Hochschule für Angewandte Wissenschaften Hamburg
Hamburg University of Applied Sciences

Hamburg University of Applied Sciences
Faculty of Life Sciences

Investigation of innate behaviour as novel endpoints for the assessment of pain in mice with acute inflammation

Bachelor Thesis

At the Department of Biotechnology

Submitted from
Jonas Ringeisen,
1919640

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Examiner: Prof. Dr. Birger Anspach (HAW Hamburg)
Examiner: Dr. Susan Boyce (Evotec AG, Neuropharmacology)

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i. Table of Content

ii. List of abbreviations

Abbreviation	Meaning
®	registered trade mark
ANOVA	analysis of variance
BALB/c	Albino inbred strain of laboratory mice
C57BL/6J	Black inbred strain of laboratory mice
CFA	Complete Freund's Adjuvant
DRG	Dorsal root ganglion
GPCR	G protein coupled receptors
HT-tube	High temperature tube
i.plt.	intraplantar (within the sole of the foot)
IFA	Incomplete Freund’s Adjuvant
IL-6	Interleukin-6
IM	indomethacin
IR	Infrared, radiation with wavelength between 700 nm to 1000 nm
MC	Methylcellulose
mW/cm²	Milliwatt per square centimetres
NSAIDs	nonsteroidal anti-inflammatory drugs
p.o.	peroral (through or by way of the mouth)
PBS	Phosphate buffered saline
PG	Prostaglandins
PKA	protein kinase A
PMN	polymorphonuclear leukocytes; granulocytes
PWL	Paw withdrawal latency
SEM	standard error of the mean
SP	substance P
TNFα	Tumour necrosis factor alpha
TRP	transient receptor potential

iii. Tables index

iv. Figures index

1 Introduction

Chronic pain affects over 11 million people in Germany each year (Wolff, et al. 2011)[1], many of whom are inadequately treated with current analgesics. The aetiology of pain is multifunctional arising from cancer, multiple sclerosis to back pain and arthritis. This not only results in chronic suffering to the patient, but also high economic costs in terms of medical bills, lost production and missed work. Therefore, there is a high medical need for the development of new and improved analgesics which reduce side effects. The International Association for the Study of Pain defines pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Merskey, et al. 1994)[2]. Acute pain results from disease, inflammation, or injury to tissues. It generally comes on suddenly after trauma or surgery, is self-limiting, and resolves within a few days. It is a proactive mechanism to prevent further injury to the individual. By contrast, chronic pain is widely believed to represent a disease itself. Chronic pain persists over several months or even years and is usually resistant to most medical treatments. Symptoms of pain can vary significantly from one disease to another and even from one patient to the next. Pain can be a dull, itchy, stabbing, shooting and burning or pins and needles sensation. The pain may be spontaneous or induced or exacerbated by movement for example an inflamed joint during walking or by heat as is the case when taking a hot shower with sunburnt skin. Spontaneous pain is the most common form of pain in patients with chronic pain. The mechanisms mediating the different types of pain are also different and so it is essential that new medications be evaluated for efficacy against all types of pain.

The methods used in laboratory animals for the detection of acute pain and hypersensitivity and to assess the efficacy of analgesics are usually based on reflexes such as tail-flick test or the paw withdraw test either in absence or presence of inflammation or following injury. Such assays were used to identify new analgesics such as COX-2 inhibitor. Nevertheless, these tests only measure evoked and not spontaneous pain. The effects of pain on the general condition of the animal are not reflected (Andrews, et al. 2012)[3]. Pain is difficult to diagnose in mice, as this species does not openly show signs of pain. This is because mice are prey animals. Thus, mice hide signs of pain or physical impairment, so they do not attract the attention of potential predators or be excluded from the group (Hutchinson, et al. 2005)[4]. The aim

of this bachelor thesis is to investigate a possible spontaneous pain model in mice, in which the recovery of an innate behaviour is the end-point. For these studies, disruption of burrowing behaviour will be assessed as a measure of spontaneous pain. Burrowing is an innate behaviour, which in the wild serves to build a shelter and gather to food. Laboratory rodents, although domesticated over many years, retain burrowing behaviour. This ethological test will be used to assess the development of spontaneous pain in mice after unilateral injection of Complete Freund's Adjuvant into the hind paw. The results will be compared to conventional pain test measuring the paw withdrawal latency following application of thermal stimulus to the inflamed paw. If the ethologic test is sensitive and robust, once established it will be used to assess spontaneous pain in mice and to evaluate efficacy of new analgesics.

1.1 Anatomy and physiology of pain

Pain is an essential physiological response that warns of danger. The reception of noxious stimuli through specialized sensory receptors (nociceptors) is referred to as nociception. Pain is the emotional experience of nociception. A noxious stimulus which triggers a nociceptor does not necessarily lead to a sensation of pain as it requires processing of the stimulus in the higher brain centres (e.g. cingulate cortex and insula) to perceive this as painful (Noback, et al. 2005)[5]. Pain is a part of the mammalian defensive system and is a protective mechanism to keep the individual from harmful situations e.g. penetrating of the skin by sharp objects, hot or cold surfaces. There are two different types of pain, somatic and visceral pain. Somatic pain arises from the joints, muscles and skin. As these contain many nociceptors, the pain is easier to locate and the intensity is well defined. By contrast, visceral organs have very few nerve fibres (nociceptors) and so visceral pain is dull and vague and not very well defined (Fein 2012)[6].

1.2 Nociceptors

Nociceptors are naked nerve endings in the epithelia: They are characterized by peripheral terminal arborisation and have a fenestrated sheath of Schwann cells and consist of numerous expansions (varicosities) which contain neuropeptides e.g. substance P. They react to noxious physical and chemical stimuli, which could lead to local injury. There are four different kinds of nociceptors, which vary depending on the sensory task and afferent nerve fibres. There are: a) mechanosensitive nociceptors which are thin myelinated A-δ fibres which respond only to mechanical stimulation; b) mechanothermal sensitive nociceptors which are thin myelinated A-δ fibres which respond to mechanical and thermal stimuli; c) polymodal nociceptors which are unmyelinated C-fibres which responds to mechanical, thermal and chemical stimuli; d) silent nociceptors which respond only to thermal or mechanical after inflammation (Fein 2012)[6]. As the following table shows, there are different types of nerve fibres related to different nociceptors. Thin myelinated A-δ fibres are responsible for mediating the fast axon reflex as these have moderate conduction velocity (Table 1). These fibres mainly convey noxious mechanical stimuli. For example, A-δ fibres are responsible for the pain experienced when one hits oneself with a hammer. While the unmyelinated C-fibres have a lower conduction velocity and activation relates to the slower burning, aching sensation that arises after touching a hot surface.

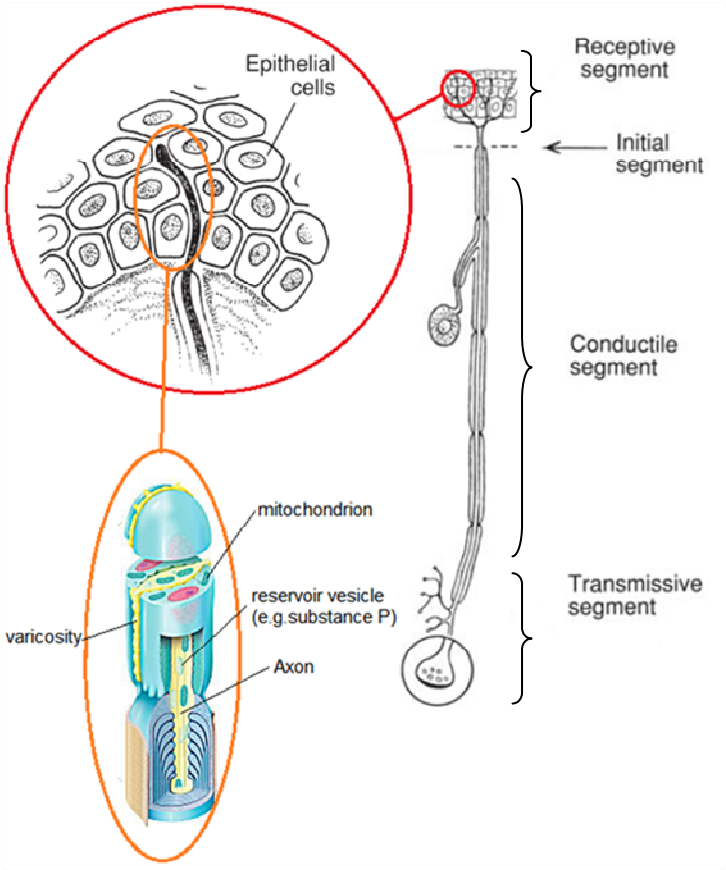

Figure 1 Open nerve ending (orange), location of nerve ending in the epethelial (red) and cell body in cranial/spinal ganglion

This figure shows the embedded open nerve ending in the receptive segment. The cell body and myelinated axon as conductive segment. And the synapses as transmissive segment (Noback, et al. 2005)[I]; (www.medical-pictures.de)[II].

Table 1 Table of nociceptors

Type of Nociceptor	Afferent nerve fibres	Transduction velocity of nerve fibres [m/s]	Diameter [µm]	Myelinated
Mechanosensitive	A-δ	10-30	2-5 µm	Yes
Mechanothermal	A-δ	10-30	2-5 µm	Yes
Polymodal	C-fibres	>3	0.2-1.5 µm	no
Silent	C-fibres	>3	0.2-1.5 µm	no

1.2.1 Signal transduction

The transduction of a noxious pain begins at the nociceptive nerve terminals. An action potentials is caused by activation of specific receptors or ion channels. The action potential is forwarded through the dorsal horn into the area of the brain (somatosensory cortex) where the signal is received and interaction with the cingulate cortex and insula contributes the emotional quality of pain. Within the somatosensory cortex, there is a good correlation of the intensity of the stimulus and the experience of the pain, but there are also regulatory mechanisms in different

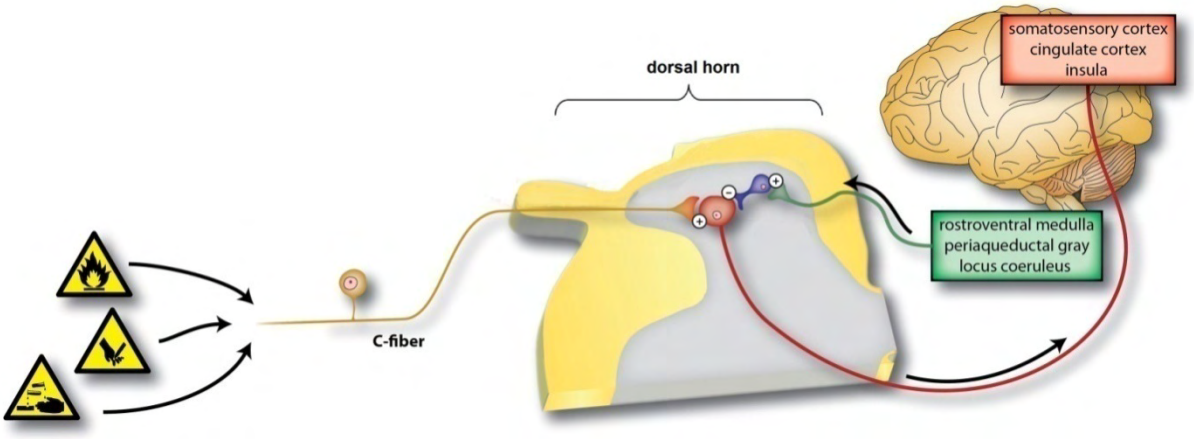

Figure 2 Pathway of sensory experience

Nociceptors are activated by intense mechanical, thermal, or chemical stimuli and transduce these signals to nociceptive neurons in the spinal cord (dorsal horn). Then ongoing via the thalamus to cortical areas generating the sensory and emotional qualities of pain. The impulse conduction through the spinal cord is subject to inhibitory and supportive influences from the brainstem (von Hehn, et al. 2012)[III].

levels of the nociceptive system. This leads to a large variation in experience of pain (Fischer, et al. 2010)[7]. An example of a regulatory system is the rostroventral medulla. It has three types of neurons, On-cells, Off-cells, and neutral cells. Off-cells are showing a temporary reduction in excitatory action right before a nociceptive reflex and have presumably an inhibitory effect. Activation of the Off-cells, for example, by morphine results in a reduction of nociception. Just before receiving a harmful stimulus, an increase in the activity was measured at On-cells. This suggests that on-cells could enhance stimulus conduction. Neutral cells do not contribute to enhancement of the excitatory drive (Urban, et al. 1998)[8].

1.2.1.1 Resting potential

To transmit any signal there has to be resting potential in the first place (Fein 2012)[6]. The difference in electric charge is the result of un-equally distributed positive and negative ions across the membrane. Those ions are sodium (Na^+) and chloride (Cl^-) and potassium (K^+). A tendency exists for the Na^+, K^+, and Cl^- ions to diffuse along concentration gradients through Na^+, K^+, and Cl^- channels. The semi permeable plasma membrane is selectively permeable through non-gated open channels. These channels are always open. They are important in determining the resting potential. The ionic concentrations on either side of the membrane are produced and maintained by a system of membrane pumps. The sodium-potassium exchange pump is an integral membrane protein that utilizes ATP as an energy source for its role in active transport. Those pumps are always active and they exchange three Na^+- ions for 2 K^+- ions 100 times per second. Due to their activity K^+- ions concentration is 30 times increased at the cytoplasm compared to extracellular space. The concentration of Na^+- ions drop to a tenth at the cytoplasm compared to extracellular space. Through the non gated channels Na^+- ions leave

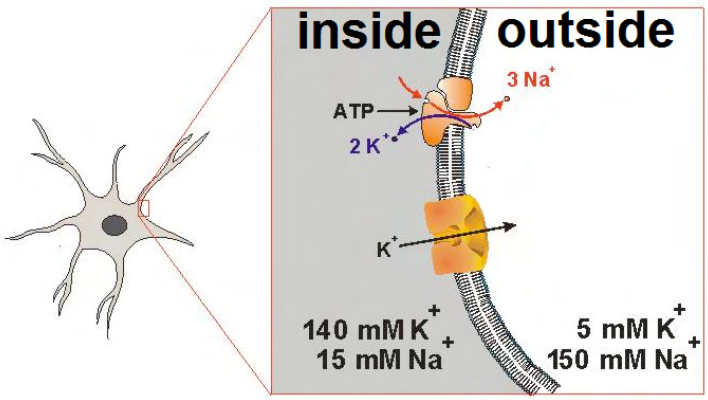

Figure 3 Resting potential

The negative resting potential is produced by active sodium-potassium pump and selectively permeable potassium channels (Lorenz 2012)[IV].

the cells and the negative membrane potential results due to a lack of positive ions (Noback, et al. 2005)[5].

1.2.1.2 Depolarisation

When the nociceptor receives a sufficiently intense stimulus, Na^+ and K^+ channels open and as result depolarization occurs. This is referred as the receptor potential (Noback, et al. 2005)[5]. The resting membrane potential which is normally set at, -70 mV is displaced to more positive values. As long as the potential remains more negative than about -55 mV, potassium flows outwardly through the still open channels. However, if the threshold voltage exceeds -55 mV, the voltage-gated Na^+-channels open. A strong Na^+-current flows into the cell and the membrane depolarizes further reaching positive value of +30 mV. The K^+-channels are still closed at this time due to their delay mechanism. After only 1-2 ms, the Na^+-channels are inactivated and the K^+-channels open. The Na^+-stream stops and K^+-ions flows from the cytoplasm into extracellular space and the membrane hyperpolarized (shifts the potential to more negative values). After the resting potential has recovered Na^+- and K^+-channels are closed. The cell is now ready to react on a stimulus again (Oh 2006)[9].

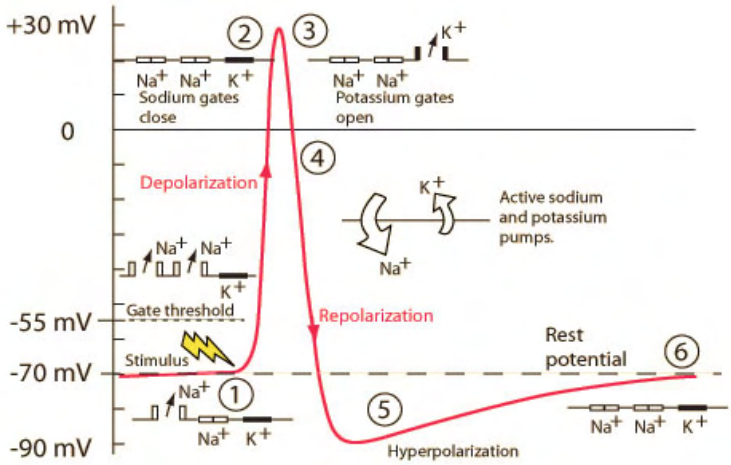

Figure 4 Depolarisation/repolarisation

(1) Stimulus occurs, a few Na^+-channels are open. Stimulus is strong enough to trigger threshold and more Na^+-channels opened. (2) Receptor potential reached maximum .All channels are closed, due to delay of K^+ channels. (3) Potassium channels are open. (4) Repolarisation, as described in 1.2.1.1 (5) Hyperpolarisation. (6) initial state, resting potential (www.hyperphysics.phy-astr.gsu.edu)[V].

1.2.2 Sensitizing of nociceptors

If an inflammation is present, even innocuous stimuli can result in painful sensations. Pronounced inflammation can cause hypersensitivity, because the polymodal nociceptors create a pain signal even at slight stimuli. This is due to a reduced excitation threshold. "In addition, noxious stimuli evoke stronger responses than in the non-sensitized state resulting in hyperalgesia." (Schaible, et al. 2011)[10]. In Figure 5, the effect of sensitization on response to pain, is displayed graphically. It is shown a left shift of the stimulus-response curve. The nociceptors respond earlier and more intensively to a stimulus. This is the result of sensitization of nociceptors but also the activation of silent nociceptors. This activation of silent nociceptors is a major contributor to the increased sensation of pain from inflammatory conditions. Moreover, as mentioned later in the text, explained with reference to the

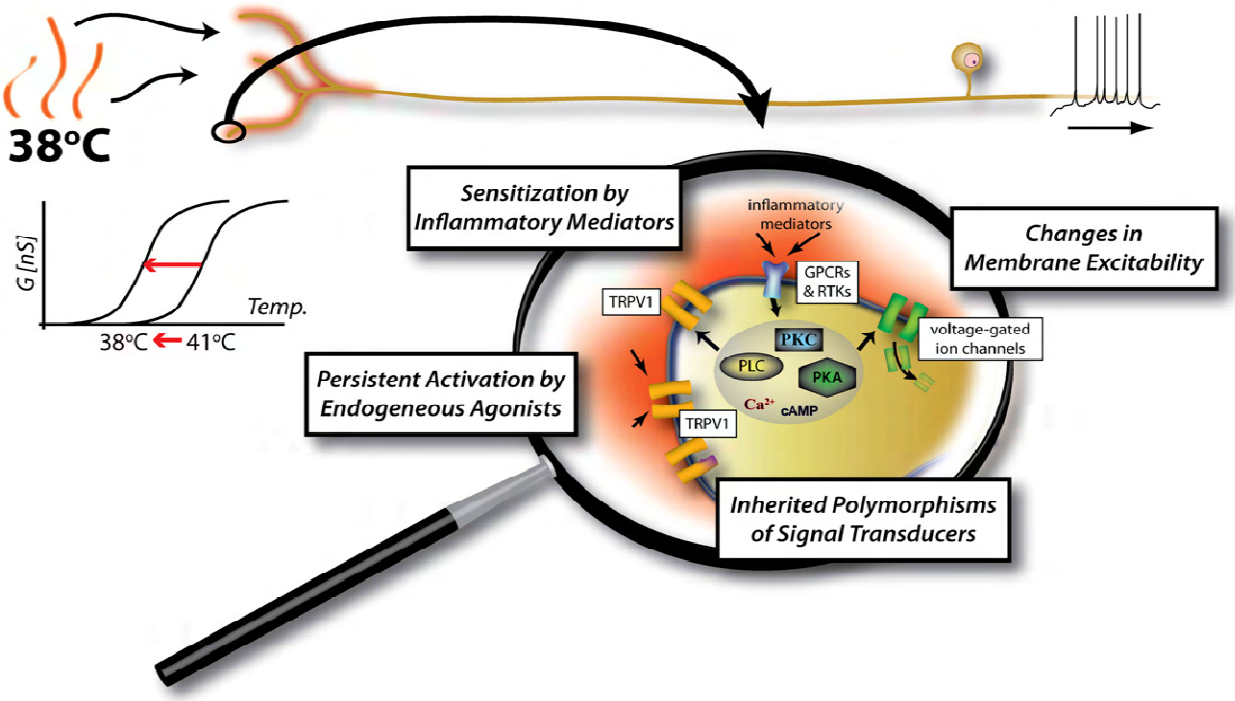

Figure 5 Sensitizing of nociceptors

An increase in the sensitivity of the peripheral nerve endings (nociceptors) contribute to faster sensation of pain, in addition to a more intense pain. This can be caused by inflammatory mediators such as shown above for example: protein kinase A, (PKA) or protein kinase C (PKC)-mediated phosphorylation of ion channels. For example the ion permeability of TRPV1 can be changed by PKA or PKC and therefore led to a sensitization (von Hehn, et al. 2012)[VI].

corresponding mediators, that peripheral sensitization is often caused by a change in the resting membrane potential. Furthermore, not only peripheral terminal nerve

endings are able of sensitizing, but also the spinal cord itself. This could be done by direct injury or through mediators. If the spinal cord is inflamed, it is called central sensitization. This may cause a change in the response to stimuli. "Typical changes of responses (neuroplasticity) of individual neurons are: a) increased responses to noxious stimulation of inflamed tissue, b) lowering of threshold of nociceptive specific spinal cord neurons (now become wide dynamic range neurons), c) increased responses to stimuli applied to non-inflamed tissue surrounding the inflamed site, and d) an expansion of the receptive fields of the spinal neurons" (Schaible, et al. 2011)[10]. Bradykinin is one of the most effective mediators of inflammation in terms of sensitization of nociceptors. The injection of bradykinin into deeper layers of the skin is causing pain and sensitivity to heat. This occurs dose-dependent and suggests that bradykinin is able to sensitize thermal nociceptors. Bradykinin is causing the release of transmitters and secondary inflammatory mediators, from neurons and immune cells. This includes substance P, interleukins, tumour necrosis factor, prostaglandins, calcitonin gene related peptide (CGRP) and acetylcholine. It was demonstrated that bradykinin sensitizes the thermally activated TRPV1 ion channel. Bradykinin also modulates other ion channels by reducing the activity of potassium channels, this causes nociceptors to be more excitable, because the resting potential is reduced (Fischer, et al. 2010)[7].

Prostaglandins (PG) are produced by cyclooxygenases. The substrate for this synthesis is arachidonic acid. Arachidonic acid is released from the cell membrane. Two PGs are the main prostaglandins types involved in inflammatory process. In humans and animals, PGE_2 and PGI_2 have been found to be efficient sensitizers in terms of sensitization of nociceptors. They have different time courses of action but both have similar effects. The main effect of PGE_2 and PGI_2 is the activation of protein kinase A (PKA) by a cascade of mechanisms. First, the coupling of PGE_2 and/or PGI_2 to GPCR, activates adenylate cyclase. This in turn releases cyclic adenosine monophosphate (cAMP) and cAMP in turn activates PKA. Finally PKA phosphorylates ion channels and affects their conductivity. The conductivity is altered by suppressed outflow of positively charged potassium ions. Again this causes a reduced resting potential and therefore a more excitable nociceptor. Arachidonic acid is also substrate for other secondary mediators such as leukotrines (Fischer, et al. 2010)[7]. Prostaglandins synthesis via cyclooxygenases 1 and 2 (COX-pathway) is the

target of non-steroidal anti-inflammatory drugs (NSAIDs), for example indomethacin and acetylsalicylic acid.

Levels of histamine in the extracellular space are significantly increased during inflammation. Mast cells are the main source due to degranulation after stimulation of substance P and other vasodilatation hormones. The sensation of itch, but not pain, is evoked by histamine (Fischer, et al. 2010)[7]. Not well understood is the underlying mechanism of the excitatory effect. To test the excitation by histamine, the Ca^{2+} influx was measured with sensitive fluorescent dye in cultured sensory neurons. Application of histamine produces temporary increase of intracellular Ca^{2+}, which leads to activation of further pathways. As final consequence, the TRPV1 channel is modulated. It is possible that the itchy sensation also derives from the modulation TRPV1 channel (Oh 2006)[9]. Whatever the role of histamine in sensitisation of nociceptors, it seems to have a rather low contribution.

Serotonin is mainly produced in the intestinal mucosa. However, since the blood-brain barrier may not be overcome, it is also synthesized in the brain. It is transported in the blood and platelets and stored in vesicles in the neurons. Serotonin is released from platelets and damaged neurons. Serotonin has a central role in development of hyperalgesia resulting from tissue injury, by activating 5-HT_{2A} receptors, at nociceptor nerve terminals. 5-HT_{2A} receptor is a G-protein coupled receptor, and its activation leads to depolarization of the resting membrane potential of acutely isolated rat dorsal root ganglion neurons. In cells exhibiting a 5-HT_{2A} -mediated response, serotonin depolarized the resting membrane potential and decreased the membrane permeability (Fein 2012)[6].

Substance P plays a central role in the development of inflammation and sensitization of nociceptors, since it triggers a multitude of different inflammatory mediators. SP can lead to both peripheral and central sensitisation. SP released from the afferent nerve terminals leads to activation of mast cells and subsequent release of histamine, bradykinin and 5-HT which in turn lead to sensitisation of nociceptors (Noback, et al. 2005)[5].

SP has the capability to make blood vessels permeable for blood and fibrin. An additional effect is the release of calcitonin gene related peptide, which causes dilatation of blood vessels. The resultant edema causes release of bradykinin and other chemical mediators, including serotonin derived from platelets, potassium ions derived from damaged tissue cells acting as activators of nociceptors. SP also underlies the development of central sensitisation in the spinal cord. Neurons expressing the SP receptor play an essential role in the maintenance of allodynia and hyperalgesia. Binding of SP to its receptor does not seem to be essential for acute hyperalgesia (Fein 2012)[6].

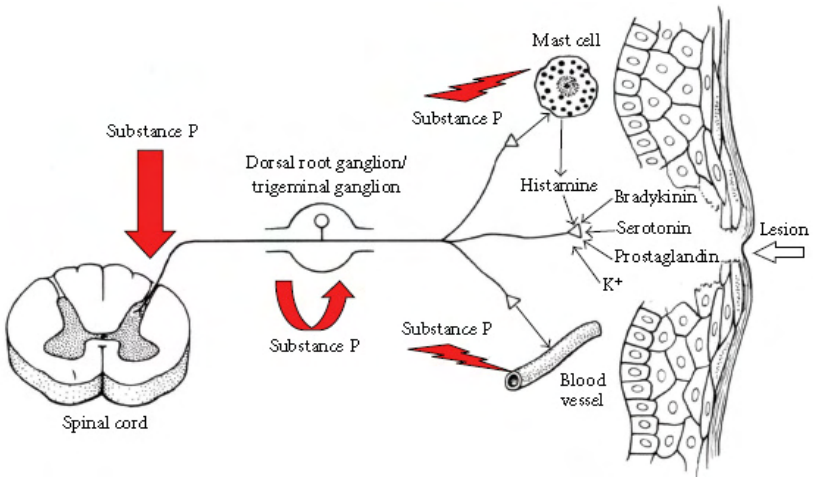

Figure 6 Substance P

Granulocytes, macrophages contain receptors for substance P. These cells produce proinflammatory mediators and cytokines after stimulation by substance P. SP itself is produced at the spinal cord and dorsal root ganglion. In addition it causes indirectly vasodilatation (Sacerdote, et al. 2012)[VII].

Tumour necrosis factor alpha (TNFα) triggers the release of an entire family of cytokines which are amongst the strongest proinflammatory cytokines. Among these interleukin (IL) 1β and IL-6 are especially potent in inducing acute and later on chronic pain. Release of TNFα ultimately leads to prostaglandin formation (Fischer, et al. 2010)[7]. IL-6 signalling pathway is in the control of leukocyte profile switching. It seems that when acute inflammation needs to resolve the IL-6 and its early form lipoxin, that controls the switch from very early PMNs to macrophage, there is the need for proinflammatory agent of degradation, particularly the removal of cytokines and that drive inflammation (Serhan, et al. 2010)[11]. "Pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα have been implicated in the induction of neuropathic and inflammatory pain states." (Fein 2012)[6]

1.3 Freund's Adjuvant

Dr. Jules Freund developed the complete and incomplete Freund's (CFA/IFA) adjuvant. Complete Freund's adjuvant contains heat killed *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate vehicle. IFA is the same mixture of oils, but without heat killed mycobacterium. Injection of the mycobacteria leads to infiltration of macrophages and other immune cells to the injection site and release of inflammatory mediators, such as described before, and induction of localised inflammation consisting of redness and swelling. Both CFA and IFA are commonly used compounds in animal models of inflammation and in vivo antibody production. For example they are routinely used as adjuvants to other immune assays to produce inflammation of the spinal cord (myelitis), bone narrow (myelitis), peripheral nerve (neuritis), uvea (uveitis), thyroid (thyroiditis) and testicles (orchitis) (Billiau, et al. 2001)[12]. In addition, they are used to investigate models of arthritis, granuloma and pain. Injection of CFA in paw of rodents is a widely used animal model to study inflammatory pain mechanisms. Additionally, the CFA model is used to investigate the acute a chronic effects of analgesic and anti-inflammatory drugs.

1.3.1 CFA induced acute inflammation

An acute inflammation is defined as immediate responses of surrounding tissues and blood vessels of the site of injury. For this studies after injection of CFA into left hind paw. Within the first few hours after injection, a complex interaction of inflammatory mechanisms is initiated involving the vascular and tissue responses as shown the mechanisms are shown in Figure 7. When damage occurs, firstly the endothelial cells are activated in first place. They are starting to express surface adhesions molecules (substance P) for leukocytes resulting in the release factors to attract granulocytes. The next step is release of proinflammatory cytokines and chemokines. The most important with respect to sensitisation of nociceptors is the release of: *bradykinin, prostaglandin, serotonin, substance P, histamine, nitric oxide Tumour necrosis factor alpha and Interleukin-6* (Fischer, et al. 2010)[7]; (Serhan, et al. 2010)[11]. The factors mentioned before are not only responsible for sensitization of nociceptors but also for opening the tight junctions of endothelial cells, resulting in vascular leakage permeable for leukocyte, granulocytes, serum proteins and oedema fluid and paw oedema. In addition, platelets form intravascular aggregates with fibrin. When all foreign components are oedema, fluid gets reabsorbed through lymphatic system and the inflammation is resolved (Serhan, et al. 2010)[11]. It is stated that there are four outcomes after an inflammation: complete convalescence, resolution with scar

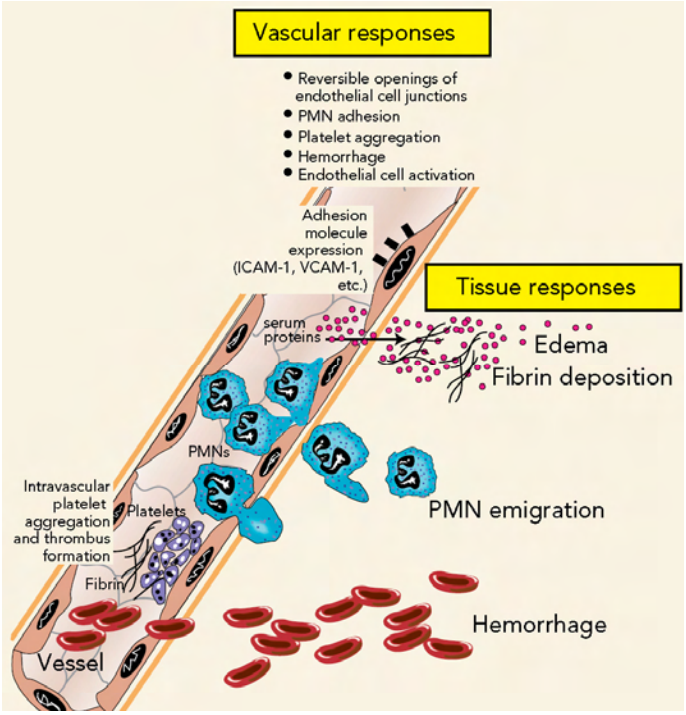

Figure 7 Acute inflammatory tissue injury

Vascular reactions, which occurs after tissue damage. These reactions are part of the immune response and inflammation reaction, and include the activation of endothelial cells and platelets, increased permeability of blood vessels, as well as the adhesion of granulocytes (PMN) to the endothelial surfaces. In addition, the surrounding tissue reacts after damage via edema, PMS transmigration of PMNs fibrin deposition also occurs and bleeding into the tissue, if the blood vessels are damaged. (Serhan, et al. 2010)[VIII].

tissue, formation of an abscess and conversion from acute to chronic inflammation (Fein 2012)[6].

1.4 Indomethacin

Indomethacin (IM) is a non-steroidal anti-inflammatory drug which was introduced in 1965, in the United States of America for treatment of rheumatoid arthritis. IM is one of the most potent nonsteroidal anti-inflammatory drugs available on the market. IM possesses approximately 10 times the analgesic potency of aspirin. However, the analgesic effect of IM is overshadowed by concern over the frequency of side effects. Indomethacin still remains one of the gold standards NSAID's used in preclinical studies (Lemke, et al. 2008)[13]. NSAIDs, including IM, interfering with inflammation via multiple mechanisms. Most important is the blocking the biosynthesis of prostaglandins through the inhibition of cyclooxygenases (COX) . There are two isoenzymes of COX, COX-1 and COX-2. COX-1 constitutionally expressed under normal conditions and play an important role on the GI-tract protecting and maintaining blood flow on the GI-tract. On the other hand, COX-2 is not found to be activated under normal conditions but has been shown to play an important role in promoting edema, fever and pain. COX-2 is activated by bradykinin in nociceptors (Fein 2012)[6]. In addition, IM also effects on immunological processes e.g. antibody production, antigen/antibody complexation, phagocytosis and has been shown to interfere with formation and release of the chemical mediators of inflammation. Furthermore it is observed that IM can be involved in the activation of the complement system (Lemke, et al. 2008)[13]. Indomethacin is a potent inhibitor of COX-1 and COX-2. Inhibition of COX-2 underlies anti-inflammatory and analgesic-effects while blocking COX-1 leads to unwanted side effects like bleedings and GI-ulcers.

1.5 Withdrawal reflex (flexor reflex)

A nociceptive withdrawal reflex occurs as a response to a noxious stimulus. For example, in response to touching a hot surface. This reflex is a protective mechanism and is automatic so that is it does not require conscious processing even though the state of mind can have a huge impact on threshold. The reflex is still present whilst sleeping. The reflex is a neural sequence. The neural sequence is organized in three steps: (1) Initializing nerve impulses by exceeding the threshold of nociceptors in the skin (2). Forwarding the signal via afferent sensory neurons to spinal cord. (3) Processing the signal at the interneurones. The impulse will be forwarded further via alpha motor neurones to motor end plate where the movement will be triggered. On the other hand there are inhibitory interneurones which interfere with the flexor reflex so that the muscle can relax. Depending upon the strength of the stimulus, the response can vary from weak twitching to an escape reflex (Noback, et al. 2005)[5].

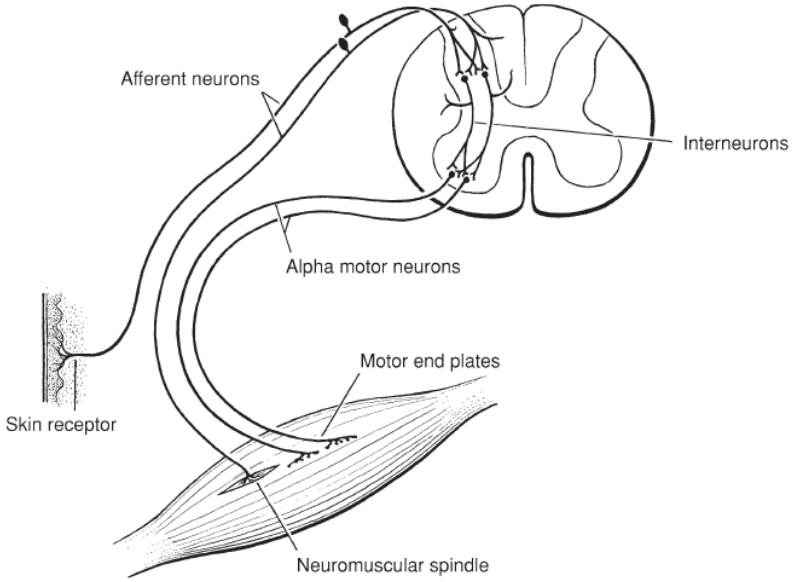

Figure 8 Withdrawal reflex

The sequence of a flexor reflex: (1) excitation by stimuli (2) forwarding / processing on / in gray matter (3) triggering of the reflex muscle (Noback, et al. 2005)[IX].

1.5.1 Paw flick test according to Hargreaves

In 1988 Kenneth Hargreaves presented a method that allowed the measurement of automated cutaneous hyperalgesia in unrestrained animals. He used a setup that consists of a glass table, individual acrylic glass chambers an infrared emitter and a control unit. The animals were set under the chambers and the hind paw was heated

by means of the infrared radiation emitter and the latency for the paw withdrawal reflex was recorded. Prior to this, no behavioural method was available to measure paw withdrawal reflex in unrestrained animals in which repeated testing could be performed. Assays that were available were the old standards. (1) The hot plate test: where the animal was placed upon a hot plate which was set to a constant temperature for example 50°C. Recorded was the time until the animal started to lick the hind paw. It was not possible to retest animals in the same experiment due to development of thermal sensitisation. (2) The tail flick/withdrawal test: where the latency for the animals to flick its tail from a radiant heat stimulus or withdraw its tail from hot water was recorded. For the later assay, animals had to be restrained which causes stress and this is known to alter nociceptive processing (i.e. stress induced analgesia). The new method has several advantages. (1) Repeated testing does not contribute to the development of hyperalgesia. (2) The thermal method showed greater bioassay sensitivity. (3) The stimuli could be applied to unrestrained animals (Hargreaves, et al. 1988)[14].

1.6 Innate behaviour

Innate behaviour has two different labels in behaviour literature. The first is the gene related behaviour. In the narrow sense, if genes change also behaviour does. The behaviour that occurs based upon the genetic code, and differences in the genetic code leads to differences in behaviour in different animals. The second meaning of innate goes a step further and it is not just about genetics relate directly to behaviour patterns but also to the inherited neural pathways. For this he used his theory of instinct. He recognized an animal's behaviour as instinctive behaviour patterns, based on neural actions. He saw instincts as more complicated reflexes. The animal did not have to learn them because they were inborn. They do not required insight or awareness. To prove his theory he raised a young animal in complete isolation so that it had no opportunity to learn behavioural patterns by observation. The animal was able to perform adult behaviour patterns normally (e.g. grooming), the behaviour was innate, the genes of the animals determined its behaviour. This experiment reaped some criticism, because the animal could mimic certain patterns of behaviour. It had, of course, deficits in interaction with conspecific. It must be accepted that genes and environment have a role in all cases (Barnard 2004)[15].

Lorenz stated in 1983 that all animals had to follow their instincts, which arises from internal stimulus production. They even have to do so if the environment does not offer opportunity.
"Mäuse müssen nagen, Hennen picken, Eichhörnchen umher hüpfen. Wenn diese Notwendigkeit unter den Bedingungen der Labor-Gefangenschaft *nicht* besteht, müssen sie es aber ebenso, und zwar deshalb, weil alle Instinktbewegungen von einer inneren Reizproduktion hervorgebracht und nur in Bezug auf das Jetzt und Hier ihrer Auslösung von Außenreizen gesteuert werden." (Lorenz 1983)[16]

1.6.1 Burrowing behaviour

Mice like to burrow and build nests where they spend their inactive hours. Although laboratory mice were domesticated over many generations they retain many behaviours of their ancestors. Some behaviours, however, were attenuated, but the instinct to burrow and build nests has remained (Hutchinson, et al. 2005)[4]. There is only little evidence of the influence of environmental conditions and genetics on the burrowing behaviour.
The Oldfield mice build an underground nest where there are several escape tunnels connected to the nest chamber. The escape routes are sealed with a thin layer of soil, only the main exit is open. Deer mice, in contrast, dig a relatively short tunnel, which then ends up in a nest chamber. In an experimental setup, twenty generations where raised under laboratory conditions were they had no possibility to create species-specific burrowing systems. Afterwards hybrids between Oldfield mice and Deer mice were bred. The burrows of those F1 hybrids, structural features of Oldfield mice burrows (several exit tunnels) were observed exclusively. Furthermore, those F1 hybrids were fertile and backcrossed with recessive Deer mice. The resulting generation build nests with both properties. It was concluded, that there must be dominant alleles in Oldfield mice which determine architecture of burrows (Hansell 2005)[17]. The most common techniques within rodents of removing excavated material is by turning round and pushing with the head and forefeet (Starck 1995)[18].

1.7 Burrowing as end-point

The burrowing assay was first developed by Robert Deacon at the University of Oxford out of the need for a mouse hoarding model. Before this model was available, the experimental setup for hoarding behaviour required a home cage and a food source far away which was linked by transition system. This was complicated to build since it needed to be mouse proof and it occupied a large area. In order to refine the experimental setup, the food source was placed in a container which was deployed within the home cage. The food pellets were found the next morning near to the entrance of the container rather than as suspected at a specific hoarding place. Further investigations revealed that the animals did not carry the pallets around but executed digging movements in order to build a burrow (Deacon 2012)[19]. Under laboratory conditions mice will naturally start to burrow. This behaviour is instinctive and has been inherited from their ancestors (Deacon 2009)[20]. In 2006, a detailed protocol was published in which Deacon described a standardized method to measure burrowing behaviour. Before that, there were different endpoints, e.g. latency until start of burrowing or complexity of burrows. The adapted method is described under 2.2.4. The assay has been utilised to examine changes in burrowing behaviour following a range of diseases: "lesions of the hippocampus and prefrontal cortex in mice, also effects of lipopolysaccharide, early stages of prion disease in mice and IL-1β in rats" (Deacon 2006)[21]. Teeling demonstrated with the burrowing test an impact on brain from systematic inflammation (Teeling, et al. 2007)[22]. Jirkof assessed post postoperative pain in mice (Jirkof, et al. 2010)[23].

2 Material and methods

2.1 Materials

2.1.1 Devices

Table 2 Compilation of devices used

Shown is a complete list of used devices during testing or preparation of experiments.

Device	Model/Serial number	Manufacturer
positive-displacement pipette 250 µl	microman	Gilson
positive-displacement pipette 1000 µl	microman	Gilson
Vortex Mixer	Vortex-Genie 2	Scientific Industries Inc
Scale	TE 1502S	Sartorius
Scale	ME235S-0CE	Sartorius
Pipettes 1000 µl	microman	Gilson
Pipettes 5000 µl	microman	Gilson
HT-pipe DN 75	-	Bauhaus
HT-socket plugs	-	Bauhaus
Plantar Test	37370	UGO BASILE S.r.l.
Acrylic glass chambers	-	AKADE Display & Design GmbH
HEAT-FLUX IR Radiometer	37300	UGO BASILE S.r.l.
Type 3 cages	-	-
High Grids	-	-
Hamilton syringe 250 µl	-	Hamilton
Animal holding cabinet	Scanbur	Scantainer
Buttoned cannula	20G/30mm	FST, Fine Science Tools GmbH
Ultrasonicator	S220	Covaris

2.1.2 Expendable materials

Table 3 Compilation of expendables used

Below is a table drawn which shows all expendables which were used.

Materials	Model/Serial number	Manufacturer
Syringe 1 ml	Omnifix®-F	Braun
Cannula	Sterican® 26G	Braun
Cannula	Sterican® 20G	Braun
Bedding material	10	Cat's Best Universal
Gravel	grain size, 2-5 mm	Vitakraft
Standard food chow	Rod16, (irradiated)	LASvendi
Pipette tips for positive displacement pipettes 250 µl	-	Gilson
Pipette tips for positive displacement pipettes 500 µl	-	Gilson
Glass vials 3 ml	548-0554	VWR International GmbH

2.1.3 Chemicals/Drugs

Table 4 Compilation of all Chemicals and Drugs used

Shown is a complete list of used all Chemicals and Drugs which were used.

Chemicals/Drugs	Serial number/Lot	Manufacturer
Phosphate buffered Saline (PBS)	H00212-3302	PAA Laboratories GmbH
Complete Freund's Adjuvant (CFA)	F 5881	SIGMA
Incomplete Freund's Adjuvant (IFA)	F 5506	SIGMA
Indomethacin	SLBB6929	SIGMA
Methylcellulose 0.5%	05420EJ	Aldrich
Ethanol 10%	-	Roth
Ethanol 70%	-	Roth

2.1.4 Animals

A total of 160 female BALB/c mice and 10 male and 12 female C57BL/6J were used in this project. BALB/c mice were obtained from Janvier (France) and C57BL/6J mice from the Universitäts Klinikum Eppendorf (UKE, Hamburg). Mice were housed in conventional clear plastic Typ 3 cages (425 mm x 266 mm x 155 mm) in groups of 5-10 per cage, unless otherwise stated, for at least 1 week before testing. Animals received standard food chow (Rod16, LASvendi) and water ad libitum. Cages were either housed in animal holding room or in Scantainers (Scanbur, Denmark) which were maintained at 24^{o}C and 60% humidity under a 12/12 h day/night light cycle from 6:00 (lights on) and 18:00 (lights off). For the burrowing experiments, animals were singly housed in Typ3 cages containing a burrowing tube (see Section 2.2.4.1 for details). The animal housing and experimental protocols were approved by the Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz, and were in accordance with German animal protection law.

2.1.4.1 Hargreaves animals

There were 90 female BALB/c mice obtained from Janvier. They were 11 weeks of age by date of delivery.

2.1.4.2 Burrowing animals

The animals were, 10 male and 12 female C57BL/6J mice, obtained from Janvier. They were 29 weeks of age by date of experiment. In addition, there were 70 female BALB/c mice obtained from Janvier. They were 11 weeks of age by date of delivery. Animals for effect of CFA-induced unilateral inflammation on burrowing behaviour with CFA 0.5 and 1 mg/ml, were significantly older (+5 weeks) than other BALB/c which were used, due to indisposition of the experimenter.

2.2 Methods

2.2.1 Dilutions and Drug preparation

2.2.1.1 CFA/IFA

Each ml of complete Freund's Adjuvant (CFA) contained 1 mg *Mycobacterium tuberculosis* (H 37RA, ATCC 25177), heat killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide monooleate (Sigma-Aldrich).Each ml of incomplete Freund's Adjuvant (IFA) contained 0.85 ml paraffin oil and 0.15 ml mannide monooleate (Sigma-Aldrich). Stock solutions were stored at 5°C.

To prepare the dosing solutions stock CFA was first mixed using a vortex then placed in an ultrasound water bath for 10-15 min at room temperature to prepare a fine suspension. For the 1 mg/ml solution concentration animals received an injection of the stock solution. The 0.5 mg/ml concentration of CFA was prepared by taking equal quantities of 500 µl of stock CFA and 500 µl of IFA using a positive displacement pipette. The 0.25 mg/ml solution of CFA was prepared by taking 250 µl of stock CFA and 750 µl of IFA using a positive displacement pipette. The solutions were mixed using a vortex before use.

2.2.1.2 PBS

Phosphate buffered saline (PBS; PAA Laboratories GmbH) was used as the vehicle for intraplantar injections.

2.2.1.3 Indomethacin

For drug studies, indomethacin was administered at 10 or 30 mg/kg orally. Doses were based on those reported in the literature to be effective in inhibiting pain responses in mice (Abu-Ghefreh, et al. 2010)[24]. Indomethacin (Sigma-Aldrich) was prepared as follows: During all experiments there were two concentrations used: 10 mg/mg and 30 mg/mg. For the 10 mg/mg concentration, 3 mg of indomethacin was weighed and merged in a vial with 2.5 ml methylcellulose 0.5%. For the 10 mg/mg concentration, 7.5 mg of indomethacin was weighed and merged in a vial with 2.5 ml methylcellulose 0.5%. Afterwards the vial was set into Covaris S220 and the following program went through:

Table 5 Covaris dilution program

Dilution program for the Covaris S220, which was used for dilution of indomethacin.

Step	Name	Time	Peak Power	Duty Factor	Cycles Burst
1	Grinding	120sec	80	50	1000
2	Delay	45sec	none	none	none
3	Sonication	150sec.	200	50	1000
4	Delay	30sec	none	none	none
5	Grinding	120sec	80	50	1000
6	Delay	45sec	none	none	none
7	Sonication	150sec	200	50	1000

2.2.1.4 Methylcellulose

Methyl cellulose solution was prepared by adding 250 ml of deionised water to 1.25 g of methylcellulose (Aldrich, 05420EJ). The mixture was heated and stirred until a clear solution was obtained. The solution was cooled and stored at 5°C until use.

2.2.2 Drug administration

2.2.2.1 Intraplantar (i.plt.) injection

The CFA dosing solutions and PBS were administered intraplantar (i.plt.). The animals were restrained and the left hind paw was held between the thumb and index finger by the person performing the injection and the leg extended. Using a 250 µl Hamilton syringe connected to a 26G needle, the needle was inserted in to the plantar surface of the hind paw to a depth of 5 mm as shown in Figure 9. Twenty (20 µl) of CFA solution or PBS vehicle was injected into the paw. The thumbnail, which locked the paw into position, was also used to support the cannula while injection. After the application was completed and the cannula removed, the paw was compressed to prevent leakage of the dosing solution. Before the animal was returned into its home cage, the success of application was visually checked. Because of the administered volume and the size of the paw, the swelling was clearly visible.

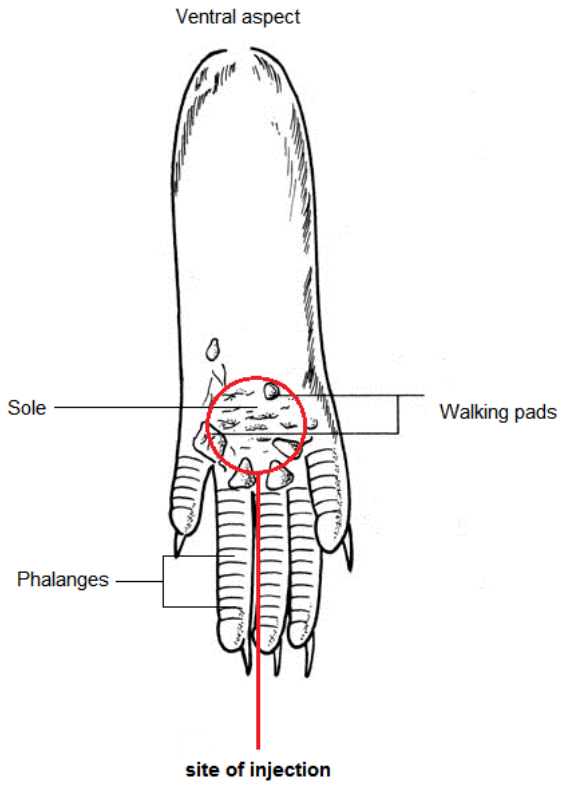

Figure 9 Right hind paw of mus musculus

The figure shows the ventral aspect of the right hind paw from mus musculus. Marked are the walking pads, the sole and the site of injection (Cook 1965)[X].

2.2.2.2 Peroral (p.o.)

Indomethacin or vehicle (0.5% methylcellulose) were administered per orally (p.o.). The animals were held in a restricted position. The head was tilted in a dorsal direction in order to bring the pharynx and the oesophagus in a straight angle. The buttoned cannula was set to the diastema so the animal had to open the mouth and swallow. While the animal was swallowing, the cannula was inserted gently to avoid perforation oesophagus. After application, the animal was returned to its home cage or onto Hargreaves apparatus. Each animal received a dosing volume of 10 ml/kg.

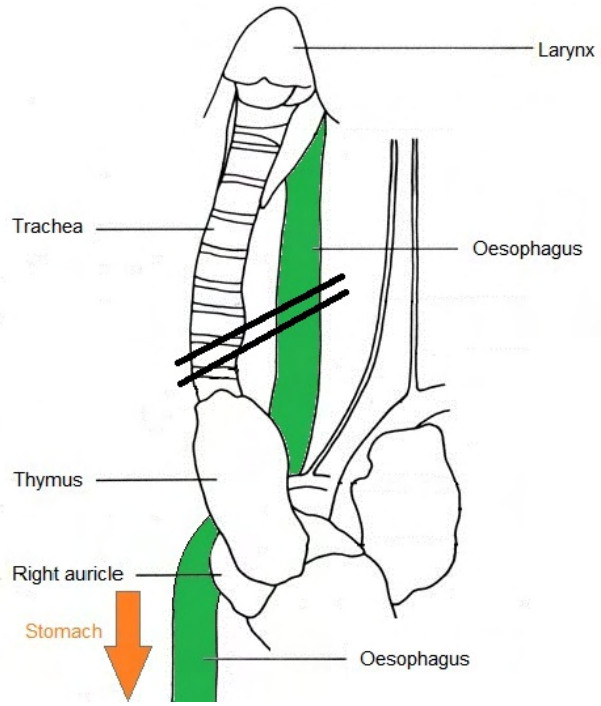

Figure 10 Neck visceria

The figure shows the inwards of the neck and internals of the thorax. Green marked is the oesophagus, which must be entered to administer the drug ore vehicle into the stomach (Cook 1965)[XI].

2.2.3 Complete Freunds adjuvant (CFA) induced thermal hyperalgesia model (Hargreaves Test)

The CFA-induced thermal hyperalgesia model is used in academia and in the pharmaceutical industry in order to understand the mechanisms of pain and assessing the in vivo efficacy of new analgesics (Hargreaves, et al. 1988)[14]. In this model, a local inflammation is induced by i.plt. administration of CFA into one hind paw. As a consequence of the inflammation, animals develop a hypersensitivity to application of painful (noxious) stimulus such as heat. In this test, the latency of an animal to withdraw its paw from a thermal heat stimulus is measured using an automated paw flick device developed by Ken Hargreaves. A reduction of the paw

withdraw latency post inflammation is taken as the development of thermal hyperalgesia (hypersensitivity).

2.2.3.1 Hargreaves apparatus

The Hargreaves device (Ugo Basile, Italy) consists of a glass table, custom made acrylic glass chambers (10 cm x 10 cm x 5 cm; Akeda, Hamburg), an infrared (IR) emitter and a control unit. A radiometer was used for adjusting and calibrating the emitter device. The IR emitter was connected with a cable to the control unit. The emitter unit was located in a cylindrical case. The cylinder contained a halogen bulb coupled to an IR-filter, which cut off the visible part of the spectrum. A quietly operating fan cooled the bulb. At both sides of the case were triggers for switching on the heating device. The heat source was positioned directly under the animals hind paw by aid of the cross (see Figure 11A). When the mouse withdraws its paw, an electronic circuitry switches off the bulb and the withdrawal latency is displayed on the device nearest 0.1 second. Before every testing day, the IR-emitters were checked with the radiometer. Two (2) tables were used each holding 10 animals so that 20 mice could be tested per day. The room temperature and humidity were noted as changes in

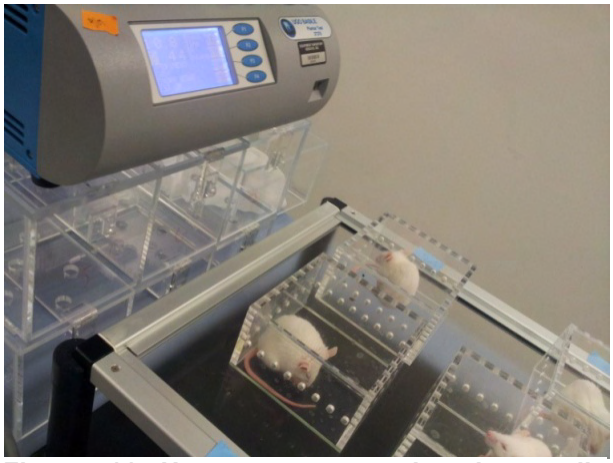

Figure 11 Hargreaves control unit, acrylic chambers and glass table

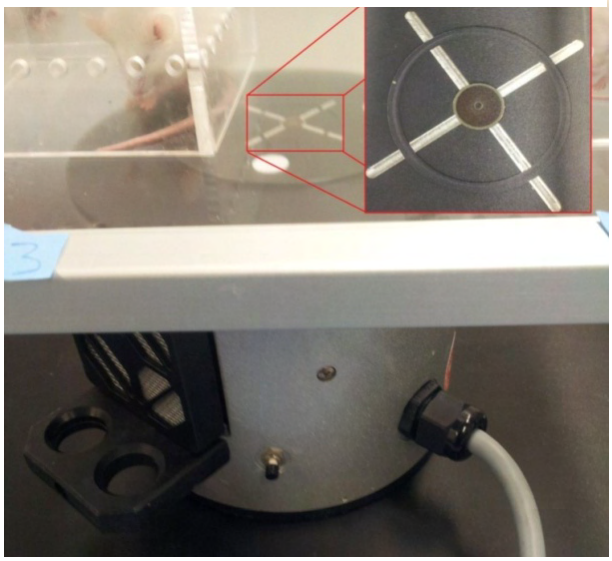

Figure 11A IR-emitter

Top: The figure shows the IR-emitter beneath the glass pane. Red marked is a magnification of the IR heat outlet. It is the smallest circle in the centre. The cross on top is to aid correct positioning of the heat source. Bottom: trigger for switching on the heat source, handle and connection cable.

the environment can alter radiant heat latency responses. After every session, the chambers were washed and cleaned with 10% ethanol. The glass tables were also washed and cleaned with 70% ethanol.

2.2.3.2 Hargreaves experiments

After delivery, animals were allowed 7-14 days acclimatisation before starting the experiments. This is important as stress-induced analgesia is known to increase in thermal nociceptive thresholds (Parikh, et al. 2011)[25]. Prior to measurement of baseline paw withdrawal latencies (PWL), mice were habituated to the acrylic glass chambers without receiving any treatments for 2 h on 2 days. A radio was played in the room to dampen any noise created by the apparatus or the experimenter. Measurement of paw withdrawal latencies: The Hargreaves apparatus was turned on. Before every testing day, the IR emitter was checked with the radiometer to validate the intensity of IR radiation. Target value was 115 mW/cm². Animals were placed on the glass tables under the acrylic chambers as shown in Figure 11 and given 2 hours to settle down. After approximately 1.75 hours, faeces were removed and the table cleaned to remove any urine and animals left a further 15 min to let them settle down after the disturbance. PWL were measured by positioning the centre of the emitter device directly underneath the hind paw. The trigger was pushed, switching on the radiant heat source. After the animal withdrew its paw, the radiant heat source was switched off and the timer stopped automatically. The latency for the animal to remove its paw from the heat source was recorded. The latency for both hind paws were measured. For a correct measurement, the animals were not allowed to move, rear, groom, sleep, sitting on their tail or scent as each of these behaviours would have led to false high readings. In addition, the paw had to contact the pane with its full area. To avoid damages at the paw a cut off time was set to 20 seconds.

If the animal urinated, the table was wiped dry and the animal allowed another 15 minutes to settle before another reading could be performed. The PWL of the animals in the adjacent chambers were not measured for a minimum of 5 minutes. Between every measurement of a paw, the animals were given at least 5 minutes.

2.2.3.2.1 Dose response effect of CFA on paw withdrawal latencies

In this experiment, 40 female BALB/c mice were used to measure the effect of intraplantar injection of different doses of CFA or PBS on PWL and to establish a time-effect curve. Baseline PWL were measured over 2 days and the mean of 4 readings was used as the PWL for each paw for each animal. After determination of baseline PWL, (as described above) animals were allocated into 4 groups each containing 7 animals ensuring that the mean baseline readings were matched across the groups. The animals then received one of the following treatments into the left hind paw: Group 1 received 0.25 mg/ml CFA i.plt., group 2 0.5 mg/ml i.plt., groups 3 a received 1 mg/ml CFA i.plt. and group 4 received PBS i.plt. and acted as a control. Every animal received a volume of 20 µl. After the administration of CFA or PBS, the animals were put back into their home cages for two hours. Animals were then placed on the glass table under the same acrylic box as for baseline readings and after 2 hours PWL were measured for both hind paws (see Figure 12). It was not possible to measure the animals sequentially according to the exact time post injection, as the animals were not always settled. Therefore, those which calmed down first, were measured first. Some of the animals did not calm down at all during the 4 hours and so measurement of PWL was not possible; these animals were excluded.

Table 6 Table of CFA concentrations in Hargreaves dose range experiments

Animals [n]	Treatment	Concentration of *m. tuberculosis* [mg/ml]	Volume [µl]
7	PBS	-	20
7	CFA	0.25	20
7	CFA	0.5	20
7	CFA	1	20

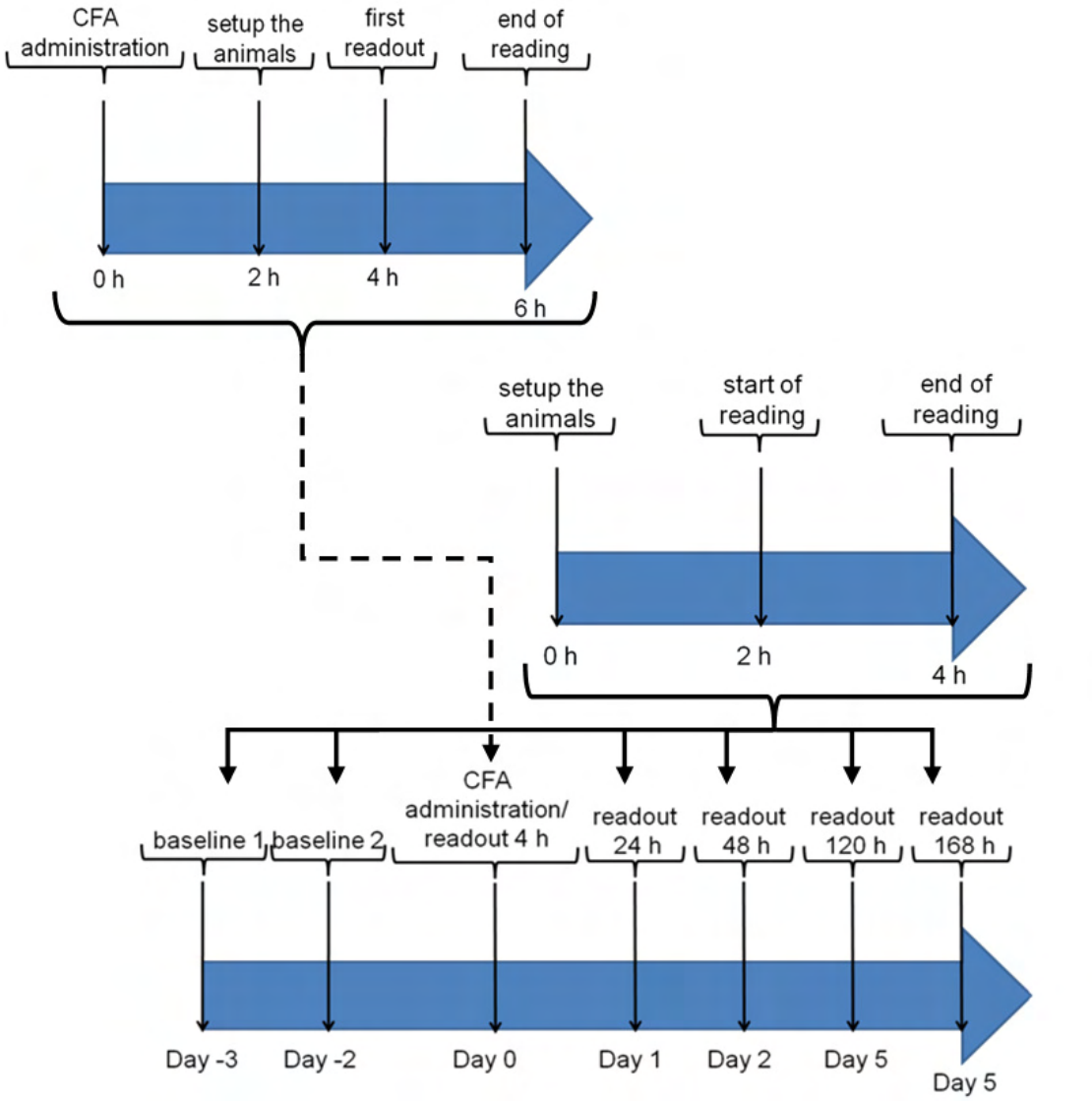

Figure 12 Experimental protocol: dose response to CFA (Hargreaves method)

2.2.3.2.2 Dose response effect of indomethacin on CFA induced thermal hyperalgesia

In this experiment, 50 female BALB/c mice were used to explore the dose response effects of indomethacin on CFA induced thermal hyperalgesia. Based on the results of the dose response effect to CFA on PWL, the dose of 0.5 mg/ml was chosen for the drug study with indomethacin as this produced the most robust response and the 48 h time point was the time of peak thermal hyperalgesia.

After determination of PWL baselines (as described in section 2.2.1.2), animals were allocated into 4 groups of 9 animals ensuring that the mean baseline response were matched across the groups. Twenty seven (27) animals received 20 µl of 0.5 mg/ml CFA i.plt. into the left hind paw; the control group received 20 µl of PBS i.plt. PWL were measured 48 h after CFA or PBS injection. Again, the animals receiving CFA were re-allocated to three groups to ensure that the degree of thermal hyperalgesia was the same across the groups. Animals then received orally vehicle (0.5% methyl cellulose, 10 ml/kg) or indomethacin 10 or 30 mg/kg p.o. as detailed in table 7.

Table 7 Table of Indomethacin concentration and treatments in Hargreaves experiments

Animals [n]	Treatment	Concentration of *m. tuberculosis* [mg/ml]	Volume [µl]	Treatment	Volume [ml/kg]
7	PBS	-	20	Methylcellulose 0.5%	10
7	CFA	0.5	20	Methylcellulose 0.5%	10
7	CFA	0.5	20	Indomethacin 10 mg/mg	10
7	CFA	0.5	20	Indomethacin 30 mg/mg	10

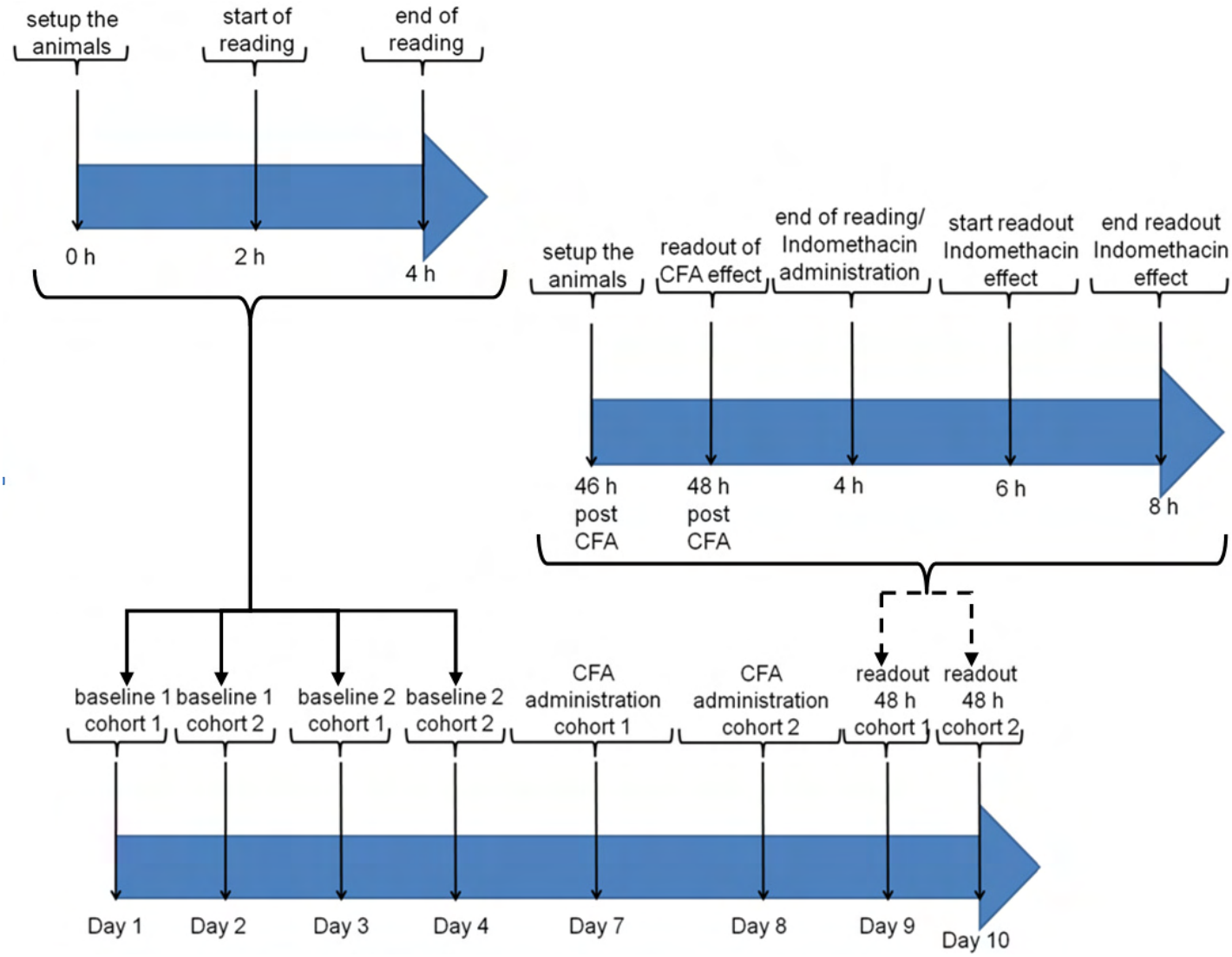

Figure 13 Experimental protocol: Dose response effect of indomethacin (Hargreaves method)

This experiment was separated into 2 cohorts due to maximum capacity of testing 20 animals per day (10 animals per glass table). The experimental protocol is shown in Figure 12.

2.2.4 Burrowing

Laboratory mice are fossorial animals and therefore naturally dig and burrow and this behaviour is highly conserved (Deacon 2006)[21]. It has been suggested that the normal expression of this behaviour indicates a global "well-being" of an animal. Burrowing behaviour can easily be measured in laboratory mice and is a simple test. A tube is loaded with bedding material, gravel, sand or similar material and placed in the animals cage. After a certain period of time, the amount of material remaining in the tube is measured. If the animal is injured or is in pain inflammation, it is expected that the animal would spend much less time burrowing and so there would be a reduction in the amount of material removed or displaced.

Recently a number of studies have explored burrowing behaviour to determine spontaneous pain in mice and rats after different perturbations. Jirkof and colleagues showed that mice after laporotomy have a deficit in burrowing behaviour and that this could be reduced by treatment with post-operative analgesics (Jirkof, et al. 2010)[23]. Similarly, a reduction of burrowing was reported in rats after administration of CFA i.plt. and to a smaller extent after peripheral nerve injury (Andrews, et al. 2012)[3]. In addition, Teeling and co-workers showed a significant suppression of burrowing behaviour in mice after intraperitoneal administration of lipopolysaccharide (Teeling, et al. 2007)[22]. In the following studies, the extent of burrowing in mice was assessed after intraplantar injection of CFA as a potential measure of spontaneous pain.

2.2.4.1 Burrowing apparatus

The device for burrowing testing was a high temperature tube (HT-tube; made of polypropylene). It was closed on one end with a socket plug. The front end of the tube was increased about 3 cm, to prevent the material from displacement, which was unrelated to burrowing. The tubes had an inside diameter of 7.5 cm. The lengths were 21.7 cm and 16.7 cm, as shown in figure 14. All tubes were weighed and numbered. All parts including the threaded screws, were obtained from the Bauhaus.

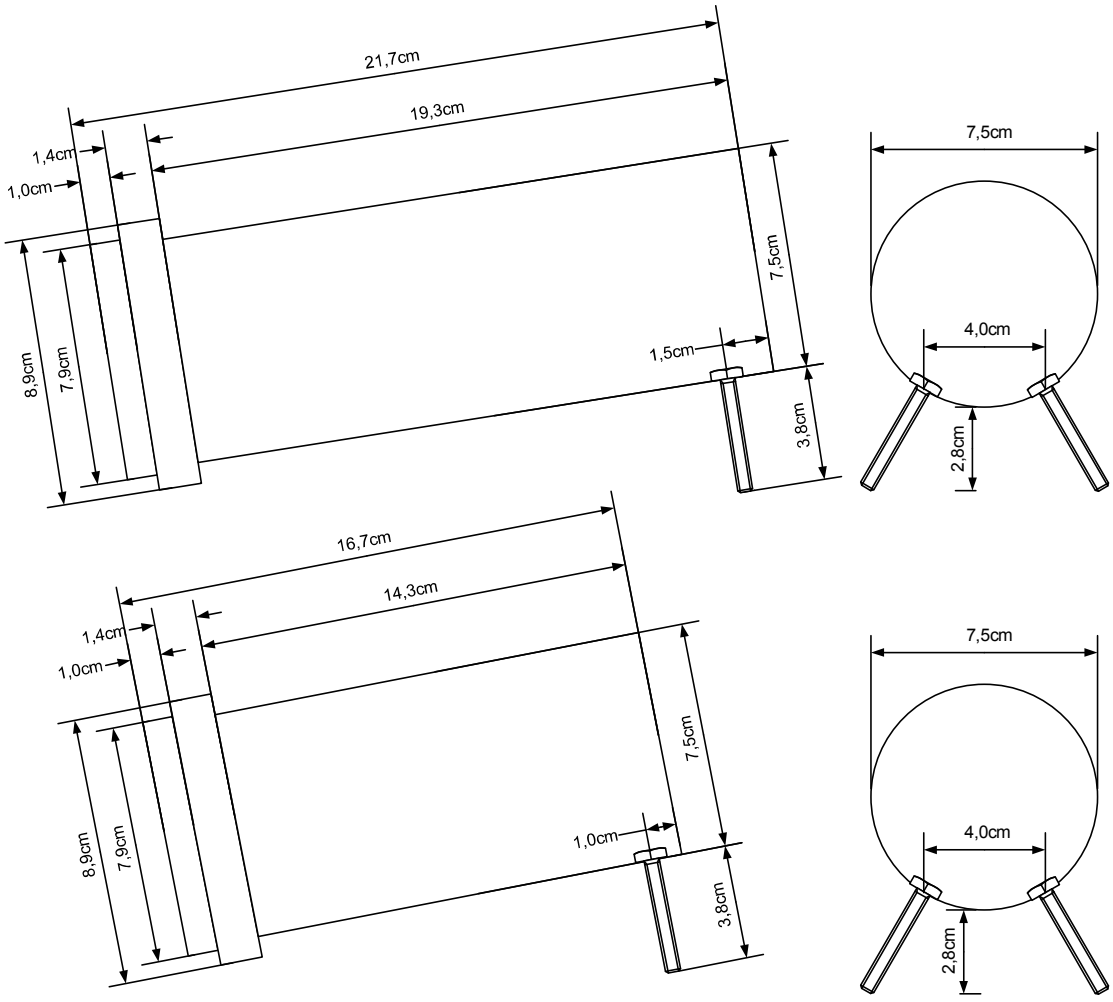

Figure 14 Measurements of burrowing devices

The figure shows the long and short versions of the burrowing devices. Shown are the front (left) and side view (right). Total length of the long version was 21.7 cm, wherein the shaft had a length of 19.3 cm. The end had total length of 2.4 cm, wherein the part with outside diameter of 8.9 cm contained a seal were the socket plugs were fitted. This end part contained also burrowing material. The front was raised with a 40 mm M5 threaded screw. To install the screw, two holes were drilled with 5 mm diameter in the tube. The holes had a distance of 4 cm. The holes were drilled 1.5 cm from the opening at the front. The short version were shortened about 5 cm. The threaded screws were 0.5 cm closer to the opening at the front, to maintain the inclination.

Two foraging materials were investigated pelleted bedding material (Cat's Best Universal 10) and gravel (Vitakraft). The pelleted bedding material had a diameter of 6 mm. The gravel had an average grain between 2 and 5 mm. Before the tubes were filled, the empty weight was noted. The labelled tubes were put on the balance and the empty weight was adjusted, so that the variations of weight were compensated. Then the tubes were filled with 425g of pelleted bedding material or 865g of gravel. The filled tube was placed in the back left corner of the cage. Placing the tube in the cage had to be done carefully so the material was not displaced. For the same reason the cage had to be held horizontally, while putting it back into the rack. Two hours after insertion of the tubes into the cage they were removed weighed and returned to the same cage without being refilled. After 24 hours, they were weighed and the tubes were refilled for the next 24 hours or the remaining material was disposed. Because of remaining dust and liquid faeces, the empty weight of the tubes was adjusted. It was essential that the tube was returned the same animal. Between experiments, the tubes were washed and sterilised. To calculate the displaced amount of material the weight at the time of measurement was subtracted from the empty weight of the tube from the previous day. This was taken as the amount of burrow behaviour.

Figure 15 Long burrowing tube

The figure shows a long burrowing tube with pelleted bedding material as filling. This animal displaced almost complete content of the burrowing tube into the cage. It can be seen the different structures of the pelleted bedding material from the tube in the front, compared to the sawdust bedding material in the back.

2.2.4.2 Burrowing experiments

After delivery, the animals were allowed 7-14 days acclimatisation before starting the experiments. A group-training phase was employed initially, wherein a group of 5 animals were exposed to one tube for 1 to 2 days. Although burrowing behaviour is normally spontaneous, there is a learned component and the learning process is reported to be enhanced by social facilitation (Deacon 2006)[21]. Animals were then separated and housed singly for the remainder of the experiments. At least 1 day was allowed before measurement of baseline burrowing behaviour after separation. Burrowing behaviour was assessed by placing the burrow containing the foraging material into the home cage typically at 4 pm each day and then measuring the tubes again at 6 pm and 24h later (4 pm the next day).This baseline was performed for each mouse and the mean burrowing calculated. The following studies were performed: (1) Pilot studies, (2) Effect of CFA-induced unilateral inflammation on burrowing behaviour and (3) Effect of indomethacin on CFA induced deficits in burrowing behaviour.

2.2.4.2.1 Pilot studies

In this experiment, 12 female and 10 male C57BL/6 mice were used to determine the optimal parameters for burrowing behaviour. This included comparing burrowing behaviour in male and female mice. In addition the effects of long versus short burrowing tubes, different foraging materials (pelleted bedding material versus gravel), were observed. Time effects were also measured. In this setting the animals did not received any treatments.

Table 8 Combinations of Animals, Tube length and filling

Sex	Length	Filling
female	long	bedding material
female	short	gravel
female	long	gravel
female	short	bedding material
male	long	bedding material
male	short	gravel
male	long	gravel
male	short	bedding material

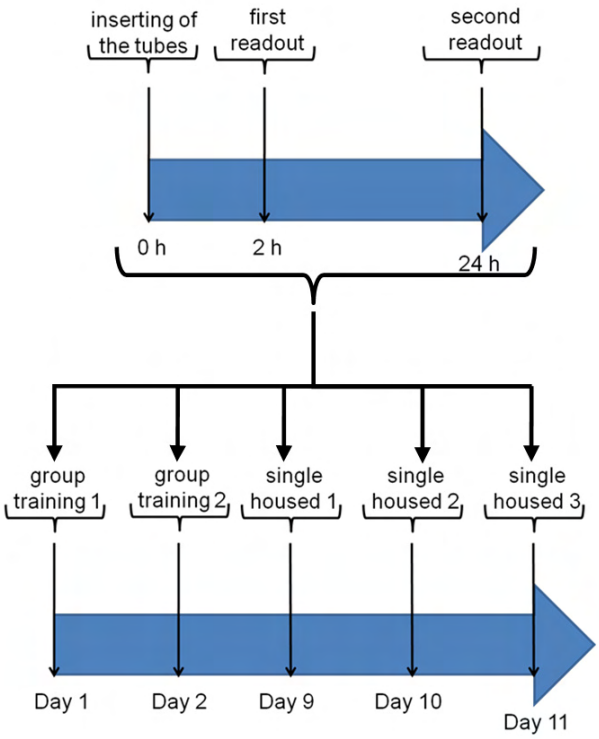

Figure 16 Experimental protocol: pilot studies

2.2.4.2.2 Effect of CFA-induced unilateral inflammation on burrowing behaviour

In these experiments, 40 female BALB/c mice were used to explore the effects of CFA induced hind paw inflammation on innate burrowing behaviour. Control animals received PBS into the hind paw. Ten (10) animals received 20 µl of 0.5 mg/ml CFA i.plt., 9 animals received 20 µl of 1 mg/ml CFA i.plt., 19 animals received 20 µl of PBS i.plt. Two animals were excluded due to lack of consistent baselines.

Table 9 Table of treatments with CFA

Animals [n]	Treatment	Concentration of *m. tuberculosis* [mg/ml]	Volume [µl]
10	CFA	0.5	20
10	PBS	-	20
9	CFA	1	20
9	PBS	-	20

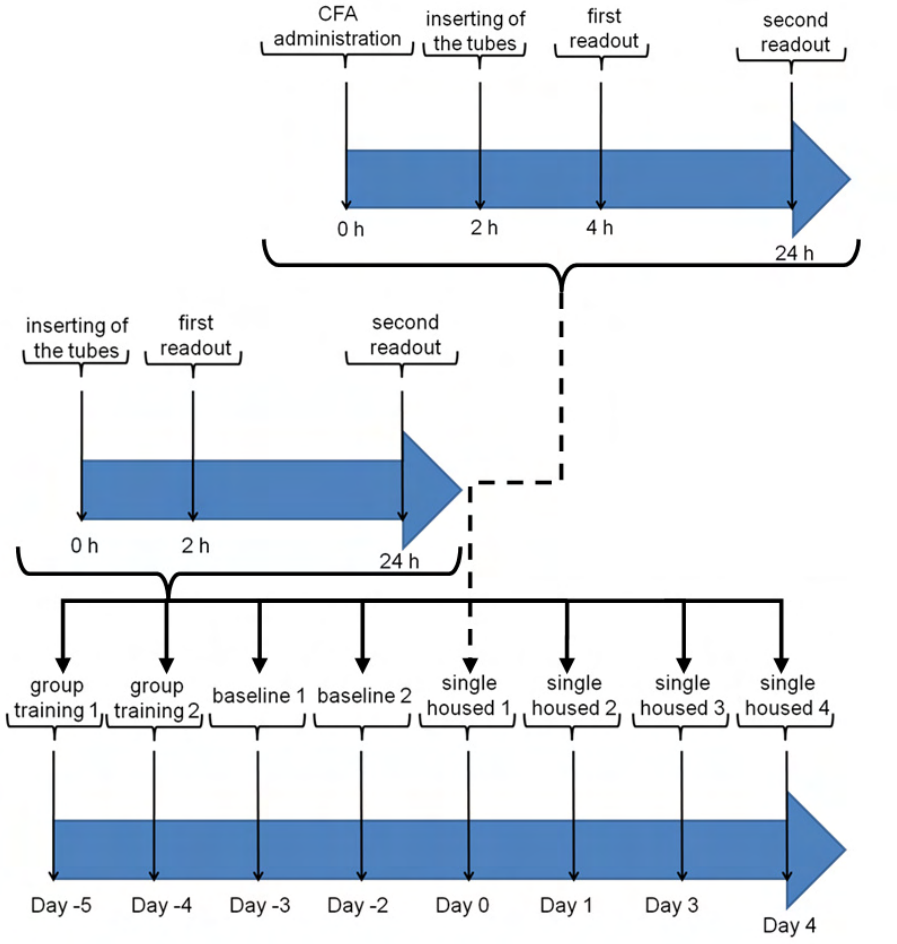

Figure 17 Experimental protocol: Effect of CFA on burrowing behaviour

NOTE: 1 mg/ml dose of CFA and PBS groups only received 1 day of training and baseline prior to CFA injection

2.2.4.2.3 Effect of indomethacin on CFA induced deficits in burrowing behaviour

In these experiments, 30 female BALB/c mice were used to explore the effects of indomethacin on innate burrowing behaviour with CFA induced deficits in burrowing behaviour. Based on the dose response curve to CFA, the 1 mg/ml dose of CFA was chosen for the drug study with indomethacin and burrowing behaviour was measured 24-48 h after CFA injection. Twenty one (21) animals received 20 µl of 1 mg/ml CFA i.plt into the left hind paw. A control group of 7 animals received 20 µl of PBS i.plt into the left hind paw. Two animals were excluded due to lack of consistent baseline responses. Burrowing behaviour was again measured from 0 to 24 h after CFA and the amount of material foraged was calculated. CFA treated animals were then allocated into 3 groups ensuring that the mean burrowing response was matched across the 3 groups. The control group (group 1) was treated with vehicle (methylcellulose, 0.5%) in a dose volume of 10 ml/kg p.o. Of the CFA treated mice, group 2 received vehicle (methylcellulose, 0.5%, 10 ml/kg, p.o.), group 3 received a dose of 10mg/kg of indomethacin p.o. and group 4 received a dose of 30 mg/kg of indomethacin p.o. as shown in table 10. Burrowing behaviour was again measured 24h later.

Table 10 Table of treatments with CFA and Indomethacin

Animals [n]	Treatment	Concentration of *m. tuberculosis* [mg/ml]	Volume [µl]	Treatment	Volume [ml/kg]
7	PBS	-	20	Methylcellulose 0.5%	10
7	CFA	1	20	Methylcellulose 0.5%	10
7	CFA	1	20	Indomethacin 10 mg/mg	10
7	CFA	1	20	Indomethacin 30 mg/mg	10

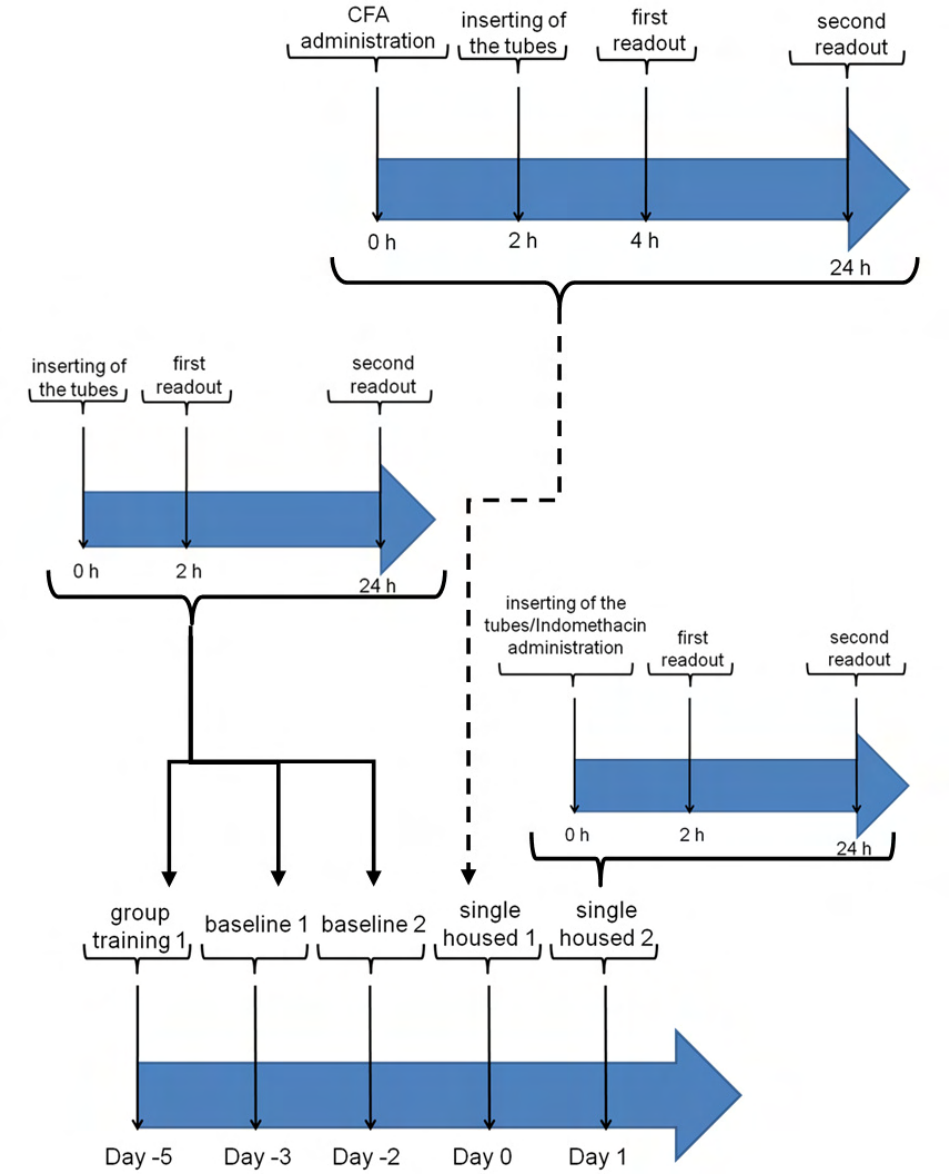

Figure 18 Experimental protocol: Effect of CFA on burrowing behaviour

2.3 Statistical Analysis

For Dose response effect of CFA on paw withdrawal latencies and Dose response effect of indomethacin on CFA induced thermal hyperalgesia, paw withdrawal latencies were presented as mean ± standard error of the mean (SEM) and were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons to determine significant differences from PBS treated mice to CFA treated mice. Pair-wise comparisons were made using the two tailed paired t-test to determine significant differences between baselines within the treatment group.

For the pilot studies, Effect of CFA-induced unilateral inflammation on burrowing behaviour and Effect of indomethacin on CFA induced deficits in burrowing behaviour, displaced bedding material or gravel were presented as mean ± standard error of the mean (SEM) and were analyzed for statistical significance by two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons for the pilot studies, Effect of CFA-induced unilateral inflammation on burrowing behaviour and Dunnet's multiple comparisons for Effect of indomethacin on CFA induced deficits in burrowing behaviour to determine significant differences from PBS treated mice to CFA treated mice.

A probability of $p<0.05$ indicates significance for all tests. Analysis was performed using GraphPad Prism version 6.02 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com

For power analysis G*Power version 3.1.7 was used. For further information see (Faul, et al. 2009)[26].

3 Results

3.1 Hargreaves

3.1.1 Dose response effect of CFA on paw withdrawal latencies

Intraplantar injection of PBS (20 µl) had no effect on paw withdrawal latencies (PWL) when measured at 4, 24, 48 120 and 168 h after injection when compared to baseline PWL or PWL of the non-injected paws (Figure 19; Table 11). In contrast, intraplantar application of 20µl of 0.25, 0.5 or 1 mg/ml of CFA into the left paw resulted in significant reduction of PWL when compared to baseline and to non-injected paw, indicating the development of thermal hyperalgesia. In addition, paw edema was not measured but observed. After 24h, differences in paw swelling PBS and CFA were present. Figure 19A and 19B shows on the ordinate are the paw withdrawal latency in seconds and on the abscissa time before and after injection in hours. All CFA treated groups showed a significant reduction in PWL at 4h after injection when compared to latencies in PBS injected animals at 4h, marked with an asterisk(*) in Figure 19B. Additionally, animals injected with 0.5 mg/ml concentration of CFA showed a reduction in the PWL at 48h after injection compared to PBS-control group, but this was not found for animals injected with 0.25 or 1 mg/ml concentrations of CFA. No significant differences in PWL were observed for any of the CFA treated groups at later time points (120 and 168h) when compared to PBS controls. However, comparing the PWL to the baseline for each treatment group showed that injection of 0.5 and 1 mg/ml concentrations produced a significant reduction in PWL at 4, 24, 48 and 120 h but not 168h; the lowest concentration 0.25 mg/ml was significant at 4h only (Figure 19B; Table 11A and 11B). Similarly, when comparing the PWL of injected and non-injected paws for individual animals treatment with 0.25, 0.5 and 1 mg/ml of CFA produced significant reductions in PWL at 4, 24, 48 but not at 120 or 168h post injection (Table 11A and 11B). There was no effect of PBS injection on PWL compared to baseline or to non-injected paws (Figure 19A; Table 11A, 11B). Comparing the PWL of non-injected paws, there was no effect

of the CFA treatments whether compared to PBS control animals at each of the time points or baseline PWL for each of the groups (Figure 19B; Table 11B).

Based on these data, the 0.5 mg/ml concentration of CFA was chosen for subsequent studies to investigate the effects of analgesics as this produced the most robust thermal hyperalgesia response; the 48h time post injection was again chosen as this was the time of maximal response. 0.5 mg/ml was more robust than 1 mg/ml, because of a presumably higher hyperalgesia, which had not only thermals aspect but rather mechanical aspects. It was observed that the mice tried to reduce contact to glass tables. Due to less applied pressure on the table, the heat transfer was reduced. This caused a false high PWL reading.

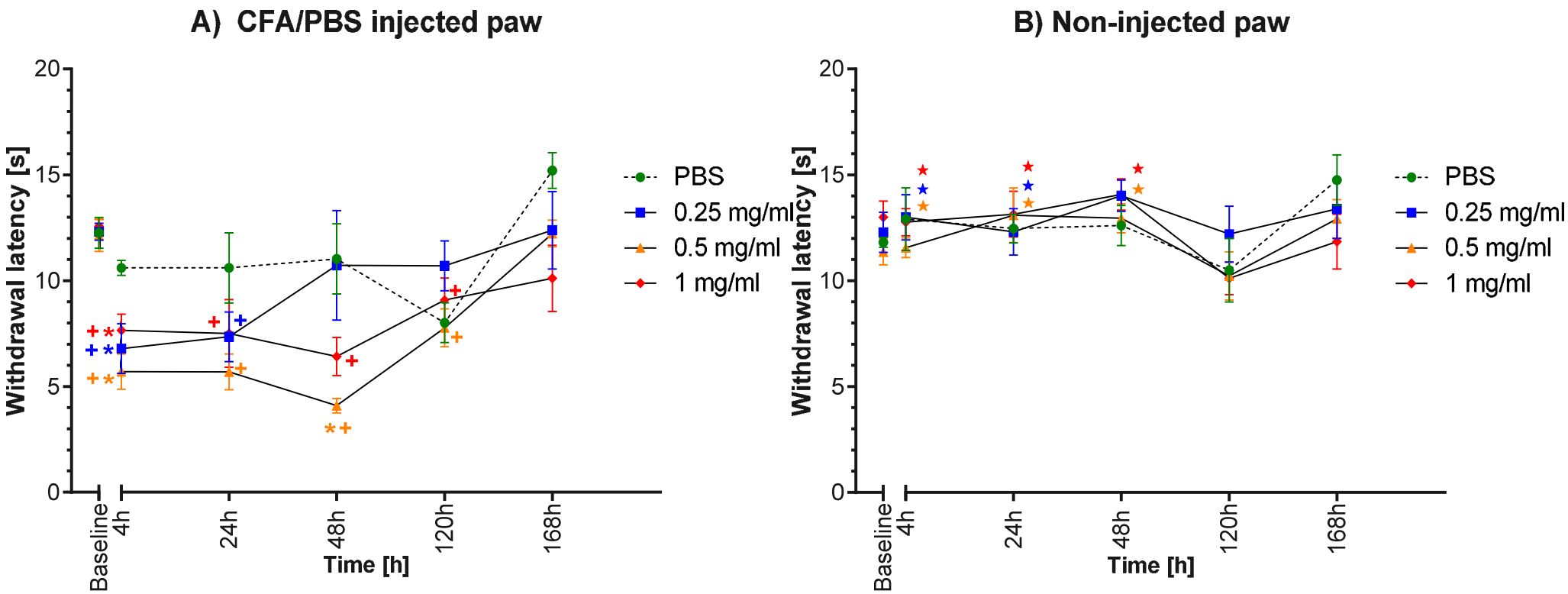

Figure 19 Dose response effect of CFA on paw withdrawal latencies

*A) Paw withdrawal latencies before were measured before (baseline) and after intra plantar injection of 20 µl of PBS or CFA at 4, 24, 48, 120 and 168h. Three different CFA concentrations were used 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml. Significant differences were determined with one-way ANOVA with $p<0.05$; CFA-treated compared to PBS are marked with *(compared with Bonferri test). CFA compared with baselines are marked with $^{+}$(compared with paired t-test). For detailed values see table 11 and 11A. B) Paw withdrawal latencies of non-injected paw, after injection of 20 µl of PBS or CFA into left paw. Significant differences between left paw compared right paw within the same CFA-group and time point ($p<0,05$), are marked with ★(compared with paired t-test). For detailed values see table 11B.*

Table 11 Statistical analysis using one-way ANOVA for the effect of CFA and PBS on thermal paw withdrawal latencies at baseline and post treatment

Time	injected			non-injected		
	p-value*		F-value	p-value*		F-value
		significant			significant	
Baseline	0.9509	No	F (3, 23) = 0.114	0.3797	No	F (3, 23) = 1.074
4h	0.0038	Yes	F (3, 23) = 5.922	0.6596	No	F (3, 23) = 0.5402
24h	0.1350	No	F (3, 23) = 2.049	0.9278	No	F (3, 23) = 0.1512
48h	0.0115	Yes	F (3, 22) = 4.649	0.4611	No	F (3, 23) = 0.8901
120h	0.2237	No	F (3, 23) = 1.570	0.5857	No	F (3, 23) = 0.6589
168h	0.0743	No	F (3, 11) = 3.045	0.3983	No	F (3, 11) = 1.078

Table 11A Statistical analysis using paired t-test for the effect of CFA and PBS on thermal paw withdrawal latencies

PWL for individual groups at each time point were compared to their respective baseline.

Time	p-value^{+}					
	0.25 mg/ml		0.5 mg/ml		1 mg/ml	
		significant		significant		significant
4h	0.0012	Yes	0.0014	Yes	0.0002	Yes
24h	0.0081	Yes	0.0017	Yes	0.0245	Yes
48h	0.5127	No	0.0002	Yes	0.0004	Yes
120h	0.3135	No	0.0216	Yes	0.0163	Yes
168h	0.8550	No	0.9868	No	0.2959	No

Table 11B Table Statistical analysis using paired t-test comparing injected and non-injected PWL

PWL for injected paws for individual groups at each time point were compared to the PWL of non-injected.

Time	p-value*					
	0.25 mg/ml		0.5 mg/ml		1 mg/ml	
		significant		significant		significant
4h	0.0079	Yes	0.0004	Yes	0.0048	Yes
24h	0.0084	Yes	0.0006	Yes	0.0023	Yes
48h	0.2172	No	0.0003	Yes	0.0005	Yes
120h	0.2835	No	0.2170	No	0.3899	No
168h	0.1836	No	0.5476	No	0.3110	No

3.1.1 Dose response effect of indomethacin on CFA induced thermal hyperalgesia

Figure 20A shows dose response effects of indomethacin on CFA induced thermal hyperalgesia. Baseline PWL before CFA injection for the treatment groups were not significantly different (Table12). Intraplantar injection of 20µl of 0.5 mg/ml of CFA into the left paw resulted in a significant reduction of PWL 48 h later compared to PBS injected control animals (Figure 20A with asterisk(*); Table 14). Also, PWL of CFA-injected animals were significantly reduced in comparison to baseline ($^{+}$) (Table 12) or the non-injected (right) paw ($\star$) (Table 12B). Intraplantar injection of PBS had no effect on PWL compared to baseline or to PWL of non-injected paws (Table 12A, 12B). Oral administration of vehicle (0.5% methyl cellulose, 10 ml/kg) in CFA injected mice had no effect on PWL measured 2-4 h later indicating that the thermal hyperalgesia was stable across this time period (Figure 20; Table 12C). By contrast, oral administration of indomethacin at 10 or 30 mg/kg attenuated CFA induced thermal hyperalgesia as shown by the partial restoration of the PWL towards baseline values but the effect failed to reach statistical significance when compared to CFA vehicle-treated control animals (Figure 20A). Significant effects were observed, however, when the PWL for both doses of indomethacin were compared to baseline post CFA values (Figure 20B) or the PWL of non-injected paw for the same group (Table 12B). There was no effect of CFA or indomethacin on the PWL of non-injected paws (Figure 20B, Table 12).

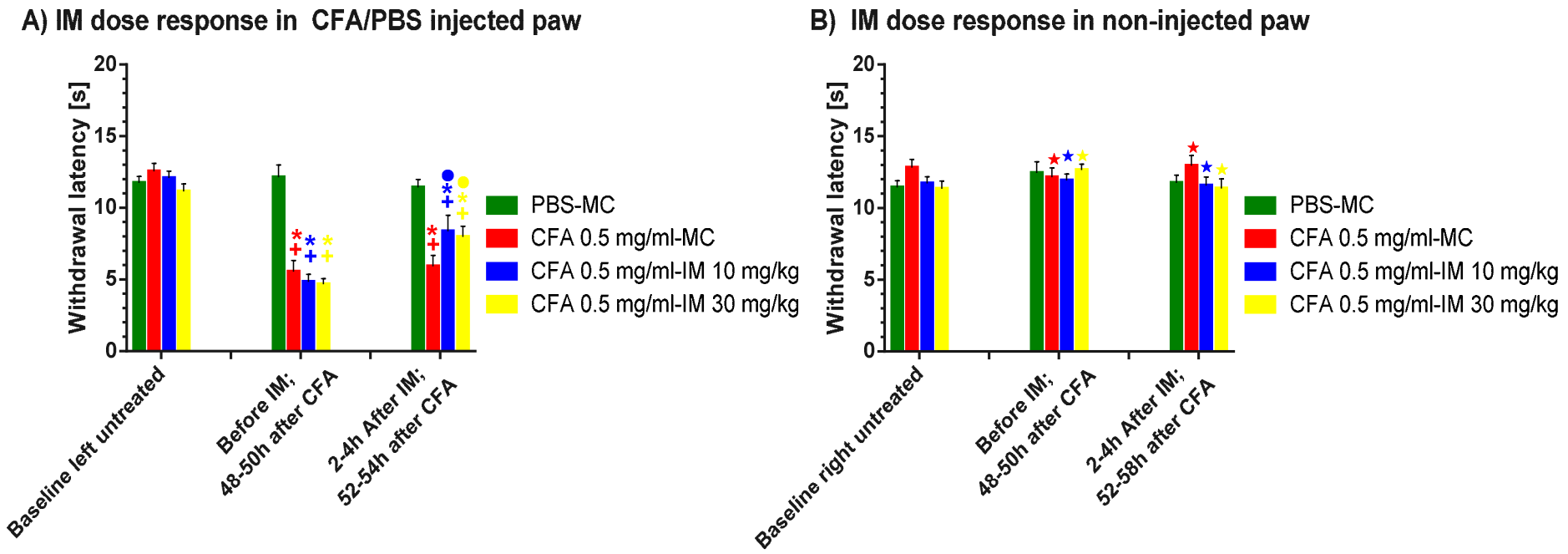

Figure 20 Paw withdrawal latencies injected (left) paw and effect of IM

*A) Paw withdrawal latencies before injection at the baseline; after injection of 20 µl of PBS or CFA at 48-50h. Two different indomethacin (IM) concentrations were used 10 mg/ml and 30 mg/ml with a volume of 10 ml/kg. Moreover, there was an untreated vehicle control group, which received PBS and methylcellulose (MC), and a treated vehicle control group, which received CFA and methylcellulose (MC). Significant differences with p<0,05, are marked with * for CFA-IM-treated compared to PBS-MC control group, with $^{+}$for CFA-IM-treated compared with their own baselines and CFA-IM-treated compared state before IM and after IM. For detailed values, see table 12, 12A and 12C. B) Paw withdrawal latencies of non-injected paw, after injection of 20 µl of PBS or CFA into left paw and after administration of IM or MC. Significant differences between left paw compared right paw within the same CFA-group and time point (p<0,05), are marked with ★. All comparisons were significant. Before IM administration, hyperalgesia was successfully established, for left and right paw comparison. IM could not restore PWL after administration, for left versus right comparison. For detailed values see table 12B.*

Table 12 Statistical analysis using one-way ANOVA for the effect of indomethacin (IM) on thermal withdrawal latencies.

The PBS vehicle, vehicle control group were compared against two IM-groups and MC vehicle control group at the same time within one site(left).

Time	injected			non-injected		
	p-value*	significant	F-value	p-value*	significant	F-value
Baseline	0.2670	No	F (3, 32) = 1.379	0.1887	No	F (3, 32) = 1.690
Post CFA	<0.0001	Yes	F (3, 31) = 28.02	0.8224	No	F (3, 31) = 0.3038
Post treatment	0.0007	Yes	F (3, 31) = 7.375	0.3105	No	F (3, 32) = 1.243

Table 12A Injected paw statistical results of paired t-test on dose response effect of IM on thermal withdrawal thresholds

The baseline of every treatment group were compared against injected paw and corresponding treatment.

Time	p-value^{+}							
	PBS-MC		CFA-MC		CFA-IM 10 mg/kg		CFA-IM 30 mg/kg	
		significant		significant		significant		significant
Post CFA	0.5762	No	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes
Post treatment	0.6875	No	<0.0001	Yes	0.0098	Yes	0.0102	Yes

Table 12B Statistical analysis using paired t-test on the effect of CFA and indomethacin on thermal paw withdrawal latencies (comparison of injected and non-injected paws)

The injected paw of every treatment group were compared against non-injected paw within same time point.

Time	p-value*							
	PBS-MC		CFA-MC		CFA-IM 10 mg/kg		CFA-IM 30 mg/kg	
		significant		significant		significant		significant
Post CFA	0.5962	No	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes
Post treatment	0.6800	No	0.0007	Yes	0.0361	Yes	0.0034	Yes

Table 12C Statistical analysis using paired t-test for the effect of indomethacin (IM) or vehicle on thermal paw withdrawal latencies

Comparison of post CFA baseline to after treatment PWL within the same group.

Time	p-value•					
	CFA-MC		CFA-IM 10 mg/ml		CFA-IM 30 mg/ml	
		significant		significant		significant
Post CFA VS Post treatment	0.7193	No	0.0171	Yes	0.0013	Yes

3.2 Burrowing

3.2.1 Pilot studies

3.2.1.1 Group-housed phase

Animals were housed in groups of 5 or 6 per cage at the start of the training phase as this was reported to lead to faster acquisition of burrowing behaviour in mice (Deacon 2006)[21]. In the group-housed phase, twenty two (22) C57BL/6J mice were used to assess different materials and tube length. Six females and five males were housed per cage. They were exposed to different materials on two consecutive days. The purpose of this phase was to investigate what would be the best material and tubes size to use for subsequent studies. Statistical analysis was not possible since there was only one measurement per tube, material and sex combination. The amount of material displaced was taken as a measure of burrowing behaviour. On the first day, none of the groups of mice displayed burrowing behaviour after introduction of the burrowing apparatus for 2h (Figure 21). However, all groups showed a high level of burrowing behaviour at 24h regardless of the material, sex or length of tube, although the amount of material displaced was less in females than males. There was a training effect regardless of materials. On the second day, all groups started to burrow within the first two hours, in contrast to the first day. Again, the males showed greater burrowing behaviour than females. Although the highest level of material displaced as in the male group with bedding material (90%) the tube was shorter than that used for the gravel material. At this point, it was not possible to make a statement about filling material due to ceiling effects. It was decided to use the longer

tubes as this would provide a larger window for detecting differences observed during the 0-24h period.

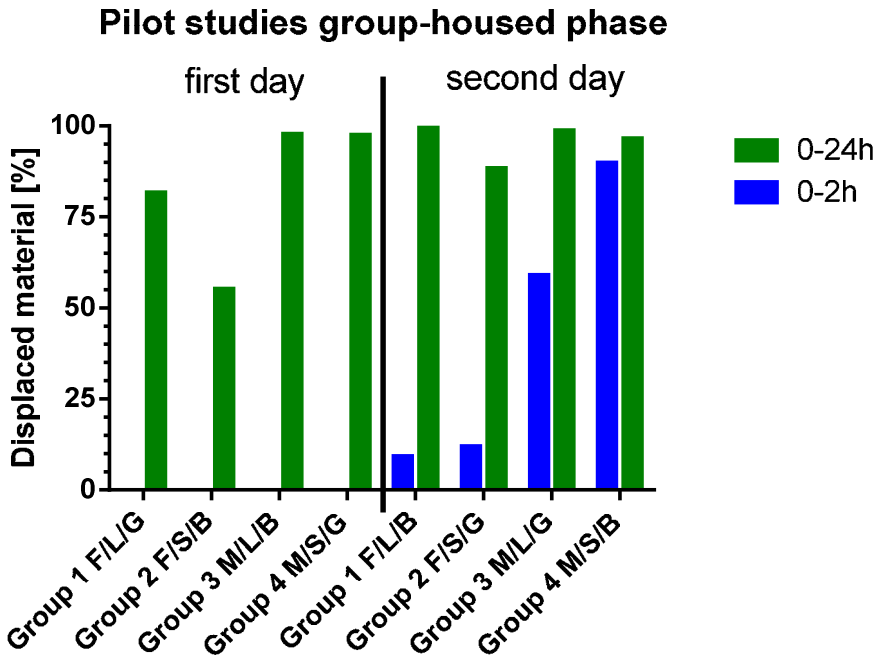

Figure 21 Pilot studies grouped housed phase C57BL/6J

Abbreviations on abscissas: F=female, M=male, L=long tube, S=short tube, G=gravel, B=bedding material. On two following days, different materials were used in different tube length. A training effect at the second day was observed. Both female groups increased burrowing performance within 2hours and 24 hours. In addition, both male groups started to burrow within the first 2 hours. Due to ceiling effects, it was not possible to define a proper filling material.

3.2.1.2 Single-housed phase

Shown in figure 22 are the mean values of displaced materials for three repeated measurements. On the ordinate, there is the displaced material in percentage plotted. On the abscesses, there are four groups plotted with different combinations of materials and sex. After completion of the group-housed phase, the mice were separated into individual cages and further studies were conducted to determine which material would be the optimal for future studies to investigate burrowing behaviour as a measure of spontaneous pain in mice after CFA hind paw inflammation. All mice were single-housed and provided with a long tube containing either bedding material or gravel. Burrowing behaviour was assessed again at 2 and 24 h after introduction of the tubes into the cage. No significant differences were found in burrowing behaviour for females using the different materials when measured at 2 hours or at 24 hours. Differences in burrowing behaviour for the 2 materials were only found in males at 2 hour time (Figure 22) as marked with asterisk(*). Again, significant differences were observed between for sex with males showing greater burrowing behaviour. It was planned that for future studies, females would be used. The reason being that if the assay would be successful, it would be used to investigate possible changes in burrowing behaviour in female BALB/c mice with experimental endometriosis. Since there were no differences in burrowing behaviour observed for bedding or gravel materials in females, bedding material was chosen over gravel for hygienic, preparation, cost and disposal reasons.

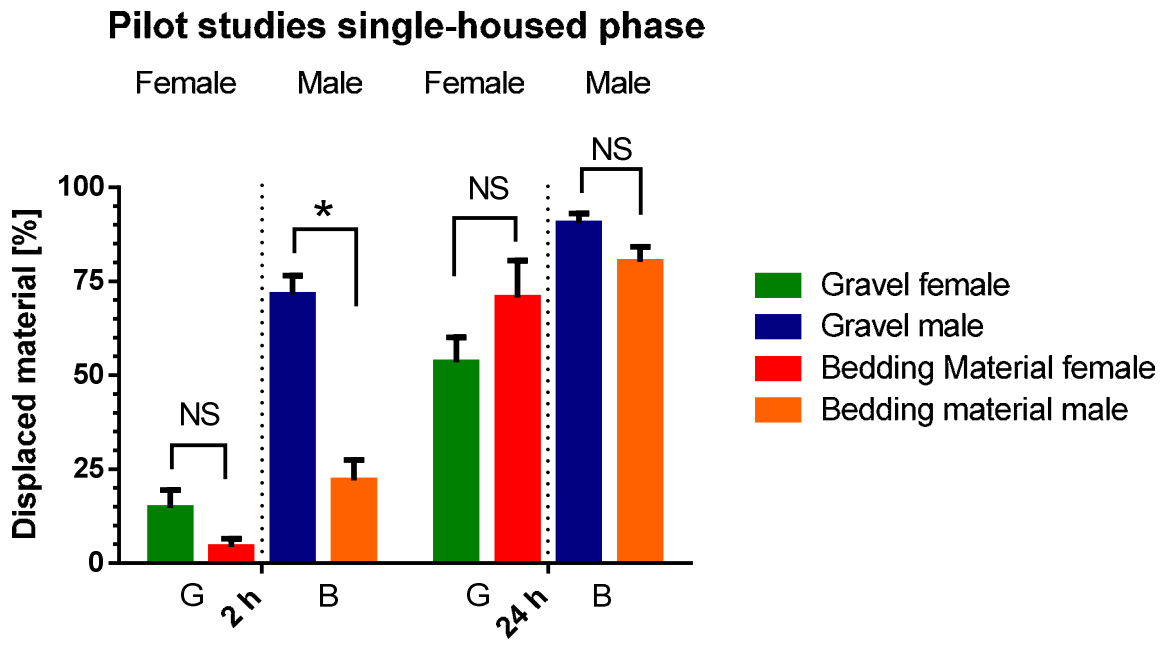

Figure 22 Pilot studies showing burrowing behaviour in single-housed

*B = bedding material, G = gravel;C57BL/6J male and female mice: Data are presented as the mean ± SEM percentage of displaced material. * p<0.05 compared to females with Dunnett's test. Significant differences with *p<0.05 compared to bedding material (males)*

Table 13 Statistical analysis using one-way ANOVA on effect of bedding material on burrowing behaviour at 2 and 4 h in single-house C57BL/6J mice.

Bedding material group were compared against Gravel group at the same time within one sex.

Time	p-value*		F-value
	Gravel V.S. Bedding material		Gravel V.S. Bedding material
Female		significant	
2h	0.0727	No	F (5, 30) = 2.271
24h	0.3765	No	F (5, 30) = 1.109
Male		significant	
2h	<0.0001	Yes	F (5, 24) = 19.30
24h	0.2664	No	F (5, 24) = 1.381

Table 13A Statistical analysis using one-way ANOVA on effect of sex on burrowing behaviour at 2 and 4 h in single-house C57BL/6J mice.

Males were compared against females at the same time within one material.

Time	p-value		F-value
	male V.S. female		male V.S. female
Gravel		significant	
2h	<0.0001	Yes	F (5, 27) = 23.96
24h	0.0014	Yes	F (5, 27) = 5.405
Bedding material			
2h	<0.0001	Yes	F (5, 27) = 9.114
24h	0.6464	No	F (5, 27) = 0.6745

3.2.2 Effect of CFA-induced unilateral inflammation on burrowing behaviour

To investigate the effect of CFA unilateral hind paw inflammation on burrowing behaviour 2 doses were chosen; 0.5 mg/ml and 1 mg/ml both doses which were associated with induction of marked thermal hyperalgesia (see 3.1.1). Baseline burrowing behaviour observed in female BALB/c mice using bedding material (approximate 75% displaced material when measured 24h after introduction into the cage) was similar to that observed in female C57BL/6J mice (See Figure 23A and 21). Intraplantar injection of PBS (20 µl) had no significant effect on burrowing behaviour in female BALB/c mice when measured at 24, 48, 120 and 168h after injection (control groups for 0.5 mg/ml and 1 mg/ml; Figure 23A). I.plt. injection of CFA (20 µl 0.5 mg/ml) had effect on burrowing behaviour at 24h to PBS control mice (Figure 23A; Table 14). Mice injected with 1mg/ml concentration of CFA showed less burrowing behaviour between 24 and 48h than PBS control mice, as seen by the reduction in percentage of bedding material displaced (Figure 23A; Table 14); no effect was apparent on material displaced at 0-2h or later time points(Figure 23B; Table 14A). There were no significant effects on burrowing behaviour between PBS and CFA-treated animals when measured during the first 2h after introduction of the tubes into the cages at any of the time points (Figure 23B). Based on the data the 1 mg/ml concentration was chosen for subsequent studies to examine the analgesic effect of indomethacin on burrowing behaviour between 24 and 48 hours as this was the time of maximum effect.

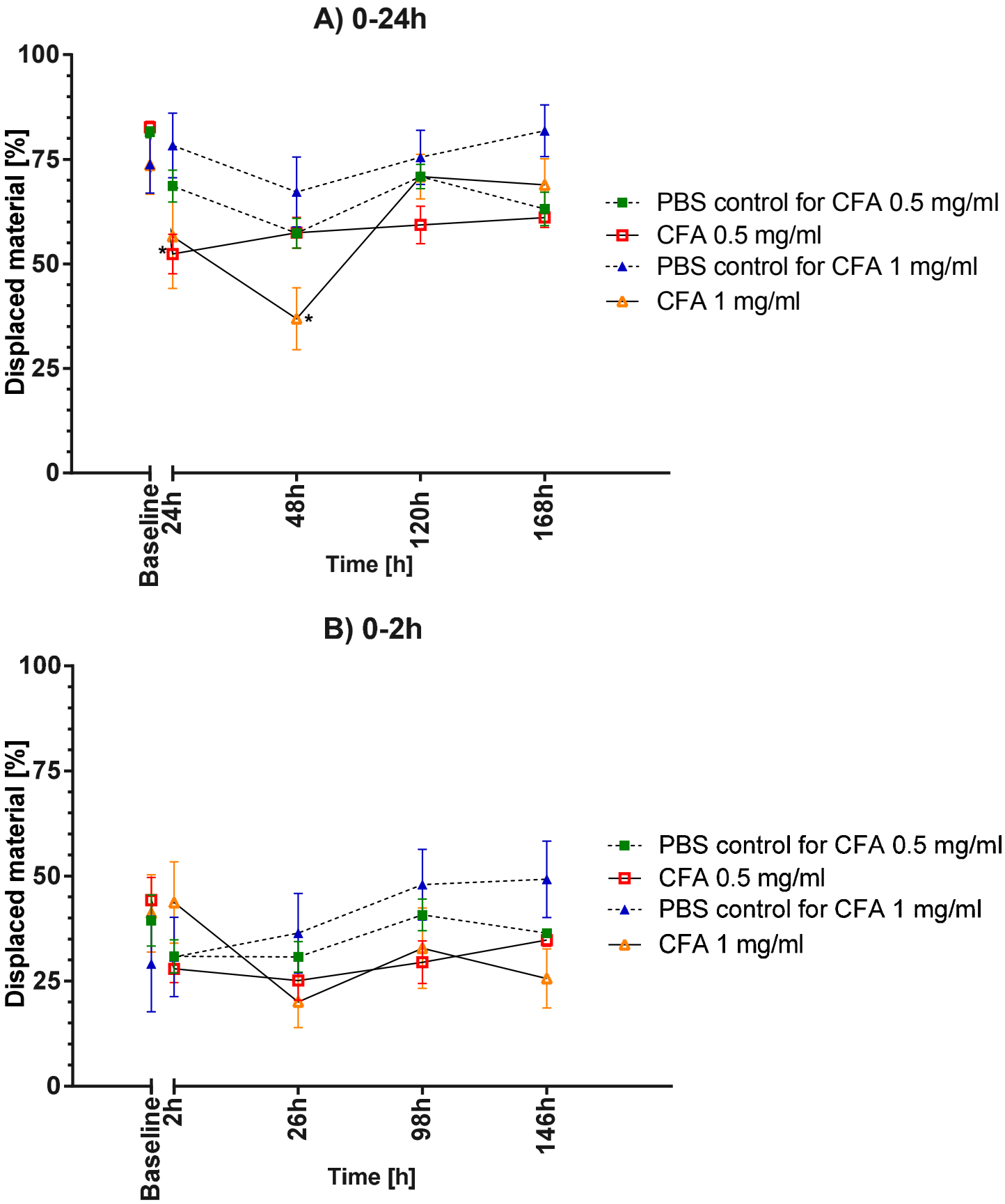

Figure 23 Effect of CFA-induced unilateral hind paw inflammation on burrowing behaviour

*A) Data are presented as mean ± SEM of percentage displaced bedding material during 0-24h period after introduction of the tube and 24, 48, 120 and 168h after CFA injection. Significant differences between CFA and PBS-control are marked with *p<0.05 compared to PBS control mice. Determined with two-way ANOVA and a Bonferri test. B) Data are presented as mean ± SEM of percentage displaced bedding material during 0-2h period after introduction of the tube and 2, 26, 98 and 146h after CFA injection. Significant differences between CFA and PBS-control are marked with *p<0,05 compared to PBS control mice. Determined with two-way ANOVA and a Bonferri test.*

Table 14 Effect of CFA-induced unilateral hind paw inflammation on burrowing behaviour 0-24h interval

P-values of Bonferri after test two-way ANOVA for 0-24h intervals

Time	CFA 0.5 mg/ml		CFA 1 mg/ml	
	p-value*		p-value*	
		significant		significant
Baseline	0.9997	No	>0.9999	No
24h	0.0061	Yes	0.2229	No
48h	>0.9999	No	0.0340	Yes
120h	0.0920	No	0.9965	No
168h	0.9965	No	0.7433	No

Table 14A Effect of CFA-induced unilateral hind paw inflammation on burrowing behaviour 0-2h interval

P-values of Bonferri test after two-way ANOVA for 0-2h intervals

Time	CFA 0.5 mg/ml		CFA 1 mg/ml	
	p-value*		p-value*	
		significant		significant
Baseline	0.9281	No	0.8816	No
2h	0.9911	No	0.8483	No
26h	0.8762	No	0.6825	No
98h	0.2604	No	0.7452	No
146h	0.9995	No	0.2962	No

3.2.3 Effect of indomethacin on CFA induced deficits in burrowing behaviour

Baseline burrowing behaviour was assessed in 28 female, BALB/c, single-housed mice for one day prior to injection of CFA or PBS. The level of bedding material displaced for this study was less approximately 50% compared to 75% of displaced material observed for previous studies (Figure 24A). Animals were allocated to treatment groups based on baseline burrowing behaviour such that the levels were the same at baseline (Figure 24A). Mice then received either PBS (20 µl) or CFA (1 mg/ml in 20 µl) and burrowing behaviour measured from 0-24h. During this time there was a small (non significant) reduction in the amount of bedding material displaced in CFA-treated compared to PBS-treated mice similar to that observed in the dose-effect study for CFA (Figure 23A and 23B). However, no reduction in percentage displaced bedding material was observed between 24-48 h in CFA injected mice that had received oral administration of vehicle when compared to PBS vehicle-treated or their own baseline values. Therefore, no deficit in burrowing behaviour was observed following CFA in this study. As a result, it is not possible to test the effects of indomethacin.

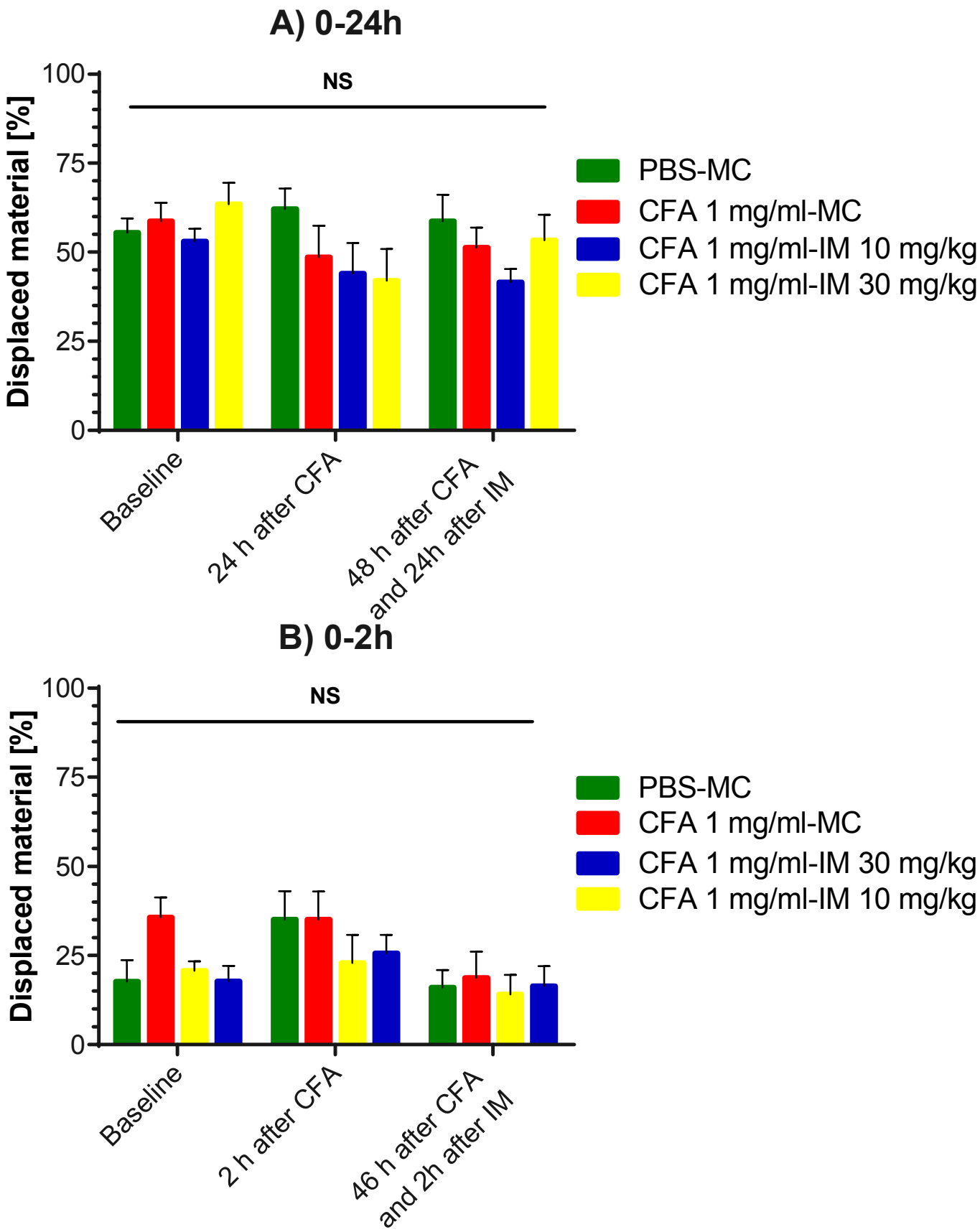

Figure 24 Effect of indomethacin on CFA induced deficits in burrowing behaviour 0-24h

A) Data are presented as mean ± SEM of percentage displaced bedding material during 0-24h period after introduction of the tube and 24, 48h after CFA and 24h IM treatment. B) Data are presented as mean ± SEM of percentage displaced bedding material during 0-2h period after introduction of the tube and 2, 4h after CFA and 2h IM treatment.

Table 15 Effect of indomethacin on CFA induced deficits in burrowing behaviour 0-24h interval

P-values of Dunnet's test ANOVA for 0-24h intervals

Time	p-value					
	CFA-MC		CFA-IM 10 mg/ml		CFA-IM 30 mg/ml	
		significant		significant		significant
Baseline	0.7174	No	0.9867	No	0.9728	No
Post CFA	0.3284	No	0.1324	No	0.0822	No
After IM treatment	0.7564	No	0.158	No	0.8858	No

Table 15A Effect of indomethacin on CFA induced deficits in burrowing behaviour 0-2h interval

P-values of Dunnet's test ANOVA for 0-2h intervals

Time	p-value					
	CFA-MC		CFA-IM 10 mg/ml		CFA-IM 30 mg/ml	
		significant		significant		significant
Baseline	0.1008	No	0.9715	No	>0.9999	No
Post CFA	0.5554	No	0.3593	No	>0.9999	No
After IM treatment	>0.9999	No	0.9922	No	0.9788	No

4 Discussion

The aim of this thesis was to investigate whether burrowing behaviour would be a suitable measure to determine spontaneous pain in mice after hind paw inflammation caused by injection of CFA and to determine if this assay is more sensitive than conventional pain tests using paw withdrawal reflex as the endpoint to assess analgesic activity. The initial studies focussed on establishing the paw withdrawal test in mice. Injection of complete Freund's adjuvant into the paw of female BALB/c mice induced local inflammation and caused a reduction in paw withdrawal latencies elicited by radiant heat using the Hargreaves setup demonstrating the development of thermal hyperalgesia. The optimal dose of CFA was 0.5 mg/ml. The thermal hyperalgesia is at least stable for up to 48 hours. Administration of the non-steroidal anti-inflammatory agent indomethacin partially reversed CFA-induced thermal hyperalgesia.

In subsequent studies to investigate burrowing behaviour, I was able to show that C57BL/6J mice display burrowing behaviour naturally when they are exposed to a filled tube. There was little difference in burrowing behaviour between males and females, although there was a tendency for males to burrow more. The degree of

burrowing was not influenced by the material, bedding or gravel, or by the length of the tube, short or long. The animals improve their burrowing behaviour over time, again regardless of sex, material and tube length. Female BALB/c mice showed a similar behaviour to C57BL/6J mice using long tubes filled with bedding material. They start to burrow when exposed to burrowing device. Burrowing behaviour in female BALB/c mice was reduced following injection of complete Freund's adjuvant into the hind paw of female BALB/c mice indicating an effect on the general well being of the mice. A higher dose of CFA (1 mg/ml) was required to induce a deficit in burrowing behaviour than induction of thermal hyperalgesia. Burrowing behaviour appears to be lower in young versus old female mice. Due to the lower level of burrowing behaviour, it was not possible to examine the effect of indomethacin on CFA induced deficit in burrowing behaviour.

4.1.1 Dose response effect of CFA on paw withdrawal latencies

Injection of PBS into the paw led to a slight but non-significant reduction in PWL compared to pre dose baseline possibly due to damage of tissue and slight oedema caused by the injection per se. Injection of CFA was associated with reddening and swelling. It appeared to be dose related but was not measured. Administration of 20 µl of CFA at a concentration of 0.25 mg/ml induced a short-lived thermal hyperalgesia, which was only observed at 4h and 24h, and the effect was variable between animals. This dose is unsuitable for investigation of chronic inflammation because the thermal hyperalgesia is too unstable. Administration of 20 µl CFA with a concentration 0.5 mg/ml induced thermal hyperalgesia which was stable up to 48 hours after injection. At 48 hours 0.5 mg/ml had the most significant reduction of PWL compared to baseline, PBS control group and contra lateral paw. Although there was a significant reduction in PWL at 120h in the 0.5 mg/ml dose group compared to baseline it is not clear if thermal hyperalgesia was present as the PBS injected mice also showed a reduction in PWL and there was no difference between PWLs between the 0.5 mg/ml and PBS-treated groups. It is not clear why there was a general reduction in PWL at this time point. Injection of the higher dose of 1 mg/ml again caused thermal hyperalgesia as seen by the reduction in PWL at 4, 24 and 48h

but the degree of hyperalgesia was less than that observed with the 0.5 mg/ml dose. This may be a consequence of a too pronounced inflammation. It was observed that the mice receiving the highest dose tried to reduce contact with the glass tables by shifting the body weight to other limbs and to lay the paw on the tail. Although the PWL readings were measured when the animals were sitting correctly on the table one can theorize that due to lower contact pressure, the heat transfer was reduced and a false high PWL was the result. As the paws were more swollen after injection of the 1mg/ml dose of CFA it can be supposed that this dose produced stronger hyperalgesia than 0.5 mg/ml dose. Based on these data it was concluded that the 1 mg/ml dose was not suitable for Hargreaves test. The present results are consistent with those reported in the literature. For example, Chen et al used 0.5 mg/ml in male mice of (4-7 months, C57BL/6J strain) and observed thermal hyperalgesia 24 hours after injection of 10 µl 0.5 mg/ml (Chen, et al. 2010)[27]. Huang et al reported a stable inflammation following injection of 20 µl 0.5 mg/ml CFA in rear paw of female albino mice (8-12 weeks, ICR strain) even though there hyperalgesia was stable up to 4 days after injection (Huang, et al. 2013)[28]. As PWL were not measured on day 4 in the present study, it is not clear if similar findings would have been found. In contrast to the present findings, Sasso et al. reported stable hyperalgesia in male albino mice (CD1 strain) for up to 14 days after intraplantar injection of 20 µl 1 mg/ml CFA (Sasso, et al. 2012)[29]. It is not clear why they would observe such prolonged hyperalgesia compared to our data and that reported by other groups. The CFA was from the supplier SIGMA. One possibility could be the strain of mice used as differences in pain thresholds have been reported between different strains of mice (Liang, et al. 2006)[30].

4.1.2 Dose response effect of indomethacin on CFA induced thermal hyperalgesia

The paw withdrawal latencies for baselines and after intraplantar injection of 0.5 mg/ml of CFA were similar to those observed in the CFA dose response experiment. Thermal hyperalgesia was successfully induced in all groups by CFA 0.5 mg/ml 48h after injection demonstrating robustness and consistency of the response. Oral administration of indomethacin caused approximately 42% for 10 mg/kg and approximately 41% for 30 mg/kg, increase in PWL. Due to variability in the responses, there was no significant difference between the PWL of CFA-MC-vehicle and CFA-IM treated groups. Nevertheless, significant differences were observed for PWL after indomethacin when compared post CFA values using paired t-test. However, there was still a significant difference between PWL baseline and contra lateral paws indicating that indomethacin only caused a partial reversal of hyperalgesia. In the present study, no significant differences were found between 10 mg/kg indomethacin and 30 mg/kg indomethacin suggesting that the maximal effect of indomethacin is produced by 10 mg/kg dose and that increasing the concentration does not have any further benefit. In order to increase the statistical significance of the effect of IM in subsequent studies, the number of animals could be raised. Based upon power analysis of the current data, using a Power of 0.95 and increasing the number of animals per group to 14 would produce a significant effect for a 40% reversal of hyperalgesia. Similar data was also reported by Sasso et al, who showed a partial recovery with 10 mg/kg indomethacin in CFA induced thermal hyperalgesia (Sasso, et al. 2012)[29]. Abu-Ghefreh et al also reported a partial recovery of thermal hyperalgesia with 10 mg/kg indomethacin in BALB/c mice but following injection of lipopolysaccharide not CFA (Abu-Ghefreh, et al. 2010)[27].

4.1.3 Pilot studies

In the pilot studies it could be shown that female and male C57BL/6J exhibit burrowing behaviour naturally and that there is learning enhancement by social facilitation as Deacon reported (Deacon 2006)[21]. Regardless of materials (bedding material or gravel) and sex and tube length, the level of burrowing behaviour was

very high in C57BL/6J. It was not possible to make a statement about the suitability of the two materials due to ceiling effects, i.e. all materials were displayed in the 24h period. Short tubes have a limited filling volume for five or six animals, so for subsequent studies long tubes were chosen. The data also suggested that males are more active than females. For example, single housed males showed significant differences at 2 hours when comparing burrowing behaviour using gravel to bedding material but this was not observed at 24h. By contrast, no differences were observed for females using the different materials whether at 2 or 24h. As a result of this study, bedding material was chosen for subsequent studies as it was more easy to handle.

4.1.4 Effect of CFA-induced unilateral inflammation on burrowing behaviour

For the studies investigating the effects of CFA on burrowing behaviour, female BALB/c mice were used. This was because if the assay was successfully established it would be used to determine possible deficits in experimental endometriosis. The data showed that baseline burrowing behaviour observed in female BALB/c mice is similar to that observed in female C57BL/6J mice using bedding material. Intraplantar injection of 0.5 mg/ml of CFA, the dose that caused significant and robust thermal hyperalgesia at 24 and 48h, caused a small significant reduction in burrowing behaviour in female BALB/c mice at 24 hours but not at 48, or later time points after CFA administration. Burrowing is performed by rearing on hind paws to get a stable stance; the forepaws are then used to remove the material. It was observed that the animals compensate while burrowing by shifting their body weight from left to right paw. This makes them capable of performing the innate behaviour and burrow bedding material while having less pain. Therefore, it was assumed that the hyperalgesia induced by 0.5 mg/ml dose of CFA may not be sufficient to produce a robust deficit in burrowing behaviour which could be used to assess the analgesic effect of indomethacin. A second study was performed to examine the effects of the higher dose of CFA, which in the Hargreaves test was observed to cause more behavioural abnormalities (lifting of the injected limb). After injection of 1 mg/ml of CFA a much larger deficit in burrowing behaviour was observed at 48h as shown by the decrease in burrowed material, the time of peak effect in thermal hyperalgesia.

This dose was chosen for subsequent experiments. The deficiency in burrowing behaviour after unilateral inflammation of the hind paw is much less than reported by other groups using different disease or injuries where burrowing behaviour appears to be more sensitive to disruption by more centralized injuries. Deacon et al showed, for example, that mice with a hippocampal lesion burrowed approximately 96% less than uninjured control mice. Furthermore, he showed that mice with developed prion disease (scrapie), burrowed approximately 85% less than uninfected control mice, 15 weeks post infection. This is about 7 weeks earlier than first appearance of clinical signs of scrapie. Jirkof and colleagues showed that mice after laparotomy have a deficit in burrowing behaviour (approximately 16% within 2h after exposition to the tube) and that this could be reduced by treatment with post-operative analgesics (Jirkof, et al. 2010)[23]. In addition, Teeling and co-workers showed a significant suppression (93% within 2-4h after introduction of the tube) of burrowing behaviour in mice after intraperitoneal administration of lipopolysaccharide (Teeling, et al. 2007)[22]. In contrast to the present findings, a reduction of burrowing behaviour was reported in rats after unilateral intraplantar administration of CFA and to a smaller extent after peripheral nerve injury (Andrews, et al. 2012)[3]. Based on this and published data it appears that burrowing behaviour is more sensitive to deficits caused by systemic disease. As experimental endometriosis is a systemic disease, it is still worthwhile to assess if burrowing behaviour could be a suitable assay to determine spontaneous pain in mice.

4.1.5 Effect of indomethacin on CFA induced deficits in burrowing behaviour

Based on the data obtained in the dose response experiment for burrowing behaviour the 1 mg/ml dose was chosen to examine the analgesic effects of indomethacin. In contrast to the previous experiment, a significant reduction in burrowing behaviour was not observed, so it was not possible to examine the effect of indomethacin. In addition, baseline burrowing was lower than in the pilot and dose range experiments. The main difference between pilot studies, dose range and indomethacin experiments was the age of the mice. The BALB/c mice used in the indomethacin experiment were 5 weeks younger than in dose range experiments and

16 weeks younger than those used in the pilot study. Since the same setup was used in all experiments this suggests that there is an age dependency to burrowing behaviour. In order to refine this part of assessment, the recommendation for future studies would be to use older female BALAB/c animals.

5 Resume

Ethological assays are commonly used in pain models with inflammation in rodents. We could show that the tried and tested Hargreaves assay works also for, adult female BALB/c with local paw inflammation induced by injection of CFA 0.5 mg/ml. A higher concentration of 1 mg/ml CFA also produced hyperalgesia but it is not suitable for the Hargreaves test of thermal hyperalgesia. This is due to avoidance attitude by shifting the body weight to other limbs. It can be theorized, that due to lower contact pressure between paw and glass table, the heat transfer was reduced and resulted in false high paw withdrawal latency. In addition, a concentration of 0.25 mg/ml CFA led to an instable hyperalgesia, which was also unsuitable for the Hargreaves test. We also were able to demonstrate a partial reversal of the thermal hyperalgesia with indomethacin 10 mg/kg whereas 30 mg/kg did not have any further benefit.

The new ethological burrowing assay, which assesses spontaneous pain and general condition, was partially successful in terms of showing a CFA effect on burrowing behaviour. The 0.5 mg/ml dose of CFA led to a reduction of burrowing behaviour but the window was too small for drug studies. In contrast, 1 mg/ml CFA led to a significant deficit in burrowing behaviour at 48 hours. However, burrowing behaviour appears to be dependent on the age of the female mice as younger animals did not show the same level of burring at baseline or deficit after CFA injection. For future studies, it is recommended that older mice be used (> 18 weeks). Another approach would be to use males as these were found to be more active than females, in order to gain stronger baselines but it is not clear if in this case that the CFA would produce a greater reduction of burrowing behaviour. Furthermore, gravel could be used as an alternative filling material as this may be more unpleasant to move or stand on with an inflamed paw due to the rough and sharp surfaces and could lead to a more significant reduction of burrowing behaviour. Based on published data, there is strong evidence that a systematic inflammation or disease produces a stronger deficit in

burrowing behaviour. This assay may therefore be a suitable ethological assay for determining spontaneous pain in experimental endometriosis in mice.

If the sensitivity of this assay is increased, with the suggestions for improvement and had proven to be robust, this assay could contribute a lot to refine future inflammation experiments, with respect to 3 R's principle (replace, reduce and refine). The animals are held in their familiar environment and are not exposed to evoked pain. In addition, a contribution to refinement is the use of mice instead of rats. This assay needs further investigation, for example with older females, the more active males, another filling material or another source of inflammation.

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