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The Evaluation of the Rat Grimace Scale and Ultrasonic Vocalisations as Novel Pain Assessment Tools in Laboratory Rats

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The Evaluation of the Rat Grimace Scale and Ultrasonic Vocalisations as Novel Pain Assessment Tools in Laboratory Rats

by

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Abstract

Measuring pain in non-human mammals such as rodents is challenging. Recently, spontaneous behaviours including facial expression and ultrasonic vocalisation have been proposed as novel, alternative measures of pain. The goal of this research was to evaluate the applicability of ultrasonic vocalisations, and the Rat Grimace Scale (a recently developed facial expression pain scale) during gas exposure (for euthanasia purposes) and in models of inflammatory and incisional pain. First, I found that ultrasonic vocalisations were emitted during carbon dioxide, but not during isoflurane exposure. Secondly, the Rat Grimace Scale was able to detect inflammatory and incisional pain in rats. Additionally, the pain score obtained using the Rat Grimace Scale corresponded with the findings of a conventional nociceptive test using von Frey filaments. My work contributes to the further understanding of ultrasonic communicative behaviour and facial expression of rats experiencing pain and the application of grimace scales in in-vivo biomedical research.

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I wouldn't be where I am if it weren't for you.

Table of Contents

List of Tables

List of Figures and Illustrations

List of Abbreviations

CFA Complete Freund's Adjuvant

RGS Rat Grimace Scale

USV Ultrasonic vocalisation

IASP International Association for the Study of Pain

FACS Facial Action Coding System

kHz kilohertz

AU Action unit

CHAPTER ONE:

INTRODUCTION

Opposed to human patients who can verbally report their experience, the evaluation of pain and pain severity in non-human animals is difficult. Especially prey species, such as rats and mice tend to mask pain to protect themselves from predation. Therefore, few specific pain behaviours have been identified in rodents. In biomedical research, where rodent models play a central role in representing human pathology, pain assessment tools are limited and often dependent on the administration of a stimulus. The widespread use of these stimulus-driven nociceptive tests has recently been questioned [1, 2]. Defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" we know that the pain experience has an emotional component. Traditional nociceptive tests that are limited to the measurement of reflexive behaviour appear to fall short in their objective of measuring this emotional component. Alternatives to these nociceptive tests have been proposed, more specifically the measurement of pain related spontaneous behaviour. Unlike traditional nociceptive tests, spontaneous behaviour can be measured using a non-invasive approach, independent of the administration of a stimulus. These measures of spontaneous behaviour are thought to reflect the pain experience including its emotional component.

Two behaviours have been proposed as measures of spontaneous pain behaviour in rodents: facial expression, and ultrasonic communicative behaviour. In this thesis I, explore the applicability and limitations of these novel pain measures.

1.1 The pain experience

Pain has an important protective function and is necessary for the survival of the individual. Pain warns of imminent or current injury, it motivates to move away from the source of the pain, to guard injured body parts and to avoid similar situations in the future. The definition of pain includes that an individual's inability to verbally communicate does not invalidate the possibility that the individual is experiencing pain. The importance of acute pain for maintaining bodily

integrity is particularly underscored in cases where normal pain sensation is absent: a report of a gene mutation in six related children described a congenital channelopathy (loss of Nav1.7 sodium channel function) causing them to be unable to detect thermal and mechanical pain [3]. The children sustained extensive painless injuries such as fractures, bruises and cuts, and were often only diagnosed because they were limping, or not using one of their limbs. Upon examination, they had normal sensory responses (touch, tickle, temperature sensation and pressure) but were unable to detect noxious insults.

1.1.1 The mechanism of pain

Harmful stimuli are detected by a variety of nociceptor terminals located in the skin or subcutaneous tissue. These terminals are the peripheral endings of primary sensory neurons (`free nerve endings`) whose cell bodies are located in the dorsal root ganglia and trigeminal ganglia. There are four classes of nociceptors: thermal, mechanical, polymodal, and a class named sleeping nociceptors. Thermal nociceptors are activated by temperatures over 45°C and under 5°C. Mechanical nociceptors are activated by pressure. Both thermal and mechanical nociceptors have thin, poorly myelinated Aδ fibers that conduct signals at moderate velocities (5 to 30 meters per second). Polymodal nociceptors are activated by mechanical, chemical, and thermal stimuli. These nociceptors have thin, non-myelinated C-fibers that conduct at slow velocities (less than 1 meter per second). The mechanism by which noxious stimuli depolarize free sensory nerve endings and generate action potentials is not known. Afferent fibers coming from nociceptive nerve endings terminate in the dorsal horn of the spinal cord (lamina I and II). These nociceptive-specific neurons project from the spinal cord to the brain through five ascending pathways: the spinothalamic, spinohypothalamic, spinoreticular, cervicothalamic, and spinomesencephalic tracts. The thalamus processes the afferent information in the lateral and medial nuclei. Additionally, nociceptive information is processed at the level of the insular cortex, which contributes the integration of the sensory, affective, and cognitive components of the pain experience. Three structures modulate the action of the cerebral cortex: the basal ganglia, the hippocampus and the amygdala help process the sensory input and associate it with emotional states, store the input as a memory, and instigate a response. Patients with lesions to the insular cortex suffer from a condition termed ‘pain asymbolia’: they can distinguish different

types of pain, but do not display the typical emotional responses, such as anger or sadness, and they do not show the distinct facial expression of pain [4].

1.1.2 Types of pain

Pain is an integrated response to a noxious stimulus, and it most often resolves after the stimulus is taken away. However, sometimes it persists even after healing is apparent. Also, pain may arise even without a stimulus or lesion present [5]. Chronic and spontaneously appearing pain, that does not seem to be associated with any tissue damage, loses its primary protective purpose. Woolf (2010) [6] underlines this functional versus non-functional categorisation of pain by creating three main types: 1. nociceptive and 2. inflammatory pain as physiological phenomena, and 3. pathological pain as an abnormal event, without protective function. Pathological pain can result from neurological damage (neuropathic pain, due to damage to the nervous system) or occur in conditions where there is no identifiable tissue damage or inflammation. In this case the pain is called dysfunctional pain, and is, for example, occurring in conditions such as fibromyalgia, irritable bowel syndrome, tension type headache, temporomandibular joint disease and interstitial cystitis) [6]. Pathological pain is the consequence of plastic changes in the nervous system caused by long term exposure to inflammatory mediators and growth factors [6]. This structural and functional reorganisation takes place in the peripheral as well as the central nervous system and facilitates the response to peripheral inputs, in other words, it amplifies sensory signals. This process occurring the spinal cord and brain is known as 'central sensitisation': the threshold for generating pain decreases and the duration, intensity and distribution of the pain increases [7]. Lastly, a type of pain exists that has no evoked nociceptive nor inflammatory origin: spontaneous pain. Although true spontaneous pain is rare (most often there is a causative stimulus), it is most often present in neuropathic conditions. Its mechanisms are yet uncertain, but are most likely associated with the spontaneous discharging of unstable axonal membranes and sleeping nociceptors [5]. Sleeping nociceptors are activated as a response to inflammation, and have spontaneous discharge in certain neuropathic conditions, such as diabetic neuropathy.

Pain is a perception: it is the product of the cerebral processing of the sensory input, and is associated with an important affective and motivational component. The pain intensity experienced by a patient is affected by psychological and situational conditions: factors such as social support, hypnotic suggestion, excitement, or distraction can significantly modulate the pain experience. Additionally, responses to the same stimulus may vary between individuals [8].
Pain was long regarded as a 'by-product' of an underlying pathology, a symptom that should be accepted and tolerated. This general opinion has shifted in the last decades: pain is now defined as a disease in its own right, and efforts to make pain alleviation or 'freedom from pain' a fundamental human right have been recently undertaken [9].
While the topic of consciousness and emotional experiences in humans is facilitated by the means of verbal communication, it has been universally agreed upon that non-human animals experience consciousness and emotions too. Artificial arousal of the cortical and subcortical networks responsible for affective states in humans generate equivalent behavioural and emotional states in non-human animals, including states of reward and punishment [10].

1.2 Assessing pain in laboratory rodents

To study the underlying mechanisms of pain, a wide array of experimental rodent models has been developed. Animal models play a crucial role in analgesic drug development and disease modeling in general. Therefore effective assessment of pain in these in-vivo assays is critical: not only for the scientific validity of the study, but also for the welfare of the animals involved. However, the assessment of pain in laboratory rodents has proven to be challenging.

1.2.1 Evoked measures of pain (algesiometric methods)

Traditionally, nociceptive tests relying on the administration of a stimulus are used to evaluate an animal's pain sensation. The animal's response to the stimulus, such as withdrawal of the limb or tail, vocalisation, struggle or avoidance behaviour, is then registered. Stimuli of various nature are used: mechanical stimuli (von Frey filament testing, Randall-Selitto paw pressure test), thermal stimuli (hot plate or cold plate test), chemical stimuli (formalin, acetic acid test) or electrical stimuli.

1.2.1.1 Mechanical hypersensitivity testing using nylon von Frey filaments

For half a century, nylon monofilaments have been used as the standard method for testing mechanical hypersensitivity in awake, unrestrained animals [11]. Typically, the sensory threshold of the hind paw (occasionally the tail, vulva or abdomen) is determined by applying a filament, or series of filaments, to the plantar surface of the foot, until a withdrawal response it registered. The prototype of these filaments was designed in 1922 by the pioneering physiologist Maximillian von Frey (von Frey, (1922) as cited in [12]). Consisting of an animal hair connected to a thistle spine, it was used to evoke itch in human patients [13]. In the 1950`s, the tool became marketed in the form of a practical set of calibrated nylon monofilaments of different thickness mounted on a handle. This set is still commercially available today and has not been significantly modified. The tool is simple and relatively inexpensive (although not everyone agrees [14]), and therefore gained widespread popularity and application in pain research. Many testing protocols on how often and in which order to apply the filaments have been reported, but none seems to be superior (see appendix Chapter 2) [14]. Automated variants of the filaments have been developed in recent years, allowing for a precise threshold determination with only one filament or 'probe' application. There is a bench top version that mechanically advances a rigid probe and applies a ramped pressure increase (Plantar Test, Ugo Basile, Gemonio, Italy), or hand held devices that contain a force transducer for accurate registration of the stimulus intensity (e.g. Electronic von Frey, Somedic, Horby, Sweden; RatMet, Topcat Metrology, UK, www.topcatmetrology.com, and Electronic von Frey filament test (Bioseb, Chaville, France). These newly developed electronic versions eliminate some of the limitations of the nylon filament set, yet weaknesses are associated with the general method.

In flexible filaments, the shape of the tip changes with the force applied, and the degree of bending. When just touching the paw, the contact area consists of the cross sectional area of the filament, a small circle. When bent and applied to flat surface (e.g. plantar surface of the rat's hind foot), the tip shape becomes the edge of the tip, a narrow bow shaped surface (Figure 1). When applied with even greater force, the tip sinks into the paw surface and the shape becomes even more complex [15]. Electronic versions have rigid probes with dome shaped tips, to avoid this issue.

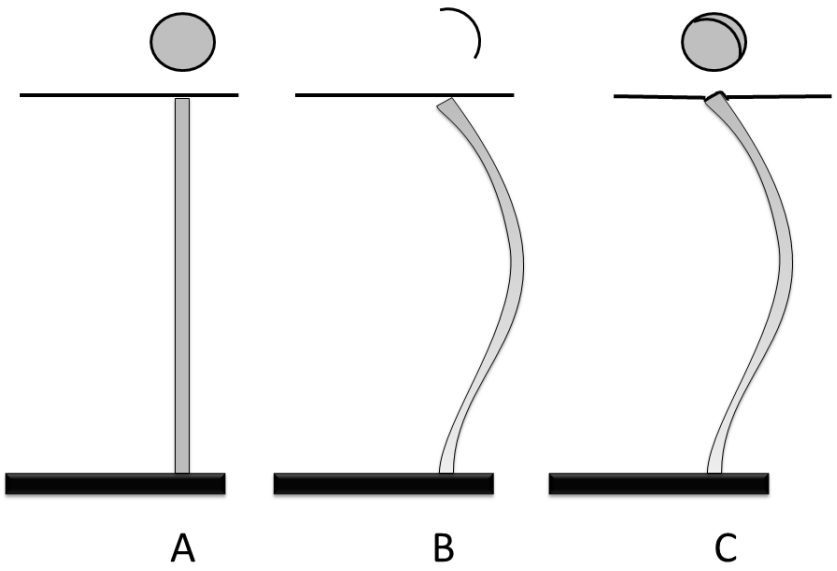

Figure 1. Graphic representation of the contact surface of nylon monofilaments under various loads. When touching the test surface without bending, the contact area consists of the cross sectional area of the filament (a circle, A). When applied until bending, the tip shape presented to the surface is a bow shape (B). Increased force application will cause the tip to sink into softer tissue and become complex in shape (C) (after Bove, 2004) [15].

The magnitude of the force applied to the paw depends on the contact surface, and is consequentially unpredictable [15]. Secondly, the rate of application determines when maximal forces of that particular filament are achieved. In slower applications, the maximal force is not reached for many seconds, while in faster applications the force develops rapidly and is can be even overshot [15]. Other user dependent application parameters such as contact time and degree of bending, but also number of stimuli for each filament, the pattern of application, and the means of analysis have not been standardised [15]. There are only limited studies that specifically describe methodology, or which particular skill or training is required. There are no studies comparing methods and results from different laboratories. Chaplan (1994) [16] has attempted standardisation including the number of trials for each filament, the pattern of application, and the means of analysis. However, this method is not universally accepted, and laboratories tend to modify the technique towards internal repeatability (within a laboratory),

rather than towards validity. Also, one can find multiple methods of analysis and the reporting of results, including 50 percent threshold interpolation, percent withdrawal to one or two filaments, and simple thresholds based upon any possible applied method. Rarely is filament contact time reported, but the difference between one second or ten seconds may mean that different nociceptors are activated (tactile versus pressure, touch versus nociception) and different sensory pathways are tested. Le Bars et al. (2001) state that therefore the stimulus is not specific for pain. Additionally, when mechanical stimuli are truly nociceptive, or applied repetitively, they potentially cause sensitisation of the tissues [17]. In this perspective, the role of the surface on which the animals are tested comes in. Rats tested on the widely used wire mesh showed larger individual variability and increased variability across different testing session, than when tested on a smooth plastic surface [12]. In an injury model, the contralateral paw showed decreased thresholds only on the wire mesh. These methodological differences make comparing the results on sensory threshold determination using von Frey filaments difficult. This seemingly important between-laboratory inconsistency appears generally accepted in the pain research community, arguing that within a given experiment these differences are of little importance as long as there is internal consistency [15].

1.2.1.2 Other nociceptive testing methods

In addition to the use of mechanical stimulation, a variety of other nociceptive tests are available. All come down to a combination of three characteristic components: the physical nature of the stimulus, the site of application, and the condition of the site of application (healthy or injured/inflamed). The first parameter is the nature of the stimulus, including its duration and intensity. Typically, stimuli will be thermal (heat or cold), electrical (shock), mechanical (e.g. von Frey filaments, pressure application) or chemical (the injection of an irritant). The second parameter is the site on the body at which the stimulus is applied. Elicited pain can be of somatic, visceral, articular, or neuropathic origin, and nociceptive tests are usually applied to a cutaneous or visceral surface. The third parameter is the stimulated site, which can be a healthy tissue or inflamed or injured body part. Despite the great variety of nociceptive tests, the information generated by these paradigms should be interpreted with caution.

1.2.1.3 Concerns regarding stimulus dependent nociceptive testing

Responses such as struggle or vocal reactions involve the engagement of supraspinal structures, whereas withdrawal can be a sole reflex, regulated via the spinal cord [17]. This indicates that tests measuring a withdrawal reflex assess nociceptive pathways, and should not be interpreted as a true pain experience. The IASP defines nociception as 'the neural process of encoding noxious stimuli', including that 'this encoding may be autonomic or behavioural', and that 'pain sensation is not necessarily implied'. Further, there is uncertainty whether the withdrawal reflexes indicate nociception, or aversive (avoidance) behaviour, that could be mistaken for pain [15]. Despite this known fact, ninety percent of behavioural animal studies published in the journal 'Pain' from 2000 to 2004 used stimulus evoked responses to document pain in rodent models (mainly thermal and mechanical hypersensitivity tests) [18]. Considered the involvement of higher brain structures (thalamic and cortical) that compose the pain experience, efforts to model human pain using only nociceptive reflex testing seems rather basic [19].

Moreover, as a result of these and other shortcomings (generally poor study design, low power, poor subject choice, non-transparent reporting, the overstatement of drug efficacy and publication bias) [20] [1], translation to human pathology has been problematic in some reported cases, e.g. [21].

Additional concerns regarding measures of evoked behaviour is that rodents as prey animals may suppress their response to an external stimulus to not reveal their weakened or injured status to predators. This, and the phenomenon of stress induced analgesia, may result in false negative experimental outcomes. Also, an animal's response to a stimulus can become modified by a learning process of the individual, after which it may try to anticipate or avoid the stimulus (aversive conditioning). Despite these known flaws, methodologies using stimulus dependent measures of pain have remained unchanged for over twenty years [2]. Based on the observation that the current animal models of pain are suboptimal, proposals for improvement have been made at different levels: refinement of current models [22], development of new models more directly applicable to the prevalent painful conditions, replacement of reflexive measures with non-reflexive (operant) measures [23], replacement of measurements of evoked responses with measurements of spontaneous behaviours [18], the use of a broader range of 'quality of life'

measures [24]. Additionally, guidelines on the reporting of research using animals, the 'ARRIVE guidelines' have been proposed and adopted by several journals [25].

1.2.2 Spontaneous behaviour as a measure of pain

1.2.2.1 The evolutionary benefit: why would animals spontaneously express pain?

To be conserved through evolution, the communication of pain in animals must have been beneficial for survival, and these benefits must have outweighed the costs. In general, an individual increases its fitness by increasing the survival success of its relatives. It has been proposed that evolution of cooperative behaviour is favoured by the chance that the favor later gets returned, in other words, the receiver may later repay the sender through reciprocity. Reports exist on empathetic helping behaviour in rats [26, 27]. Langford et al. (2006) have suggested that mice can be affected by the pain status of a familiar conspecific [28], and that female mice prefer to maintain close proximity to other mice in pain [29]. Other examples of communicating one's affective state that is beneficial for both the sender and the receiver can be seen in infant rats eliciting maternal care [30] or in the response of a colony to a rat's warning calls when a predator is nearby [31]. Rodents are highly gregarious, and it is conceivable that social interactions are mediated through different means of communication, including attending to facial expression, and the vocal expression of conspecifics.

1.2.2.2 The facial expression of pain

When a human experiences pain this is typically characterised by a change in the person's facial expression. The human pain grimace has been a topic of extensive research in medicine and has been proposed as a potential measure of the severity of pain the patient is experiencing [32, 33]. The evaluation of facial expressions has proven to be a useful tool to evaluate pain and consequently the patient's need for analgesic treatment, especially in neonates and speech impaired individuals who have no possibility of verbally communicating their wishes. Human facial expressions have been extensively documented in the Facial Action Coding System (FACS) [34]. FACS is considered an accurate and reliable method for describing changes in the

appearance of the face. Similarly, a Neonatal Facial Coding System describes nine facial movements that have been consistently associated with pain in newborns (brow lowering, eyes squeezing shut, deepening of the nasolabial fold, open lips, vertical and horizontal mouth stretch, cupping of the tongue, chin quiver and lip pursing) [35, 36], which can be used as a pain scale to evaluate pain severity in neonates. Such facial changes result from individual or combined muscle actions, referred to as 'action units'. In 1872, Charles Darwin [37] proposed that all mammals are able to express emotions in the form of facial expressions. He further claimed that the ability to perform facial expressions was innate and largely involuntary in all mammals, and that each species has its own repertoire. Based on the idea that facial expressions in animals indicate the affective component of pain just like in humans, facial features that are specific for pain have recently been identified in other mammalian species. Pain scales that evaluate changes in facial expression (so called 'grimace scales') have been developed for mice, rats, rabbits and horses [38-41]. These grimace scales aim to assess pain severity, which is intended to allow for a more objective decision on analgesic intervention. A potential cage-side pain assessment scale like a grimace scale is allegedly easy, quick to use, offers an effective and specific estimate of spontaneously occurring pain, and offers the opportunity for immediate intervention. Pain left otherwise untreated will not only compromise animal welfare, but the altered physiology of research subjects experiencing pain will negatively affect the study results. The question arises: can we justify and validate an approach developed for humans to other mammals? Further, what are the challenges and limitations of this cross-species approach?

1.2.2.2.1 Interpreting human facial expression as social communicative behaviour

The experience of pain in humans is often accompanied by a specific facial expression, a so called pain grimace. The neural centres for the production of movement in the face are the facial nucleus of the pons, and the facial area of the motor cortex. The facial nucleus regulates spontaneous, involuntary (i.e. emotional) facial movement while voluntary movements are controlled by the facial region of the motor cortex [42]. Recent work has shown that the volume of the primate facial nucleus is largest in humans and great apes and smaller in other primate taxa. Additionally, these species show a greater organization of the primary motor cortex face area, which points to greater (voluntary) fine motor control of facial mimetic musculature [43].

From an early age, humans appear to develop a sensitivity to observable pain in others [44] and eventually develop a highly refined ability to recognise pain and deduce its intensity from facial changes of the sufferer [45]. Changes in mimetic musculature in humans in pain are thought to be a means of non-verbal communication, with the purpose of eliciting sympathy and establishing interpersonal ties. Expressions of pain solicit attention and help of others, in an evolutionary perspective seen as an attempt to increase chances of survival and reproduction (fitness) for the pain sufferer but also the interpreter of the expression [46]. In the pain sufferer (the sender), an initial nociceptive stimulus is translated (encoded) into a facial expression. This message is subsequently interpreted by a receiver. There are consequently three levels involved that are partially under conscious control of the individuals involved: a) the experience of pain, b) the encoding as expressive behaviour (grimacing) in the sender, and c) the decoding, permitting the receiver to interpret and draw inferences about the pain of the sender (Figure 2.) [33]. Intrinsic factors influencing intensity of the initial noxious stimulus include changes in neural transmission (e.g. due to age or disease), disposition of the individual and cognitive factors (e.g. anxiety, stress, strong emotions). Extrinsic factors include exogenous compounds such as analgesics, or experience (e.g. stress induced analgesia) and expectation. A pain stimulus must reach a certain intensity, indicated as a threshold, before it will be evident as a facial expression [33]. An individual may be experiencing pain of substantial intensity in a passive fashion, without facial changes. The threshold at which pain will become visible in the face is variable between individuals, and likewise, the extent of the changes in facial musculature that are needed for the receiver to detect pain are also individually variable [33]. Additionally, an individual may attenuate or exacerbate his or her facial expression, which may complicate the interpretation. Therefore, it is of great importance to understand that the expression as well as the interpretation are subject to error and bias.

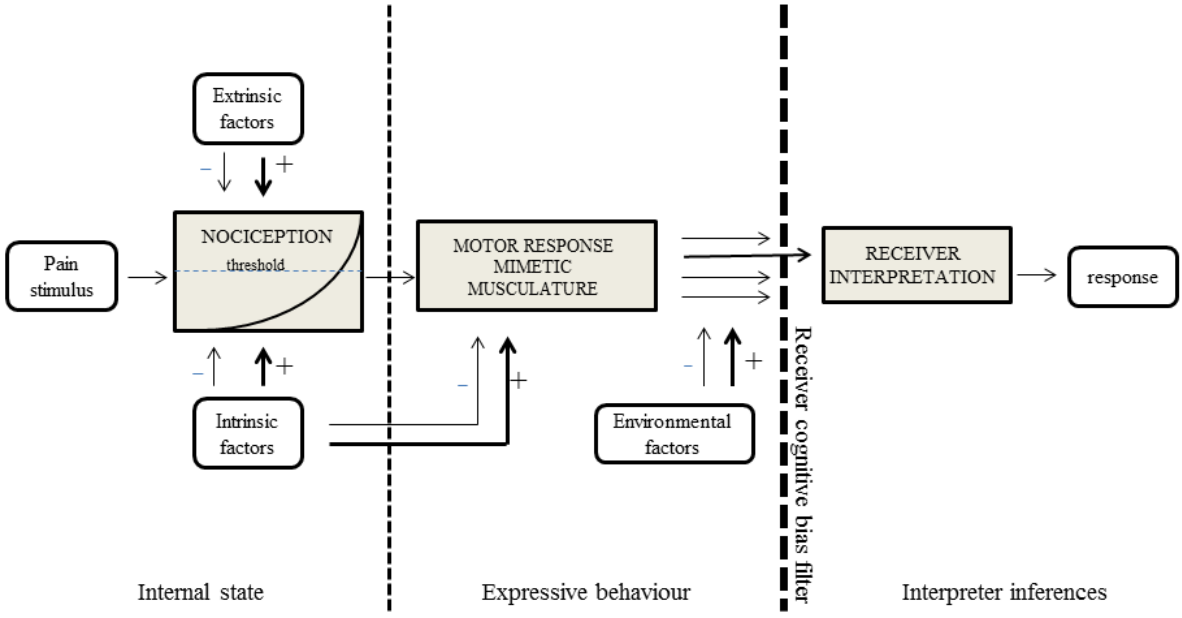

Figure 2. **A model of pain expression through facial musculature**: A nociceptive stimulus can cause facial expression if the threshold is exceeded. Intrinsic factors (e.g. age, disease,..) and extrinsic factors (e.g. analgesics) can exacerbate (thick arrows) or suppress the experience (thin arrows). Facial expression, which is in its turn subject to environmental factors (e.g. threat, cultural influence, social situation), can be interpreted by a receiver. On the observer end, the interpretation of the signal is subject to personal cognitive bias. (adapted from Prkachin, 1995) [33]

1.2.2.2.2 The features of human pain expression

The current literature has described multiple features of the facial musculature ('action units') that are unique to pain and that can be distinguished by the human eye. Interestingly, the pain expression appears to be similar across different experimentally induced painful stimuli (e.g. electric shock, cold, pressure or ischemia) [47]. The action unit that occurs most reliably is orbital tightening, leading to narrowing of the eyelids and a concurrent raising of the cheeks. Subsequently, the eyebrows are lowered and the bridge of the nose wrinkled, as well as the upper lip raised. Ultimately, the eyelids may close entirely. These typical pain features have some similarities with displays of fear, anger and sadness, but overlap is minimal [48].

In terms of interpretation on the receiver side, Prkachin et al. [33] used the FACS pain scale in patients with shoulder injuries and found that the relationship between FACS-coded facial displays, self-reports of pain and observer judgements about the pain of patients with shoulder injuries was correlated only when the pain was severe. It appears that the skills and experience of the clinician on the receiver end can lead to judgement errors concerning the sufferer's internal state [45]. A filter or cognitive bias in the receiver may tune out certain signals, weigh them differently or misconstrue their meaning, which has potentially far reaching consequences for the patient, leading to over or underestimation of the pain severity. In human studies, observers tend to systemically underestimate the pain of patients [49]. This underestimation bias seems to be dependent on previous experience: observers who had lived with someone suffering from chronic pain tended to underestimate the pain less than observers with no experience. In contrast, clinicians that routinely work with pain sufferers and experienced nursed showed an exaggerated underestimation bias compared to observers less experienced with pain patients [33, 49].

1.2.2.2.3 Facial expression of pain in rodents, rabbits and horses

Although human facial expressions of pain are well understood and described, current knowledge on other species is in its infancy. Communicating pain may be of similar purpose in the animal kingdom, benefiting both sender and receiver to respectively solicit and receive help and increase chances of survival by receiving warnings of imminent danger. Based on this rationale, facial expression pain scales have currently been developed for a number of species [38-41]. Considering the need of objective measures of spontaneous pain, particularly in rats and mice as subjects of biomedical research, a cage-side measure of pain severity in species that rely on human caretakers for their welfare could increase both quality of life of experimental animals as well as outcome quality and advancement of medical research that relies on these animal models. Because of our historically poor ability to recognise pain in non-human species and the need for more objective measures of spontaneous pain, animal pain scales have quickly gained popularity in biomedical, veterinary and animal welfare research.

Pioneers in developing animal facial pain scales, Langford et al. (2010) used a model of 0.9% acetic acid intraperitoneal injection causing abdominal pain and writhing behaviour in mice to

assess facial pain expression [38]. The animals were videotaped before and thirty minutes after injection and cropped still images of the face from each video were evaluated for five action units. Three of these facial features are similar to the human Facial Action Coding System: orbital tightening (narrowing of the eyelids), nose bulging, (described as 'skin extension on the bridge of the nose') and cheek bulge (protruding cheek muscle). Additionally, the study showed ear and whisker position to be informative indicators of pain, specifically ears pulled back and sideways, and the whiskers positioned either backward or forward opposed to the head. To obtain a total score for an image, each separate action unit (AU) was assigned a 0, 1 or 2 depending on their appearance, 0 being 'not present', 1 meaning 'moderately visible' and 2 'severe'. A total average out of 2 yields the Mouse Grimace Scale (MGS) score for the image. Observers were highly successful and scored 97 percent of the images from animals in pain significantly higher than images from animals that had not received a painful injection. The scale was subsequently used in fourteen other preclinical pain assays. Most, but not all assays that produced pain of moderate duration (consisting of intra-abdominal, intra-plantar or intra-cystic irritant injection) caused a significant increase in the MGS score. Scores were higher when irritants were administered in deep tissues (joints, viscera), which was attributed to the difference in affective experience between short and prolonged pain, or deep and superficial pain. The MGS did not detect changes in facial expression in mice subject to pain assays of longer (days) or shorter (minutes) duration. The importance of pain severity was explored by the administration of different dosages of the irritant. The MGS increased linearly with dosage, but only in models where there were no other overt pain behaviours (e.g. intra-articular zymosan). In models where other overt behaviour became more obvious with increasing dose (such as writhing due to acetic acid) no observable MGS score increase was found. It is possible that the grimacing was not detected, or the animals did not grimace during the exhibition of other behaviours. The latter would be unusual, considered that no upper threshold of pain intensity has been identified in humans nor in animals above which grimacing would be attenuated. A dose dependent decrease of MGS was observed after administration of morphine sulfate, supporting the face validation of the MGS, suggesting facial changes can be attributed to pain [38].

The mouse grimace scale was consequently applied to a laparotomy model, with the intent to evaluate the intensity and duration of postoperative pain, and the efficacy of analgesic protocols

for the treatment of postoperative pain [50]. Mice that underwent a laparotomy appeared to be in pain until 36 to 48 hours after surgery, with highest MGS scores at 8 to 12 hours post-surgery. Surprisingly, animals that underwent a surgery in the evening had higher MGS pain scores than animals that had surgery in the morning, suggesting a circadian rhythm effect. The study states that mice are potentially experiencing more pain in their active phase (dark time). However, another possibility might be that mice are less likely to grimace during light periods when there is more exposure to potential predators. Another possibility is that during daytime, which is the time during which mice are inactive, facial expression may be attenuated because mice are resting or sleeping. A mean difference in MGS score around 0.5 (i.e. postoperative score was 0.5 higher than baseline on a scale of 2) was found for animals that underwent laparotomy without analgesic, which is the same as was reported in Langford (2010) [38]. One hour after the intervention, there was also a statistically significant increase in MGS score in the control group receiving isoflurane anesthetic only, mainly due to orbital tightening, which was attributed to the prolonged sedative effect of the anesthetic. The MGS subsequently responded to a dose dependent alleviation of postoperative pain with buprenorphine, and with high dosages of carprofen and ketoprofen (2 to 4 fold of commonly used doses). Acetaminophen did not reduce MGS scores at any dose, even though acetaminophen reportedly reduced mean different MGS scores in a zymosan intra-articular assay with fifty percent (Langford 2010) [38]. Potentially acetaminophen is more effective in reducing arthritic pain than visceral pain caused by laparotomy. After vasectomy, the MGS showed a decrease after the use of buprenorphine and meloxicam, and the MGS score was also correlated to other measured pain behaviour: wound licking, grooming and a composite pain score was positively correlated with MGS, rearing was negatively correlated with the MGS score [51].

A modified version of the MGS was later described for research using rats (Rat Grimace Scale, RGS) [39]. The RGS is composed of only four action units as two action features from the MGS, nose and cheeks, occurred simultaneously and were merged into one. The facial expression of rats subject to three pain assays (intraplantar Complete Freund's Adjuvant, intra-articular kaolin and laparotomy) was video recorded and evaluated according to the same principles as the MGS. In this paper, developed in the same research group as the MGS, a score of "1" indicated either moderate appearance of the action unit, or equivocation over its presence or absence. Mean

difference scores were not reported, rather, absolute values for RGS scores were given. A similar average increase to what was reported previously for mice (a 0.5 increase out of 2 after surgery or injection) is seen in these models, followed by a time dependent decrease and return to baseline values over time, as would be expected as the tissues recover from the insult. Despite small variability within groups, there is a noticeable RGS score difference of 0.2 between the different treatment groups scored at baseline, which may reflect normal biological variation within a rather small sample (n = 6-10). Low scores greater than zero at baseline are inevitable when using a scale that is a composite of four or more individual action units. In a non-painful state these action units can be observed occasionally in isolation at a low intensity (score of 1 due to e.g., blinking of the eyes). However, the origin of the variability at the baseline of this study is not addressed and underlines the need for a species and maybe even strain specific (and potentially sex specific?) baseline grimace scale score. The RGS appeared responsive to morphine (at doses equal to or larger than 2 mg per kg) after intraplantar CFA injection, decreasing the score to baseline (before intervention) values.

A laparotomy model with extensive anesthetic period (2 hours of isoflurane) that consisted of an abdominal incision and additional muscle retraction was used in a study assessing postoperative pain and its consequences to spatial memory in aged rats [52]. There was no intra-operative muscle retraction used in the original RGS paper. The version of the grimace scale that was applied had linguistic modifications made that could potentially induce a bias. Using the original 0,1,2 scale, a 0 was assigned to action units indicating ‘no pain’, a 1 for ‘moderate pain’ and a 2 for ‘intense pain’, this in contrast to the original ‘(action unit) absent, moderately present, and obviously present', which only indicates the status of the action unit, not the assessed state of the animal. Even though the scoring was done by an experienced evaluator who was blinded to the study treatment, inferences can be drawn and a bias introduced by the observer when asked to assign pain severity rather than action unit appearance. The reported feature ‘nose and cheek fluttering’, is originally described as ‘nose and cheek flattening’ and it remains unclear if the correct feature was scored. Rats undergoing laparotomy had a postoperative increase of RGS score of around 1.0 on a 2.0 scale, which is higher than reported previously [39]. This greater postoperative increase in this study could not be attributed to the extended isoflurane exposure, as the isoflurane control group had no increase RGS score 2 hours post-surgery. In contrast with

the expectation derived from data from mice from Langford et al. [38], who reported an increased MGS score 1 hour after isoflurane exposure alone. It is potentially the additional intra-operative muscle retraction in this study that caused increased pain and RGS scores. Morphine at 0.8mg/kg successfully decreased RGS scores to baseline values postoperatively, in contrast to Sotocinal et al. [39], who reportedly needed 2 mg/kg or larger doses of morphine to obtain a significant decrease in RGS. Additionally, the group receiving a local anesthetic ropivacaine at the incision site, and ketoprofen at the rather high dose of 40 mg/kg showed no increase in RGS postoperatively [52, 53]. Previously, Matsumiya et al. reported a significant decrease of MGS after laparotomy with a 20 mg/kg dose [50].

The RGS scores showed large increases when applied to a rat model of orthodontic tooth pain [54]. Closed coil springs were placed between the incisors and first molars of rats, mimicking clinical orthodontic forces. Ten minute videos were obtained before and up to 14 days after placement of the springs, that were loaded at three different force levels (20, 40 and 80g). A control group was implanted with non-loaded springs, as well as an analgesic group receiving morphine. The authors chose to report the mean difference in RGS score at all time points, and did not report variability in the data in the form of standard errors or standard deviation. This suggests that there was variability in the scores at the baseline. Post implantation of the springs, RGS scores showed a mean increase of up to 1 (on a scale of 2) in the group with the highest load on the springs on day 1 and day 3 compared to the control group. The control group had increased RGS scores compared to baseline throughout the experiment, likely as a result of discomfort from the intra-oral springs. Morphine (3 mg/kg) decreased RGS scores to baseline values in animals with loaded springs. The authors correlated RGS patterns with previously determined c-Fos levels in the trigeminal nucleus, and P2X3 receptor levels in trigeminal ganglia in previous studies on the same model. Previous behavioural data collected post spring implantation did not correlate with RGS scores: increased post-operative face grooming normalised after day one in previous studies, whereas RGS remained significantly higher until after day 3 [54].

Simultaneous use of overt behaviour scores (i.e. scores of observable behaviour such as rearing or grooming) or composite scales such as used in Leach et al. [51] that have been correlated with

pain behaviour may add validity to the findings obtained with grimace scales. This approach was used in the development of the Horse Grimace Scale (HGS) [41]. The HGS consists of six facial action units scored on still images grabbed from videos made from horses before and after castration. Three features are similar to action units used in other species: orbital tightening, ears backward and strained masseter muscles (cfr. cheek flattening). Other AUs specific for the horse consist of tension above the eye, mouth strained and tense chin, and strained nostrils and flattening of the profile. Two treatment groups (either a single or repeated analgesic administration) and a control group were implemented and compared at different time points at three behavioural levels: facial expression through HGS, pain behaviour according to the Composite Pain Scale (CPS) for horses (previously used to evaluate orthopedic, visceral and somatic pain) [55] and overall behaviour (exploration, alertness, grooming). The authors were able to correlate an increased HGS score to an increase in CPS, and an expected decrease in exploratory behaviour and alertness. No difference was detected between analgesic treatment groups, with any assessment method [41].

A more extensive approach was used by Keating et al. [40] when assessing pain in rabbits undergoing an ear tattooing procedure. A rabbit grimace scale (RbtGS) was developed based on four action units: orbital tightening, cheek flattening, pointed nose and whisker change. The changes in RbtGS were correlated to changes in arterial blood pressure, serum corticosterone level, home cage behaviour (grooming, moving and rearing), heart rate, and overt behaviours during the procedure, such as vocalisation and struggling against gentle manual restraint. All parameters were evaluated with and without the use of the local anesthetic EMLA cream (Eutectic Mixture of Local Anesthetics, lidocaine/prilocaine). Tattooing without EMLA cream resulted in more struggling behaviour and vocalisation, greater facial expression scores of pain, higher peak heart rate, as well as higher systolic and mean arterial blood pressure compared to all other treatments. This multifactorial approach strengthens the correlation between pain and the outcome of grimace scale scores, but further studies are needed to determine whether rabbits display ‘pain faces’ when experiencing pain of longer duration.

In order to be useful measures of pain, pain scoring scales such as grimace scales should be evaluated for reliability (whether it produces similar results under consistent conditions, i.e.

measurement error), sensitivity (being able to identify small changes), and validity (the ability to measure what it is supposed to measure). Recently, Oliver et al. (2014) [56] tested reliability (internal consistency, intra- and inter-rater reliability) of the Rat Grimace Scale and determined an 'analgesic intervention score' (AIS; equal to 0.67) based on expert opinions on rat pain states. The study showed that the RGS scoring has a very good inter- and intra-rater reliability (0.85 and 0.83 respectively).

1.2.2.3 Ultrasonic communication in rats

Rats are highly social animals and vocal communication is another important feature of their interactions. Not only do rats vocalise at frequencies within the human hearing range, they also have a repertoire of calls that extends beyond our auditory range ('ultrasonic') at frequencies above 20 kilohertz (kHz).

With the development of condenser microphones and software that allows spectrographic analysis of the sound, ultrasonic vocalisation (USV) recordings have gained much wider application than what was previously possible with bat-detectors (devices that convert ultrasonic echolocation sounds into audible signals). The recording of USVs [38] has been commonly applied in rodent ecology and behaviour research, fear studies, pharmacological studies (USVs are a highly translational animal model of emotion in drug abuse studies) and, with variable success, in pain studies [57-59].

Ultrasonic vocalisations can be recorded from rats in a variety of semi-natural and laboratory settings. Calls typically occur at three distinct frequencies: between the age of 1 to 20 days, rat pups will vocalise at 40 kHz when they are removed from the nest, or as a response to other unfamiliar environments [60-63]. These pup calls have two remarkable effects on adult rats: they 1) elicited maternal retrieving behaviour and 2) inhibited aggression from other rats. Adult rats will vocalise at frequencies around 22 kHz, or 50 kHz [64]. Twenty two kHz calls are predominantly emitted when a rat perceives a threat, or anticipates an unavoidable aversive event [65]. These 'low frequency calls' are emitted by adult rats and range between 18 and 32 kHz with a duration of 300 to 3000 milliseconds. They have simple spectrographic shapes (unmodulated, meaning that a constant frequency was maintained throughout each call) and come in bouts of three to five calls [66]. The overt behaviour that rats exhibit during these calls

is visually discernable as the rats maintain an immobile and tense posture, where short inspirations are followed by long expirations [67]. Twenty two kHz calls have been recorded during a wide variety of social situations. Male rats emit these calls during sexual behavior and intraspecific agonistic encounters [68, 69]. The calls emitted by the defeated rat reduce the aggressive approaches from the opponent [70]. Twenty-two kilohertz vocalisations have been mostly reported in behavioural studies using 'visible burrow' systems, and more specifically to research predator-prey interactions [71]. Rats living in colonies in burrows emitted 22 kHz vocalisations upon presentation of a cat, and continued doing so up to 30 minutes after removal of the predator.

Twenty two kHz USVs used as warning calls benefit all members of the colony, informing them about potential approaching danger. Playback of 22 kHz calls resulted in increased alertness, defensive behaviour such as freezing, and decreased emerging in rats that hear the calls. Additionally, individually housed rats did not vocalise when presented with a predator, which underlines the social purpose of this behaviour [72] [73]

In contrast, 50 kHz calls have been associated with positive social interactions. They are shorter than alarm calls (3-300 milliseconds), occur at a frequency range between 30 and 100 kHz and are therefore also called high frequency calls or 'chirps' [74]. Rats chirp during rough-and-tumble play, and during playful 'tickling' by an experimenter [75] [76], during mating and courting behaviour [68], and during aggression and submission [77, 78] [79]. Knutson et al. [80] proposes the idea that these calls reflect a generalised appetitive motivation for rewarding situations. Panksepp (2000) [76] takes it one step further and boldly states that the chirping at 50 kHz represents laughter, a behaviour that to date only has been registered in primates yet [81] as cited in [76]. Playback of 50 kHz calls encourages exploratory behavior in receiver rats [82].

In general, USVs are thought to be elicited by an emotional experience and are therefore representative of the animal's positive (50 kHz) or negative (22 kHz) affective state [83, 84]. All mammalian brains are supposed to have circuits that facilitate emotional experiences as these are evolutionary conserved [83, 85]; an argument that supports the statement that USVs are an expression of emotion [84].

1.2.2.3.1 The use of ultrasonic vocalisations in pain studies

Among multiple studies reporting on the use of USVs as a measure of pain, some found USVs to be a good representative of pain [57, 86] while others did not find a correlation between pain state and the emission of USVs [59, 87]. Whether USVs can be used as a measure of pain duration and severity seems to depend on a combination of the following factors: 1. the severity and 2. duration of the pain model ('nociceptive insult'), 3. the social situation and testing environment (alone or during interaction with another rat), 4. the rat strain, 5. the sound recording methods, 6. whether a stimulus is given to elicit calls, and the type of this stimulus.

An early study investigated audible and ultrasonic vocalisations using electrical shock to evoke calls from rats. The animals would emit one or two audible calls, followed by ultrasonic calls in half of the cases [88]. The two audible calls were thought to represent the human 'double pain phenomenon' (fast Aδ myelinated fiber transmission, followed by slower non-myelinated c-fiber transmission). The USVs were emitted at 22 kHz: flat, unmodulated spectrographic shapes (i.e. maintaining the same frequency throughout each call) and maintained in a typical repetitive pattern ('trains') for several minutes. After lidocaine treatment, the second audible calls were diminished, but lidocaine did not affect USV emission [88]. When morphine was used, USV intensity decreased significantly [89].

Ultrasonic vocalisations indicated a pain state in rats with lipopolysaccharide induced intracranial hypersensitivity (migraine). The animals emitted significantly more 22 kHz USVs after being stimulated with air puffs [90]. Air puffs to the body or face of the rat are known to consistently elicit 22 kHz USVs [91, 92].

Other studies differed from these as they did not use a stimulus to elicit a response but instead registered spontaneously emitted USVs from a subject presumed to be in pain during a social interaction with another rat. Rats with an arthritic stifle joint (intra-articular Freund's adjuvant) presented with a larger, healthy conspecific vocalised significantly more often than rats that were not in pain [93]. They emitted long pulse type 22 kHz USVs of 1000 to 3000 milliseconds during this interaction. The vocalisation was negatively correlated with concurrently measured exploration, grooming, and locomotor behaviour: besides emitting more USVs, arthritic rats explored less, and were more immobile than control rats. Both morphine (central analgesic) and aspirin (peripheral analgesic) reduced the number of vocalisations. Morphine was administered at a non-sedative dose that caused no alterations in motor behaviour. Even though morphine is

known to modulate affect, the USVs were also suppressed by aspirin, indicating that it is the analgesic properties of the drug causing the decrease in USVs, and not the central modification by morphine [93]. It cannot be ruled out that the affective modulation of morphine can contribute in vocalisation behaviour. The authors believed that arthritic rats used the USVs as a communication method to minimize or avoid painful contact with the other rat. Because the rats did not vocalise from pain alone, the USVs from these animals were interpreted as an integrated defensive response to exteroceptive stimuli (pain or fear caused by the other rat), but not to interoceptive noxious stimulation (the joint pain). This suggests that a noxious stimulus or a potentially noxious stimulus is needed to elicit USVs from an animal in pain [93], and that another animal may have to be present. The analgesic effect of three non-steroidal anti-inflammatory drugs was able to attenuate the increase in USVs from rats in pain presented with a conspecific (stimulus rat) [94].

The emission of ultrasonic vocalisations has also been used in chronic pain models. A social situation similar to the paired study on arthritic rats [93] was used in a study that assessed the ultrasonic response in animals with neuropathic diabetes, carrageenan induced inflammation, and Freund's adjuvant induced arthritis. Treated rats as well as control rats vocalised without significant difference upon presentation with a conspecific. When the rats were alone, none of them vocalised. In this study, changes in other behavioural parameters like immobility, exploration, interaction and grooming did reflect a pain state, but no direct link could be established between these and USVs [87].

The general weakness of the previous studies is that they lack standardised experimental conditions making it difficult to compare studies. To minimize variability from different sources, some studies chose to restrain the animals during the application of the stimulus and recording of USVs. Even though the authors claim that the restraint did not cause stress induced analgesia or spontaneously emitted USVs [57], this method of restraining an animal while being tested introduces a confounding stress factor which may have an effect on vocalising behaviour [95]. Under conditions of stress an animal's normal reaction to pain such as a withdrawal reflex of vocalisation behaviour could be disadvantageous. During stress, these reactions to pain are suppressed so that attention could be directed to more adaptive behaviors. For example, when a laboratory animal is exposed to a new or aversive stimulus, the animal's sensitivity to other

painful stimuli is reduced. The duration of this stress-induced analgesia may range from minutes to hours, depending on the nature and severity of the stimulus [4]. Additionally, information obtained from ultrasonic vocalisations is probably most valuable when recorded in a spontaneous fashion (not evoked by a stimulus), and from animals that are free moving and exhibiting natural behavior, without restraint and with minimal interaction with the experimenter.

In contrast, several studies did not find that USVs were a reliable measure of pain. Wallace et al [59] looked for the relationship between USVs and overt behaviour during the application of mechanical and thermal stimuli. Models of somatic pain (intraplantar formalin inflammation), visceral pain (turpentine induced cystitis), arthritic and neuropathic pain (partial sciatic nerve ligation) were used. Unfortunately, very little information was given on the sound recording hardware and methods in this study. No USVs were recorded in any of the experiments from any animal, except one. This rat vocalised at an unusually high rate. The outcome of this study underlines the need of more detailed reporting of methods. At this point one cannot judge if there were truly no USVs or if this outcome could be due to experimenter error. Bearing in mind the social communication of rats, it is unusual for animals to be absolutely silent during the entire recording period. If there were truly no USVs occurring, a reason could be the rather low dose of formalin injected (50 microliter of a 5% solution), compared to another study that did record USVs reliably after formalin injection in the paw of rats. In the latter experiment, increasing concentrations of formalin were stepwise injected. USVs were noted mostly during the interphase of the formalin test, a quiescent period typically occurring 10 to 20 minutes after injection, after 50 microliter of 12.5% formalin was injected. Morphine suppressed these USVs in a naloxone reversible manner [86].

The temporal progression of spontaneous pain was assessed using USVs in mice suspected to experience cancer pain [96]. The authors showed that mice expected to be in pain following the induction of osteosarcoma only differed from healthy ones in vocalising behaviour if they were sufficiently accustomed with the testing chamber (hence experiencing minimal novelty stress). As tumor invasion progressed, the number of emitted USVs progressively increased. The USVs were reduced by fentanyl administration, an analgesic shown to reduce mechanical allodynia in mouse cancer pain [96]. A similar observation was made in mice with a spared nerve injury that showed an increased rate of vocalisation: here, gabapentin decreased the ultrasonic vocalisation

rate at one and two weeks after the injury. Gabapentin inhibited mechanical allodynia determined by a nociceptive test [96].

It appears that no consensus has been reached on the use and sensitivity of ultrasonic vocalisations as a measure of pain state or pain severity. A lack of standardised models and generally poor reporting of the methodologies make it difficult to compare published work or draw conclusions on the matter.

1.2.2.3.2 Ultrasonic vocalisations during gas exposure

The majority of laboratory rodents, including rats, will be euthanised upon completion of the experiment, because a humane endpoint is reached, or because they are surplus to the needs of the facility. For its simplicity, low cost and good safety properties, gradual fill of a closed chamber with carbon dioxide is a conditionally approved and widely used method of euthanasia. It is however known that even at low concentrations, carbon dioxide causes aversion in rats. Approach-avoidance studies show that even at relatively low concentrations rats will abandon a safe, dark chamber for a bright lit area [97], or leave a testing room that has food rewards [98-100]. Based on human reports, this behaviour may be due to a feeling of breathlessness in carbon dioxide. Carbon dioxide also causes a stinging sensation in the nasal and conjunctival mucosae when it forms carbonic acid in contact with fluid [101, 102]. At higher concentrations (from 40 percent) carbon dioxide causes loss of consciousness, followed by cessation of breathing and, ultimately, cardiac arrest. The administration of anesthetic gases such as isoflurane to induce anesthesia before euthanasia has been put forward as an alternative, more humane method [103]. Isoflurane appears less aversive in approach-avoidance studies [104, 105].
No studies have been published yet that use USVs to assess aversive or pain states during gas exposure.

1.2.2.4 Other proposed spontaneous measures of pain

Non-invasive behavioural methods of measuring pain have the benefit of having a wide field of application (biomedical, veterinary and welfare research) and reduced ethical concerns compared

to stimulus induced behaviours. Besides the previously discussed USV and grimace scales, other measures have been successfully used as specific measures of pain. The monitoring of naturally occurring behaviour such as locomotor activity (spontaneous as well as running wheel activity and gait analysis) [106], sleep [107] and burrowing behaviour [108, 109], but also simple behaviours such as spontaneous foot lifts [110] and home cage activity monitoring [67, 111] are on the rise. Many techniques that have been used often involve retrospective analysis and therefore do not allow changes to the analgesic regimen (e.g. administration of rescue analgesia). In this perspective, measures that allow real time assessment of pain behaviour of the animal in the home cage have been favoured for further development.

1.3 Conclusion

The literature on the facial expression of non-human mammals as a measure of pain is sparse and besides the original papers, few or no reports on the use of such grimace scales have been published. Prior to a general acceptance of the method, further exploration of facial pain scales in mammals is in place. In regards to the literature on ultrasonic communication in rats experiencing pain, it is unclear whether the behaviour can be used as a measure of pain state. From current knowledge, it appears that standardised social settings, and potentially a stimulus are needed in order to apply USVs reliably as an estimate of pain or pain severity.

This study was designed to further explore the possibilities and limitations of these novel pain measures. In particular, the following questions were asked:

1. Can the Rat Grimace Scale successfully detect changes in the facial expression of rats in different models of pain?

2. How does the generated RGS score relate to the results of a stimulus-driven nociceptive test in the same animals?

3. Can ultrasonic vocalisations provide a useful measure of aversion during carbon dioxide and isoflurane euthanasia practices?

In Chapter 2, question 1 and 2 will be addressed. The Rat Grimace Scale and a concurrent nociceptive test (von Frey filaments) will applied to three pain models: intraplantar injection of carrageenan, intraplantar injection of Complete Freund's Adjuvant (CFA), and a plantar incision as a model of post-operative pain. These pain paradigms were chosen based on the following criteria: the use of the model must be previously standardised, the model must be commonly used in pain research, and must include the development hypersensitivity that can be quantified with von Frey filaments. It must be simple to create, with minimal variability.

Subcutaneous injections of carrageenan, a linear sulphated polysaccharide extracted from red algae, and Complete Freund's Adjuvant (CFA), a solution of inactivated Mycobacterium tuberculosis emulsified in mineral oil, are characterised by an acute, transient peripheral inflammation through activation of pro-inflammatory cells [112, 113]. A similar inflammatory reaction occurs as a response to tissue trauma in rats with a plantar incision. Pro-inflammatory cells release local inflammatory mediators (substance P, glutamate, prostaglandins, histamine, and serotonin) which in their turn cause the five typical characteristics of inflammation: redness, swelling, heat, loss of function, and also pain. Inflammatory mediators sensitize primary afferents, resulting in the development of primary hyperalgesia [114]. Additionally, activation of early genes like c-Fos (which leads to changes in the synthesis of neuropeptides in the dorsal horn of the spinal cord) [115] as well as central activation of the descending system from the locus coeruleus [116], indicate that hyperalgesia has peripheral but also central regulating mechanisms.

In Chapter 3, two euthanasia techniques, exposure to increasing concentrations of carbon dioxide, and the allegedly less aversive isoflurane gas exposure were assessed using ultrasonic vocalisations as a measure of affective state, aversion or pain (question 3).

Statement of author and co-author contribution

Chapter 2: Daniel Pang and Debbie De Rantere conceived, designed and coordinated the experiment, analysed the data and drafted the manuscript. Debbie De Rantere, Chelsea Schuster and Julie Reimer performed the experiments and collected the data, including animal handling, video taping, image selection and scoring. All authors read and approved of the final manuscript.

Chapter 3: Daniel Pang conceived the project. Jessie Chisholm and Debbie De Rantere coordinated and performed the experiments, and collected the data. Debbie De Rantere analysed the data. Daniel Pang, Debbie De Rantere and Jessie Chisholm drafted the manuscript. Aleksandra Krajacic proofread the manuscript and coordinated the experiments. Nicole Fernandez performed the cytology. All authors read and approved of the final manuscript.

CHAPTER TWO : The application of the Rat Grimace Scale as a measure of pain in three experimental models and its relationship to a conventional nociceptive test

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1.4 Introduction

The evaluation of facial expression in non-human mammals as a measure of spontaneous behaviour has found recent application in pain research [38-41]. Particularly in prey species such as rats and mice, in which specific pain behaviours are difficult to identify [117], non-invasive measures of pain have the potential for widespread application in biomedical and veterinary research.

Human faces are known to reflect a wide array of states and emotions including pain, and human facial expressions have been extensively documented [34]. Pain assessment based on facial expression is especially useful to assess the need for analgesic treatment in patients with limited ability to verbally communicate their needs, such as neonates or patients with an altered state of consciousness [118, 119]. Based on evidence from human medicine, extrapolating the concept that facial expressions are a reflection of the affective ('emotional') component of the pain experience, facial features that are specific for pain have recently been described and composed into pain scales, so-called 'grimace scales', for several non-human species. [38-41]. The idea that non-human animals can communicate their emotions through facial expressions is not new. As early as 1872, Charles Darwin proposed that all mammals are able to translate emotional experiences into facial expressions [37]. He further proposed that the ability to perform facial expressions was innate and largely involuntary in all mammals, and that each species has its own repertoire.

In pain research employing rodents, traditional stimulus-based nociceptive tests are still the most prevalent measures of pain status [17]. Due to their perceived efficacy in evaluating properties of novel analgesic drugs, or the clinical features of painful conditions, these tests are widely used and accepted in pain research. Nociceptive assays typically consist of the application of an acute stimulus (thermal, mechanical, electrical or chemical), to which the animal's responsive

behaviour, usually in the form of a withdrawal, is subsequently measured. These withdrawal responses, however, are reflexes regulated via the spinal cord [2, 17]. Therefore, tests measuring a withdrawal reflex, such as von Frey filament application, assess nociceptive pathways only and should not be interpreted as measures of pain. This limitation is framed by the definition of nociception, which, according to the International Association for the Study of Pain (IASP), includes that the encoding of noxious stimuli may be autonomic or behavioural and that pain sensation is not necessarily implied. Further, there is uncertainty whether the observed withdrawal reflexes indicate nociception, or avoidance behaviour as a result of aversion to the stimulus (the tendency to avoid or withdraw from a situation that is associated with a noxious stimulus) [15]. Avoidance behaviour could occur with repeated testing and could be mistaken for a nociceptive response (e.g. responding to touch that precedes noxious increase in pressure) [15]. By recognising the importance of the affective component of pain, efforts to model human pain limited to spinal reflex testing will generate limited information.

In response to these limitations, facial expression scales have been developed to improve pain evaluation by moving towards non-invasive, spontaneous behavioural measures of pain that are not stimulus dependent or limited to a spinal reflex [1, 2]

The Rat Grimace Scale (RGS) used in this study consists of four facial "action units" (orbital tightening, nose and cheek appearance, ear position, and whisker position) that are scored on a 0 to 2 scale for their presence in digital video frames [39]. As an aid to the interpretation of the RGS score, an 'analgesic intervention score' (AIS) has recently been derived [56]. This score is the threshold above which presence of pain is probable.

In our study, we aimed to determine the relationship between a spontaneous measure of pain (RGS) and a stimulus-based test of mechanical hypersensitivity (von Frey). The RGS and concurrent von Frey test were applied in three common pain models: intraplantar Complete Freund's Adjuvant (CFA), intraplantar carrageenan and a plantar surgical incision. The concurrent application of a nociceptive test allows the first comparison to be made between traditional (stimulus-evoked) and novel methods (grimace scale) of pain evaluation.

We expected an increase in RGS score and hypersensitivity to develop in all models, with a null hypothesis that there was no relationship between RGS score and paw withdrawal thresholds.

1.5 Methods

All experiments were approved by the University of Calgary Health Sciences Animal Care Committee, Calgary, Canada and conducted in compliance with protocols AC13-0161, AC13-0115, AC13-0124 and AC11-0024. The Animal Care Committee operates under the auspices of the Canadian Council on Animal Care.

1.5.1 *Animals*

A total of 52, 8 week old, male Wistar rats were obtained from Charles River, Canada. The animals were housed in pairs in standard cages (47 x 25 x 21 cm) with bedding consisting of wood chips and shredded paper, and a plastic tube for enrichment. A light cycle of 12 hours lights on/12 hours lights off was maintained in a temperature and humidity controlled room (23˚C; 22% humidity), with lights on at 7:30 am. Laboratory rat pellets (Prolab 2500 Rodent 5P14, LabDiet, PMI Nutrition International, St Louis, MO) and tap water were available ad libitum.

1.5.2 *Animal handling*

All animals were allowed to acclimatise for five days at the university rodent housing facility after arrival from the breeding facility. Thereafter, the rats were handled daily by a female experimenter (DDR) in the behaviour suite where all further testing would take place. During these handling sessions, each rat was gently held and frequently placed in the video box and on the mechanical hypersensitivity testing platform, and given a food reward (generic brand honey oat cereal loops). Animals were considered to be habituated and ready for experiments when they voluntarily moved from the experimenter's hand into the video box or onto the testing platform, and readily ate the reward. On average, two to five days were needed to obtain this behaviour, depending on time spent with the animals per day and individual variation.

Animals were randomly allocated to one of five treatment groups: intraplantar carrageenan (n = 12); intraplantar Complete Freund's Adjuvant (n = 10); plantar incision (n = 10); an anesthetic control group (receiving isoflurane anesthesia only, n = 12), or a saline control group (anesthetic + intraplantar saline injection, n = 8). Sample sizes were derived from observed variability in a

previous publication using the RGS [39]. Each animal underwent one treatment. One animal was excluded from the study because its baseline paw withdrawal threshold was below the normal range (< 15 g). All injections and surgeries were performed in the morning and testing carried out during the lights on period of the light cycle.

1.5.3 *Inflammatory pain assays: Carrageenan and Complete Freund's Adjuvant (CFA) induced paw inflammation*

General anesthesia was induced with each animal placed individually in an induction chamber (5% isoflurane in 1L/min oxygen). Following loss of the righting reflex, the animal was removed from the box and anesthesia was maintained via a nose mask (2% isoflurane in 1L/min oxygen). With the rat in sternal recumbency, the left hind paw was extended caudally and placed on a non-sterile surgical drape. The plantar aspect of the paw was wiped with 70% ethanol. Each rat received a single 100 microliter injection of 1 percent λ-carrageenan (w/v, Sigma-Aldrich, St.Louis, MO, dissolved in saline), injected subcutaneously in the plantar surface of the left hind paw [113].

Animals treated with CFA (n = 10) received an injection of 150 microliter of a 50 percent CFA (Sigma-Aldrich, St.Louis, MO, diluted in saline) deposited subcutaneously in the plantar surface of the left hind paw (After Sotocinal, 2010) [39]. Care was taken to insert the needle (25G) at the same location proximal to the footpads, and advanced 6 millimeters under the skin before depositing the compound. To prevent leakage from the injection site and to promote even spread of the injectate, digital pressure was applied and the paw gently massaged. The animal was then recovered from anesthesia and returned to its home cage. Animals were weighed and checked daily for signs of weight loss, infection at the injection site or systemic illness. A localised redness and swelling were seen, but no other symptoms such as skin ulceration or sloughing were documented.

The extent of edema formation resulting from carrageenan-induced inflammation was quantified using digital calipers (after Hargreaves et al. [1988])[120]. The dorsoplantar paw diameter was measured after each video recording. Paw hypersensitivity was assessed using von Frey filaments and a RGS video taken before and at distinct time points after the injections. For carrageenan treated animals: 1h (video only), 3h, 6h, 9h, 24h (8 animals). Four additional

animals were added to this cohort upon observation that paw withdrawal thresholds had not returned to baseline at the 24-hour time point. These animals were tested at the original time points: 1h (video only), 3h, 6h, 9h, 24h, and at additional time points (48h and 168h [7 days]) after injection. Test times for CFA treated animals were 1h (video only), 4h, 6h, 24h, 48h and 168h (7days) after injection. The choice of assessment times was based on previous studies using mechanical hypersensitivity testing or RGS [121] [39].

1.5.4 *Incisional Pain assay*

General anesthesia was induced and maintained as described above. With the rat in sternal recumbency, the left hind paw was extended caudally and placed through a non-sterile surgical drape. The plantar aspect of the paw was prepared with a chlorhexidine solution and wiped with 70% ethanol. With a size 11 surgical blade, a one centimeter longitudinal incision was made through skin, fascia and muscle, starting five millimeters from the proximal edge of the heel and extending toward the phalanges (after Brennan [2010]) [122]. Light pressure was applied with a cotton tip applicator to stop any hemorrhage, and the skin apposed with two simple interrupted sutures of 5-0 polydioxanone (PDS II, Ethicon, Inc. Johnson&Johnson NJ, USA). The wound site was covered with a polymyxin B, neomycin, and bacitracin combination antimicrobial ointment (BNP, Vetoquinol, QC) after which anesthesia was discontinued. The animal was returned to its home cage to recover. The total procedure took less than ten minutes and all animals recovered uneventfully from the anesthetic within two minutes. The animals were weighed and incisions checked daily post-operatively. RGS scores and paw hypersensitivity was assessed before, and at distinct time points after the surgery: 1h, 3h (video only), 6h, 9h (video only), 24h, 48h, 72h, 96h and 120h post-operatively.

1.5.5 Anesthetic control group and saline control group

For the anesthetic control group, general anesthesia was induced and maintained as described above. Each rat was placed in sternal recumbency and the left hind paw was extended caudally. Anesthesia was maintained for 2 minutes, the anesthetic time required for injection of carrageenan or CFA solutions. The animals were then allowed to recover and returned to their

home cage. For the saline control group, after induction of anesthesia, the plantar aspect of the paw was wiped with 70% ethanol and a single 150 microliter injection of sterile NaCl 0.9% administered subcutaneously in the paw.

1.5.6 *Mechanical hypersensitivity testing*

Paw withdrawal thresholds were determined using a set of hand-held calibrated nylon 'von Frey' filaments (TouchTest sensory evaluator, North Coast, Gilroy, CA). Each animal was individually placed under a small clear plexiglass box on a customised platform (after Pitcher, 1999) [12], consisting of a 3 millimeter thick plexiglass surface with 1.5 millimeter diameter holes and 5 mm between holes throughout the entire area of the platform. The up-and-down method described by Chaplan [16] was modified to minimize filament trials and avoid a learning process associated with repeated testing. Starting with a thin filament predicted to be below the expected threshold, filaments were presented in an ascending fashion: each filament was applied once and held buckled against the paw for one second. A positive response was recorded when the animal withdrew its foot during application or during removal of the filament. After identifying the two filaments that straddled the threshold, four more filaments were tested, oscillating around the threshold. Each filament was presented once, at intervals of approximately ten to twenty seconds. In the incisional model, filaments were applied not on the incision, but adjacent to the incision site. In the injection models, filaments were applied to the mid-plantar area of the paw. If the rat exhibited behaviour that interfered with the withdrawal response (walking, rearing or grooming) during the application of the filament, the result was discarded and the same filament retested. An upper cut off value between 15 and 26 grams was applied, as thicker filaments lifted the animal's foot.

1.5.7 *Digital video recording and image capturing*

Each rat was videotaped for ten minutes at each time point. Two high definition cameras (Panasonic HC-V720 HD video camera) were used with the animal placed in a purpose built clear plexiglass video chamber (W 14 cm x L 26.5 cm x H 20.5 cm). The experimenter left the room after the recording was initiated and did not re-enter before the recording was completed.

Video footage was later reviewed using Windows Media Player (Microsoft Corporation, Redmont, WA). No images were collected during the first minute of the video recording. Still frames were determined to be useful if the animal was stationary, facing the camera, and not exhibiting other specific behaviours (grooming, rearing or sleeping). Three still frames in which all facial features were visible and not blurred by a motion artefact were selected from each ten minute video. Images were then pasted into a numbered Powerpoint (Microsoft Corporation, Redmont, WA) presentation with a black background. The images were enlarged and cropped to eliminate other body parts and surrounding details, so that only the rat's face with eyes, ears, nose and whiskers were fully visible, one image per slide.

1.5.8 *Rat Grimace Scale scoring*

All images were randomly presented to a blinded scorer and scored according to the Rat Grimace Scale. Four 'action units': orbital tightening, nose and cheek appearance, ear position, and whisker position) were appointed a 0, 1 or 2 depending on whether these a) were absent (scoring a 0), b) were moderately appearing (scoring 1), or c) were obviously present (scoring 2)[39]. An average score (out of a total of 2) was calculated for each of the still frames, and for each video.

1.5.9 *Statistical analyses*

Data analyses were performed using IBM SPSS Statistics 21 (IBM, Armonk, NY) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). For within group comparisons repeated measures ANOVA was used followed by a post-hoc test with p-value correction (Bonferroni). Assumptions for repeated measures ANOVA were met with the data approximating a normal distribution pattern and the independent variable consisted of more than two matched groups (time points). Corrections offered in the SPSS software (Greenhouse-Geisser correction) were used if the assumption of sphericity (equality of variances) was not met within a dataset.

A two-way parametric ANOVA was applied for between group comparisons (treatment versus controls). A planned post-hoc test (Bonferroni) was applied to time points where the score was significantly different from baseline. Mean differences or correlations were said to be

statistically significant if the computed two-tailed p-value was equal to or smaller than 0.05. Data are presented as mean ± SEM.

1.6 Results

These data show that in all models evaluated, the highest levels of paw hypersensitivity coincided with the highest RGS scores, illustrating that the RGS is a useful tool for measuring pain in all three models of inflammation (CFA, carrageenan, incision).

1.6.1 *Complete Freund's Adjuvant induced inflammatory pain*

In CFA treated animals, significant increases of the RGS score (0.83 ± 0.11) compared to baseline (0.38 ± 0.06) were observed at 6 hours after injection of CFA ($F = 2.44$, df 6, $p < 0.05$, Figure 3.). At this time, the mean RGS score of CFA treated animals was also significantly higher than saline and anesthetic control groups ($p < 0.05$ and $p < 0.01$, respectively). At 24 hours, the RGS score returned to baseline ($p > 0.05$). The RGS score did not increase in the saline and anesthetic control groups ($F = 0.94$, df 6, $p = 0.47$ and $F = 0.98$, df 6, $p = 0.44$, respectively).

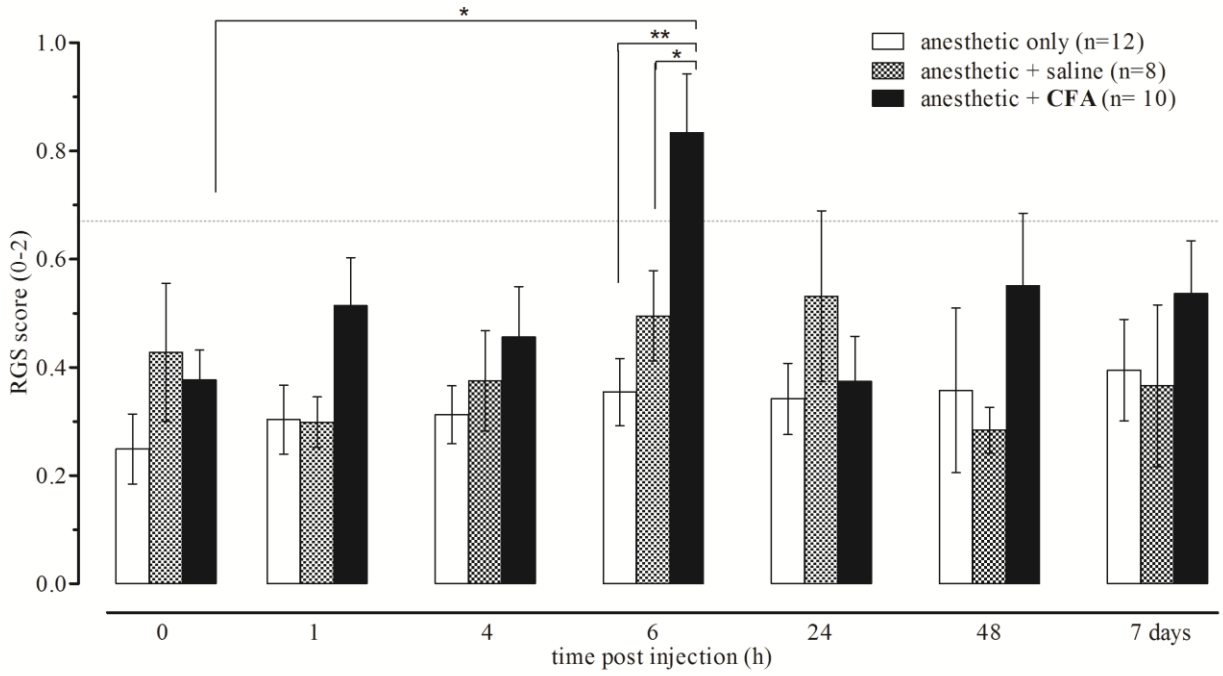

Figure 3 Rat Grimace Scale score of Complete Freund's Adjuvant injected animals and control animals. At 6 hours, significant increases of the RGS score (0.83 ± 0.11) compared to baseline (0.38 ± 0.06) were noted (* $p < 0.05$) (n = 10). At this time point, the mean RGS score was also significantly higher than saline injected (n = 8) and anesthetic only (n = 12) control groups (* $p < 0.05$ and ** $p < 0.01$, respectively). The RGS did not increase in the saline and anesthetic control groups ($p = 0.47$ and $p = 0.44$, respectively). The analgesic intervention score of 0.67 (indicated by a horizontal dotted line) is exceeded at 6h post-injection. Data are mean ± SEM.

The 6-hour peak in the RGS score coincided with the lowest measured paw withdrawal thresholds (5.9 ± 0.78g, Figure 4). Paw withdrawal thresholds continued to be significantly decreased at all time points after injection until 48 hours after injection ($F = 19.56$, df 5, $p < 0.001$). After 7 days, paw hypersensitivity returned to baseline values ($p > 0.05$). Withdrawal thresholds in the saline injection and anesthetic control groups did not decrease during the testing period and remained within the normal range ($p = 0.39$ and $p = 0.57$, respectively).

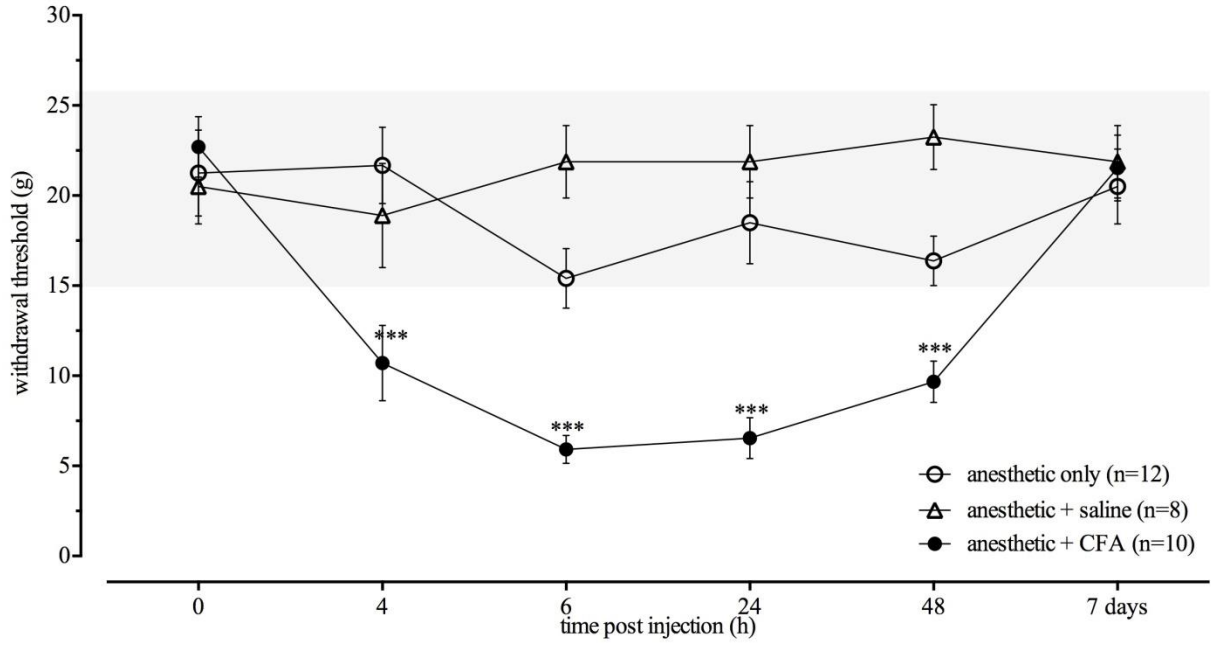

Figure 4. Ipsilateral paw withdrawal thresholds for Complete Freund's Adjuvant treated and control animals. Paw withdrawal thresholds are significantly decreased at all time points after injection until 48 hours after injection (*** $p < 0.001$)(n = 10). Withdrawal thresholds in both control groups did not decrease during the testing period ($p = 0.39$ and $p = 0.57$ respectively). Peak hypersensitivity was reached at 6 hours after injection (5.95 ± 0.42). A grey band indicates the withdrawal threshold range of healthy animals. Data are mean ± SEM.

1.6.2 Carrageenan induced inflammatory pain

The RGS score of carrageenan treated animals (n = 12) was significantly increased at 6 hours (0.79 ± 0.11) and 9 hours (0.78 ± 0.11) after injection compared to baseline (0.32 ± 0.09; F = 2.22, df 7, p < 0.001 at both time points, Figure 5). At 6 hours, the RGS score of carrageenan treated animals was significantly higher than the RGS scores of animals in saline and anesthetic control groups (F = 4.47, df 7, p = 0.025 and p = 0.039, respectively). At 9 hours after injection, the RGS score in the carrageenan treated group was significantly higher than the RGS score from rats in the anesthetic control group (0.31 ± 0.09, p = 0.008). At 24 hours after carrageenan injection, the RGS score returned to baseline (p = 0.9). The RGS score did not increase in saline and anesthetic control groups (F = 0.94, df 6, p = 0.47 and F = 0.98, df 6, p = 0.44, respectively).

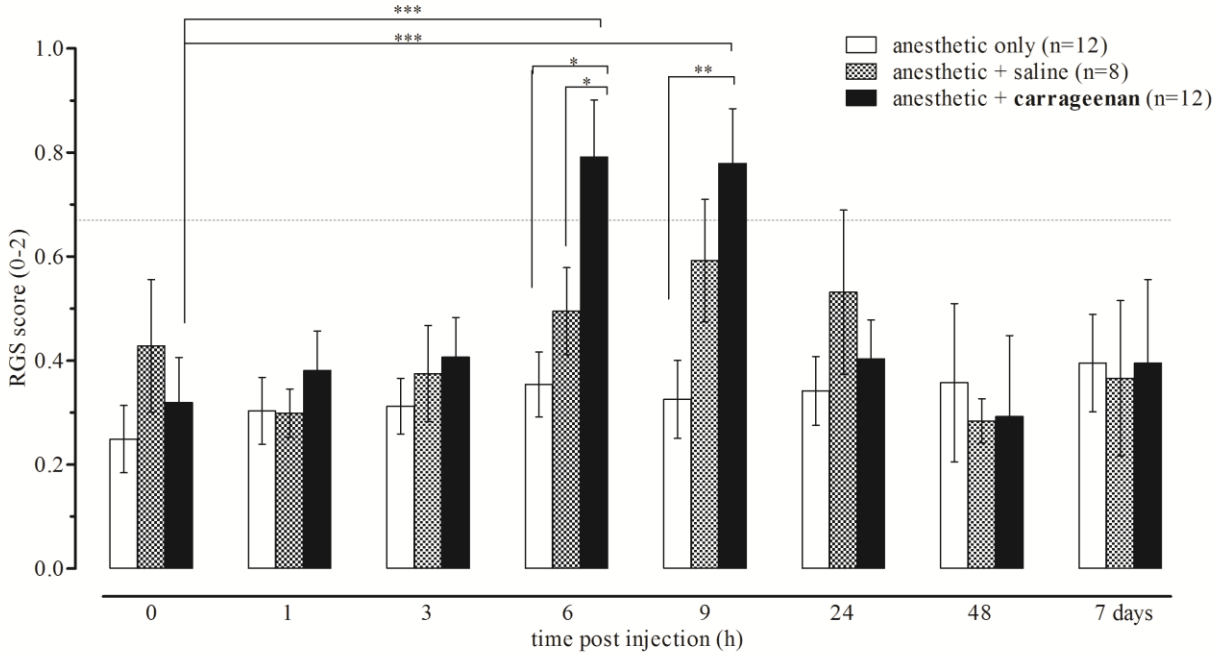

Figure 5. Rat Grimace Scale score of carrageenan treated animals and controls. Rats injected with carrageenan show increased RGS scores at 6 and 9 hours after injection (0.79 ± 0.11 and 0.78 ± 0.11 respectively), compared to baseline values (0.32 ± 0.09) (*** p < 0.001)(n = 12). The RGS score is significantly higher than the score of the saline control group and the anesthetic control group at 6 hours (*p = 0.025 and p = 0.039 respectively). The analgesic intervention score of 0.67 (indicated by a horizontal dotted line) is exceeded at 6 hours and 9 hours post-injection. Data are mean ± SEM.

The mechanical hypersensitivity testing results reflected a similar pattern whereby the paw withdrawal threshold decreased over time, with the peak hypersensitivity evident at 6 hours after injection (6.57 ± 1.24g, $F = 18.83$, df 6, $p < 0.001$, Figure 6.). The paw withdrawal threshold was significantly reduced at all time points after injection ($p < 0.001$) until 7 days after injection when the threshold returned to baseline values ($p > 0.05$). Withdrawal thresholds in the saline and anesthetic control groups did not decrease during the testing period ($p = 0.39$ and $p = 0.57$, respectively).

Peak edema formation following injection occurred at 6 hours after carrageenan injection. Paw diameter before injection was 7.39 ± 0.23 mm, increasing post-injection to 9.47 ± 0.36 mm at 6 hours. At all time points after injection, paw diameter was significantly increased ($F = 12.99$, df 5, $p < 0.01$ [1, 6, 9], $p < 0.001$ [24h], and $p < 0.05$ [48h]). At 7 days after injection, paw diameter had returned to baseline ($p > 0.05$).

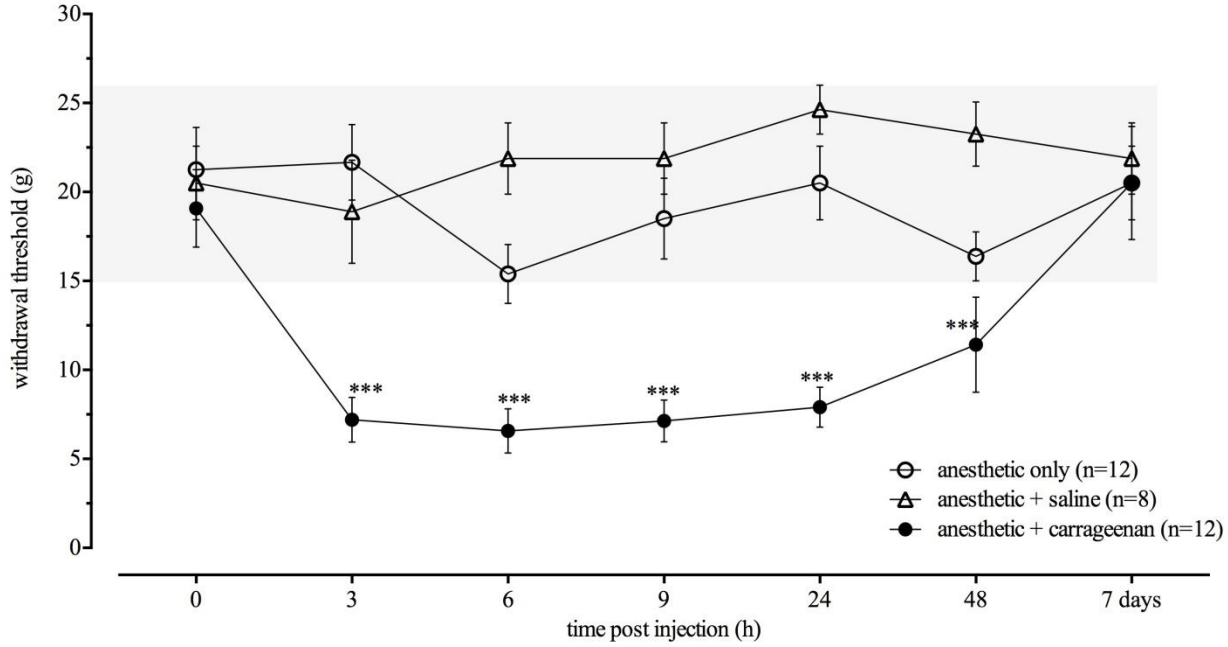

Figure 6. Ipsilateral paw withdrawal thresholds of carrageenan treated animals and control animals. The paw withdrawal threshold is significantly decreased at all time points after injection (*** p 3h, 6h, 9h, 24h and 48h < 0.001) until 7 days after injection (n=12). Withdrawal thresholds in both control groups does not decrease during the testing period ($p = 0.39$ and $p = 0.57$ respectively). Peak hypersensitivity is reached at 6 hours after injection (6.57 ± 1.24g). A grey band indicates the withdrawal threshold range of healthy animals. Data are mean ± SEM.

1.6.3 *Incisional post-operative pain*

In this model of post-operative pain, RGS scores were significantly higher at 6 hours (0.74 ± 0.12) and 9 hours (0.68 ± 0.09) after surgery compared to baseline (0.28 ± 0.06, F = 1.79, df 9, p < 0.05 at both 6 and 9 hours, Figure 7.). At 6 hours post-incision, the RGS score of the incision group was significantly higher than the anesthetic control group (F = 3.14, df 9, p = 0.014). The RGS score of anesthetic control animals did not increase (p = 0.44). At 24 hours post-incision, the RGS score returned to baseline (p > 0.05).

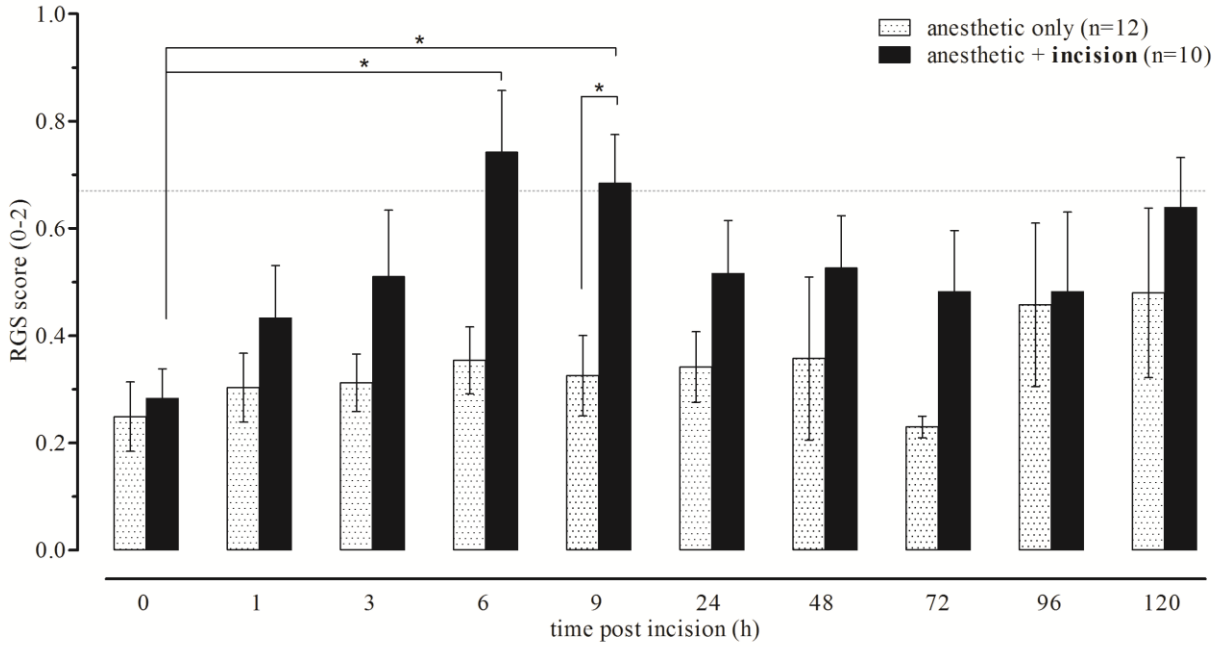

Figure 7. Rat Grimace Scale score of animals with a plantar paw incision. RGS scores increase after surgery and are significantly higher at 6 and 9 hours (at 6 hours: 0.74 ± 0.12 and at 9 hours: 0.68 ± 0.09) after surgery (* p < 0.05 at both time points) compared to baseline score (n = 10). The RGS score in the group that received an incision is also significantly higher than the anesthetic control group at the 6 hour time point (* p < 0.05). The analgesic intervention score of 0.67 (indicated by a horizontal dotted line) is exceeded at 6h and 9h post-injection. Data are mean ± SEM.

The paw withdrawal thresholds decreased significantly for 3 days after incision of the paw (F = 51.70, df 6, p < 0.001 [6, 24 and 48 h], Figure 8.), with the lowest values reached 24 hours after

surgery (5.55 ± 0.90g). Four days after the incision, the paw withdrawal threshold returned to baseline ($p > 0.05$ [96 and 120h]). Withdrawal thresholds of animals in the anesthetic control group did not change during the testing period ($p = 0.57$).

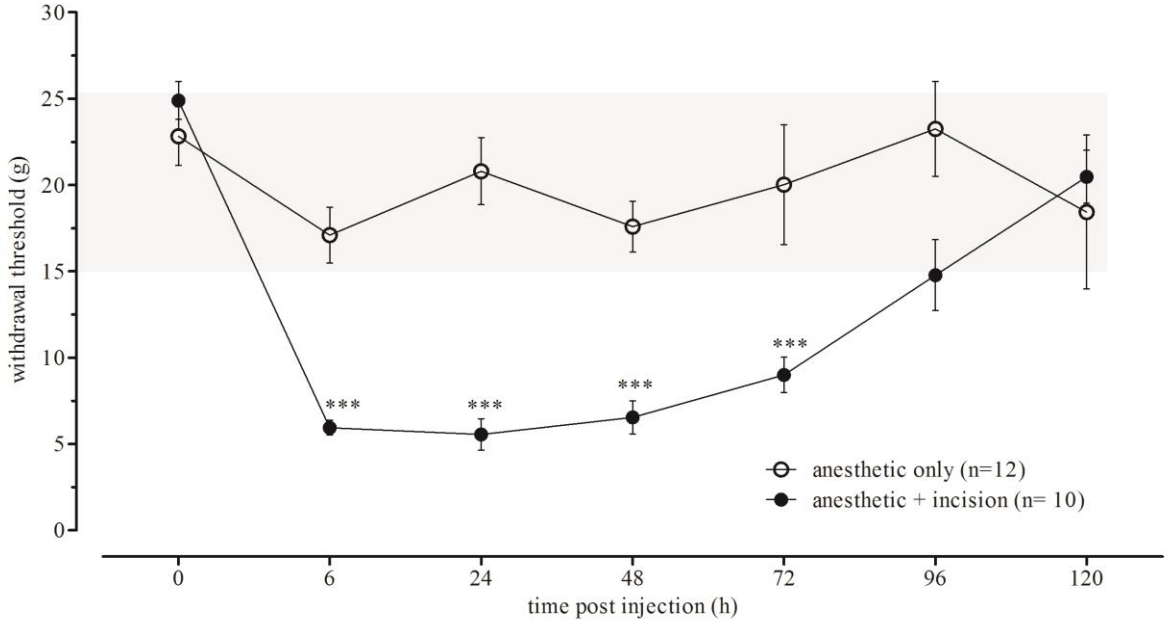

Figure 8. Ipsilateral paw withdrawal thresholds of animals with plantar paw incision and control animals. The thresholds decrease significantly for 3 days after incision of the paw compared to baseline (at baseline, thresholds are considered normal between 15 and 26g) (*** $p < 0.001$), with the lowest values at 24 hours after surgery (5.55 ± 0.90 g) (n = 10). Withdrawal thresholds of animals in the control group that receive only an anesthetic do not change during the testing period and remain within the normal range. A grey band indicates the withdrawal threshold range of healthy animals. Data are mean ± SEM.

1.7 Discussion

The application of the RGS with the parallel use of a nociceptive assay allows for the assessment of the relationship between a traditional nociceptive test and a recently proposed measure of spontaneous behaviours associated with pain. Our findings show that: 1. the highest RGS score and paw hypersensitivity coincided in time, indicating that the RGS was effective in detecting pain of inflammatory origin resulting from the three models studied, 2. The duration of measurable hypersensitivity exceed that of an increase in RGS score, 3. the scores in saline and anesthetic control groups were not elevated at any of the time points.

The pattern of responses recorded from the RGS and mechanical hypersensitivity testing was similar across the different pain models studied. Following an initial alignment of peak response to each assay, RGS scores returned to baseline levels. This was in contrast to mechanical hypersensitivity testing, in which hypersensitivity remained for a longer time following the experimental insult. Therefore it appears that at later time points, rats did not exhibit changes in facial expression associated with pain, but hypersensitivity remained. Following an initial alignment of the peak response to CFA injection, RGS scores returned to baseline. This was in contrast to mechanical hypersensitivity testing, in which hypersensitivity remained present beyond the 48-hour time point. Carrageenan induced inflammation caused significant increases in RGS scores at 6 and 9 hours after injection, agreeing with previous observations that the peak inflammatory response occurs approximately 6 hours after injection [114]. Again, the duration of mechanical hypersensitivity persisted beyond the duration of significant increases in RGS scores, with measurable differences present up to 3 days after insult. A similar pattern was observed in the post-incisional model, where the decline in RGS scores towards baseline levels following incision was more rapid than the decline in withdrawal responses to mechanical hypersensitivity testing, where significant hypersensitivity remained up to four days post-incision. Current literature supports the idea that mechanical hypersensitivity following CFA injection lasts for extended periods of time (up to several weeks) [123, 124]. In the incisional model our observation that the significant decrease observed in the withdrawal response lasts for up to 3 days agrees with the original work of Brennan et al. (1996)[125]. Carrageenan induced mechanical hypersensitivity has been reported to reach maximal levels at 5 hours after injection [121]. The variation in the pattern of response and duration of perceived hypersensitivity in these studies may result from differences in testing methodology and tools used (e.g. incremental stimulus presentation versus up-and-down method, hand held filaments versus electronic von Frey device), sex [126], or strain [127]. Hence, the comparison of methods in the scientific literature is limited by substantial variability and absence of standardisation in reporting [128]. The advent of the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines has sought to raise awareness of this issue and implement a more structured, standardised approach to reporting [128].

Of these models, CFA induced inflammatory pain has been previously assessed with the RGS, and our data confirms these findings [39]. Furthermore, the point at which a peak in RGS scores was attained (6 hours post-injection) coincided with crossing a recently-derived analgesic intervention score [56]. This threshold, determined as scores greater than 0.67, represents the point above which the presence of pain is probable. This pattern of peak RGS scores crossing the threshold of the analgesic intervention score was repeated in both the carrageenan and post-incisional models.

Our results indicate that when pain is present, mechanical hypersensitivity is also present. In contrast, hypersensitivity can be detected in the absence of pain. In the presence of an induced inflammatory response, inflammatory pain (reflected by an increase in RGS score) is measurable and present for a well-defined period of time before returning to baseline levels over the following 24 hours. However, when the inflamed area is mechanically stimulated, significant increases in hypersensitivity can be identified for an extended period, up to several days.
It is clear that stimulus-dependent nociceptive tests and tests measuring spontaneous pain behaviour register different concepts. The RGS score reflected pain caused by the inherent inflammatory reaction, while the mechanical nociception test registered the response to an extrinsic insult to the inflamed tissue. Neither test is a substitute for the other. Instead, investigators must carefully define their research question and select the pain model and assessment method(s) that are most suited to the question they seek to answer.

1.8 Conclusions

The RGS is able to detect pain resultant from inflammation caused by intraplantar CFA, carrageenan or a plantar incision. Pain assessed by using the RGS coincides with the development of hypersensitivity determined with von Frey filaments. Hypersensitivity, a stimulus dependent response, can be elicited for extended periods of time without pain being present.

1.9 Appendix: Repeated mechanical hypersensitivity testing with von Frey filaments causes rapid conditioning in healthy and injured laboratory rats.

1.9.1 Introduction

Mechanical hypersensitivity testing using nylon von Frey filaments is a common nociceptive test in the field of pain research. The technique consists of a series of calibrated filaments mounted on a handle that are applied to the foot or body part of interest until an observed response in the form of a withdrawal is registered. There are several methods describing how to apply the filaments, but there appears to be no evidence which one of these is superior [14]. In the simplest technique, one begins with the application of the thinnest filament of the set. If no response is noted, the next thicker filament is tested. The pain threshold is considered the force of the filament which causes the withdrawal [129]. Similarly, one can start with the thickest filament, and go down in filaments successively, until the animal does not respond with withdrawing the paw. The corresponding filament force determines the pain threshold [130]. A third methodology begins with the application of a middle filament: with a positive response testing is continued with thinner filaments, with a negative response testing is continued with thicker filaments, until a change in response is noted. The threshold is then determined as described above, or the testing can continued for four additional stimuli after the first change in response occurs [16, 131]. This method is referred to as the up-and-down method by Chaplan et al. [16]. The pattern of responses obtained by this method is converted to the fifty percent withdrawal threshold using look-up tables provided by Dixon (1980) [132]. The methodology used by Chaplan et al. has recently been modified into a more practical ‘Simplified Up and Down Method’ [133]. Where it previously varied how many filament trials each animal would receive, the simplified method reduces the minimal required number of filament applications to a standard of five trials, eliminating the variability in trial numbers and consequent variability in threshold measurement between study subjects. The study also proposes the use of an adjustment factor, which eliminates the need of the look-up table. Finally, there are methods where each filament is presented a number of times. In the case of, for example, ten presentations, the number of positive responses is then multiplied by ten and reported as a percent response. For each animal,

ascending filaments are tested either until the maximum cut-off filament is reached, or until a filament strength was reached that caused a 100 percent response [16].

Mechanical hypersensitivity testing in a rat model of incisional pain and control animals was performed by a novice experimenter (author). Unexpected results caused by the use of a repetitive testing method, and potential operator inexperience caused a training effect in the animals that dramatically affected experimental outcome. With a simple modification of the method no conditioning to the test stimulus occurred.

1.9.2 Methods

1.9.2.1 Animals

Fourteen male Long-Evans rats (original method) and 22 male Wistar rats (modified method), aged nine to ten weeks old were obtained from Charles River, Canada. Housing and handling was as described in Chapter 1.

1.9.2.2 Original testing protocol

Each animal was individually placed under a small clear Perspex box on a customised platform (after Pitcher, 1999) [12]. A set of nylon 'von Frey' filaments (TouchTest, North Coast, Gilroy, CA) was applied using the following testing protocol (original method): starting at the low end of the set, filaments were presented three times each in an ascending fashion: each filament was applied and held for one second during each application. The absence of a response in three trials with the same filament was interpreted as a negative response and prompted the use of the next thicker filament. A positive response was acknowledged when the animal lifted its foot, flinched or retracted its paw during application or during removal of the filament. One withdrawal response during these three trials was sufficient to be called a positive response and no further trials were done with the respective filament. After identifying the two filaments that straddle the threshold, four more filaments were tested. Following Dixon (1980) [132], a next thicker filament was tested following a negative response (O) and the next thinner filament following a positive response (X). The tabular value of the response pattern to these filaments (e.g.

OXOXXO) was looked up in a table designed by Dixon (1980) [132] and the estimated response threshold was interpolated using the formula:

threshold = $(10^{(X_f + k\delta)}) / 10{,}000$

where X_f = value (in log units) of the final von Frey hair used; k = tabular value for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli. Filaments were applied not on the incision, but immediately adjacent to the incision. If the rat started walking or exhibited any other locomotor behaviour involving moving of the hind feet during the stimulus, the result was discarded and the same filament retested.

A value anywhere between 15 and 26 grams was respected as upper limit, as thicker filaments passively lifted the animal's foot and would cause unnecessary stimulation of the nociceptors. Testing was performed during day time of the circadian cycle, between 9 and 11 am, with the exception of the first day post-surgery, where tests were performed in the afternoon (6 and 9 hours after intervention).

1.9.2.3 Modified method

The original method described was customized to minimize filament trials and avoid a learning process in the subjects. Each filament was presented only once, at intervals of at least ten seconds (10 to 20s), allowing the resolution of the behavioral responses to the previous application. Additionally, no up-and-down method was pursued for baseline testing. For baseline testing, the animals were tested with incremental filament thickness starting from the low end of the filament set until a withdrawal was noted. Animals reaching the 15g or 26g filament were included in the study and no further filaments tested. Similarly, postoperative testing was discontinued as soon as the 15g threshold was exceeded. The threshold was deemed 'normal' at this point.

1.9.3 Results

Animals that received a plantar incision and control animals that were only anesthetised were repeatedly tested with nylon von Frey filaments to identify their paw withdrawal threshold with and without the development of mechanical hypersensitivity. With the original testing method,

animals that received an incision had significantly decreasing withdrawal thresholds compared to their baseline values ($F = 5.80$, df 6, $P < 0.001$ (except at 24 hours: $P < 0.01$) at all testing times after surgery (Figure 9). In the same animals, the contralateral paws also had decreased withdrawal thresholds compared to their baseline values at all time points except 6 hours post-op ($F = 5.91$, df 6, p 6h = ns, P 24h < 0.001, P 48h < 0.001, P 72h < 0.01, P 96h < 0.001, P 120h < 0.001). In control animals, thresholds had also decreased significantly at all measured time points after surgery ($F = 10.06$, df 6, $P < 0.001$ except at 6h: $P < 0.05$). Withdrawal values remained low beyond the 120 hour time point in ipsilateral and contralateral paws in the treated group and also in the control group. A two-way ANOVA did not detect significant differences between ipsilateral and contralateral paw and control group ($F = 0.73$, df 6, $P = 0.50$).

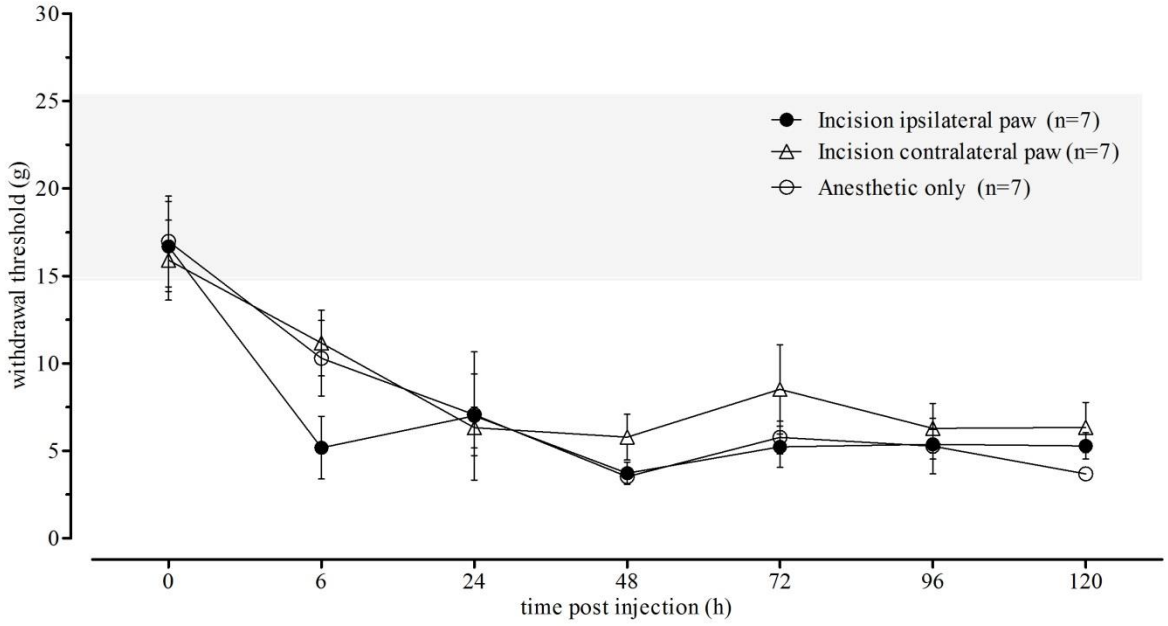

Figure 9. Withdrawal thresholds of the ipsilateral and contralateral paw of animals that received a plantar incision and of control animals using a methodology with repeated filament testing. Withdrawal thresholds are significantly reduced in both injured animals' ipsilateral and contralateral foot, and control animals ($P < 0.01$)

With the modified technique, which entailed less trials per filament, more time between trials and a simplified baseline screening, similar results were obtained in treated animals on the ipsilateral foot: baseline values were similar (original method mean 18.87g ± 2.64 SEM, versus baseline modified method mean = 24.90g ± 1.1g SEM). At subsequent time points, treated

animals had significantly decreased paw withdrawal thresholds compared to baseline (F=51.70, df 6, p<0.001). After 4 days, the paw sensitivity had returned to normal values. The contralateral paw of treated animals did not show decreased withdrawal thresholds at any of the time points after surgery. Control animals did not have decreased withdrawal thresholds at any of the time points and remained within normal range (Figure 10.).

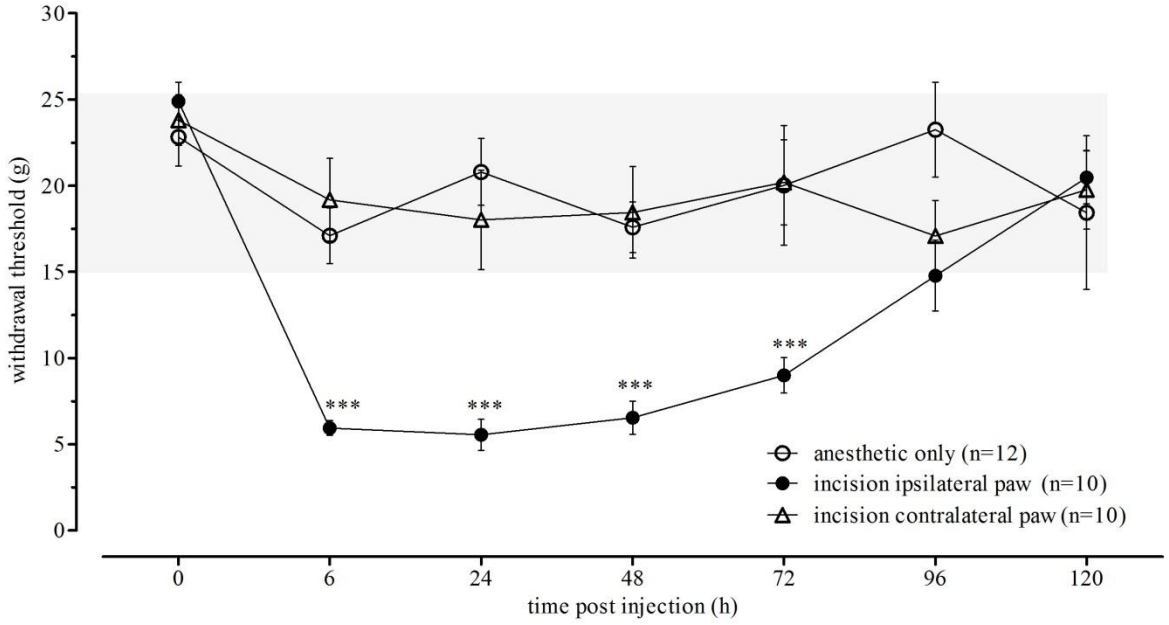

Figure 10. Withdrawal thresholds of the ipsilateral and contralateral paw of animals that received a plantar incision and of control animals using a modified method that minimises filament application. Withdrawal thresholds are significantly reduced in injured animals' ipsilateral paw (p<0.01), but not in the contralateral paw and not in control groups.

1.9.4 Discussion

This study revealed an unexpected decrease in paw withdrawal thresholds in the contralateral paw of animals with an incision and in control animals that were repeatedly tested with von Frey filaments. A slight modification of the methodology did not lead to decreased thresholds, indication that with less trials, this effect could be avoided for multiple testing sessions (7) over multiple days (7).

There are three explanations for decreased withdrawal thresholds with repeated testing, each of which could occur simultaneously. Physiological phenomena could explain our findings. Through accumulation of inflammatory mediators at the level of the spinal cord, repeated application of a stimulus can sensitise peripheral receptors or cause central sensitisation [17]. This facilitation will cause exaggerated responses that negatively affect test results. Secondly, the animals could learn to anticipate the test. Cognitive responses to repeated testing can develop extremely rapid, according to LeBars et al (1994) [17]. In the Randall-Selitto paw pressure test, baseline withdrawal thresholds were significantly decreased in healthy rats in the second test session and decreased more in the third and fourth testing session. Additionally, only after three testing sessions (three days), the withdrawal threshold of healthy animals had stabilised and a significant difference could be distinguished between healthy and injured animals (bradykinin injection) [134]. When using heat or pressure, thermoreceptors and mechanoreceptors are recruited before the nociceptors. This sequence of a conditioning stimulus before a conditioned stimulus is used to study anticipation of pain [17], and conditioning with von Frey filaments (with touch as the conditioning stimulus) is a viable possibility. Thirdly, Chaplan et al. (1994) showed the impact of experimenter experience on withdrawal thresholds. The test results of three investigators, one with extensive experience, one with intermediate experience, and one with no prior experience with the testing procedure were assessed in regards to stability of the threshold with repeated testing. Experimenters with no or intermediate experience measured significantly decreased withdrawal thresholds in healthy rats after 2 to 3 testing sessions (2 to 3 days) [16].

1.9.5 Conclusion

The unexpected decrease in withdrawal thresholds in non-injured animals and contralateral paws of injured animals has most likely a multifactorial explanation: conditioning of the animals to the experimental procedure, sensitisation (cfr. decreased threshold in contralateral paws) and operator inexperience could have caused the outcome. The modified testing protocol, which significantly decreases the number of trials per filament and testing session, as well as filament trials per time window (more time left in between), delays the onset of a conditioning effect and slows sensitisation. The author gained experience as experiments progressed, which also may have contributed to obtaining stabile thresholds with the modified methodology. Lastly, it cannot

be ruled out that strain differences played a role in the responses to the two testing methods. There are however, to our knowledge, no previous reports on this.

CHAPTER THREE

Carbon dioxide, but not isoflurane, elicits ultrasonic vocalisations in female rats

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1.10 Introduction

With over 2.5 million animals being used annually in Canada and the European Union, rats are one of the most common species in biomedical research [135, 136]. The great majority of these animals will be euthanized using an overdose of carbon dioxide gas. Despite evidence from behavioural studies showing that carbon dioxide gas is aversive to rats [104, 105] the practice remains popular because it is cheap, effective, widely available, and poses a minimal health risk to personnel. Euthanasia of rats with isoflurane as the sole agent [137] or as part of a two-stage process [138] has been deemed acceptable. Current national guidelines are largely based on evidence from approach avoidance studies, with the unavoidable limitation that data cannot be collected between the onset of aversion and loss of consciousness.[98, 104] Vocalising in the ultrasonic range is a strategy rats have developed to adapt to high predatory pressure. Therefore ultrasonic vocalisation (USV) allows communication with conspecifics but is inaudible to many predators.[139] USVs in rats have been shown to reflect a negative state (such as pain or distress) [86] and may provide a valuable tool for identifying pain and distress during euthanasia.[140] In general, lower frequency USVs (18–32 kHz, so-called ‘22 kHz calls’) have been associated with negative states, and higher frequency USVs (32–92 kHz, so-called ‘50 kHz calls’)with positive states. Lower frequency USVs act as alarm calls and have been associated with pain, distress, and fear [86, 141]. For these reasons, the recording of USV has been suggested as a measure of pain and fear in laboratory animals [2, 142]. Oliveira and Barros assessed USV as a behavioural measure of pain and recorded a significantly increased number of low frequency USVs from rats during the formalin test. During exposure to carbon dioxide with rats, at a rate of 17.25% chamber volume per minute, Niel and Weary showed an increase in the occurrence of USVs [140]. By contrast, high frequency USVs (50 kHz calls) have been recorded during purported positive states such as tickling and mating [143, 144]. We conducted a pilot

study to evaluate the application of USV recording as a reflection of pain or distress (or both) experienced by rats during exposure to carbon dioxide or isoflurane, building on previous work by Niel and Weary [140].

1.11 Methods

Nine female Sprague–Dawley rats (Health Sciences Animal Resource Centre, University of Calgary, Calgary, Canada) between the ages of 7 to 9 weeks old and weighing 195–312 g were used in this experiment. The animals were housed in groups of two or three in a standard rat cage (47x25x21 cm) with commercially available wood shavings (Aspen chip, NEPCO, Warrensburg, NY, USA) and plastic tubes for enrichment. The rats received water and food (Prolab 2500 Rodent 5P14, LabDiet, PMI Nutrition International, St Louis, MO, USA) ad libitum and were kept on a 12 h light–dark cycle (lights off at 19:00 h). All the experiments were performed between 15:00 h and 18:00 h with a minimum of 24 h between treatments to allow the rats to recover. Six animals were exposed to each gas on different occasions. The order of these treatments was determined by a random draw. Three other rats received only carbon dioxide as a treatment. Each animal was tested individually and exposed to the gas in a purpose made closed Perspex test chamber (3000mL volume) while remaining within sight of its cage mate(s) throughout the experiment. The test chamber had three openings fitted for instrument connection (microphone, gas analyzer and gas inflow tube). The following standardized protocol was used: 5 min acclimatization period in room air, then 5 min with oxygen inflow at one litre per minute (L/min), which equals 30% chamber volume per minute (CV/min), and finally (once oxygen concentration had returned to 21%) exposure to the treatment agent. Carbon dioxide (100%) or isoflurane (2.5% carried in oxygen) was delivered at a flow rate of 1.0 L/min (30% CV/min). Sound recordings were performed using an ultrasound microphone (Condenser ultrasound microphone and UltraSoundGate CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) from the time the animal was placed in the test chamber. Carbon dioxide and oxygen were delivered with agent-specific calibrated flowmeters, and the isoflurane and oxygen concentrations were monitored with a calibrated gas analyzer (Datex Ohmeda s/5 monitor, GE Health Care, Waukesha, WI, USA). The experiment was terminated and the animal was allowed to recover following confirmation of a loss of righting reflex by tilting the test chamber until the animal was

in dorsal recumbency. This was performed once gross purposeful movement ceased and the animal became recumbent in the test chamber. Vaginal swabs were taken during recovery and smears were prepared for cytological examination (slides were air-dried and stained with Diff Quik) to determine if there was a correlation between the stage of oestrous cycle and the presence or absence of USVs. All recordings were visually inspected twice for USV identification by a blinded observer. Vaginal smears were also evaluated by a blinded observer. Descriptive statistics are reported and data are shown as median and range. The decision to use female rats was driven by a wish to reduce animal use. It was preferable to use surplus stock from our institutional colony rather than order in animals from an external source. Consequently, this limited availability to female animals. This experimental protocol was reviewed and approved by the Health Sciences Animal Care Committee at the University of Calgary, Canada, which operates under the auspices of the Canadian Council on Animal Care. Control recordings made during the acclimatization period and oxygen inflow (performed before each gas exposure) resulted in one rat vocalising once during both oxygen exposures. Data from this animal were not included in the analysis as conclusions could not be drawn from the presence or absence of any calls during the subsequent treatment (isoflurane or carbon dioxide) phase. It would be impossible to interpret such behaviour as being due to the different treatments and not the sound of gas inflow.

1.12 Results

Treatment	Number of animals vocalising	Number of calls	Frequency, kHz	Duration, seconds
room air	0 out of 8	0	NA	NA
oxygen	0 out of 8	0	NA	NA
isoflurane	0 out of 6	0	NA	NA
carbon dioxide	8 out of 8	23	51 (30-70)	0.05 (0.014–0.26)

Table 1. **Occurrence and properties of ultrasonic vocalisations** during exposure to room air, oxygen alone, isoflurane (2.5% carried in oxygen) and carbon dioxide (100% carbon dioxide at a fill rate of 30% chamber volume per minute) in female rats.

Exposure to isoflurane did not elicit USVs from any rat (0 out of 6 animals). By contrast, during exposure to carbon dioxide, we recorded USVs from all animals (8 out of 8 animals). Of these, a median of two calls per rat (range 1 to 8) were recorded. The frequency ranged from 30 to 70 kHz (median 51 kHz) with a median duration of 0.05 s (0.014 to 0.26 s). The onset of USVs ranged from 81.9 to 152.1 s (median 133.8 s) after carbon dioxide gas flow began. With the 1 L/min flow rate employed and chamber volume of 3L, this equated to a calculated chamber concentration change of greater than 95% (more than three time constants). The results are summarized in Table 1 and a typical USV is shown in Figure 11. Vaginal cytology revealed that the vocalising rats were at various stages in the oestrous cycle and that there were no associations with the occurrence of USVs.

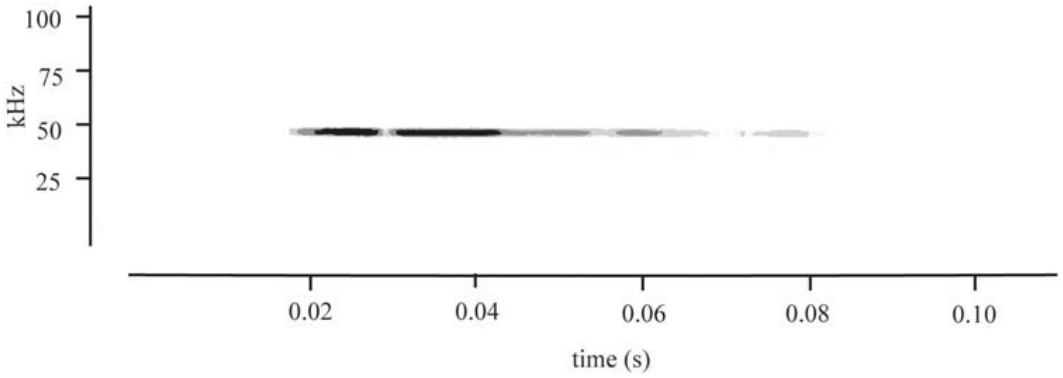

Figure 11. **Example of a typical ultrasonic vocalisation emitted by a female rat exposed to 100% carbon dioxide at a fill rate of 30% chamber volume per minute**. All rats exposed to carbon dioxide gas (8 out of 8 animals) produced ultrasonic vocalisations. No rats (0 out of 6 animals) exposed to isoflurane (2.5% carried in oxygen at a fill rate of 30% chamber volume per minute) produced ultrasonic vocalisations.

1.13 Discussion

To our knowledge, these preliminary data show, for the first time, that female rats vocalise during exposure to carbon dioxide but not when exposed to isoflurane. Though 22 kHz calls are

usually associated with negative states such as distress or pain, our findings in the context of evidence from approach avoidance studies indicate that USV in a higher frequency range may also be reflective of these states. Work by Niel and Weary using adult male Sprague–Dawley rats, has reported findings similar to our data, with 50 kHz calls being identified in response to carbon dioxide exposure [140]. Additionally, a series of experiments by Wohr et al. have shown that 50 kHz calls are not strictly attributed to positive experiences but are also emitted when rats are separated from a cage mate, during an open field test, elevated plus maze test and introduction to a novel cage [145] These data indicate that 50 kHz calls are not restricted to positive states. Taken together with the results of approach avoidance studies showing that isoflurane is aversive [104, 105] our data indicate that it may be a preferable alternative to carbon dioxide. This work does not allow generalization to male animals or other rodents, and further work is necessary to address these issues in addition to determining whether different administration techniques or alternative agents provide more humane alternatives. As millions of rodents are euthanized by carbon dioxide each year the implications are widespread.

1.14 Appendix: Rats are able to emit 22 kHz and 50 kHz ultrasonic vocalisations during isoflurane exposure

1.14.1 Introduction

We have shown the use of ultrasonic vocalisations (USVs) as a measure of aversion or pain during gas exposure for euthanasia purposes [146]. The animals vocalised during carbon dioxide exposure but did not emit vocalisations in isoflurane. The question arises whether these rats were physically able to vocalise during isoflurane, given that isoflurane at subanesthetic doses may alter behaviour. The anesthetic effect of isoflurane could cause sedation and/or laryngeal muscle relaxation. Alternatively, it is possible that the physical properties of the carrier gas (oxygen) mixed with the isoflurane could alter the airway dynamics, and prevent the rats from vocalising.
An experiment was set up to test the hypothesis: rats are able to emit USVs during isoflurane exposure. Animals were exposed to increasing concentrations of isoflurane after they were stimulated to emit USVs.

1.14.2 Methods

1.14.2.1 Animals

Twenty male Wistar rats, aged 9 to 10 weeks old were used. Housing and handling were as previously reported in Chapter 2.

1.14.2.2 USV induction

The animals were individually placed in a clear red Perspex box and allowed to acclimatise for two minutes. The box was constructed with openings for gas inlet, a gas analyser sampling line (Datex Ohmeda s/5 monitor, GE Health Care, Waukesha, WI) and a USV microphone (Condenser ultrasound microphone and UltraSoundGate CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany). Sound and video recording as well as continuous gas analysis was initiated at the start of the experiment. Air puffs were used to provoke ultrasonic calling in rats following a

technique adapted from [91, 92]. A Picospritzer equipped with a pressure valve for pressure control was used to deliver air puffs (Parker Intangibles, Cleveland, OH). Duration and pressure of the air puffs were set at 400 milliseconds and 90 psi respectively. Air puffs were delivered from a 1.5 millimeter internal diameter plastic straw located 95 cm distal from the solenoid valve and were aimed at the rat's face or neck region. Puff administration was manually controlled by a push button. During a single trial, one air puff was delivered every 15 seconds until the rat began to vocalise. In our experience, with this protocol approximately 50 percent of male Wistar rats will start vocalising within a five minute testing window, and without further intervention, the animals are likely to continue to emit USVs for up to one to two minutes. Once vocalising was induced, the lid of the box was closed and 2% (n=4) or 5% (n=16) isoflurane in oxygen (at a flow rate of 1 l/min) delivered to the chamber. Video and sound recordings and in-chamber gas analysis were continued until loss of consciousness (loss of righting reflex). The animals were then removed from the box, transferred to an anesthetic nose mask with 2% isoflurane and experiments continued as planned (CFA, carrageenan or saline injection, or anesthetic control). If there was no vocalising response after five minutes of stimulation (20 puffs), anesthesia was induced and experiments continued as planned.

The time point and isoflurane concentration at which the last vocalisation was emitted was visually determined during the experiment from real-time display of ultrasonic sound recording, and verified on the video footage. The gas analyser was not included in the experimental set up when one rat (rat C) vocalised during isoflurane: the concentration during which this animal vocalised was determined retrospectively using video footage from this experiment and isoflurane concentration curves obtained from repeated experiments using identical settings (5% isoflurane in 1 l/min oxygen).

1.14.3 Results

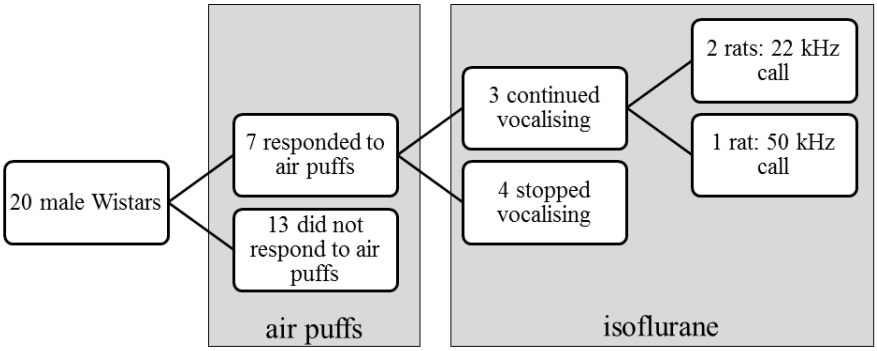

Table 2. The response of 20 male Wistar rats to air puff stimulation followed by isoflurane exposure.

Out of 20 tested rats, 7 started vocalising in the 22 kHz range with air puff induction (7/20). Four of these animals immediately interrupted the vocalising with the initiation of the isoflurane (4/7). Three animals continued to vocalise during the gradual fill of the chamber with isoflurane. Two of them continued to emit calls during 2% isoflurane administration (2/3) (Table 1): the isoflurane concentration was 0.65% (animal A) and 0.94% (animal B) when these animals emitted the last call. Calls from animal A were the expected 22 kHz long calls, but rat B emitted an additional modified 50 kHz call after the 22 kHz calls. In 5% isoflurane, one animal emitted 22 kHz calls up to 2.7% isoflurane (C) (Figure 12.).

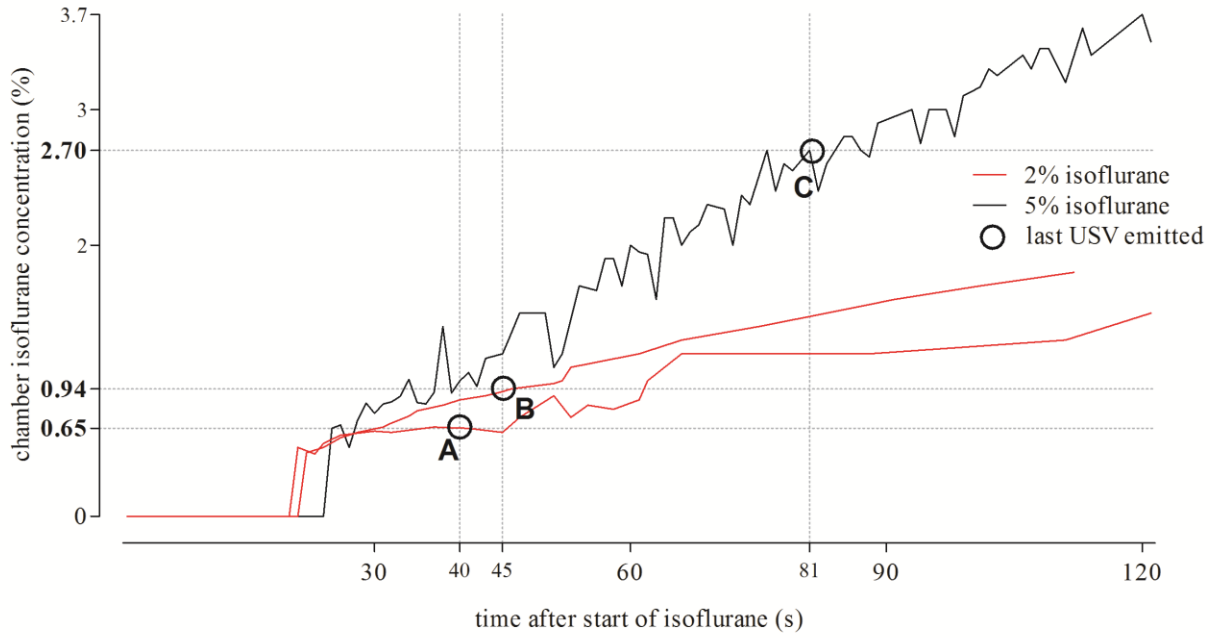

Figure 12. Isoflurane concentration in a closed chamber and the occurrence of ultrasonic vocalisations. Isoflurane administered in oxygen at a flow rate of 1 liter per minute and vaporiser setting of 5% isoflurane (black) and 2% isoflurane (red). The occurrence of the last registered ultrasonic vocalisation is indicated by a circle with rat identification (A,B or C).

1.14.4 Discussion

The results show that male Wistar rats are able to emit 22 kHz calls in isoflurane concentrations up to 2.7%. Some rats stopped emitting USVs when the isoflurane was initiated. We speculate that it is the distinct smell of isoflurane [147] that alerted the animals and made them redirect attention away from the air puff. It was previously noted that rats would interrupt vocalising behaviour with movement or sound made by the experimenter in the room. It is therefore possible that a novel stimulus such as a smell could have the same consequences. Similarly, the sensation of gas entering the chamber could be a reason for cessation of vocalising.

Our results concur with previous reports, including place preference studies (light/dark box and food reward), indicating that isoflurane may be less aversive than carbon dioxide [104, 148].

Ultrasonic vocalisations are a potential measure of negative state caused by aversion to a stimulus.

CHAPTER FOUR

Discussion

Nociceptive tests have been used in the study of pain for over half a century, but the wide spread utility of these techniques has recently been put into question [1, 2]. Given that pain is an emotional experience, traditional nociceptive tests appear to fall short in their objective of measuring the full pain experience. These tests often measure spinal reflexes only and do not take account of a higher-level processing of the signal, and therefore should not be interpreted as measures of pain. As a proposed alternative, there are calls for the use of spontaneous behavioural measures of pain. Unlike nociceptive tests, spontaneous behaviours can be used non-invasive, independent of an external stimulus and are thought to reflect the pain experience, including its emotional component, as a whole.

Two distinct behaviours have been proposed as measures of spontaneous pain behaviour in rodents: facial expression, and ultrasonic vocalisation. In this study, I set out to explore the possibilities and limitations of these novel pain measures. In particular, I was interested in answering the following questions:

1. A. Can the Rat Grimace Scale (RGS) successfully measure facial expression of rats in different models of pain?

   B. How does the generated facial pain score relate to the stimulus evoked response from a nociceptive test in the same animals?

2. Can ultrasonic vocalisations provide a useful measure of aversion during euthanasia practices?

1.15 The Rat Grimace Scale

1.15.1 Can the Rat Grimace Scale be successfully applied in different models of pain?

In the past, the Rat Grimace Scale has proven to be a tool that can detect pain from laparotomy [39, 52, 53], intraplantar CFA [39], intra-articular kaolin [39] and orthodontic forces [54]. The results of my study show that the Rat Grimace Scale was able to detect pain of different

inflammatory origin. First, I partially replicated the work of Sotocinal et al. [39] by applying the Rat Grimace Scale to a model of Complete Freund's Adjuvant induced local inflammatory pain. CFA is known to cause an acute inflammatory immune response that peaks after 24 hours and lasts for 7 days [113]. Because there is evidence that rodents may suppress facial expression with the presence of a human (more so with males than females) [149], I implemented additional standardization by using a strictly quiet environment without experimenter presence to videotape rats before and after intervention. Through personal communication with Dr. Sotocinal at McGill University, I was able to replicate her work using an identical methodology on the same rat sex and strain. My study showed significant increases in RGS score and highest RGS score at the same time point 6 hours after injection). My results, however, did not show significance at more than one time point after surgery. It appears that a slightly greater variation in the data obtained in our study prevented me from concluding that the RGS was increased at time points other than 6 hours after injection. Interestingly, both this study and the original found the pain score to be the highest at 6 hours, whereas the CFA model is known to cause peak inflammation at 24 hours. Thse data are potentially based on physiological rather than behavioural parameters, such as edema formation. But, as this report mentions, hyperalgesia does not necessarily coincide with edema [113]. Likewise, pain may not coincide with physiological parameters of inflammation.

With the implementation of control groups (animals receiving an anesthetic alone and animals receiving an anesthetic and a saline injection), further validation of the grimace scale was possible. Animals in these control groups did not show increased RGS scores at any of the time points. This suggests that the RGS increased because the animals experienced pain from the intervention. It can be noted, however, that the animals receiving a saline injection trended to have higher mean RGS scores than the group that was anesthetised only. It is possible that the RGS detected pain the animals experienced from the saline injection, but because the perceived effect was small, conclusions could not be drawn from the data.

The RGS was similarly applied to animals receiving a carrageenan injection. The carrageenan model differs from CFA with the formation of edema. Also, carrageenan induced inflammation is of shorter duration: it peaks at 6 hours after injection and lasts for 24 hours [113]. In this model, peak RGS score was determined at 6 hours and significantly increased scores at 6 and 9

hours. Here, the highest RGS pain score coincides with the expected peak inflammatory response and edema [113].

The RGS also detected incisional pain. Similar to previous models, peak RGS scores were detected around the 6 hour time point. According to previous work that used a cumulative pain score based on foot position in this model, the animals had increased pain scores after surgery, highest immediately after and remaining significantly elevated for up to 4 days [125]. Although not significantly, the RGS also trended to remain high after the 6 hour peak score, for over the 5 day time course of the experiment.

Interestingly, at the time points where the RGS was significantly higher than baseline, the previously determined analgesic intervention score (AIS) was also exceeded [56]. The scores we identified as peak pain scores also called for analgesic treatment based on the findings of this study. In sample sizes up to n = 10, with a known or expected variability and expected baseline RGS values, we can state the following: when a significantly elevated RGS score is identified, it is very likely that this score has exceeded the AIS. In other words, based on these findings, the RGS detects pain that is severe enough to require analgesic treatment.

1.15.2 How does the generated facial pain score relate to the stimulus evoked response from a nociceptive test in the same animals?

All inflammatory responses in our models caused substantial hypersensitivity measured with von Frey filaments. In CFA injected animals, withdrawal thresholds remained significantly decreased for longer than 48 hours. This agrees with the previously reported data [123] where withdrawal thresholds remained decreased for 2 weeks after CFA injection. (It needs to be emphasised though, that due to the variety of methodologies and tools available, no direct comparisons between the findings of different studies can be made). The registered nociceptive thresholds remained significantly decreased over the time course of the experiment, in contrast to the RGS scores, which were only elevated at one measured time point (6 hours).

In carrageenan injected animals, the von Frey threshold remained decreased beyond 48 hours after injection. This is longer than the increase in RGS score, which was only registered at 6 and 9 hours after injection. A similar withdrawal threshold pattern was found in the incisional model, where von Frey thresholds were decreased immediately after surgery and remained low until day

4, whereas RGS score normalised after 9 hours after surgery. A previous study using the incisional model showed that withdrawal thresholds had returned to baseline at day 3 [125]. Overall, our study showed decreased withdrawal thresholds that were similar to previously published studies, and this hypersensitivity occurred sooner and lasted much longer that the perceived RGS score increases.

Conceivably, during the nociceptive test, the animals were exhibiting a nociceptive response to a stimulus, but were not experiencing pain without the stimulus. In other words, the nociceptive test created a nociceptive event (and maybe pain as well) with the application of the filaments. Without the stimulus, the animals may have been able to minimise pain in the injured foot by guarding and reduced weight bearing on the injured paw.

On the other hand, it is also possible that the animals were in pain for longer than the RGS reflected, but the RGS was not sensitive enough to detect this. From these results, however, we can tell that elevated RGS scores are predictive of decreased paw withdrawal (pain with nociception), but that decreased withdrawal thresholds can exist without RGS scores being elevated (acute nociception does not indicate constant pain). Interestingly, upon closer examination of the source of variability in the RGS data, individual differences lie mainly in the time point at which an individual expressed pain. Indeed, with the exception of two animals in the carrageenan cohort, all rats exceeded a score of 0.75 (a two or threefold of their baseline scores) and seemed to experience pain at at least one time point in the study. This indicates that the RGS was successful in detecting pain, but not at consistent time points and underscores the importance of evaluating individual animals rather than cohorts.

From the results it is clear that stimulus-dependent nociceptive tests and tests measuring spontaneous pain behaviour register different concepts. The RGS score reflected pain caused by the inherent inflammatory reaction, while the mechanical nociception test registered the response to an extrinsic insult to the inflamed tissue. Therefore, neither test is a substitute for the other: that is, there are no replacements or `better alternatives`. Instead, researchers must carefully define their research question and select the pain model that is most suited to the question they seek to answer. For example, mechanical nociceptive tests are fully applicable in models that research allodynia, where innocuous mechanical stimuli are the cause of pain. A measure like the

grimace scale may application in laboratory animal welfare research, such as the development of analgesic protocols for rodents.

1.15.3 General thoughts on the use of facial expression scales

1. Facial pain scales are said to have multiple benefits, including that they are easy and quick to use and apply our tendency to focus on the face when estimating pain [38]. In my study, the Rat Grimace Scale was used retrospectively on video still frames and its use as a cage-side/real-time pain assessment tool was not evaluated. In studies using video footage to assess facial expression, substantial time needs to be spent frame-grabbing and scoring images. Also, I believe that thorough training is required for a novice experimenter to effectively apply the scale.

2. As we understand from human research, methods using facial expression are particularly sensitive for introduction of bias. At the level of the sender (the animal), intrinsic and environmental factors will influence the grimacing behaviour, potentially causing a suppression of grimacing. For example, an experimenter present in the testing suite can be perceived as a potential threat and cause a suppression of facial expression of pain in the animal as it attempts to mask its vulnerable physical state. Studies have shown that olfactory stimuli from males cause stress analgesia [149]. Hence it is of great importance that these details are reported in the methodology, as long as it is not understood how and when animals may exhibit this pain-masking behaviour. On the receiver side (the observer), bias can be introduced by personal experience as has been shown in human clinicians. Alike human physicians, experimenters familiar with animals in pain may be prone to underestimate pain severity [32], whereas experienced grimace scale users may become more sensitive to subtle changes. The role of this training effect and the influence of previous personal experience on the outcome of grimace scale scoring should be further examined. Another source of bias can derive from attributing suggestive terminology to action features, as was noted in Chi et al. [52]. Blinded scorers applying the grimace scale as a tool should be unaware of the underlying cause or purpose of the score, i.e. action units (AUs) should be scored ‘absent, present, or obviously present’. All

terminology suggesting pain, such as ‘severe’ or ‘mild pain’ must be avoided because this would suggest how other AUs in the same image should be scored.

3. With the development of grimace scales, facial expressions of pain in animals have been assumed to occur on a continuous scale. Stating that pain ranging from mild to severe can be detected [38], the scales presume that mild pain will be represented by mild increases in score. From human medicine, nonetheless, we know that pain below a certain threshold will not cause grimacing [33] and that the nociceptive input must exceed a certain intensity for it to be reflected in the face. This may apply to animals as well.

1.16 Ultrasonic vocalisations

1.16.1 Can ultrasonic vocalisations provide a useful measure of aversion during euthanasia practices?

Ultrasonic vocalisations (USVs) are known to be a means of communication used by rodents in a wide range of situations [64]. They are believed to be elicited by emotional experiences, and the distinct frequencies at which these calls are emitted are seemingly related to either the positive (50 kHz) or negative (22 kHz) affective state of the animal [83, 84].

Gradual fill of a closed chamber with carbon dioxide is a conditionally accepted method for the euthanasia of laboratory rats. The method is widely used because it is cheap and operator safe, and it is procedure easy to perform on a large number of animals. Current research, however, has shown that carbon dioxide is aversive to rats [97-100], and the administration of anesthetic gases such as isoflurane to induce anesthesia before euthanasia has been put forward as a less aversive alternative [103-105].

Consequently, the question was asked if a negative emotional state as a part of the aversion that rats would experience in carbon dioxide and/or isoflurane, also results in the emission of ultrasonic calls.

We found that all rats emitted USVs exclusively during carbon dioxide exposure. The registered calls were not emitted at the expected 22 kHz (the frequency previously associated with negative affective state), but were emitted at 50 kHz (associated with positive affective state). The

spectrographic shape of these calls, on the other hand, was non-modulated (‘flat’), meaning that the frequency was maintained throughout the call, without change. This characteristic belongs to 22 kHz calls. Previous studies also found that rats emitted 50 kHz calls during carbon dioxide [140], and situations that are not related to a positive experience (such as separation from a cage mate, in an open field test or elevated plus maze test, and during introduction to a new environment [140, 145].

Considering the increasing evidence that carbon dioxide exposure causes significant distress, our results suggest that not only frequency, but the shape of the call may carry information on the affective state of the animal. From our study results, it appears that the distinction of ultrasonic vocalisations and their direct reflection of affective state solely based on frequency may be too rigid, and that spectrographic shape of the call may play a vital role in the interpretation as well.

1.17 Future directions

An interesting route for further research is to assess facial expression during the nociceptive test. We can hypothesise that with mechanical stimulation of inflammatory tissue causes nociception and pain. The RGS could be used to determine if the test of mechanical hypersensitivity causes a strictly nociceptive response or also a temporary pain experience. In the experimental design of this thesis, nociceptive testing was always performed after the RGS video was taken, hence no information was collected on the pain experience during or right after nociceptive testing.

Secondly, the implementation of an analgesic control group in the experiment would further validate the Rat Grimace Scale. If the RGS specifically measures pain, it would be expected that with an effective drug regimen, the RGS score of animals receiving an analgesic will be significantly decreased compared to animals that do not receive an analgesic, and be potentially as low as baseline values.

Thirdly, experimenter skills to visually detect facial changes and consistency in applying the RGS increase with repeated exposure to large image databases of healthy and injured animals. A RGS score learning curve can be generated, and from here, an ideal number of images required to adequately train a novice scorer.

Ultimately, it appears from our data that certain action units contribute more extensively to the grimace scale score of animals in pain, and others seems less predictive of the animal`s status.

Currently, all action units (eyes, ears, nose, whiskers) are equal contributors to the total score. This may be a disadvantage, considering that the RGS score is an average of all four AUs. If one AU is not predictive of pain, it will wrongly decrease or increase overall scores. Adding weighing factors to the action units may make the RGS more sensitive to detecting pain within a certain model. Weighing the separate AUs can be model specific, and features may be less or more predictive depending on the type of pain (e.g. neuropathic versus visceral pain versus arthritic pain, etc.).

1.18 Conclusions

The Rat Grimace Scale is a novel method of assessing pain severity of laboratory rats in different pain paradigms. It was successfully applied to identify local inflammatory pain from various origins in male Wistar rats. Non-invasive measures of pain that do not rely on an external stimulus, such as the RGS, are not replacements for nociceptive tests, but can exist alongside conventional nociceptive testing. With the development of animal grimace scales, a new realm of information becomes available to the researcher; and careful choices on which method(s) of pain assessment to apply should depend on the questions that need to be answered.

Non-modulated ultrasonic vocalisations support previous research that carbon dioxide is a method of euthanasia that is likely to be aversive, and that isoflurane may be a better alternative. Care should be taken with the interpretation of the call frequency; the spectrographic shape of the call may be of equal importance.

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