardboard tube and hazelnut spread in their home cage overnight before FET. After the FET lasting 5 minutes with all arms open, the animals were trained for 8 days (see Figure 7). As described in 2.8.2, the following measures were taken: latency 1 (= animal leaves start), latency 2 (= target visit), arm visits and wrong target visits. At day 11 a probe trial starting completely randomized from the south or the north and without targets (lids and cardboard tubes missing) was completed in three minutes per animal. The probe trial was recorded and analysed using the video tracking system ANY-maze. The time spend in target arm and in wrong target arm was measured.

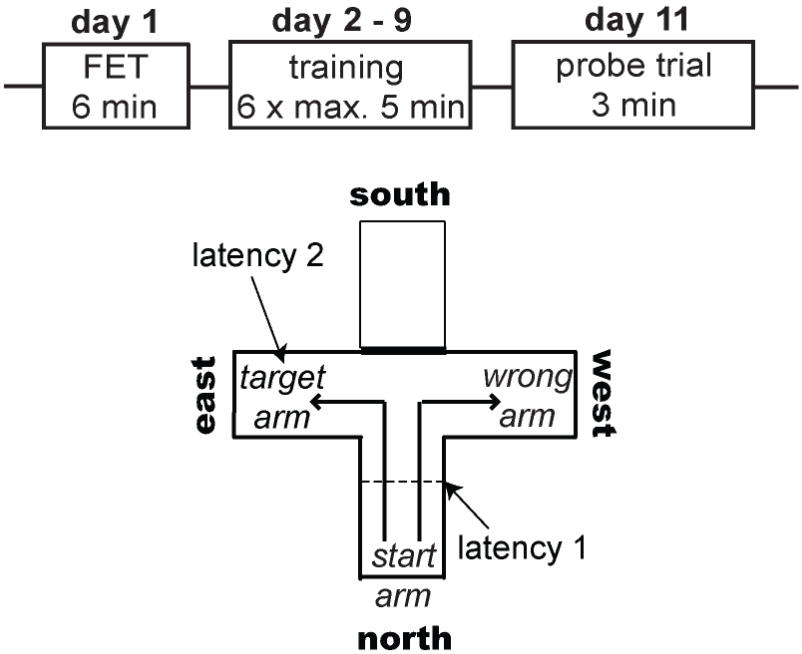


Figure 7: The animals (n = 7) were trained for 8 days. In contrast to experiment 2 the animals were allowed to enter a cardboard tube, while eating from the hazelnut spread. In the wrong arm the cardboard tube was closed and the cup remained empty.

2.8.4 Experiment 4: CPP with an alternating or a simultaneous training

During the FET the animals (n=16) started from the centre and were allowed to explore the arms in the north and the south for 6 minutes. The FET was recorded and analysed by the video tracking system ANY-maze (as described in2.4). After FET the animals were divided in 2 groups to undergo 2 different training protocols both lasting for 3 days (see Figure 8): An alternating training for group 1 and a simultaneous training for group 2 (see 2.8.3). In both protocols hazelnut-spread was used as target as described above. The target was located in the south for 4 animals and another 4 animals could find it in the north with complete randomization of the target between both groups.

During the alternating training the animals had to start from the centre and there was only one arm open (north or south). The animals were trained in the target arm at first and in the wrong arm in a second run every day. Every sequential run lasted for 3 minutes.

The group for simultaneous training performed one training per day (three minutes) with both arms (target arm and wrong arm) open (see Figure 8).

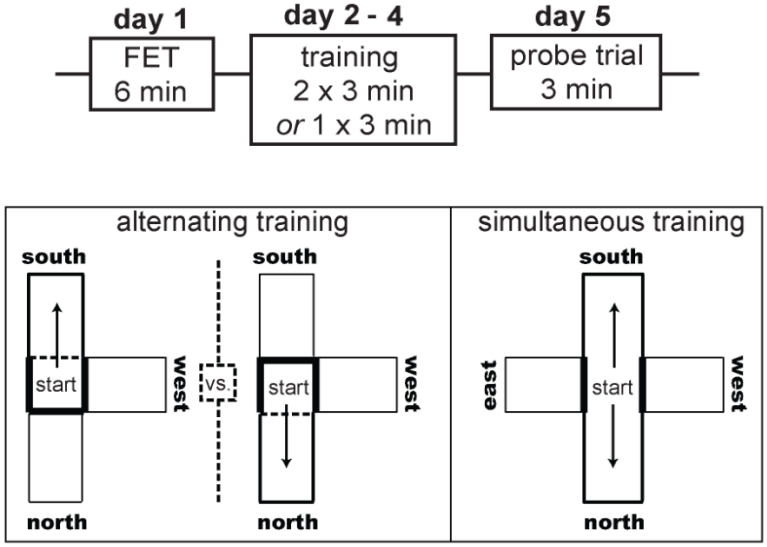


Figure 8: During FET two groups of 8 animals explored the northern and southern arm for 6 minutes. One group was trained in an alternating way which means that one arm was open and the other was closed during the training sessions (target or wrong arm). This alternating protocol was started with the target arm every day and the wrong arm was open during the second run (2 runs per day lasting 3 minutes each). The other group was trained with 2 open arms (simultaneous training lasting 3 minutes). For both groups the target (hazelnut spread) was located in the south (for 4 animals of each group) or in the north (for remaining animals of each group). The probe trial (3 minutes) started from the shortened western arm at day 5.

At day 5 the probe trial (3 minutes) was performed starting from the shortened western arm (see Figure 9) for both groups. The probe trial was also recorded and evaluated by ANY-maze video tracking.

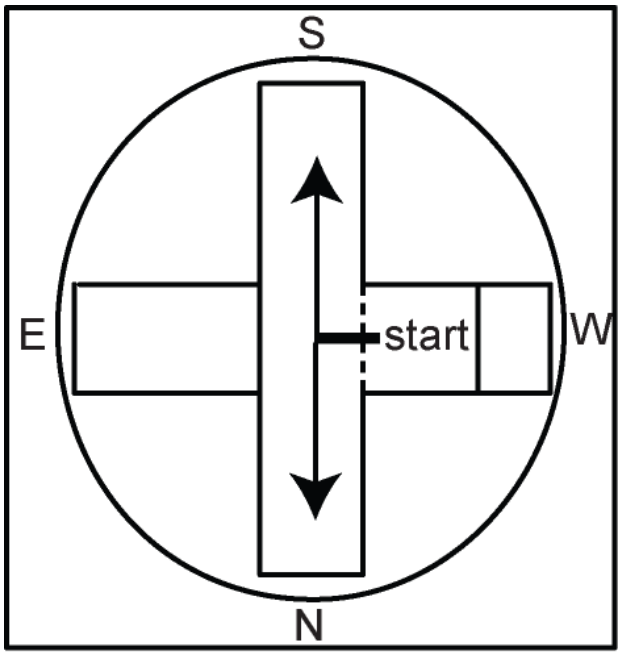


Figure 9: Set-up during probe trial

2.8.5 Experiment 5: CPA induced by AA injection

This protocol run in the Tolman maze was operated as an alternating training to ensure the pairing of the injection to the corresponding arm. The FET lasted 6 minutes and started from the centre with the arms in the south and the north open. During the training the animals (n=5) underwent 2 runs per day lasting 8 minutes for 3 days. Mice were injected i.p. with saline (0.01 ml / mg bodyweight) in the first and with AA 0,9 % (0.01 ml / mg bodyweight) in their second run. The arms were completely randomized: 2 animals received AA 0,9% in the north and 3 animals in the south. The animals started from the centre during the training sessions. The probe trials at day 5 and 8 (FET and probe trial were recorded for video tracking) started from the shortened western arm lasting 3 minutes (see Figure 9 and Figure 10).

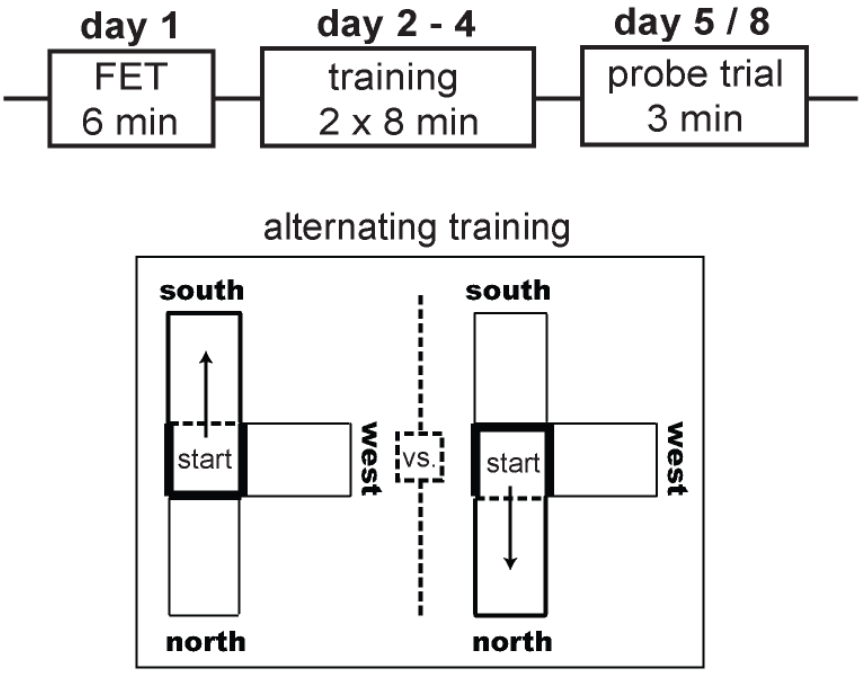


Figure 10: After FET (6 minutes) the animals (n=5) were trained daily in 2 runs (8 minutes per run) for 3 days. Training consisted of i.p. injections of saline (first run) or AA 0,9 % (second run) while the pairing of injected solution and trained arm was completely randomized. The probe trial (3 minutes) started from the shortened western arm at day 5 and was repeated on day 8.

2.8.6 Experiment 6: CPA with prolonged daily training

The experiment started with a FET (6 min – see2.8.5). The pairing of AA to north / south was completely randomized for 6 animals and 2 additional mice received AA in their preferred arm according to the FET. During the training for 4 days every animal received AA 0,9 % and saline i.p. two times (2 days saline and 2 days AA) in an alternating way: Half of them received saline (0.01 ml / mg bodyweight) first and the other half started with AA 0,9 % (0.01 ml / mg bodyweight) injections (counterbalanced). After injection the animals started from the centre of the maze and could explore either the southern or northern arm for 30 minutes. The probe trial at day 6 (FET and probe were recorded for video tracking) started from the shortened western arm (see Figure 9) and lasted 3 minutes.

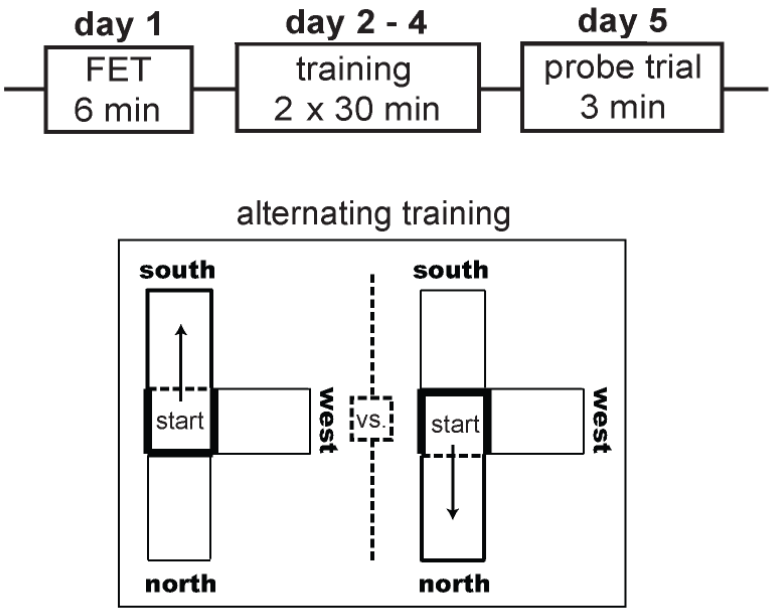


Figure 11: After FET (6 minutes) the animals (n=7) were trained daily in 2 runs (30 minutes per run) for 3 days. Training consisted of i.p. injections of saline (first run) or AA 0,9 % (second run) while the pairing of injected solution and trained arm was completely randomized. The probe trial (3 minutes) started from the shortened western arm at day 5.

2.8.7 Experiment 7: CPA and metamizol analgesia

The animals (n=20) were divided into two groups: one group receiving saline s.c. and the other group receiving the non-steroidal anti-inflammatory drug (NSAID) metamizol before being injected with AA 0,9 %. The s.c. Injections of metamizol were done 30 minutes prior to the i.p. injections of AA 0,9 %. The animals were anaesthetised with isoflurane for every injection (i.p. and s.c.). While this protocol was run in the maze for 2 weeks 10 animals including 5 saline and 5 Metamizol treated mice per week were trained per week. After FET (6 minutes per animal) the training lasted for 3 days. The training started with the saline i.p. injections in the morning and AA 0,9 % was injected for the second run in the afternoon. Each run lasted 8 minutes. The controls received saline and the treatment group received 300 mg / kg metamizol s.c. 30 minutes prior to the i.p. injection of saline / AA 0,9 %. 0.01 ml / mg bodyweight was the general injection volume. The pairings of AA 0,9 % to the south or the north were completely randomized. These runs were started from the centre of the cross maze, while only one arm was open (see Figure 12). The probe trial started from the shortened western arm at day 5 (see Figure 9). FET and probe trial were recorded and analysed by ANY-Maze as described before.

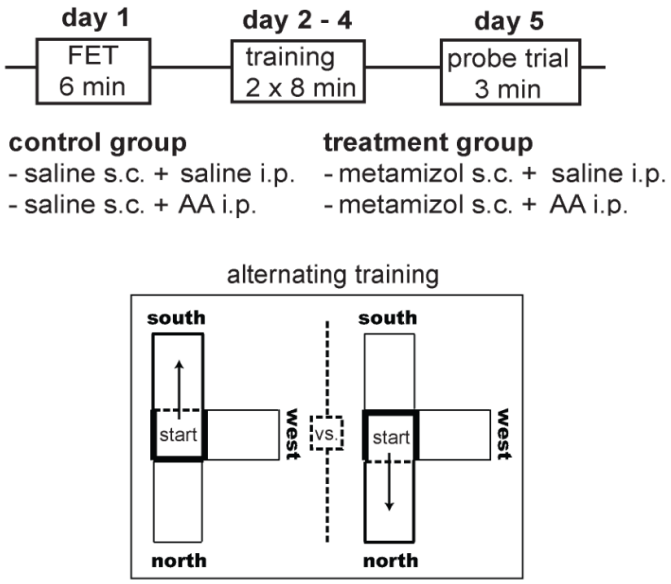


Figure 12: After FET (6 minutes) one group (n = 8) was s.c. injected with saline and the other group (n = 8) with metamizol (300 mg / kg) 30 minutes prior to the i.p. injections of saline or AA 0,9 % (under isoflurane narcosis). Every run lasted for 8 minutes and the pairings of AA 0,9 % to the south or the north arm were completely randomized per animal.

2.8.8 Experiment 8: CPP by cocaine

FET was performed with 8 animals exploring the northern and southern arm for 6 minutes. The pairing of cocaine / saline to north / south was completely randomized except of 2 animals that showed a preference of more than 60 percent for the northern or southern arm according to the FET, and therefore were injected with cocaine in the less preferred arm. During the training, which lasted for 4 days, every animal received cocaine and saline i.p. 2 times (2 days saline and 2 days cocaine) in an alternating way. Half of the group received saline (0.01 ml / mg bodyweight) on the first day and the other half started with cocaine (15 mg / kg bodyweight) on the first day (counterbalanced). After the injections the animals were placed in one arm of the maze for 30 minutes (see Figure 13). The probe trial at day 6 (FET and probe were recorded for video tracking) started from the shortened western arm (see Figure 9) and lasted 3 minutes.

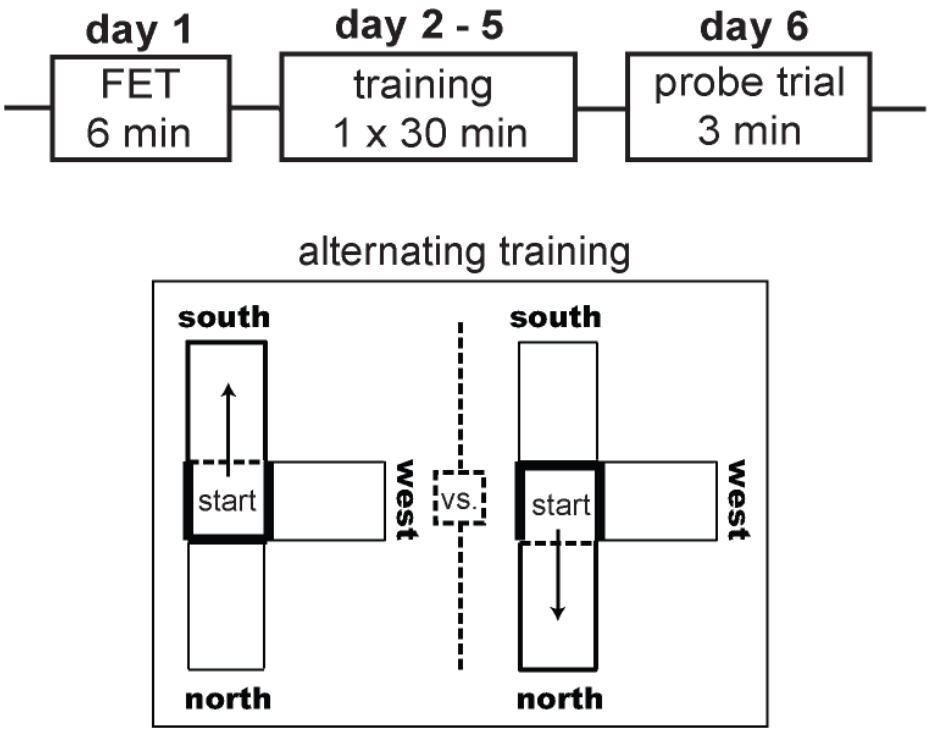


Figure 13: After FET (6 minutes) every animal (n = 8) received cocaine and saline 2 times (2 days saline and 2 days cocaine – one i.p. injection per day) in an alternating way for 4 days. Half of the group received saline at the first day and the other half started with cocaine (15 mg / kg bodyweight) at the first day in a counterbalanced design. The cocaine injection was paired with the arm that was less preferred during FET. After the injections animals were placed in one arm of the maze for 30 minutes.

2.9 Statistics

GraphPad Prism (San Diego, California) was used for statistical analysis and illustration of data. Adobe Illustrator CS5 was used for design and layout of the figures. The experimental data were analysed by Student's *t* test. Statistical significance was accepted if $p < 0,05$.

3 Results

3.1 Experiment 1: CPA protocol in the three chamber box paradigm

Both concentrations of AA induced typical nociceptive writhing behaviour approximately 2 - 4 minutes after the i.p. injection. 2 animals showing freezing (one in AA 0,5 % and one in AA 0,9 % group) during the probe-trial were excluded. As shown in Figure 14 all animals taken together show a CPA after treatment. They spent significantly ($p = 0,0042$; paired t-test) less time in the AA paired compartment.

The mean variation of time spent in the AA paired compartment was 21,1 % for the mice treated with AA 0,5 % and 26,96 % for the mice treated with AA 0,9 %, with the AA 0,9 % treatment revealing a significant difference (Figure 15).

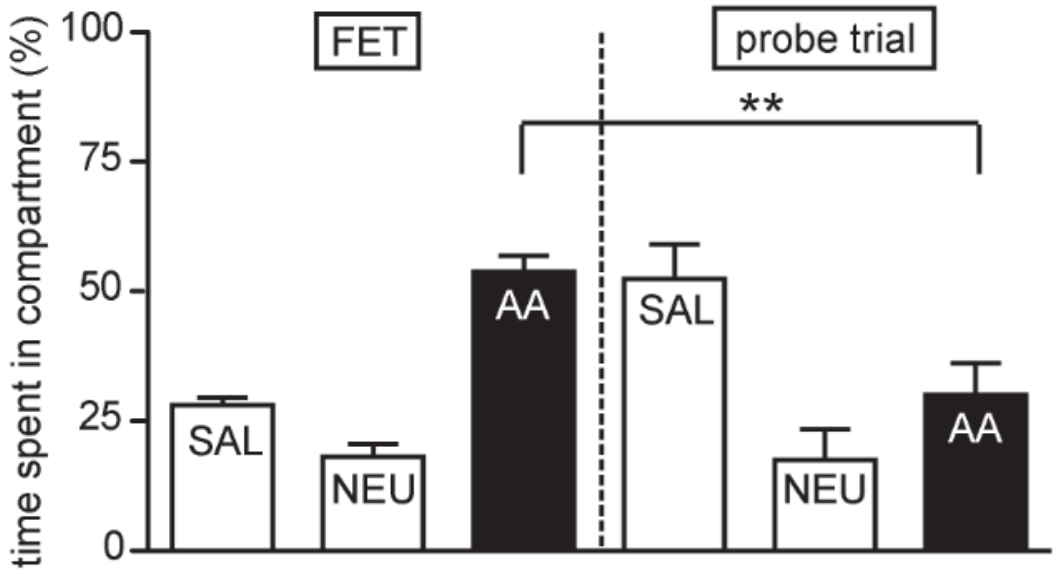


*Figure 14 The time spend in each compartment during FET and probe trial with pooling of data from AA 0,5 % and AA 0,9 % injected mice. ** p = 0,0042*

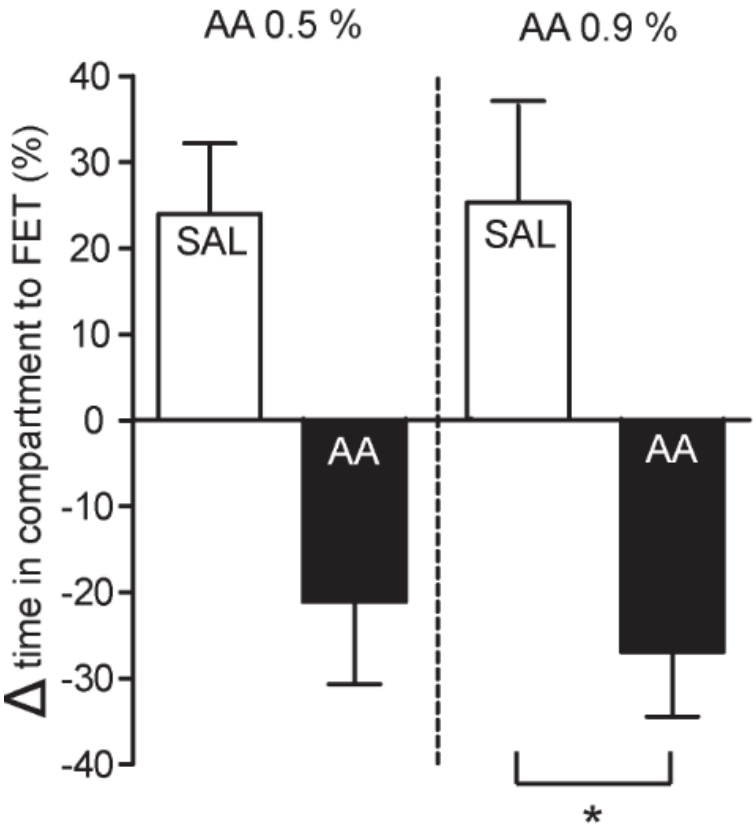


*Figure 15: A significant difference of time spent in the AA paired compartment was observed in the AA 0,9 % treatment group. * p = 0,0320 (paired t-test)*

3.2 Experiment 2: CPP by sucrose solution

In contrast to the three chamber box task the animals had to use extra-maze cues for orientation in this experiment (see Figure 4). As described in 2.8.2, the animals were rewarded with a sucrose solution supported by a water deprivation protocol.

After 3 days of training the animals did not show a preference and one animal did not drink from the sucrose solution at all. From experimental day 2 to day 3 latency 1 (average time an animal needed to cross a line located 50 cm from the end of the start-arm) did increase about 50 percent. On the 5 following consecutive days of training only a slight increase of latency 1 was observed (Figure 16A). While latency 1 did increase over the time, the animals remained in start-arm for a mean of 105,9 seconds and it took them approximately 170

seconds to drink from the sucrose solution (Figure 16B). The number of total arms visited decreased over time (Figure 16C).

Accuracy increased from day 2 – 6 but still did not achieve the expected 83 % (5 accurate trials out of 6). There was no individual animal that accomplished the task by performing 5 accurate runs of 6 per day over the testing period. Basically, the accuracy was around chance level and therefore the experiment has not been proceeded after day 7 (Figure 16D).

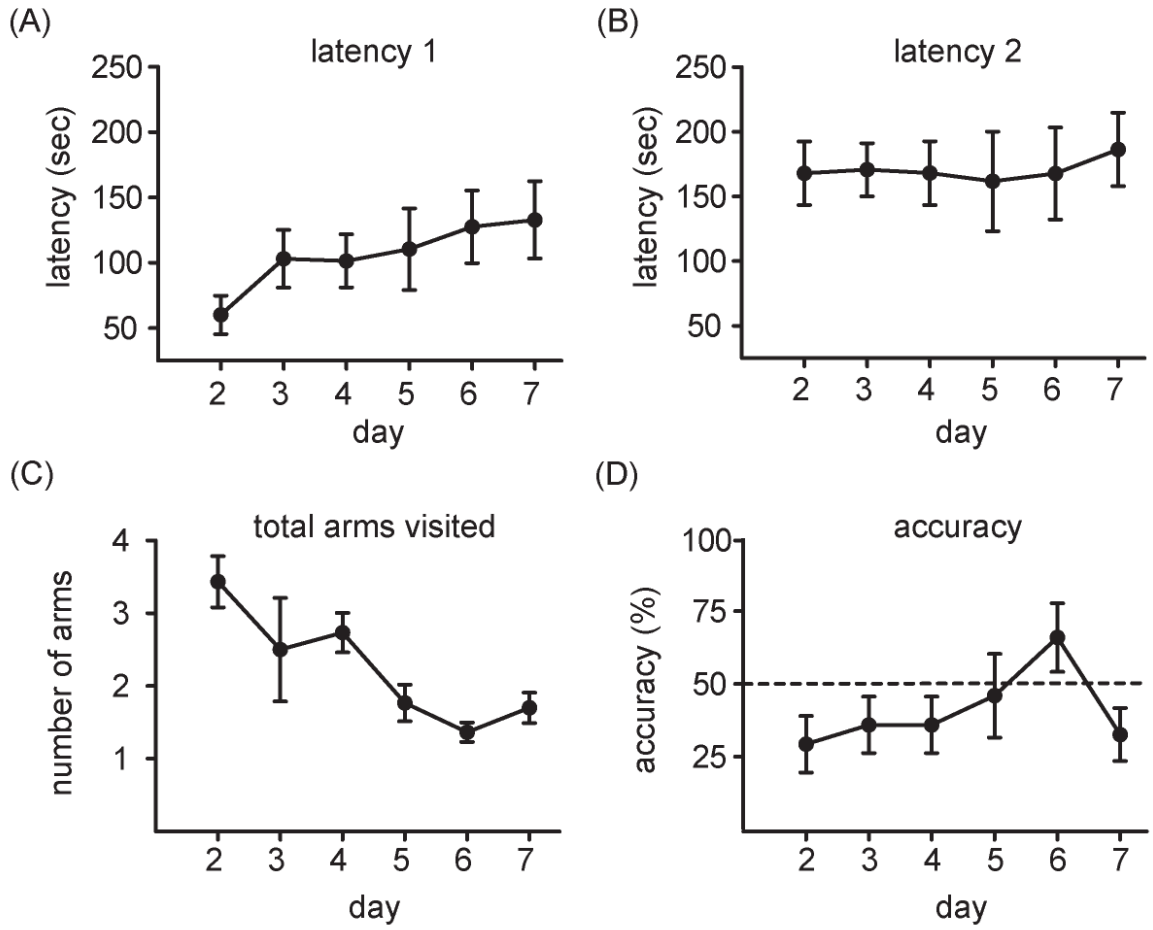


Figure 16: ***(A)*** *The average time the animals needed from start to cross the line at latency 1 is shown per day.* ***(B)*** *The average time the animals needed from start until drinking the sucrose solution is shown per day.* ***(C)*** *Number of total arms visited shown per day.* ***(D)*** *Percentage of accurate trials shown per day.*

3.3 Experiment 3: CPP by hazelnut-spread

In this modified CPP protocol the animals were habituated to the reward (hazelnut-spread) used as target in a pre-test trial overnight. In addition, the animals were allowed to enter cardboard tubes located at the end of an arm to minimize distraction, while they were consuming their reward.

The mean latency 1 was reduced from 105,9 seconds in the prior experiment (see 3.2) to 28,6 seconds (Figure 17A). Latency 2 was also reduced from 170,4 seconds observed in the protocol using sucrose solution to 51,6 seconds (Figure 17B). This may indicate a higher reward preference of hazelnut-spread over sucrose. The trend of total arms visited was analogous to the first experiment and in both protocols the mean number of arms visited decreased below the value of 2 at day 4 of training (Figure 17C). The accuracy increased every day until training day 6 and became asymptotic to about 70 % (Figure 17D). However, this cohort also failed to achieve the required accuracy of 83 % (5 accurate trials of 6 trials a day). At day 6 a mean accuracy of 73,8 % (4,428 accurate trials of 6 per animal) was achieved. Training was stopped after day eight.

Considering the fact that this cohort did achieve an accuracy of 73,8 %, it was likely that they showed spatial orientation in the maze, although they failed the accuracy criteria properly. To probe spatial orientation, we implemented a probe trial without the target and cardboard tubes and measured the time the trained animals spent in each arm. As shown in Figure 18 this cohort of 7 animals trained for 8 days displayed significant place preference for the target arm (paired t-test $p = 0,0368$) as they spent more time in it.

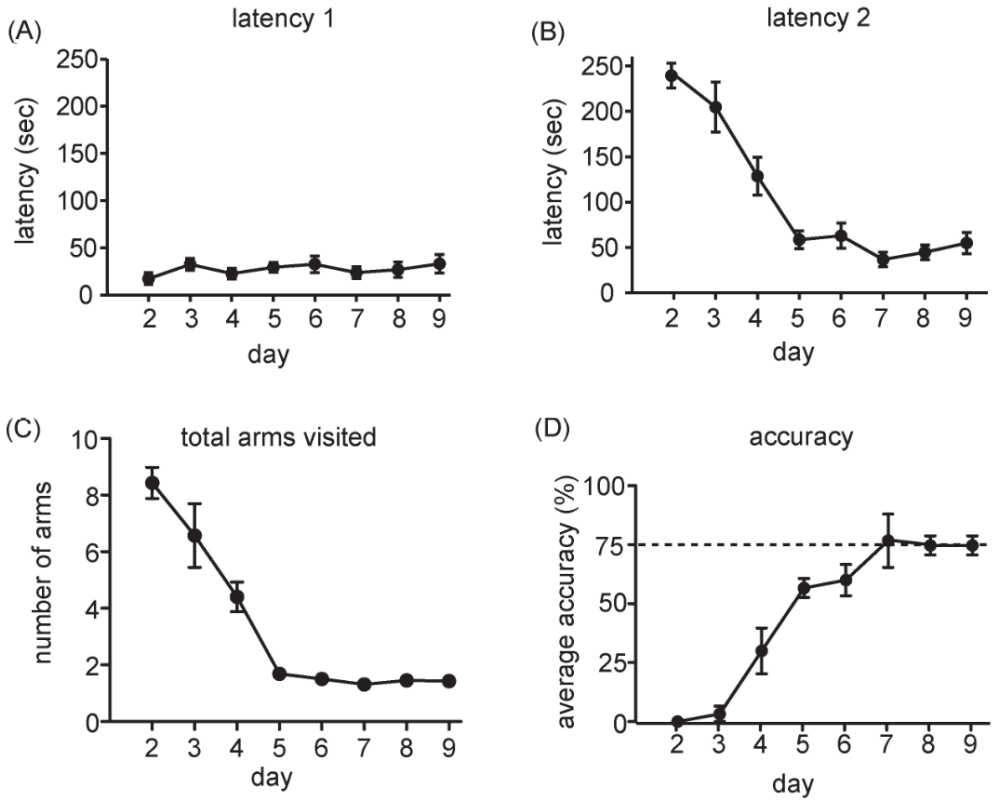


Figure 17: ***(A)*** *The average time the animals needed from start to cross the line at latency 1 is shown per day.* ***(B)*** *The average time the animals needed from start until consuming hazelnut-spread is shown per day.* ***(C)*** *Number of total arms visited shown per day.* ***(D)*** *Percentage of accurate trials shown per day.*

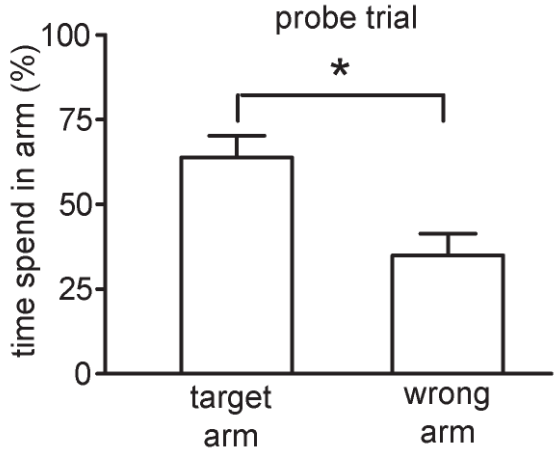


*Figure 18: Animals displayed a significant preference for the target arm * p = 0,0368 (paired t-test).*

3.4 Experiment 4: CPP after an alternating or a simultaneous training

To compare an alternating and a simultaneous protocol two groups of animals were trained at the same time: The first group (n = 8) was trained simultaneous and the second group was trained sequentially for 3 days (Figure 8).

The time the animals spend in the area, where the target respectively the wrong target used to be during training was measured in the probe trial (defined as last fifth of the arm). The group that was trained in an alternating way spent significantly (p = 0,0393) more time in target area (Figure 19).

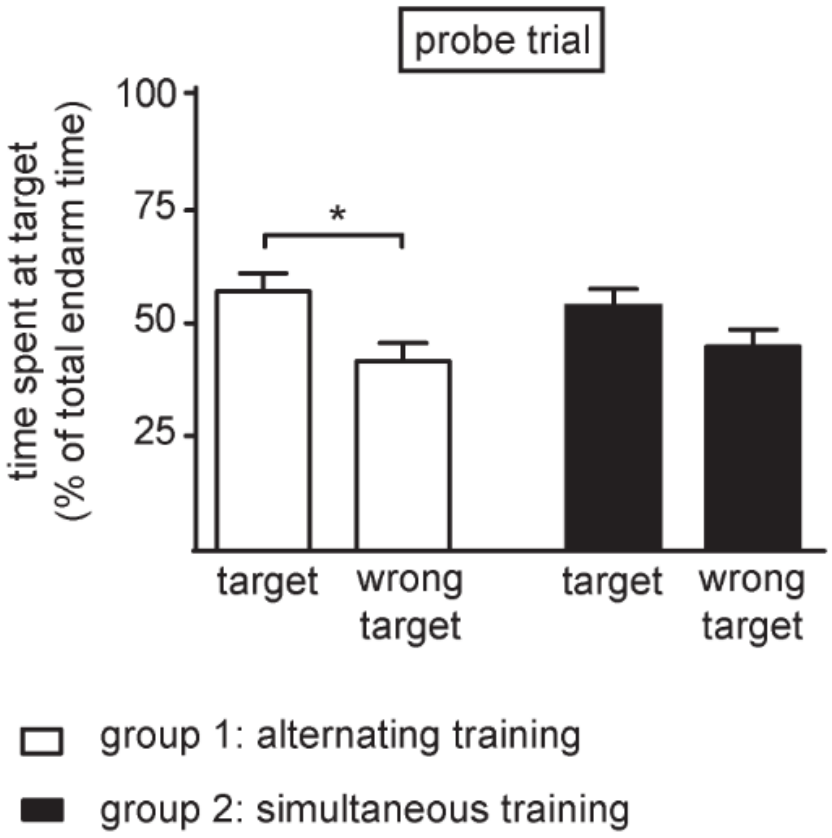


Figure 19: The percentage of time spent at target and wrong target is shown. Only mice in the alternating training group displayed a significant preference for the target area. ***** *p = 0,0393 (paired t-test)*

3.5 Experiment 5: CPA induced by AA injection

This CPA protocol (as described in 2.8.5) was modified from the alternating CPP training with AA 0,9 % injections being performed instead of reward presenting. During probe trial the animals spend a mean of 27,6 % in the AA paired arm compared to 53,3 % during the FET [p = 0,0253] (Figure 20A). Therefore, the animals showed place avoidance in the spatial orientation task analogous to the results observed in the three chamber box. The cohort failed to reproduce significant CPA in a second probe-trial done 3 days after the first one (Figure 20B).

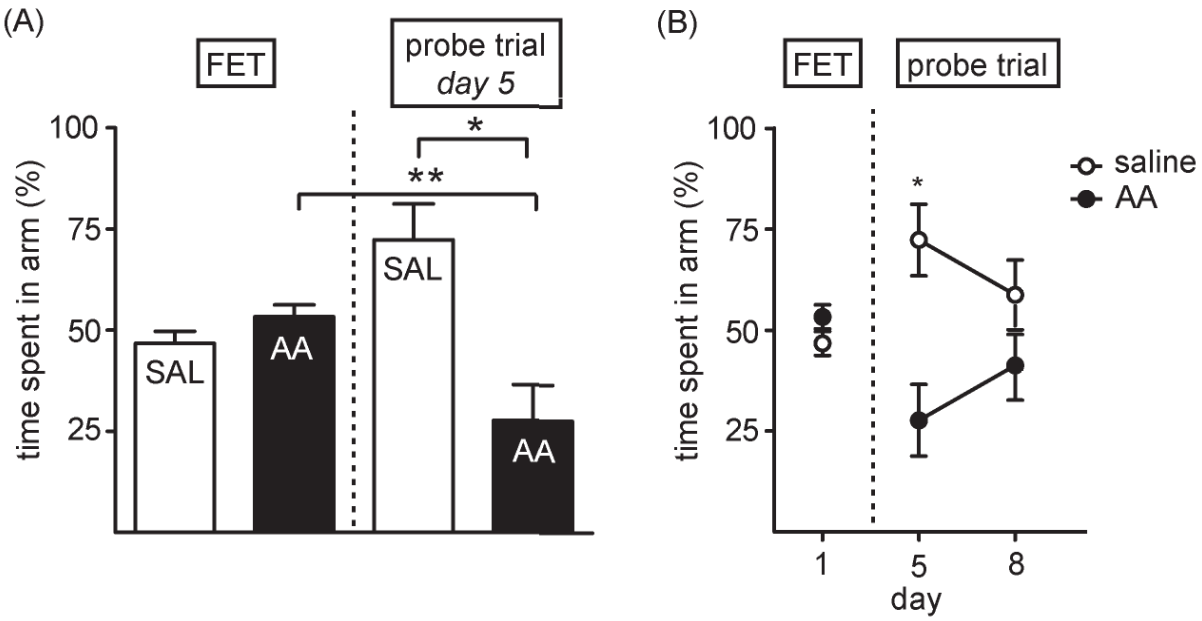


Figure 20: ***(A)*** *Animals spent significantly less time in the AA-paired arm during the probe trial on day 5 expressed as percentage of total time spent in arms.* ***(B)*** *The animals didn't show a significant place avoidance during the repetition of probe trial at day 8. * p = 0,0329; ** p = 0,0253 (paired t-test)*

3.6 Experiment 6: CPA with prolonged daily training

The CPA protocol was performed as in exp. 5, but modified by extending the time per run from 8 to 30 minutes during the training. The results (see Figure 21) revealed that the animals significantly preferred the AA-paired arm during the probe trial: They spent 69,8 % of the time in the AA-paired arm against 30,2 % in the saline paired arm (p = 0,0441).

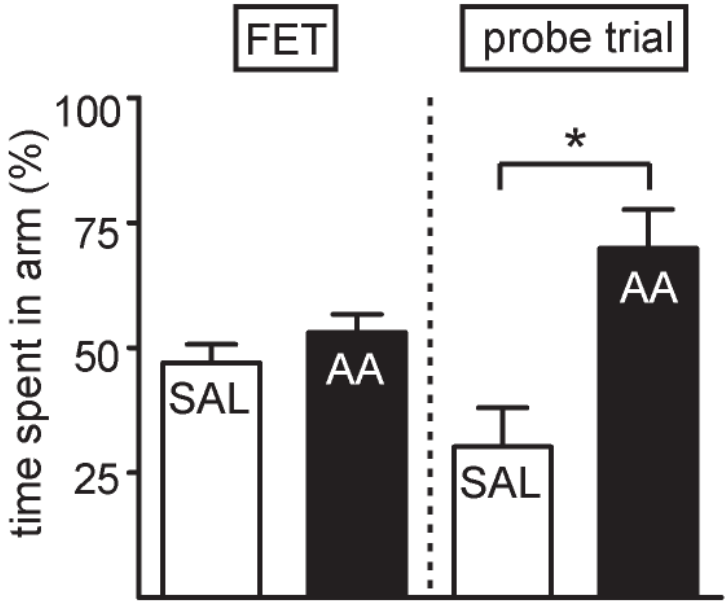


*Figure 21: Animals spent significantly less time in the saline paired arm during the probe trial on day 5 expressed as percentage of total time spent in arms. * p = 0,0441 (paired t-test).*

3.7 Experiment 7: CPA and metamizol analgesia

Another batch of animals (divided in two groups) was trained in the same way as described before (Experiment 5), but one group received the NSAID metamizol (300 mg per kg, s.c.) while the other group received saline. All s.c. injections were done 30 minutes before the i.p. injection of AA 0,9 %. In the control group, the animals stayed 64,1 % of the time in the saline paired arm and 35,9 % in the AA paired arm during the probe trial (p = 0,1607). The metamizol treated mice stayed 55,4 % in the saline-paired arm and 44,6 % in the AA-paired arm during the probe trial (p = 0,3362). One animal died and two animals had been excluded due to freezing in the probe trial.

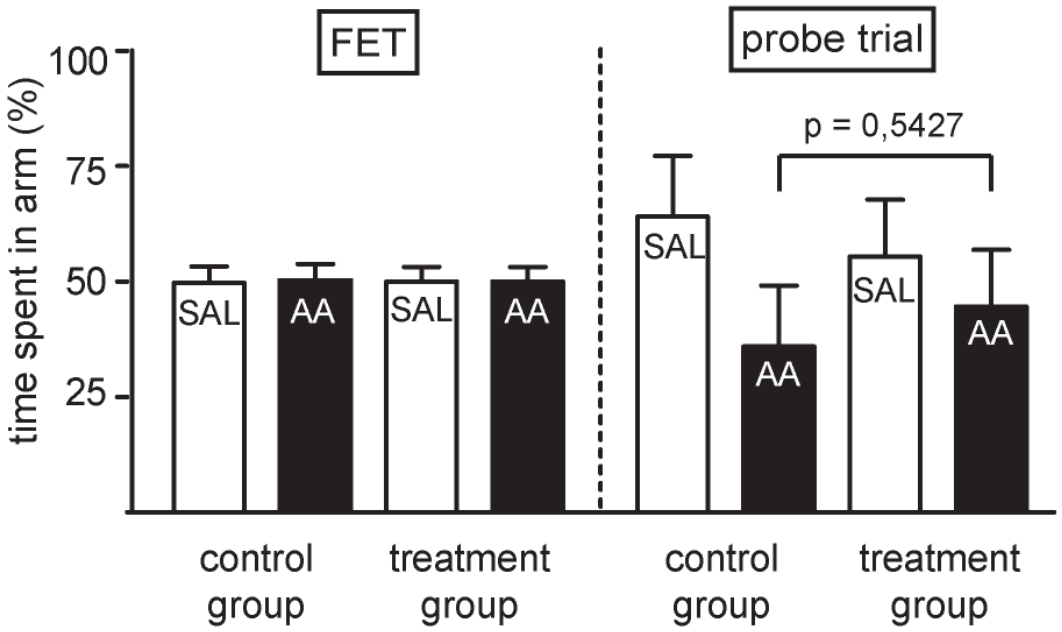


Figure 22: During the probe trial there was a trend towards CPA in the control group. Metamizol treatment did not reverse the place aversion in the treatment group (p = 0,5427; unpaired t-test).

3.8 Experiment 8: CPP by cocaine

As described in literature cocaine administered between 5-20 mg/kg can be used for place conditioning in a three chamber box-test (dela Cruz et al., 2009). Here a dose of 15 mg / kg animal was applied in the Tolman maze. In the probe trial, animals spent a mean of 42,4 % before and 57,6 % after training in the cocaine-paired arm (see Figure 23), representing significant place preference (p = 0,0083, paired t-test).

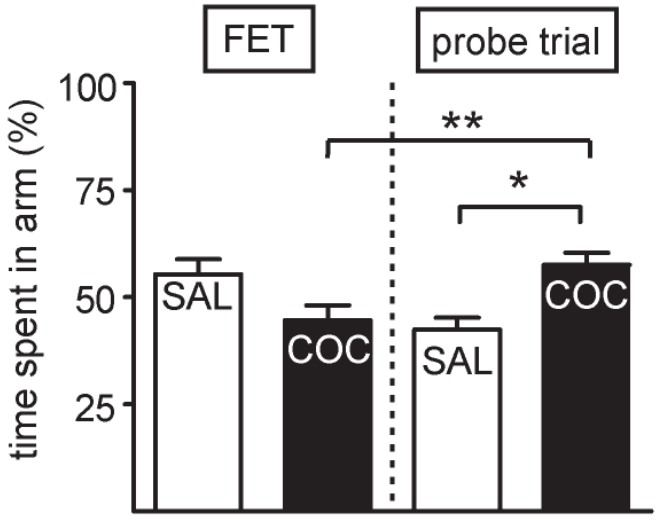


*Figure 23: The animals spent significantly more time in the cocaine paired arm than the saline paired arm (percentage of total time spent in both arms) * p = 0,0296; ** p = 0,0083 (paired t-test).*

4 Discussion

4.1 General aspects

As new methods and new approaches opened another view on visceral nociception since the 1990s it became more obvious that there are differences in the background of visceral pain processing besides the overlaps with the somatic nociceptive system.

There is a link between cognitive processing and visceral pain, but basic research is confronted with the problem to find a reliable and valid model for a standardized evaluation. Basic research uses simplified methods like the shuttle-box test (a two- / three-compartment place conditioning apparatus), which measures the escape latency from the compartment paired with a painful stimulus (Mauderli, Acosta-Rua, & Vierck, 2000). Operant procedures using the shuttle-box can be regarded as a well-established experimental setting offering a lot of advantages because it is highly reproducible, objective, shows a high level of reliability and the results can be compared easily. Facing these advantages of such a classical conditioning model it is reasonable to use it for example in research of the motivational respectively aversive effects of drugs and other pharmaceutical substances. On the other hand it is obvious that such experiments are limited in their external validity for the testing of higher cognitive processing. The contradictory issue about classical place avoidance tests using painful stimuli is that they primarily measure withdrawal-based behaviours, which means that there is possibly no higher cerebral processing involved. Withdrawal of a painful stimulus (e.g. moving into another compartment or stepping on a platform) as an innate behaviour rather represents the sensory-discriminative component of pain processing than the affective-motivational one. The experiments described here are based on the work of Edward C. Tolman and his spatial learning experiments with rats. He was able to prove that the animals use distal spatial cues to navigate in a clear-class maze. Rodents showed spatial orientation in his experimental setting (Tolman, Ritchie, & Kalish, 1946), and since an integrated brain function including neocortex and limbic regions is necessary to reach such a complex task this kind of spatial orientation paradigm was a promising experimental design

to address the affective-motivational component of visceral pain. As described before (see2.3) the animals were faced to a Tolman maze in T-configuration, and due to this setting they had to decide between two options: The basic assumption was that a naive animal will decide by chance to go left or right after leaving the start-arm. After a few days of training using rewarding or painful stimuli paired with a particular location there should be a change in the animal's motivational state and their choice for a preference / avoidance of an arm due to the establishment of memory.

4.2 Discussion of experiments

4.2.1 Experiment 1

In this experiment the aim was to prove that mice, notably C57BL6 mice, learn CPA through noxious visceral stimulation. There are several studies that could prove CPA due to visceral pain in rats following the three chamber box paradigm (Yan et al., 2012; Cao, Zhang, Yan, Chen, & Li, 2012; Deyama et al., 2009; Korzeniewska-Rybicka & Plaznik, 2001; Wang, Chai, Wu, & Wang, 2007).

As described in literature the writhing behaviour like lengthwise stretches of the body with concave arching of the back and characteristic hind limb extension shown by the animals did start within 5 minutes after injection. This behaviour occurred independently from the 2 concentrations of AA. Both groups spent more time in the saline paired compartment after treatment which was highly significant for the AA 0,9 % treatment group (see Figure 14 and Figure 15). Therefore, it can be assumed that visceral pain leads to a measurable place avoidance in C57BL6 mice whereas the animals treated with AA 0,5 % failed to show a significant result even though the quality of the writhing behaviour after the injection was the same in both groups. This kind of highly aversive treatment causes stress and anxiety (Zhong et al., 2012), which was apparent in both groups as one animal showed freezing after AA injections independent from the concentration of AA and the compartment where they received the injection. According to Collier et al. (1968) there are about 16 % of the mice non-responders after being injected with AA 0,5 %. Additionally, they were able to show that the rate of non-responders can be reduced by using higher AA concentrations (Collier,

Dinneen, Johnson, & Schneider, 1968). According to these results an AA concentration of 0,9 % was used in all following tests.

4.2.2 Experiment 2

In this experiment spatial orientation of C57BL6 was tested in a Tolman maze. The animals needed a relatively long time to leave the starting area which was increasing every consecutive day while the total time needed to reach target (latency 2) almost remained the same. Therefore the time needed from latency 1 till target (latency 2) was decreasing by more than 50 percent over the six days of training (from a mean of 125 s to 50 s). The C57BL6 kept exploring in the open field like setting of the Tolman maze which is consistent with the assumption that C57BL6 mice have a pronounced disposition for exploration behaviour although they are confronted with a rather unfamiliar environment like the open field (Brown, Corey, & Moore, 1999). It was important to note that the experimental environment was tolerable for the animals and didn't seem to be aversive to them by nature.

Although a decrease of latency 1 and latency 2 suggested a learning process, the data rather suggested that the animals habituated to the apparatus as described by Whishaw and colleagues (Whishaw, Haun, & Kolb, 1999): Besides a reduction of open field activity over time the animals spend more time in their “home-base” area. In addition the rodent's shift in behaviour towards longer time sitting immobile once they are familiar with the environment described by Whishaw et al. (1999) could explain the results (increase of latency 1). The total arms visited in each run showed a decrease from a mean of 3,5 to 1,4 per run. Together with an accuracy around statistical chance level this decrease of arms visited is more consistent with habituation than target oriented movement due to spatial orientation.

Besides habituation the fact that the animals were not attracted as expected by the target (sucrose solution) became obvious. Target visits were counted only when the animal actually drank from the solution. The animals did not drink from the solution every time they reached the target area and one animal avoided to drink from it at all. Therefore the motivation to drink from the sucrose solution was not sufficient. To increase motivation the water deprivation protocol could be intensified or possibly switched into a food deprivation

protocol but these treatments are stressful and therefore it would not be possible to control their influence on the animals' cognitive performance.

Some pitfalls in the realization of the first experiment need to be noted. A long latency 1 (up to 120 seconds which means up to 40 percent of the total time) can partly be considered as a problem of the paradigm because there was no sufficient stimulus to reinforce the animals to leave the starting position. Rodents prefer to stay close to corners, walls and in a safe home base. As described by Kleinknecht et al. (2012) this problem could be solved by flooding the maze with water and offering a rescue platform (Kleinknecht et al., 2012). This kind of water-maze task is elegant for spatial orientation tasks but the water as a strong stimulus would interfere with any other stimuli offered at the same time. Another problem is the nature of the reinforcing reward to change the animal's motivational state. A practicable method used in pharmacological experiments with C57BL6 mice where mice receive different drugs per oral administration was to hide the substance in hazelnut cream (Ingberg et al., 2011; Jacobsen et al., 2013). Ingberg et al. compared different administration regimes for 17β-estradiol in ovariectomized mice and the group receiving 17β-estradiol per oral administration in hazelnut cream showed a steady concentration within the physiological range in a timespan of five weeks. These results indicated that C57BL6 mice were highly attracted by hazelnut cream. Therefore the protocol was modified and hazelnut spread was used as target instead of sucrose in the following CPP experiments. Cressant et al. (2007) postulated that mice steadily show a tendency to "explore environments recurrently" what makes it difficult to show accurate results in behavioural tasks (Cressant et al., 2007).

4.2.3 Experiment 3

The results deducted from the modified protocol with hazelnut spread supported observations from the first experiment that the animals showed appropriate exploration behaviour and no freezing. The animals made use of the options to enter the open cardboard tube at the target as well as licking from the hazelnut cream. According to the reduced latency 1 (see Figure 17) the aim to arouse attention and motivation by offering objects at the end of both arms (target arm / wrong arm) and hazelnut cream was achieved. Furthermore the animals visited less than 2 arms per run from day 5 on. The small difference between latency 1 and 2 may indicate that the animals were more focused to reach the target (note that the animals needed a minimum of 50 seconds (average) from L1 to L2 in the first experiment).

The increasing accuracy achieved a stable state of 75 percent from day 7 and the following days. This value can also be expressed as an average of 4,5 accurate trials out of 6. Therefore, there were animals reaching the originally estimated 5 of 6 accurate trials. Nevertheless a higher accuracy was observed with the WCM by Kleinknecht et al. (2012). The fact that errors can be made without consequence and that there is an individual variation of attention and motivation may influence these measures.

Finally a probe-trial was performed in addition to the measurement of the accuracy. The probe trial (preference test) is an evaluation tool implemented in CPP experiments, for example in the shuttle-box or the Morris water maze as described by Cunningham et al. (2006) or de la Cruz et al (2009)(Cunningham et al., 2006; dela Cruz et al., 2009). The advantage of measuring the preference compared to the accuracy is that it is possible to evaluate behavioural response. The animals showed a significant preference for the target arm during the probe trial (see Figure 18) indicating that the mice navigate through the maze using external, spatial cues and memorise them.

4.2.4 Experiment 4

It was necessary to operate the training sessions in closed compartments to confine spatial cues to rewarding or noxious stimuli. In experiment 1 and 2 the animals were allowed to move freely to evaluate their progress in daily performance. After evidence has been generated that the animals were able to navigate by the use of distal cues an alternating training (using the same reward) with closed compartments was evaluated in the Tolman maze. Another question was to evaluate an adequate duration for the training to generate a measurable place preference in the probe trial.

As shown in Figure 19 the alternating training is practicable and the animals displayed a significant place preference although the starting point differed between the training and the probe trial. The other group of animals which underwent a simultaneous training (less time, less runs per day and less open arms compared to experiment 2) failed to show a significance in their side preference.

4.2.5 Experiment 5

In the next step the alternating CPP protocol had to be transferred into the CPA protocol using the visceral pain-inducing stimulant acetic acid. The mice showed writhing after the i.p. injections of AA 0,9 % and a significant CPA was found in the probe trial at day 5 (see Figure 20).

In the follow up to investigate the persistence of the shown CPA over the time with a repetition of the probe trial at day 8 mice did not show a significant avoidance again. The probe trial (actually a FET) at day 5 where the mice were exposed to the setting without experiencing the visceral pain stimulus again may induce extinction of the CPA memory.

4.2.6 Experiment 6

In this experiment a prolonged protocol was chosen to enable a less stressful treatment because the handling to move the animal from the home cage to the maze and the i.p. injections were reduced to only take place once per day. Another rational could be that the subjects were able to perceive their environment more detailed due to the longer sessions. Amazingly, the result of this modification (see Figure 21) was that the animals spent significantly more time in the compartment paired with AA 0,9 %. Since it has been described in literature that the frequency of the observable writhing behaviour peaks after 5 – 10 minutes and decreases afterwards (Collier et al., 1968) a possible assumption could be that the acute pain decreases, too. Certainly, it has to be mentioned that the fact that we don't know the exact duration of the pain sensation experienced after the injection is a weak point of this test. An attempt to explain the animals preference for the AA-paired arm could be that they experienced pain relieve in this compartment due to the fact that they spend a longer time in the maze, referring to the article published by Leknes et al. (2013) to introduce the phenomena that pain could be experienced as pleasant under some circumstances. Healthy volunteers (average age 25) were separated in a relative relief group and a control group. Moderate and intense pain was caused by a thermal resistor applying 48,9 ° C respectively 53,3 ° C at the volar aspect of the left arm. In the relative relief context moderate pain was the best outcome (alternative: intense pain) while moderate pain was the worst outcome in the control context. The participants had to evaluate their sensations in a hedonic scale from pleasant to painful. Here the pain relief group paradoxically rated moderate pain as pleasant

(instead of less painful) despite the intrinsic averseness of pain. Additionally skin conductance and brain activity were compared while both groups experienced moderate pain. At the same intensity of pain sensation the physiological measures did show significantly higher values in skin conductance and higher activities of dorsal anterior cingulate cortex (dACC) within the control group (by fMRI imaging). Taken together these findings showed that it was possible to pair moderate pain with an rewarding outcome in the relative relief setting (Leknes et al., 2013). In accordance with these results the place preference shown in this experiment could originate from the rewarding component of pain relief after AA 0,9 % has induced a very intensive, but remittent pain sensation.

4.2.7 Experiment 7

This protocol introduced metamizol analgesia (s.c. injection) and its effects on the CPA. The pharmacological intervention was intended to suppress the CPA induced by AA 0,9 %. 2 injections were necessary for every animal before each run and therefore the animals were injected 4 times a day. The animals were anaesthetised with isoflurane before each injection to reduce the stress-level during the repeated procedures.

In the probe trial, the animals in the control group showed no significant CPA. Furthermore, the metamizol group spent more time in the AA paired arm then the control group, but these results were not significant.

The aim to reduce stress induced by the repeated injections by the usage of the volatile anaesthetic isoflurane could not be achieved. Isoflurane seems to interfere with the animal's cognitive function and abilities in spatial orientation. Culley et al. (2004) showed in their spatial orientation experiments with rats being exposed to isoflurane that the animals showed impairments in the acquisition of spatial memory within a period of two weeks. Independently from the animal's age less correct choices were made and it took them more time to solve an already learned spatial orientation task in a radial 12-arm maze (Culley et al., 2004). Another work published from D. Lin and Z. Zuo 2011 revealed similar data concerning the cognitive impairments of rats after treatment with isoflurane and additionally, they were able to show decreased neuronal density in the CA1 region of the hippocampus (Lin & Zuo, 2011). Besides the inconsistent data there are strong hints for the neurotoxicity of isoflurane shown in literature (Jevtovic-Todorovic et al., 2003) (Wise-Faberowski et al.,

2005). On the other hand there is evidence that isoflurane has no significant effects upon cognitive function in mice (Butterfield, Graf, Ries, & MacLeod, 2004) while Butterfield and colleagues have anaesthetized the C57BL6 mice after each training. Therefore the time between the narcosis and the next training respectively testing session seems to be of high relevance. A possible assumption would also be that the animals were not able to show their full spatial performance because they got awake from isoflurane narcosis shortly before testing. These facts shown together with the problem that it remains unclear how long the directly affecting side-effects of isoflurane (like confusion, dizziness, vertigo etc.) do persist indicate that the application of isoflurane acts as a confounder in spatial orientation tasks. Furthermore isoflurane seems to influence pain perception by sensitising the TRPV1 channel.

In summary, it is difficult to control the effects of isoflurane upon cognitive function and the perception of pain. Facing these facts it doesn't seem useful to establish isoflurane in behavioural experiments with rodents. Therefore other ways to reduce the animal's stress-level during treatment and testing have to be chosen. This uncovers a major challenge about behavioural assessment in rodents: Every experimental design should be optimized towards a high level of stress reduction for the animals. The handling of the animals and the injections were the most vulnerable steps in these experiments and the experience in experiment 6 very much indicates that handling and injection of the animals should be reduced to an absolute minimum to gather reliable data.

4.2.8 Experiment 8

This experiment aimed to deliver more evidence to confirm the findings shown in experiment 6 that a protocol of prolonged training sessions also leads to a CPA respectively a CPP. As described in 4.2.6 a possible explanation for the findings in experiment 6 could be that the animals experienced pain relieve as a rewarding stimulus and therefore preferred for the pain-paired compartment. Accordingly, it should be possible to use the same prolonged protocol for the training of another cohort of animals using an indisputable rewarding stimulus. The rewarding properties of cocaine were described in various publications using different animal models also involving mice as subjects (Munoz-Cuevas et al., 2013). Literature shows a wide range of dosing of cocaine in successfully operated CPP protocols:

10 – 30 mg per kg (see dela Cruz et al., 2009; Munoz-Cuevas et al., 2013). Therefore, cocaine was assumed to be an appropriate stimulus for a CPP experiment.

The present data (see Figure 23) showed that a prolonged protocol including longer sessions in a lower frequency during the training works with C57BL6 mice in a spatial orientation task, although it was pharmacologically supported by cocaine.

4.3 Translational outlook

Data deducted from the experiments above shows that mice navigate through a maze using and memorize external spatial cues and successfully establishing a CPA by a visceral pain-inducing stimulant. Finally these findings must be considered in a translational context.

Functional magnetic resonance imaging (fMRI) studies in patients suffering of irritable bowel syndrome (IBS) yield evidence that there are structural changes related to visceral pain in various areas of the brain including medial and lateral prefrontal regions, thalamus, HPC or the periaqueductal grey (PAG) (see Kwan et al., 2005). Rectal balloon distension was used as a painful visceral stimulus in these fMRI studies with IBS patients and it was possible to prove that these patients displayed abnormal brain responses to painful visceral stimulation compared to healthy volunteers. CL Kwan et al. (2005) demonstrated that the medial thalamus and the hippocampus show pain related responses during painful rectal distension in IBS patients while the healthy control croup did show pain related activity in the right anterior insula and the right ACC (Kwan et al., 2005). In another oxygen 15 labelled water PET study the alteration of regional cerebral blood flow during rectal distension in IBS patients, UC patients and healthy controls was compared. It turned out that there was more activity in parts of the PFC and the PAG of the healthy controls compared to the IBS patients. However, IBS patients showed greater activation of the amygdala, rostroventral ACC, and dorsomedial frontal cortical regions. Mayer et al. (2005) puts it straight that affective networks show more activity in IBS patients while the regions of anti-nociception are less active (Mayer et al., 2005). Finally, a study where the changes in neural activity in brains of IBS patients undergoing cognitive behavioural therapy (CBT) was examined, has to be mentioned. After treatment the patients showed a reduction of baseline activity in the parahippocampal gyrus and inferior portion of the right ACC. Furthermore, they showed improvements in GI symptoms, anxiety and worry. The authors assumed that the CBT caused changes in the activity of the relevant brain regions leading to a reduction of attention to visceral stimuli or visceral-specific anxiety. Therefore learning plays an important role in the perception of visceral pain (Lackner et al., 2006) and affective factors could determine a deficiency in anti-nociceptive response in IBS patients. These insights allow the definition of the brain regions where the alteration of affective-motivational processing of visceral pain is located, but the underlying molecular and neurobiological processes remain elusive.

Another challenge is to address the issue of the origin of the detected changes: Do they originate from abnormal efferent input or from an altered processing of the efferent input?

Furthermore it is important to understand the underlying molecular pathways for pharmacological approaches. For example antidepressants are used to treat functional gastrointestinal disorders (Ford et al., 2014), but the question whether they directly influence the processing of visceral pain or just address global distress in these patients have not yet been answered. For instance, patients treated with amitriptyline displayed “reduced pain related cerebral activations in the perigenual ACC and the left posterior parietal cortex” during rectal balloon distension under exposure to auditory stress at the same time (Morgan et al, 2005). Under these circumstances it is difficult to causally determine the effects of the pharmacological intervention by detecting changes in cerebral activation.

These examples show that functional magnetic resonance imaging is a helpful tool to observe structural or functional changes in the brain of patients suffering of (chronic) visceral pain. However it leads to a lot of new questions which cannot be examined in human subjects. Therefore the experiments of the present work implementing affective components and context dependent learning in a mouse model of visceral pain could offer new translational approaches to learn more about the interaction between the complex endogenous pain modulation system and influencing factors like environmental context, cognition or affect. We were able to prove that mice show a change of motivational state after experiencing visceral pain in a certain context and therefore this experimental design could be an approach to clarify the underlying molecular mechanisms leading to neurobiological changes in the brain as shown in previous fMRI studies, for instance. After successfully establishing an animal model it could be possible to analyse the neurobiology of affective and cognitive components of visceral pain by using molecular methods and pharmacological approaches.

5 Summary

Research about the processing of visceral pain is important to understand pathomechanisms of gastrointestinal diseases. There are various experimental animal models of visceral pain for rodents like the acetic acid writhing test, the cerulein-induced acute pancreatitis or the colorectal distension test. The main caveat for these models is that they mostly show the sensory discriminant components of pain and reflex-related behaviours after receiving a strong painful stimulus. However visceral pain and especially chronic visceral pain cause strong affective and cognitive reactions and most of these tests do not recapitulate this pain component. Therefore, these animal models of visceral pain are limited if it comes to translation from bench to bedside. To assess higher brain processing in animal studies of visceral pain tests which include emotion and memory are needed. The corticolimbic system as the center of anxiety, fear and aversive memories does play an important role in the development of pain related behaviours. Spatial orientation is a central task of the limbic system as well. If the painful stimulus is presented in a context of spatial orientation it is possible to simulate the learning of pain related behaviours in rodents.

The present work shows that inbred mice are able to navigate through a maze using external spatial cues and memorise them. If mice experienced visceral pain in a spatial orientation task, they displayed increasing conditioned place avoidance suggesting the evolvement of pain memory. Based on these findings it is possible to study complex, cognitive pain related behaviours in a mouse model. This sets the basis of studying the underlying neurophysiological changes of pain learning and memory. We achieved a model of visceral pain in laboratory animals including a complex visceral pain memory and emotion representation, which can be used to examine brain mechanisms of visceral nociception and to evaluate novel analgesic substances.

6 References

Binkofski, F., Schnitzler, A., Enck, P., Frieling, T., Posse, S., Seitz, R. J., & Freund, H. J. (1998). Somatic and limbic cortex activation in esophageal distention: a functional magnetic resonance imaging study. *Annals of Neurology*, *44*(5), 811–5. doi:10.1002/ana.410440516

Breivik, H., Collett, B., Ventafridda, V., Cohen, R., & Gallacher, D. (2006). Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. *European Journal of Pain (London, England)*, *10*(4), 287–333. doi:10.1016/j.ejpain.2005.06.009

Brown, R. E., Corey, S. C., & Moore, A. K. (1999). Differences in Measures of Exploration and Fear in MHC-Congenic C57BL / 6J and B6-H-2K Mice, *29*(4), 263–271.

Burgess, N., Maguire, E. A., & O'Keefe, J. (2002). The human hippocampus and spatial and episodic memory 1. *Neuron*, *35*(4), 625–641. Retrieved from ISI:000177521000005

Butterfield, N. N., Graf, P., Ries, C. R., & MacLeod, B. A. (2004). The Effect of Repeated Isoflurane Anesthesia on Spatial and Psychomotor Performance in Young and Aged Mice. *Anesthesia & Analgesia*, 1305–1311. doi:10.1213/01.ANE.0000108484.91089.13

Cao, B., Zhang, X., Yan, N., Chen, S., & Li, Y. (2012). Cholecystokinin enhances visceral pain-related affective memory via vagal afferent pathway in rats. *Molecular Brain*, *5*, 19. doi:10.1186/1756-6606-5-19

Carr, D., Cohen, M., Devor, M., Dworkin, R., Greenspan, J., Jensen, T., King, S., Koltzenburg, M., Loeser, J., Merskey, H., Okifuji, A., Paice, J., Serra, J., Treede, R.D., & Woda, A. (2011). Pain Terms. *Classification of Chronic Pain, Second Edition*. Retrieved from http://www.iasp-pain.org/files/Content/ContentFolders/Publications2/ClassificationofChronicPain/Part_III-PainTerms.pdf

Cervero, F. (1994). *Sensory innervation of the viscera: peripheral basis of visceral pain. Physiological reviews* (Vol. 74). Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8295936

Cervero, F., & Laird, J. M. (1999). Visceral pain. *Lancet*, *353*(9170), 2145–8. doi:10.1016/S0140-6736(99)01306-9

Collier, H. J., Dinneen, L. C., Johnson, C. A., & Schneider, C. (1968). THE ABDOMINAL CONSTRICTION RESPONSE AND ITS SUPPRESSION BY ANALGESIC DRUGS IN THE MOUSE BY. *British Journal of Pharmacology*, *32*, 295–310.

Cressant, A., Besson, M., Suarez, S., Cormier, A., & Granon, S. (2007). Spatial learning in Long-Evans Hooded rats and C57BL/6J mice: different strategies for different performance. *Behavioural Brain Research*, *177*(1), 22–29. doi:10.1016/j.bbr.2006.11.010

Culley, D. J., Baxter, M. G., Yukhananov, R., & Crosby, G. (2004). Long-term impairment of acquisition of a spatial memory task following isoflurane-nitrous oxide anesthesia in rats. *Anesthesiology*, *100*(2), 309–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14739805

Cunningham, C. L. (2014). Genetic Relationship Between Ethanol-Induced Conditioned Place Preference and Other Ethanol Phenotypes in 15 Inbred Mouse Strains. *Behavioral Neuroscience*, *128*(4), 430–445. doi:10.1037/a0036459

Cunningham, C. L., Gremel, C. M., & Groblewski, P. A. (2006). Drug-induced conditioned place preference and aversion in mice. *Nature Protocols*, *1*(4), 1662–1670. doi:10.1038/nprot.2006.279

dela Cruz, A. M., Herin, D. V., Grady, J. J., & Cunningham, K. A. (2009). Novel Approach to Data Analysis in Cocaine Conditioned Place Preference. *Behav Pharmacol.*, *20*(8), 720–730. doi:10.1097/FBP.0b013e328333b266.Novel

Deyama, S., Katayama, T., Kondoh, N., Nakagawa, T., Kaneko, S., Yamaguchi, T., Yoshioka, M., & Minami, M. (2009). Role of enhanced noradrenergic transmission within the ventral bed nucleus of the stria terminalis in visceral pain-induced aversion in rats. *Behavioural Brain Research*, *197*(2), 279–83. doi:10.1016/j.bbr.2008.08.024

Ford, A. C., Quigley, E. M. M., Lacy, B. E., Lembo, A. J., Saito, Y. A., Schiller, L. R., Soffer, E.,Spiegel, B., & Moayyedi, P. (2014). Effect of antidepressants and psychological therapies, including hypnotherapy, in irritable bowel syndrome: systematic review and meta-analysis. *The American Journal of Gastroenterology*, *109*(9), 1350–65; quiz 1366.

doi:10.1038/ajg.2014.148

Grotewold, S. K., Wall, V. L., Goodell, D. J., Hayter, C., & Bland, S. T. (2014). Effects of cocaine combined with a social cue on conditioned place preference and nucleus accumbens monoamines after isolation rearing in rats. *Psychopharmacology*, *231*(15), 3041–3053. doi:10.1007/s00213-014-3470-0

Häbler, H., Jänig, W., & Koltzenburg, M. (1990). Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. *The Journal of Physiology*, 545–562. Retrieved from http://jp.physoc.org/content/425/1/545.short

Ingberg, E., Theodorsson, A., Theodorsson, E., & Strom, J. O. (2011). Methods for long-term 17 β -estradiol administration to mice. *General and Comparative Endocrinology*, *175*(November), 188 – 193. doi:10.1016/j.ygcen.2011.11.014

Itzhak, Y., & Martin, J. L. (2002). Cocaine-induced conditioned place preference in mice: induction, extinction and reinstatement by related psychostimulants. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *26*(1), 130–4. doi:10.1016/S0893-133X(01)00303-7

Jacobsen, K. R., Fauerby, N., Raida, Z., Kalliokoski, O., Hau, J., Johansen, F. F., & Abelson, K. S. (2013). Effects of buprenorphine and meloxicam analgesia on induced cerebral ischemia in C57BL/6 male mice. *Comparative Medicine*, *63*(2), 105–13. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3625051&tool=pmcentrez&rendertype=abstract

Jevtovic-Todorovic, V., Hartman, R. E., Izumi, Y., Benshoff, N. D., Dikranian, K., Zorumski, C. F., Olney, J.W. & Wozniak, D. F. (2003). Early Exposure to Common Anesthetic Agents Causes Widespread Neurodegeneration in the Developing Rat Brain and Persistent Learning Deficits, *23*(3), 876–882.

Karimi, S., Radahmadi, M., Fazilati, M., Azizi-Malekabadi, H., & Alaei, H. (2014). Roles of the Nucleus Accumbens (Shell) in the Acquisition and Expression of Morphine-Induced Conditioned Behavior in Freely Moving Rats. *International Journal of Preventive Medicine*, *5*(3), 262–268. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4018634/

Kleinknecht, K. R., Bedenk, B. T., Kaltwasser, S. F., Grünecker, B., Yen, Y.-C., Czisch, M., & Wotjak, C. T. (2012). Hippocampus-dependent place learning enables spatial flexibility in C57BL6/N mice. *Frontiers in Behavioral Neuroscience*, *6*(December), 87. doi:10.3389/fnbeh.2012.00087

Korzeniewska-Rybicka, I., & Plaznik, A. (2001). Role of serotonergic and noradrenergic systems in a model of visceral pain. *Polish Journal of Pharmacology*, *53*, 475–480.

Koster, R., Anderson, M., & De-Beer, E. J. (1959). Acetic acid for analgesic screening. *Federation Proceedings*, *18*, 412–418.

Kwan, C. L., Diamant, N. E., Pope, G., Mikula, K., Mikulis, D. J., & Davis, K. D. (2005). Abnormal forebrain activity in functional bowel disorder patients with chronic pain. *Neurology*, *65*(8), 1268–1277. doi:10.1212/01.wnl.0000180971.95473.cc

Lackner, J. M., Lou Coad, M., Mertz, H. R., Wack, D. S., Katz, L. A., Krasner, S. S., Firth, R., Mahl, T. C., & Lockwood, A. H. (2006). Cognitive therapy for irritable bowel syndrome is associated with reduced limbic activity, GI symptoms, and anxiety. *Behaviour Research and Therapy*, *44*(5), 621–638. doi:10.1016/j.brat.2005.05.002

Leknes, S., Berna, C., Lee, M. C., Snyder, G. D., Biele, G., & Tracey, I. (2013). The importance of context: When relative relief renders pain pleasant. *Pain*, *154*(3), 402–410. doi:10.1016/j.pain.2012.11.018

Lin, D., & Zuo, Z. (2011). Isoflurane induces hippocampal cell injury and cognitive impairments in adult rats. *Neuropharmacology*, *61*(8), 1354–1359. doi:10.1016/j.neuropharm.2011.08.011.Isoflurane

Mauderli, A. P., Acosta-Rua, A., & Vierck, C. J. (2000). An operant assay of thermal pain in conscious, unrestrained rats. *Journal of Neuroscience Methods*, *97*(1), 19–29. doi:http://dx.doi.org/10.1016/S0165-0270(00)00160-6

Mayer, E. A., & Tillisch, K. (2011). The brain-gut axis in abdominal pain syndromes. *Annual Review of Medicine*, *62*, 381–96. doi:10.1146/annurev-med-012309-103958

Mayer, E. A. (2000). The neurobiology of stress and gastrointestinal disease. *Gut*, *47*, 861–869.

Mayer, E. A., Berman, S., Suyenobu, B., Labus, J., Mandelkern, M. A., Naliboff, B. D., &

Chang, L. (2005). Differences in brain responses to visceral pain between patients with irritable bowel syndrome and ulcerative colitis. *Pain*, *115*(3), 398–409. doi:10.1016/j.pain.2005.03.023

Mayer, E. A., Bradesi, S., Chang, L., Spiegel, B. M. R., Bueller, J. A., & Naliboff, B. D. (2008). Functional GI disorders: from animal models to drug development. *Gut*, *57*(3), 384–404. doi:10.1136/gut.2006.101675

Morgan, V., Pickens, D., Gautam, S., Kessler, R., & Mertz, H. (2005). Amitriptyline reduces rectal pain related activation of the anterior cingulate cortex in patients with irritable bowel syndrome. *Gut*, *54*(5), 601–607. doi:10.1136/gut.2004.047423

Munoz-Cuevas, F. J., Athilingam, J., Piscopo, D., & Wilbrecht, L. (2013). Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference. *Nat Neurosci*, *16*(10), 1367–1369. Retrieved from http://dx.doi.org/10.1038/nn.3498

Ness, T. J. (1999). Models of Visceral Nociception. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, *40*(3), 119–128.

Phillips, M. L., Gregory, L. J., Cullen, S., Coen, S., Ng, V., Andrew, C., Giampietro, V., Bullmore, E., Zelaya, F., Amaro, E., Thompson, D.G., Hobson, A.R., Williams, S.C., Brammer, M., & Aziz, Q. (2003). The effect of negative emotional context on neural and behavioural responses to oesophageal stimulation. *Brain : A Journal of Neurology*, *126*(Pt 3), 669–684.

Prus, A. J., James, J. R., & Rosecrans, J. A. (2009). *Methods of Behavior Analysis in Neuroscience. 2nd edition. Chapter 4.* (J. Buccafusco, Ed.). Boca Raton (FL): CRC Press/Taylor & Francis.

Roesler, R., Schröder, N., Vianna, M. R. M., Quevedo, J., Bromberg, E., Kapczinski, F., & Ferreira, M. B. C. (2003). Differential involvement of hippocampal and amygdalar NMDA receptors in contextual and aversive aspects of inhibitory avoidance memory in rats. *Brain Research*, *975*(1-2), 207–213. doi:10.1016/S0006-8993(03)02656-8

Sengupta, J. N., Saha, J. K., & Goyal, R. K. (1990). Stimulus-response function studies of esophageal mechanosensitive nociceptors in sympathetic afferents of opossum. *Journal of Neurophysiology*, *64*(3), 796–812. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2230925

Sora, I., Wichems, C., Takahashi, N., Li, X., Zeng, Z., Revay, R., Lesch, K.P., Murphy, D.L., & Uhl, G. (1998). Cocaine reward models : Conditioned place preference can be established in dopamine- and in serotonin-transporter. *Proc Natl Acad Sci U S A.*, *95*(June), 7699–7704.

Stevenson, G. W., Bilsky, E. J., & Negus, S. S. (2006). Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. *The Journal of Pain : Official Journal of the American Pain Society*, *7*(6), 408–416. doi:10.1016/j.jpain.2006.01.447

Tammpere, A., Brusberg, M., Axenborg, J., Hirsch, I., Larsson, H., & Lindstrom, E. (2005). Evaluation of pseudo-affective responses to noxious colorectal distension in rats by manometric recordings. *Pain*, *116*(3), 220–226. doi:10.1016/j.pain.2005.04.012

Tolman, E. C., & Honzik, C. H. (1930). Introduction and removal of reward, and maze performance in rats. *University of California Publications in Psychology*, *4*, 257–275.

Tolman, E. C., Ritchie, B. F., & Kalish, D. (1946). Studies in spatial learning. I. Orientation and the short-cut. *Journal of Experimental Psychology*, *36*, 13 – 24.

Tzschentke, T. M. (1998). Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Progress in Neurobiology*, *56*(6), 613–672. doi:http://dx.doi.org/10.1016/S0301-0082(98)00060-4

Vlaeyen, J. W., & Linton, S. J. (2000). Fear-avoidance and its consequences in chronic musculoskeletal pain: a state of the art. *Pain*, *85*(3), 317–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10781906

Wang, H.-C., Chai, S.-C., Wu, Y.-S., & Wang, C.-C. (2007). Does the medial thalamus play a role in the negative affective component of visceral pain in rats? *Neuroscience Letters*, *420*(1), 80–4. doi:10.1016/j.neulet.2007.04.064

Whishaw, I. Q., Haun, F., & Kolb, B. (1999). Analysis of Behavior in Laboratory Rodents BT - Modern Techniques in Neuroscience Research. In U. Windhorst & H. Johansson (Eds.), *Modern Techniques in Neuroscience Research* (pp. 1243–1275). Berlin, Heidelberg: Springer Berlin Heidelberg. doi:10.1007/978-3-642-58552-4_44

Wise-Faberowski, L., Zhang, H., Ing, R., Pearlstein, R. D., & Warner, D. S. (2005). Isoflurane-induced neuronal degeneration: an evaluation in organotypic hippocampal slice cultures. *Anesthesia and Analgesia*, *101*(3), 651–7, table of contents. doi:10.1213/01.ane.0000167382.79889.7c

Zhong, X.-L., Wei, R., Zhou, P., Luo, Y.-W., Wang, X.-Q., Duan, J., Bi, F.-F., Zhang, J.-Y., Li, C.-Q., Dai, R.-P., & Li, F. (2012). Activation of Anterior Cingulate Cortex Extracellular Signal-Regulated Kinase-1 and -2 (ERK1/2) Regulates Acetic Acid-Induced, Pain-Related Anxiety in Adult Female Mice. *Acta Histochemica et Cytochemica*, *45*(4), 219–225. doi:10.1267/ahc.12002

7 Table of Figures

Figure 10: After FET (6 minutes) the animals (n=5) were trained daily in 2 runs (8 minutes per run) for 3 days. Training consisted of i.p. injections of saline (first run) or AA 0,9 % (second run) while the pairing of injected solution and trained arm was completely randomized. The probe trial (3 minutes) started from the shortened western arm at day 5 and was repeated on day 8.

Figure 11: After FET (6 minutes) the animals (n=7) were trained daily in 2 runs (30 minutes per run) for 3 days. Training consisted of i.p. injections of saline (first run) or AA 0,9 % (second run) while the pairing of injected solution and trained arm was completely randomized. The probe trial (3 minutes) started from the shortened western arm at day 5.

Figure 12: After FET (6 minutes) one group (n = 8) was s.c. injected with saline and the other group (n = 8) with metamizol (300 mg / kg) 30 minutes prior to the i.p. injections of saline or AA 0,9 % (under isoflurane narcosis). Every run lasted for 8 minutes and the pairings of AA 0,9 % to the south or the north arm were completely randomized per animal.

Figure 13: After FET (6 minutes) every animal (n = 8) received cocaine and saline 2 times (2 days saline and 2 days cocaine – one i.p. injection per day) in an alternating way for 4 days. Half of the group received saline at the first day and the other half started with cocaine (15 mg / kg bodyweight) at the first day in a counterbalanced design. The cocaine injection was paired with the arm that was less preferred during FET. After the injections animals were placed in one arm of the maze for 30 minutes.

Figure 14 The time spend in each compartment during FET and probe trial with pooling of data from AA 0,5 % and AA 0,9 % injected mice. ** p = 0,0042

Figure 15: A significant difference of time spent in the AA paired compartment was observed in the AA 0,9 % treatment group. * p = 0,0320 (paired t-test)

Figure 16: **(A)** The average time the animals needed from start to cross the line at latency 1 is shown per day. **(B)** The average time the animals needed from start until drinking the sucrose solution is shown per day. **(C)** Number of total arms visited shown per day. **(D)** Percentage of accurate trials shown per day.

Figure 17: **(A)** The average time the animals needed from start to cross the line at latency 1 is shown per day. **(B)** The average time the animals needed from start until consuming

hazelnut-spread is shown per day. **(C)** Number of total arms visited shown per day. **(D)** Percentage of accurate trials shown per day.

Figure 18: Animals displayed a significant preference for the target arm * p = 0,0368 (paired t-test).

Figure 19: The percentage of time spent at target and wrong target is shown. Only mice in the alternating training group displayed a significant preference for the target area. * p = 0,0393 (paired t-test)

Figure 20: **(A)** Animals spent significantly less time in the AA-paired arm during the probe trial on day 5 expressed as percentage of total time spent in arms. **(B)** The animals didn't show a significant place avoidance during the repetition of probe trial at day 8. * p = 0,0329; ** p = 0,0253 (paired t-test)

Figure 21: Animals spent significantly less time in the saline paired arm during the probe trial on day 5 expressed as percentage of total time spent in arms. * p = 0,0441 (paired t-test).

Figure 22: During the probe trial there was a trend towards CPA in the control group. Metamizol treatment did not reverse the place aversion in the treatment group (p = 0,5427; unpaired t-test).

Figure 23: The animals spent significantly more time in the cocaine paired arm than the saline paired arm (percentage of total time spent in both arms) * p = 0,0296; ** p = 0,0083 (paired t-test).

8 List of Abbreviations

AA ..... acetic acid
ACC ..... anterior cingulate cortex
AP5 ..... 2-amino-5-phosphonopentanoic acid
CBT ..... cognitive behavioural therapy
CPA ..... conditioned place aversion
CPP ..... conditioned place preference
dACC ..... dorsal anterior cingulate cortex
FET ..... free exploration trial
fMRI ..... functional magnetic resonance imaging
GI ..... gastrointestinal
HPC ..... Hippocampus
i. p. ..... intraperitoneal
IASP ..... International Association for the study of Pain
IBS ..... irritable bowel syndrome
MPI ..... Max-Planck-Institut
NSAID ..... non-steroidal anti-inflammatory drug
PAG ..... periaqueductal grey
PET ..... positron emission tomography
PFC ..... prefrontal cortex
s.c. ..... subcutaneous
TRPV1 ..... Transient Receptor Potential Vanilloid 1
UC ..... ulcerative colitis
WCM ..... water-cross maze

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