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Abstract

The aversive aspect of pain constitutes a major burden on pain patients. This drives the development of rodent pain aversion assays for preclinical studies. We developed two paradigms for studying aversion, using a punctate heat probe (PHP): real-time place avoidance (RTPA) and heat escape threshold (HET). The differential effect of morphine on pain was also examined. Finally, opioid-induced hyperalgesia (OIH) modelled generalised pain.

Inflammatory pain was induced by intraplantar injection of carrageenan (CAR) and OIH by intrajugular infusion of remifentanil. Heat-RTPA and HET tests were conducted in two-chamber behaviour arena with mesh floor. RTPA scores compared time spent in the 'pain'-chamber (stimulation of CAR-injected paw) during baseline and test sessions. HET was evaluated by applying heat incrementally starting at 41.1°C (i.e., increased following escape or vice versa).

RTPA and lowered HET were detected in CAR-rats and counteracted by application of systemic morphine. Putative pain aversion was compared to nocifensive-reflexive data from Hargreaves tests. Thereby, morphine selectively attenuated aversion while preserving sensorimotor function at a cut-off dose (1mg/kg). Rats with OIH showed lower HETs, bilaterally, compared to control rats in the HET paradigm.

The PHP can reproducibly and quantifiably measure aversion in two original paradigms. Future studies can use the HET in optogenetic experiments or RTPA for pharmacological studies, for basic research and drug screening, respectively. The PHP can also be used for *in vivo* electrophysiological studies of neuronal circuits. Perspective investigations with local application of compounds in the CNS can shed light on neuronal mechanisms for alleviation of pain aversion.

Der aversive Aspekt von Schmerzen stellt eine große Belastung für Schmerzpatienten dar. Dies treibt die Entwicklung von Nagetier-Schmerzaversionsassays für präklinische Studien voran. Wir entwickelten zwei Paradigmen zur Untersuchung der Aversion unter Verwendung einer punktförmigen Wärmesonde (PHP): Real-Time Place Avoidance (RTPA) und Heat Escape Threshold (HET). Die differentielle Wirkung von Morphin auf den Schmerz wurde ebenfalls untersucht. Schließlich wurde mit der Opioid-induzierten Hyperalgesie (OIH) der generalisierte Schmerz modelliert.

Entzündliche Schmerzen wurden durch intraplantäre Injektion von Carrageenan (CAR) und OIH durch intrajuguläre Infusion von Remifentanil induziert. Hitze-RTPA- und HET-Tests wurden in einer Zweikammer-Verhaltensarena mit Netzboden durchgeführt. RTPA-Scores verglichen die Zeit, die in der "Schmerz"-Kammer (Stimulation der CAR-injizierten Pfote) während der Grundlinie und der Testsitzungen verbracht wurde. HET wurde bewertet, indem Wärme schrittweise, beginnend bei 41,1°C, appliziert wurde (d.h. Erhöhung nach Flucht oder umgekehrt).

RTPA und erniedrigte HET wurden in CAR-Ratten nachgewiesen und durch die Verabreichung von systemischem Morphin ausgeglichen. Die vermeintliche Schmerzaversion wurde mit nozifensiv-reflexiven Daten aus Hargreaves-Tests verglichen. Dabei dämpfte Morphin selektiv die Aversion, während die sensomotorische Funktion bei einer Cut-off-Dosis (1mg/kg) erhalten blieb. Ratten mit OIH zeigten im Vergleich zu Kontrollratten im HET-Paradigma beidseitig niedrigere HETs.

Das PHP kann die Aversion in zwei ursprünglichen Paradigmen reproduzierbar und quantifizierbar messen. Zukünftige Studien können den HET in optogenetischen Experimenten oder RTPA für pharmakologische Studien, für die Grundlagenforschung bzw. das Wirkstoffscreening, einsetzen. Das PHP kann auch für in vivo elektrophysiologische Studien von neuronalen Schaltkreisen verwendet werden. Perspektivische Untersuchungen mit lokaler Applikation von Substanzen im ZNS können Licht auf neuronale Mechanismen zur Linderung der Schmerzaversion werfen.

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Contents

VIII.28. "Pain is an evil either to the body - let the body then denounce it […] - or to the Soul; but the Soul can ensure her own fair weather and her own calm sea, and refuse to account it an evil. For every conviction and impulse and desire and aversion is from within, and nothing climbs in thither."

IV.7. "Efface the opinion, I am harmed, and at once the feeling of being harmed disappears; efface the feeling, and the harm disappears at once."

Marcus Aurelius, Meditations

1. Introduction

Pain as a general phenomenon is a personal experience with evolutionary significance. Pain is a clinical syndrome that stems from multiple aetiologies in a heterogenous population (Larson et al., 2019). Somatosensation is crucial for the health, wellbeing and homeostasis of all organisms. In humans, somatosensation comprises the modalities: touch, proprioception, pressure, temperature, pain, and itch. Peripheral sensations further contribute to behaviours ranging from social interaction to danger avoidance (Harding et al., 2020). Pain sensation is important for avoidance of damage to tissues and healing; notwithstanding its homeostatic value – pain entails emotional suffering (Elman & Borsook, 2018). In fact, chronified pain (lasting longer than six months) has lost its evolutionary significance and beneficial value; thus, causing a great burden of pandemic enormity for society, healthcare systems, and the industry (Katz et al., 2015).

1.1. Peripheral Processing of Nociception

Peripheral somatosensory information is transmitted in the peripheral nervous system (PNS) by specialised receptors on primary afferent neurons (Harding et al., 2020). The peripheral neural apparatus reacts to noxious (injurious or potentially injurious) stimuli and alerts the organism (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Most investigations have studied cutaneous sensibility by electrophysiological recordings from single nerve fibres. Skin sensory receptors are the best studied receptors and allow for conducting correlative psychophysical studies between species and draw powerful inferences. Moreover, cutaneous pain has substantial clinical significance (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Information that travels along primary afferent fibres (with cell bodies in the dorsal root ganglia) reaches the dorsal horn of the spinal cord (Harding et al., 2020). Nociception includes afferent activation in neuronal pathways responsible for detection or reflexive response to noxious stimuli (Elman & Borsook, 2018). Primary afferent fibres are classified into four types, depending on their myelination and conduction velocity (Aα, Aβ, Aδ, and C); originally, they were also subdivided based on their modalities of sensory information (Harding et al., 2020; McGlone & Reilly, 2010; Roberts & Elardo, 1986). Aα and Aβ fibres are responsible for the transmission of proprioception. Aβ fibres conduct mechanical information. Aδ and C fibres carry nociceptive and thermal information. In addition, nociceptors can be subclassified based on their response properties to distinctive molecular markers (membrane receptors) (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Potential injurious agents for living organisms can be in thermal, mechanical, and chemical forms of energy, and nociceptors are usually responsive to several modalities (polymodal), while some are more specialised. Thus, nociceptors exhibit sophisticated biology and heterogeneity. Each nociceptor possesses a respective receptive field that has been standardly localised by use of mechanical stimuli. Subsequently, investigators applied additional stimulus modalities to develop a nomenclature characterising C-fibre mechano-heat-sensitive (CMH) nociceptors and A-fibre mechano-heat-sensitive (AMH) nociceptors (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). It was shown later that, most mechano-heat-sensitive nociceptors were also responsive to chemical stimuli, which characterised CMH and

AMH as polymodal nociceptors (Davis et al., 1993; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Assigning a particular stimulus modality to nociceptors is often biased by the failure to apply stimulation at appropriate intensity (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). This bias has been exemplified following the description of mechanically insensitive afferents (MIA), which were overshadowed by the more prominent and easier to detect mechanically sensitive afferents (MSA).

1.1.1. C-Fibre Nociceptors

CMHs are widespread dermal afferents and their stimulation evokes burning pain sensation (Cavanaugh et al., 2009; Harding et al., 2020; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013), information about itch, and low-threshold, pleasant mechanical stimuli (Harding et al., 2020; Jing Huang et al., 2018; Ikoma et al., 2006; Olausson et al., 2002; Wooten et al., 2014). The area of the C-fibre receptive field correlates with the size of the animal; e.g., macaque monkey: 15-20mm^2 (LaMotte & Campbell, 1978) and human 100mm^2 (Schmidt et al., 2002). Non-human primate thermal experiments have shown that the C-fibre response increases monotonically with stimulus intensity over 41-49°C; which reflected the human pain threshold (LaMotte & Campbell, 1978; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Stepped heat stimulation causes two types of heat responses: quick C (QC) with peak discharge during the rising phase; and slow C (SC) with discharge peak during the plateau phase (Johanek et al., 2008; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Electrophysiology and thermal modelling studies have shown that CMHs' heat threshold depends upon the temperature at the penetration of the receptor and not the rate of temperature increase (Tillman et al., 1995). Moreover, heat stimuli transduction into action potentials occurs at various skin depths for different CMHs (Tillman et al., 1995; D. Yarnitsky et al., 1992); and suprathreshold responses of CMHs vary directly with the rate of increase in temperature (Tillman et al., 1995; D. Yarnitsky et al., 1992). Importantly, when skin is stimulated with stepped heat, the temperature increases in the subsurface levels more slowly due to thermal inertia. The difference between surface temperature and the temperature at the receptor depth correlates directly with depth and indirectly with time. CMH terminals vary widely in depth and the actual heat thresholds are reached only when the temperature increase rate is very gradual or when the stimulation duration is very long (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). When tested with gradually increasing or prolonged temperature application; the heat threshold of CMHs lies within the tight margin between 39 and 41°C (Tillman et al., 1995).

C-fibres are the smallest primary afferent fibres and are not myelinated, thus, their conductance is the slowest. C-fibres also have the highest activation threshold and select for nociceptive or 'painful' stimuli. Aδ- and C-fibres can be termed collectively: nociceptors or 'pain fibres' (D'Mello & Dickenson, 2008). The stimulation pattern also plays a pivotal role in the determination of CMHs' response. Sensitisation and fatigue are commonly detected artefacts (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Fatigue is exemplified by the lower response to the second of two identical heat stimuli; and depends upon the interval between stimuli. This effect has also been recorded in human subjects (LaMotte & Campbell, 1978) and also following stimulus of different modality (cross-modal mechanical or electrical first stimulus) (Peng et al., 2003; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.1.2. A-Fibre Nociceptors

Another classification of A-fibres subdivides them into four categories: alpha (α), beta (β), gamma (γ), and delta (δ). Aα-fibres are not involved in pain processing and are the major transmitter of proprioceptive information (Harding et al., 2020; Maxwell & Bannatyne, 1983; Maxwell & Riddell, 1999; Mears & Frank, 1997). Aβ-fibres have large diameter and are highly myelinated. They have low activation thresholds and respond to light touch and convey tactile information to their central terminals (D'Mello & Dickenson, 2008). Aβ- fibres transmit the bulk of innocuous tactile information (vibration, pressure, touch, and texture) (Burke et al., 1975; Cavanaugh et al., 2009; Harding et al., 2020). Aδ-fibres have smaller diameter and are only thinly myelinated, thus slower-conducting than Aβ-fibres. Their activation thresholds are also higher than Aβ-fibres. Aδ-fibres carry a mixture of noxious and innocuous tactile and cold information (Arcourt et al., 2017; Harding et al., 2020; Koch et al., 2018; L. Li et al., 2011). Aβ- and Aδ-fibres can respond to thermal and mechanical stimuli (D'Mello & Dickenson, 2008). The conductance capacity of Aδ-fibre afferents is considered more robust than C-fibres but of similar character. Their discharge frequencies are also higher and they supply more input to the central nervous system (CNS) (Slugg et al., 2000).

There are two types of A-fibre nociceptors (D. D. Price & Dubner, 1977; Treede et al., 1998). Type I fibres are polymodal nociceptors and respond to mechanical, thermal, and chemical cues (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Type I fibres have high thermal thresholds (>53°C) when short-duration stimuli are applied (Burgess & Perl, 1967). Threshold determination with long-duration heat stimulation of type I AMHs has reported 45-50°C range (Treede et al., 1998). Type I AMHs can be histologically identified in glabrous and hairy skin (Slugg et al., 2000). Type II A-fibre nociceptors have poor response properties to mechanical stimulation, which complicated early identification studies. Modern electrophysiology has revealed that in fact type I and II A-fibre nociceptors have similar distribution in the hairy skin of primates but do not occur in the glabrous integument of the palm (Davis et al., 1993). Type I fibres evoke a gradually increasing response to heat stimulation and sensitise to burn and chemical injury, thus mediating hyperalgesia (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Type II fibres are similar to CMHs in their response to heat stimulation: with a pattern of an early peak frequency and gradually adapting response (Tillman et al., 1995). Type II A-fibres are the putative nociceptors responsible for signalling 'first pain' sensation in response to heat (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.1.3. Nociceptors and Thermal Pain

Microneurographic studies in awake human individuals have permitted to correlate the discharge of afferent fibres and the reported sensations of the subjects. Thereby, a microelectrode was inserted percutaneously into the fascicles of nerves (e.g., superficial radial nerve). It has been concluded that, nociceptors in humans and monkeys demonstrate similar properties. The function of CMHs to carry painful information has been supported by experiments where intraneural stimulation of CMHs in humans elicited pain (Torebjörk & Ochoa, 1980); activation of CMHs in awake humans was achieved at temperatures identical to the temperatures reported as painful (Van Hees & Gybels, 1981). Finally, individuals rated painful sensations over the temperature range 39-51°C and the recorded CMHs responses were found linearly correlated (Torebjork et al., 1984).

Importantly, human pain thresholds were reported as the level at which pain sensation was first reported when heating the skin linearly (Marstock technique) (Fruhstorfer et al., 1976). Yet, faster rates of

temperature shift cause the estimation of lower heat pain thresholds (Tillman et al., 1995; David Yarnitsky & Ochoa, 1990).

In glabrous skin of the palm, CMHs, warm fibres, and type I AMHs conduct painful signals (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). CMHs and warm fibres carry the response to short-duration heat (≤5 seconds) at temperatures around the pain threshold (i.e., ca. 45°C) (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Warm fibres are highly responsive to gentle warming of the skin and conduct warmth sensation (Darian-Smith et al., 1979). Nevertheless, warm fibres do not respond monotonically over noxious temperature ranges (45-49°C). Moreover, psychophysical studies in humans validate monotonic increase of pain with stimuli between 40 and 50°C. As CMHs respond monotonically over the same range, they are considered the principal mediator of heat pain to the glabrous skin of the palm (LaMotte & Campbell, 1978).

Experiments in 1981 with long-duration, intense heat stimulation (53°C, 30 seconds) of the glabrous skin of the palm of monkeys and humans, compared the activation of CMH and AMH fibres (Meyer & Campbell, 1981). Human subjects rated the sensation of pain at ca. 20 units on the scale from 1 to 30. Yet, when individual fibres were examined, it was found that CMHs discharged within the first 5 seconds of the stimulus and then adapted to a lower level until the end of the 30 second trial. On the contrary, type I AMHs were unresponsive before the 5 second mark and then discharged vigorously until the end of stimulation (Meyer & Campbell, 1981).

Indeed, nociceptor activation may serve as a good estimate for pain response to heat but this is not always true. Sometimes, low-level discharges in nociceptors do not lead to sensation (Cervero et al., 1993; Van Hees & Gybels, 1981). There are central mechanisms at play, such as: attentional and emotional states, that certainly serve a crucial role in pain perception and the involvement of nociceptors thereof. Furthermore, clearly, pain signalling can be achieved via different receptors and their involvement may be critical for pain perception (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.2. Central Processing of Pain

Historically, the involvement of brain regions in pain perception has been elusive. Already in 1911, Head and Holmes have shown that patients with impairments of the cerebral cortex were still able to perceive pain (Head & Holmes, 1911). In contrast, their contemporaries Dejerine and Roussy, described syndrome of central post-stroke pain in 1906 (Klit et al., 2009). By the 20th century, it was evident that the composite nature of pain comprises both sensory features and emotional and motivational components (Casey et al., 2000). By the late 1980s, it was clear that several regions of the brain were involved in pain processing; namely: the limbic (M. Catherine Bushnell et al., 2013), paralimbic (Ong et al., 2019), and sensory areas (anterior cingulate cortex, insular cortex, and primary and secondary somatosensory cortices) (M. C. Bushnell et al., 1999). Nevertheless, scarce animal and human data of cerebral cortex involvement in pain perception sustained the view that pain was exclusively a subcortical phenomenon (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.2.1. Spinal Cord Mechanisms of Pain

The spinal dorsal horn is divided into six parallel laminae, that extend from the superficial to deep dorsal horn (D'Mello & Dickenson, 2008; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The first classification was performed in cat spinal cord slices and depended upon size and packing density of the neurons (cytoarchitectonics) (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The dorsal horn has long been recognised as a key relay for somatosensory processing (Harding et al., 2020; Molander et al., 1984; Rexed, 1952). This speculation was further supported by the advent of the Gate Control Theory, proposed by Wall and Melzack in 1965 (Melzack & Wall, 1965). They proposed the existence of a system that permits innocuous stimuli to affect the transmission of noxious stimuli to the brain and that these stimuli were not separated into distinct networks (Harding et al., 2020). They postulated the existence of inhibitory interneurons in the superficial dorsal horn, which control the incoming sensory signals prior to their transmission to the brain (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Since then, the spinal cord dorsal horn has been subject to extensive research. The dorsal horn consists of four types of neurons: (i) the central terminals of primary afferent axons, which penetrate into different zones based on diameter and type of stimulus to which they respond; (ii) interneurons, which remain within the spinal cord, either terminating locally or extending into other spinal segments; (iii) projection neurons that proceed rostrally in white matter and eventually reach the brain; and (iv) descending axons from higher brain regions that modulate nociceptive information transmission (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

Most C-fibres arborise the superficial laminae I-II, with few extending into deeper layers (Cavanaugh et al., 2009; Harding et al., 2020). Aα-fibres arborise mainly laminae IV-VI and the ventral horn; whereby, contributing to sensory-motor loops (Harding et al., 2020; Maxwell & Bannatyne, 1983; Maxwell & Riddell, 1999; Mears & Frank, 1997). Aβ-fibres relay signals to the cuneate and gracile nuclei via the dorsal column; they also collateralise into the dorsal horn, arborising laminae III-V (Cavanaugh et al., 2009; Harding et al., 2020; Niu et al., 2013). Aδ-fibres mainly terminate in laminae I and V. There is also a part of Aδ-fibres that conduct low-threshold mechanosensation and terminate within lamina III (Harding et al., 2020). The dorsal horn is subjected to descending modulation from higher brainstem regions (e.g., rostral ventromedial medulla and locus coeruleus); these connections mainly serve inhibitory function (Gebhart, 2004; Harding et al., 2020; Ren & Dubner, 2002). Brainstem opioidergic, adrenergic, and cannabinergic descending modulation is able to inhibit dorsal horn neurons, reduce primary afferents' release of neurotransmitters, and ultimately affect pain behaviours (Harding et al., 2020; Junting Huang et al., 2019; Ossipov & Gebhart, 1986; Porreca et al., 2002). Additionally, descending serotonergic inputs play a complex role and transmit primarily pro-nociceptive information (Heinricher et al., 2009). The exact system-level functioning of the descending modulation of dorsal horn sensory processing is still unclear (Harding et al., 2020). Figure 1 depicts a schematic representation of the fundamental interconnectedness of the nociception relay system (Harding et al., 2020).

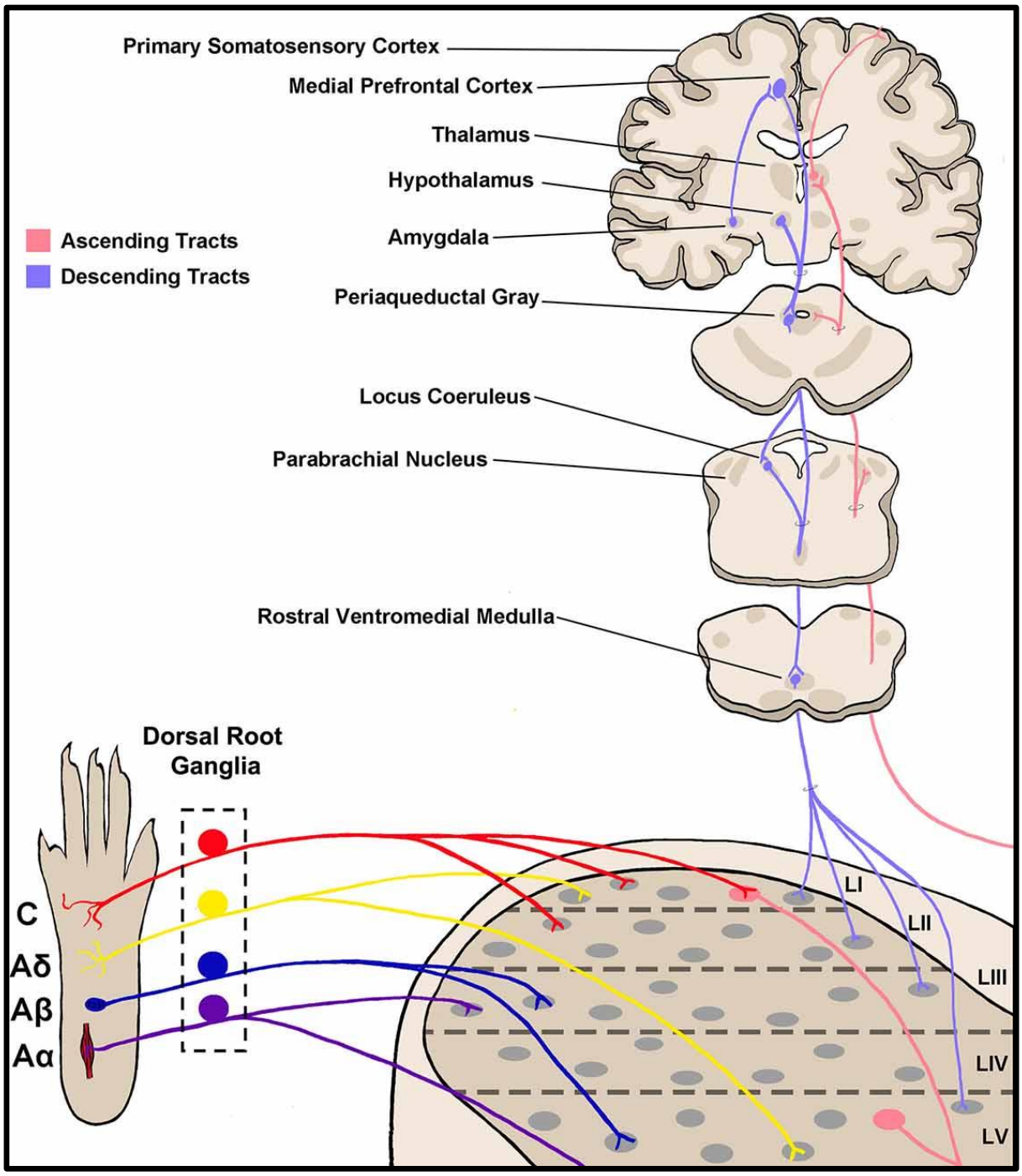

Figure 1. Peripheral and central networks involved in somatosensation. Primary afferent fibres transmit information to the spinal dorsal horn. The four types of primary afferent fibres (Aα, Aβ, Aδ, and C) are classified based on transduction velocity and somatosensory modality. These fibres arborise specific laminae of the spinal dorsal horn (Koch et al., 2018; McGlone & Reilly, 2010; Roberts & Elardo, 1986). Subsequently, projection neurons form ascending tracts that reach the brain (Niu et al., 2013). Thereby, axons decussate at the level of the spinal cord and ascend contralaterally towards the thalamus and areas in the brainstem. Descending projections target superficial dorsal horn laminae and lamina V (J. -F Bernard et al., 1993; D'Mello & Dickenson, 2008). Figure adapted from (Harding et al., 2020).

1.2.2. Ascending Projection Systems

As abovementioned, information about noxious events is transmitted from peripheral nociceptors through to the dorsal horn, and ascends in nociceptive pathways to the brain. The most prominent ascending nociceptive pathways reach the forebrain *via* the lateral spinothalamic tract and indirect spinobulbar projections that passage through brain stem homeostatic sites. The cortex receives nociceptive information *via* several areas in the thalamus (e.g., posterior part of the ventral medial nucleus; ventral caudal part of the medial dorsal nucleus; and ventral posterior inferior nucleus) (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Most findings associating certain pathways with pain processing derive from anatomical and functional properties, and studies in behaving animals and human patients (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The most important ascending pathways involved in pain processing reach sites in the brain stem and thalamus (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Other sites (e.g., cerebellum, lateral reticular nucleus, inferior olive, and tectum), primarily integrate sensory-motor information. The ascending tracts that convey painful information to the brain are direct thalamic projections: spinothalamic tract (STT) and trigeminothalamic tract; direct projections to medullar and brain stem homeostatic regions: spinomedullary and spinobulbar projections; and direct hypothalamic and ventral forebrain projections: spinohypothalamic tract (SHT) (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Indirect pathways that reach the forebrain *via* the brain stem include: the post-synaptic dorsal column and the spinocervicothalamic pathway. Similarly, pathways originating from the trigeminal sensory nuclei in the medulla represent facial structures (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.2.3. Pain Aversion Circuits

The responses to an acute nociceptive stimulus include freezing, pallor, diaphoresis, hypertension, tachycardia, tremulousness, and preponderance of the adrenomedullary, as related to noradrenergic stimulation (Elman & Borsook, 2018; Feinstein et al., 1954; Goldstein, 1995). This is considered an adaptive reaction as epinephrine improves memory consolidation (Elman & Borsook, 2018; McIntyre et al., 2012), and augments coping with extreme situations by enhancing 'gating' (i.e., activating descending pathways) of noxious stimuli from reaching conscious awareness (Elman & Borsook, 2018; Mendell, 2014). Hence, to instate swift and automated responses to hazardous situations, a large proportion of the nociceptive system remains sub- or unconscious (Baliki & Apkarian, 2015; Elman & Borsook, 2018).

Theoretically, pain has been subdivided into three distinctive categories. In 1968, a seminal paper by Ronald Melzack and Kenneth Casey observed pain as a sum of the 'sensory-discriminative' aspect which consisted of the sense of the intensity, quality, duration, and location of pain; the 'affective-motivational' aspect, responsible for the unpleasantness of pain; and the 'cognitive-evaluative' quality of pain which was determined by higher cognition represented by cultural values, distraction, appraisal, and hypnotic suggestion (Moayedi & Davis, 2013).

Pain transpires once modulatory gating is exceeded; thus, in acute occasions, attention is drawn to the bodily effects of the noxious stimulus. Chronic pain, conversely, is frequently experienced in the absence of palpable tissue injury (Elman & Borsook, 2018). Regardless the cause, pain aversion represents non-nociceptive ill feelings. This reverberates the core phenomenological status of pain as the most potent signal of imminent harm (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Underscoring this emotive content are two principal features: conscious recognition of unpleasantness and induction of behaviours

which aims to terminate current and reduce future painful occurrences (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Most recently, great advances have been achieved in understanding the peripheral and spinal mechanisms of nociception (Basbaum et al., 2009; Hunt & Mantyh, 2001; Woolf & Ma, 2007). Yet, very little is known about the ways in which the brain integrates this input and initiates pain perception and behaviours (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). A systems-level understanding of pain behaviour requires a perception that traverses three distinct levels, proposed by David Marr in 1983 (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The first level represented the computational problem facing organisms in pursuit of self-preservation; the second level comprised the solution in algorithmic terms, used by the organism; and finally, the third level represented the way solutions are implemented in the brain as neuronal networks, neurotransmitter systems, and other processes. Ultimately, pain phenomenology derives from processes that subsume all three levels (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The pain system is a complex of perceptual, cognitive, and affective processes and involves coordinated engagement of manifold systems. Moreover, behaviour can be manifested highly variably depending upon the injurious agent and various environmental and physiological contexts (Eccleston & Crombez, 1999; Fields, 1999; Donald D. Price, 2000; Villemure & Bushnell, 2002; Wiech et al., 2008). Notwithstanding, the existence of core processes that surpass the diversity of pain experiences is not questioned (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

Regardless the type of noxious stimulation, hardwired neural networks facilitate suitable physiological responses that start as adaptive behavioural and autonomic changes within the acute context, and transcend into learned avoidance behaviour (Chiang et al., 2019). The literature provides a large and steadily growing body of work that points to the parabrachial complex (PBN) as an evolutionarily conserved hindbrain structure that links an array of threats to a fitting configuration of physiological and behavioural responses (Jean Francois Bernard & Bandler, 1998; Chiang et al., 2019; Fuller et al., 2011; Fulwiler & Saper, 1984; Kaur et al., 2013; Morrison & Nakamura, 2011). The parabrachial nucleus (PBN) has established as a ‘hub’ for pain, aversion, and homeostatic control due to its extensive involvement in homeostasis under stressful or threatening circumstances (Chiang et al., 2020; Palmiter, 2018; Saper, 2016); food neophobia, hypercapnia, protective behaviours (Campos et al., 2018; Chamberlin & Saper, 1998; Chiang et al., 2020; Kaur et al., 2013, 2017); and regulation of ingestive behaviour and thermoregulation (Chiang et al., 2019). Moreover, the PBN has been indicated as integral in nociceptive behaviour and long-term behavioural alteration in response to pain (Campos et al., 2018; Chiang et al., 2020; Han et al., 2015). Thus, the PBN must process convoluted exteroceptive and interoceptive signals and evoke autonomic regulation of behaviour (Chiang et al., 2019, 2020).

The PBN is a cluster of neurons, inclosing the superior cerebellar peduncles in the dorsolateral pons. In rodents, the PBN can be divided into more than a dozen subnuclei, based upon cytoarchitecture (Chiang et al., 2019; Fulwiler & Saper, 1984). The medial PBN (mPBN) comprises of neurons of similar size and morphology; while, lateral PBN (lPBN) comprises several homogenous assemblages. The cytoarchitectonic division of neurons in the m- and l-PBN are also associated with differential connectivity and neurochemistry (Chiang et al., 2019). The lPBN is densely innervated by ascending spinal projection neurons; while the same input is sparse in the medial and dorsal PBN segments and overlapping the visceral afferent input from the solitary nucleus (Burton et al., 1979; Craig, 2003). LPBN afferent projections are weakly topographically organised (Feil & Herbert, 1995). Spinal projections to the lPBN originate mainly in

lamina I, and sparsely in laminae IV-VI. Rodent studies have shown that the lPBN is robustly connected to brain stem reticular formation cells that control homeostasis and autonomic integration (Chamberlin & Saper, 1992). The lPBN gives rise to projections to the hypothalamus, amygdala, midline and intralaminar thalamus, and parts of the ventrobasal thalamus that relays to the insular cortex for visceral sensory activity (J. -F Bernard et al., 1993). Nociceptive projections from the lPBN to the amygdala or hypothalamus have response characteristics similar to lamina I neurons and allow for nociceptive activity, forebrain autonomic, neuroendocrine, and emotional control integration. Lamina I input allows for nociceptive activity, forebrain autonomic, neuroendocrine, and emotional control integration (J. F. Bernard & Besson, 1990). Figure 2 depicts a schematic of the lPBN neuronal pathways (Chiang et al., 2019).

Most lPBN neurons are glutamatergic, with a small but significant population of GABAergic neurons (Geerling et al., 2017). A vast array of peptides colocalises with both populations, such as: calcitonin gene-related peptide (CGRP), substance P, neurotensin, and dynorphin (Chiang et al., 2020). An estimate of 80% of all lamina I ascending projection neurons that reach the lPBN express neurokinin 1 (NK1) receptor for substance P (D'Mello & Dickenson, 2008; Todd, 2002). Substance P is a neuropeptide expressed by nociceptive afferents and thereby signifies these cells' responsiveness to noxious stimuli (D'Mello & Dickenson, 2008; Doyle & Hunt, 1999; Mantyh et al., 1997). Lamina I cells which express NK1 project to the thalamus, periaqueductal grey, and particularly to the lPBN (D'Mello & Dickenson, 2008; Todd, 2002).

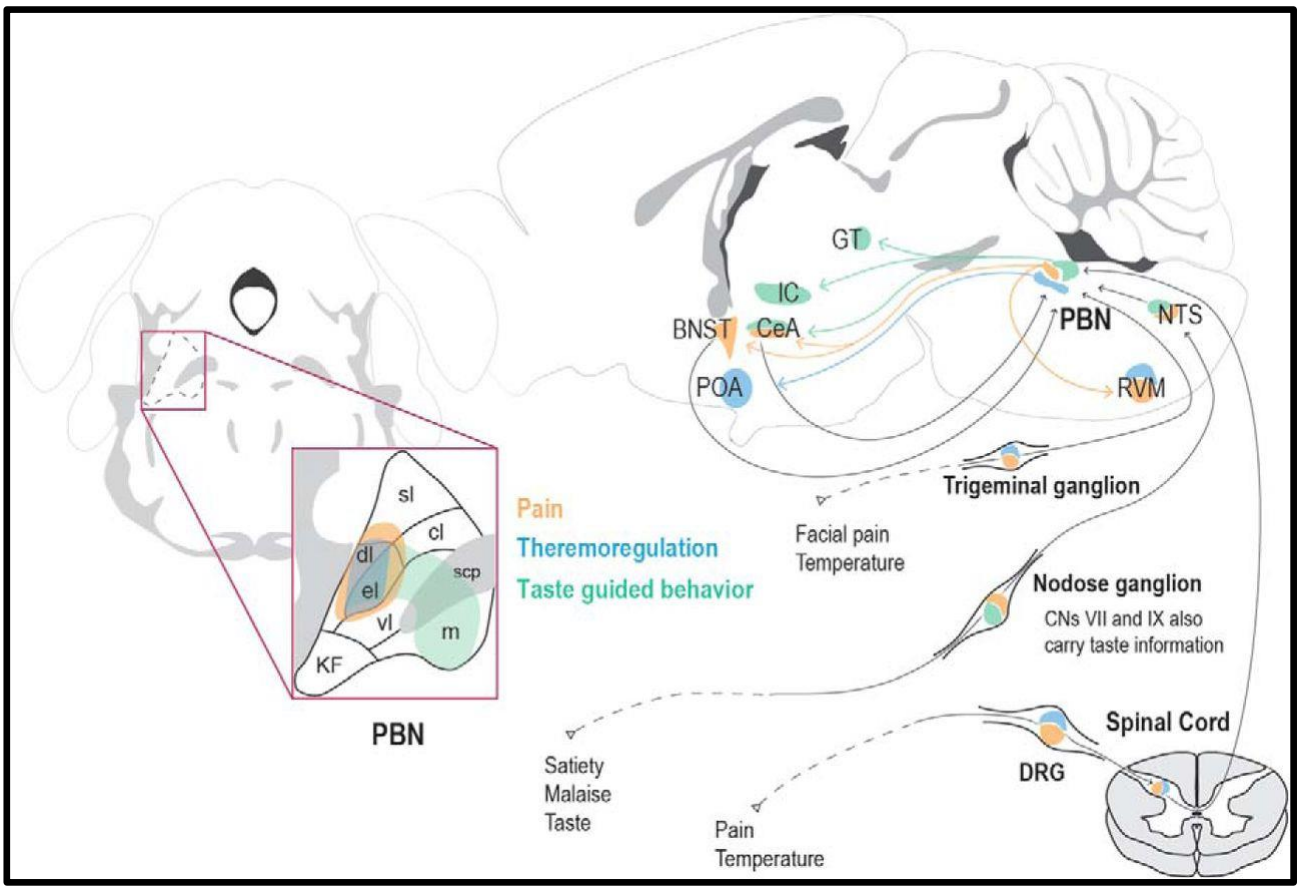

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Figure 2 The parabrachial complex as a hub for pain aversion. Afferent sensory information about temperature, noxious stimuli, and taste from primary neurons reaches distinct subnuclei within the PBN. The mPBN, processes gustatory information. The lPBN mediates extero- and intero-ceptive information that corresponds to painful (orange) or thermal (blue) neuronal innervation. The PBN is subdivided into: sl, superior lateral; vl, ventral lateral; cl, central lateral; m, medial; dl, dorsal lateral; el, external lateral. CeA, Central amygdala; KF, Koellliker Fuse; POA, preoptic area; scp, superior cerebellar peduncle; IC, insular cortex. Figure adapted from (Chiang et al., 2019).

1.3. Opiates and Their Effect on Pain Aversion

Clinical reports have evidenced that opioid-induced pain modulation has differential effect upon the sensory and emotional aspects of pain. As previously described (Chapters 1.2.1 and 1.2.2), pain is transmitted *via* secondary neurons that terminate in supraspinal structures. Many of these loci express high levels of opioid receptors (Jensen, 1997; Vogt et al., 1995). Moreover, many of these areas overlap the limbic system and particularly the Papez neuronal circuit (*cf.* MacLean (Chapman, 1996)); which is extensively involved in the processing of emotions (Jensen, 1997). The differential effect of morphine was supported by studies with chronic pain patients and volunteers for acute thermal stimuli delivered with a contact thermode (Kupers et al., 1991; D. D. Price et al., 1985). In both studies participants were administered morphine sulfate intravenously. The former study tested four doses (0.00; 0.04; 0.06; 0.08 mg/kg) and the latter study – 0.3 mg/kg morphine or placebo saline. Both studies used affective visual analogue scale (VAS) responses and revealed dichotomous effect of morphine upon the affective and sensory modalities of pain. Finally, human imaging studies have also reported a more selective action of morphine upon the medial pain system than the lateral pain system (Simon & Hiller, 1973; Vogt et al., 1995). Altogether, data from clinical studies, report the ability of morphine to alleviate the affective component of pain; while the sensory modality remained intact and was affected at higher doses. This observation was extended to several forms of chronic or acute pain and causal agents (e.g., heat-induced first and second order pain and central and peripheral neuropathic pain) (Kupers et al., 1991; D. D. Price et al., 1985).

Seminal experiments which employed two types of anti-nociceptive responses in animal models; namely, reflexive measures (e.g., tail flick following stimulation, Section 1.4) or organised types of pain responses (e.g., paw licking, flinching, guarding, favouring, Section 1.4), have investigated the effect of opioids on aspects of behaviour, controlled supraspinally; such as: attention, aversion, or affect (Jensen, 1997). In 2008, van der Kam *et* al., compared reflexive and aversive responses of rats following application of systemic morphine (van der Kam, Vry, et al., 2008). In the study, morphine was administered to animals at doses from 0.01 to 10 mg/kg. The animals were treated intraplantaly with carrageenan to induce an inflammatory pain model and tested in two behavioural paradigms. The paradigms compared on the one hand the reflexive response to acute noxious stimulation and on the other hand the learned putatively-aversive behaviour in a conditioning paradigm (van der Kam, Vry, et al., 2008). Interestingly, the findings of the study reported rescue of pain aversive behaviour at a minimal effective dose (MED) of 0.03 mg/kg; that dose stood below the dose required to activate the rewarding system (at 1 mg/kg) and affect reflexive reactions (also at 1 mg/kg) (Rutten et al., 2011; van der Kam, Vry, et al., 2008). Moreover, the findings from the van der Kam animal study were in agreement with preclinical and clinical studies, reporting a negative shift in the doses activating reward in a pain state (Narita et al., 2005; Suzuki, 2001; Suzuki et al., 1998).

1.4. Methods Used to Evaluate Pain Behaviours in Rodents

Studying pain in humans is difficult to perform, subjective, and limited by ethical considerations. Therefore, the pathophysiological mechanisms of pain are investigated in animal models, mostly in rats and mice (Deuis et al., 2017; Mogil, 2009). Anthropomorphisation of the perception of pain in animal models is subject to challenges, uncertainty, and criticism. Indeed, nociception is an essential physiological function but reactions to stimuli do not necessarily indicate pain experiences. (Deuis et al., 2017; Sandkühler, 2009). Therefore, pain cannot be measured in animals directly; the putative pain experience is inferred from pain-like behaviours like withdrawal of a body part, reduced ambulation, agitation, increased grooming, and vocalisations. Consequently, nociception and pain must be related to non-communication and communication subjects, respectively (Deuis et al., 2017). Moreover, as pain cannot be inferred directly, analgesia and analgesic interventions cannot be considered; rather, anti-nociception and anti-nociceptive interventions can be (Deuis et al., 2017). This caveat has motivated the development of indirect methods to quantify and evaluate pain-like behaviours in a sensitive, reproducible, specific, and reliable fashion (Deuis et al., 2017; Mogil, 2009).

Most behavioural methods provide data that is often variable between laboratories and somewhat subjective (Deuis et al., 2017). This is usually the case because behavioural responses tend to lie on a spectrum of intensity but are scored as binary (present/absent). Moreover, experimenters cultivate individual insight into what constitutes a behaviour on the spectrum and this causes variability between laboratories. This issue is often alleviated by using appropriate randomisation, allocation concealment, blinding and standardised protocols and procedures (Deuis et al., 2017; Hirst et al., 2014). Nociception in rodents can be measured with two types of behavioural methods: stimulus-evoked and non-stimulus-evoked.

1.4.1. Stimulus-evoked Nociception

1.4.1.1. Mechanical Stimuli

Pain-like behaviours in response to mechanical stimuli, their presence and extent, are commonly determined using manual or electronic von Frey or the Randall-Selitto (R-S) test. The von Frey test was developed to evaluate allodynia in mice and rats, and has been established as the gold standard for determination of mechanical thresholds in these species (Deuis et al., 2017). During the experiment, animals are placed in small enclosures with penetrable bottom (usually a mesh). Then, monofilaments are applied perpendicularly to the plantar surface of the hind paw until buckling of the filament is observed. At buckling, the monofilament delivers a pre-determined force for 2-5 seconds (Deuis et al., 2017). A positive response is reported if the animal exhibits nocifensive behaviours: brisk paw withdrawal, licking or shaking of the paw, during or immediately after application of the stimulus (Deuis et al., 2017). Standard, commercial monofilament kits contain filaments with pre-determined forces and the increment is logarithmic and not equally spaced (Deuis et al., 2017). An alternative to the force discrepancy is the electronic von Frey where the force is applied increasingly by a single non-bending filament and measures on a continual scale (Deuis et al., 2017). Disadvantages of the von Frey method arise from the variable number of measurements per animal in different testing protocols that use the up-down method and the prerequisite of repeated, time-intensive measurements, which promote sensitisation or learnt responses (Deuis et al., 2017).

The Randall-Selitto (R-S) test is a paw pressure test commonly used to evaluate response thresholds to mechanical pressure stimulation and measure mechanical hyperalgesia (Deuis et al., 2017; Randall & Selitto, 1957). In this test, a mechanical calliper is used to apply increasing pressure to the surface of the paw or tail until withdrawal or vocalisation occurs (Deuis et al., 2017). The R-S test is more suitable for rats, as it involves heavy physical restraining which is not tolerated by mice (Anseloni et al., 2003; Deuis et al., 2017; Minett et al., 2011, 2014; Santos-Nogueira et al., 2012). The R-S produces reliable data only after the animals have been extensively habituated to the restraining method and apparatus, which is very time consuming. Moreover, the withdrawal response is visually detected by the experimenter and can lead to subjective measurement of the threshold and some researchers argue that withdrawal threshold can be considered a measure of spinal reflex, not relevant for investigations of pain aversion; and thus favouring vocalisations (Deuis et al., 2017; Kayser & Christensen, 2000; Santos-Nogueira et al., 2012; Charles A. Winter & Flataker, 1965).

1.4.1.2. Heat Stimuli

The tail flick test was first developed in 1941 and involved the use of heat stimulus to the tail of mice or rats to measure the time required for the animal to 'flick' or twitch its tail (D'Amour & Smith, 1941; Deuis et al., 2017). There are two alternatives for application of heat: either radiant heat centred on the tail, or immersion of the tail into a hot water bath. Both require restraining of the animal (Deuis et al., 2017). While quick and easy to conduct, behavioural responses to the tail flick test can be observed in spinally transected rats; implying that the tail withdrawal response was in fact spinal reflex and not indication of pain behaviour involving higher brain centres (Deuis et al., 2017; Irwin et al., 1951). Additional drawbacks of the tail flick test arise from the variation of the skin and ambient temperature, the location of stimulus application, and learnt avoidance behaviours. (Berge et al., 1988; Deuis et al., 2017; Yoburn et al., 1984).

The hot plate test was first developed in 1944 and used to estimate heat thresholds in rats and mice (Deuis et al., 2017; Woolfe & MacDonald, 1944). Tests that utilise application of heat to the hind paws are considered to involve supraspinal pathways, as spinally-transected rats failed to withdrawal the hind limbs in the hot plate test (Deuis et al., 2017; Giglio et al., 2006). The traditional hot plate test requires the animal to be placed onto a metal plate that is heated to a constant temperature between 50 and 55°C; and the time required to observe nocifensive behaviour is recorded (response latency or number of responses) (Deuis et al., 2017). Depending on animal species and strain an ethogram with a minimum of 12 different nocifensive behaviours have been described; such as: sniffing, stamping of the legs; leaning; jumping; licking; freezing; and grooming (Deuis et al., 2017; Espejo & Mir, 1993). The hot plate test associates with a number of drawbacks, namely: confounding effect of learnt behaviour, all four paws are exposed to the plate surface which interferes with results of unilaterally treated animal (Deuis et al., 2017; Menéndez et al., 2002).

Finally, the Hargreaves test was first described in 1988 and used radiant, infrared focused beam stimulation to quantify heat thresholds in the hind paws of rats or mice (K. Hargreaves et al., 1988). Thereby, an animal is placed into a small enclosure with transparent floor and stimulation of either hind paw commences with radiant, infrared heat source. The heat intensity of the beam focused onto the plantar surface of the hind paw increases over time and the time taken to withdrawal is recorded manually as the withdrawal latency. The starting intensity of the beam must be adjusted in naïve animals to produce withdrawal latencies of 10-

12 seconds (Deuis et al., 2017). This method allows for establishment of internal control by testing both ipsi- and contra-lateral hind paws of test animals. Moreover, the method does not require restraining the animals which reduces stress and time of the experiment (Deuis et al., 2017). The Hargreaves test records the withdrawal latency of the paw but does not report an exact temperature applied to the skin (Deuis et al., 2017).

1.4.1.3. Cold Stimuli

Similar to the hot plate test, the cold plate test, monitors an animal's response to noxious temperature. In the cold plate test the metal surface is pre-cooled to -5 to 15°C and the animal's nocifensive reactions (jumping, licking, shaking) determine the response time (Allchorne et al., 2005; Deuis et al., 2017). This test can be used with rats and mice and provides an indirect measure of cold-induced hypersensitivity.

The acetone evaporation test investigates pain-like behaviours generated by evaporative cooling and measures cold hypersensitivity (Colburn et al., 2007; Deuis et al., 2017; Vissers & Meert, 2005). The test is performed in an enclosure with mesh floor where acetone is dabbed or sprayed onto the plantar surface of the hind paw; thereby, producing cooling to 15-21°C (Deuis et al., 2017; Leith et al., 2010). There are two ways of experimental scoring, namely: recording the withdrawal latency of the stimulated paw, or scoring the severity of the response (e.g., 0, no response; 1, brisk withdrawal or flick; 2, repeated flicking; 3, repeated flicking and licking; (Colburn et al., 2007)) (Deuis et al., 2017). The drawbacks of the acetone test are: response may be too fast and require slow motion video recording; acetone produces nocifensive responses in naïve animals despite its innocuous character (most likely due to olfactory or auditory cues (Deuis et al., 2017).

1.4.1.4. Chemical Stimuli

Certain chemicals may act as irritants and cause pain by local administration to skin or organs. The mode of action may either be by direct activation of free nerve endings of nociceptors and/or by promoting inflammatory or toxic reactions. The effect duration is usually protracted, as opposed to acute physical stimulation (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Most investigations study the immediate effect (e.g., licking, favouring, flinching, biting) upon the affected paw, and quantification utilises alterations in threshold to mechanical or thermal stimulation, as described above (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

The formalin test is the most commonly used method and causes irritation and pain-like behaviour in a number of species, including humans and rodents (Raboisson & Dallel, 2004; Tjølsen et al., 1992; Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The formalin test is characterised by its two-phasic action, namely: an acute first phase during the first 10 minutes, following intraplantar or intradorsal injection into rat's hind paw; and late, second phase: 20-60 minutes post-injection. Typically, the test concludes 1 hour post-injection but attenuated hypersensitivity has been reported to last for up to 4 weeks and inflammation progresses past behavioural data collection (Fu et al., 2001).

The writhing test consists of injection of a strong irritant (e.g., acetic acid) directly into the peritoneum of an animal that causes writhing and stretching which are quantified based on the number of episodes or accumulated time of the behaviour. This test is rarely used as the strain differences in sensitivity may be significant (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

Topical application or injection of substances can induce transient pain and subsequent hypersensitivity (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). A good example of this approach is the intraplantar injection of capsaicin in rat or mice's hind paw. Capsaicin selectively activates the transient receptor potential vanilloid 1 (TRPV-1)-expressing nociceptors and experiments with this compound can validate mechanistic and translational hypotheses in several species, such as: rat, mouse, monkey, and humans (Butelman et al., 2004; Chizh et al., 2007; Joshi et al., 2006).

Additional compounds that can be injected into animals' tissues and produce inflammatory reactions are the complete Freund's adjuvant (CFA); kaolin-carrageenan; or carrageenan (CAR) alone. The inflammatory reaction observed following their injection causes the development of oedematous hindpaws (particularly noticeable and prolonged for CFA).

1.4.2. Non-Stimulus Evoked Nociception

Stimulus-evoked tests that measure nociception in animal models often face poor translation into the clinic. Therefore, spontaneous pain tests incorporating an efficacious endpoint, model human conditions more precisely and increase the clinical significance of animal experimentation (Deuis et al., 2017; Mogil, 2009).

Grimace scales provide a mean to score the subjective intensity of pain (Deuis et al., 2017; Langford et al., 2010). This method scores facial features correlated to the severity of perceived 'pain'. The scale is accurate but only at high levels of nociception (e.g., writhing in response to intraperitoneal application of a strong irritant; second phase of the formalin test; post-surgical pain; or intraplantar injection of zymosan or mustard oil). While a 'pain face' can be observed in tests of medium length (e.g., acetic-acid induced writhing, post-surgical pain, second phase of formalin test, and after intraplantar injection of zymosan or mustard oil); this method is not appropriate for short-lasting nociception (e.g., tail clip or flick tests) or long-lasting (e.g., chronic constriction or spared nerve injury) (Deuis et al., 2017; Langford et al., 2010). The grimace scale method faces limitations in that investigators need extensive training and practice and incorporate high degree of subjectivity and variability (Deuis et al., 2017).

Rodent burrowing is a spontaneous and self-motivated behaviour and suitable as proxy for nociception (Andrews et al., 2012; Bryden et al., 2015; Jirkof et al., 2010). The standard assay measures the amount of burrowing substrate removed from a predefined burrow. Namely, prolonged and later onset burrowing, indicates hyperalgesic state. An advantage of this method is the objective end of the experiment and requires minimal experience and invasion from the experimenter. Variations of the classical burrowing experiment exist, namely: nesting construction, marble burial test and food hoarding (Deuis et al., 2017).

Weight bearing and gait analysis can be used as surrogate measure of nociception. The static weight bearing (incapacitance) assays report the weight distribution between the hind paws of an animal placed onto inclined holder equipped with two independent pressure sensors. This test is suitable only for unilateral hind paw pain models (Deuis et al., 2017). Gait analysis allows the detection of changes in limb locomotion and positioning (Deuis et al., 2017). Both tests were originally designed to improve translation into the clinic; yet, correlative studies failed to reflect parameters from rodent (anti-)nociception studies to von Frey test results (Deuis et al., 2017).

Pain-like states can be achieved in behaving animals with models of neuropathic pain. Neuropathic pain is caused by pathology of the nervous system, such as: infection (e.g., herpes zoster), nerve trauma,

compression, and autoimmune disease (Colleoni & Sacerdote, 2010). Neuropathic pain models can be significantly protracted and even irreversible. Neuropathic pain is caused by damage or disease and often following trauma or metabolic derangement, exposure to toxins, ischaemia. The most common approaches to induce neuropathic pain employ peripheral nerve injury models, anti-cancer-therapeutics, and artificially-induced diabetes (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). A commonly used polyneuropathic model is the streptozotocin-induced diabetic neuropathy model. As neuropathy is a dose-limiting side effect of chemotherapy, standardly used anti-cancer agents can be used to induce polyneuropathic pain in laboratory animals; namely: vincristine, paclitaxel, cisplatin, oxaliplatin (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). Alternatively, mononeuropathic models of pain exist that target a single nerve, most often the sciatic nerve (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013). The most commonly used models are: the chronic constriction injury (CCI) model; the spinal nerve ligation (SNL) model; and the partial sciatic nerve ligation (PSL) model (Wall, P. D., McMahon, S. B., & Koltzenburg, 2013).

1.4.3. Conditioning Paradigms

The vast majority of pre-clinical pain research focuses on reflex-based withdrawal responses or mammal nocifensive behaviours following acute noxious stimulation. Yet, this approach has shown little predictive validity and poor translation into the clinic (J. X. Li, 2013). Great criticism has been directed toward withdrawal- or reflex-based measurements; therefore, several conditioning paradigms have been proposed for the measurement of the affective-motivational dimension of pain (J. X. Li, 2013). Motivation is the driving force that incentivises organisms and presents vigour to overcome obstacles and achieve goals. Motivated behaviour has multiple behavioural and neural components that can be separated into two categories: reward-gain based and aversion avoidance/escape-based motivation (Kelley, 2004; Natsubori et al., 2017; Robinson & Berridge, 2013; Salamone & Correa, 2002; Tsutsui-Kimura, Bouchekioua, et al., 2017; Tsutsui-Kimura, Takiue, et al., 2017). The former behaviour entails the presentation of a positive/desirable occasion or opportunity; while the latter is instigated by a negative/undesirable predicted or actual event (Campese et al., 2016; Elliot, 1999; Elliot & Thrash, 2002; Tsutsui-Kimura, Bouchekioua, et al., 2017).

The first reports of established conditioned place preference (CPP) were published in 1976 and reported ability of rats to discriminate between two conditions (Weissman, 1976). CPP is a well-validated and simple test that measures ongoing and paroxysmal spontaneous pain (J. X. Li, 2013). The generated place preference to a compartment paired with an analgesic compound is assumed to represent ongoing or spontaneous non-evoked and non-reflexive pain (Gregory et al., 2013). Conditioned place aversion (CPA) was first used to evaluate the emotional component of pain in 2001 (Johansen et al., 2001). Unlike CPP, during CPA tests, investigators actively administer a painful stimulus (e.g., algogenic chemicals) to the animal and associative learning is produced by pairing of this stimulus to a specific location (J. X. Li, 2013). CPP and CPA are fundamentally different in that CPA questions the aversive quality of the present pain; while CPP questions the existence of pre-existing (ongoing) pain. Therefore, CPA can only be conducted with short-lasting painful manipulations (J. X. Li, 2013).

The place escape/avoidance paradigm was described by LaBuda and Fuchs in 2000, and was used to measure aversion to pain (Labuda & Fuchs, 2000). This paradigm makes use of rodents' natural avoidance of open bright spaces and pairs the protected dark chamber with aversive stimulation. The time spent in the bright chamber serves as a proxy for pain aversion (Labuda & Fuchs, 2000). Thereby, animals are placed in a

novel behaviour arena and allowed to freely move. The arena is placed over a metal grid floor that allows for the stimulation of the hind paws of the animal. Following induction of unilateral plantar pain model, stimulation of the hind paw commences with a certain mechanical pressure stimulus applied with a vF monofilament. The two chambers are assigned as pain-paired (i.e., stimulation of the affected paw) and pain-free (i.e., stimulation of the unaffected contralateral paw). Over the course of the experiment the animals develop a dislike to one side of the chamber. Naturally, rodents prefer closed dark spaces and therefore the pain-paired chamber is usually covered and not illuminated; while the pain-free chamber is brightly illuminated and open. This contributes to the robustness of the results, but implies an additional conflict between natural instincts and learned pain-aversive behaviour.

1.5. Rationale and Synopsis of the Project

The main purpose of developing tests that measure nociception and anti-nociception in model organisms is the elucidation of fundamental neuronal mechanisms that underlie pain processing and to assess various compounds for analgesic properties in the hope for innovation in the pain alleviation field and translation into the clinic. Current animal models have been widely criticised and deemed inappropriate, not sensitive enough, or ineffective to study pain (Bars et al., 2001; J. X. Li, 2013; Mogil, 2009; Mogil & Crager, 2004; van der Kam, De Vry, et al., 2008). Contemporary preclinical nociception research is hindered by the ineptness of the commonly used paradigms to distinguish sensory and affective pain perception. This, by far, has been caused by the use of reflex-based evoked nocifensive paradigms with poor predictive validity. Certainly, pain is an amalgam of its sensory and affective aspects and should be treated and studied as an entirety, but it is worthwhile to improve and develop tests that study pain affect. Certain compounds have been shown to illicit far superior relief of affective pain, compared to sensory (e.g., 30-fold difference for morphine, (van der Kam, De Vry, et al., 2008)); therefore application of appropriate tests may broaden the variety of molecular foci and introduce or reinstate compound classes for treatment of pain.

The main subject of this project focused upon development of a set of test paradigms that assay aversion to pain in rats. For that purpose, a novel device was designed and produced. Results produced with the punctate heat probe (PHP) were compared with the current leading paradigm: the place escape avoidance paradigm (PEAP). Subsequently, the PHP was implemented in original tests for measurement of pain aversion. Thereby, pain aversion was inferred both by the time spent in a chamber paired to thermal stimulation of an intraplantar pain model; and by reduction of thermal thresholds required to induce complex behaviour. Ultimately, a differential effect of morphine upon the sensory and aversive pain was documented within the novel paradigms. Finally, a different pain model was used to confirm the applicability of the novel test paradigms and the PHP. Overall, a robust method for examination of the aversive component of pain was developed, examined, and confirmed effective.

2. Materials and Methods

2.1. Materials

All consumables, substances, and technical equipment used in this project are listed in Table 1.

Table 1. List of consumables and technical equipment

	Device/reagent/material	Vendor	Cat. #
Consumables			
1	Neutral wipes	Penaten	3319NC-B2
2	Gloves StarGuard Touch	Starlabs	SG-T-L
3	Λ-Carrageenan	Sigma	BCBP8978V
4	Dimethylsulfoxide (DMSO)	Sigma	BCBQ8161V
5	AP-5	Sigma	207-987-9
6	CNQX	Abcam	APN10087-2-11
7	LPS form *E.coli* 0111:B4	Sigma	011M4008V
8	Pentobarbital	Exagon	0919803AE
9	Diethylether	Roth	5920.3
10	Ethanol	Roth	CP43.3
11	Distilled H_2O	Qualilab	2156754
12	Acetic acid glacial	Fisher Chemical	1853615
13	Syringes 0.01-1 mL	Braun	20H17C8
14	Needle 24G x 1’’; 0.55 x 25mm BL/LB	Stericom	19K23C8
15	Needle 30G x ½’’; 0.30 x 12 mm BL/LB	Braun	19D01C8
16	Physiologic solution	Fresenius kabi	19LG12GA
Animal Medications			
1	Baneocin-Salbe (Bacitracin Zinc/Neomycin)	Sandoz	474919A
2	Bepanten Plus Crème (Dexpanthenol (5%)/ Chlorhexidine-Dihydrochlorid (0.5%))	Bayer	1-19149
3	Fenistil	GSK	1800738
4	1 mg Dimetindenmaleat	Ugo Basile	37450-275
Equipment			
1	Sonicator	Bandelin	DT-52-H
2	Scales	Ohaus	DX224
3	Chemical hood	Schneider	EN14175
4	Vaporiser	Penlon	
5	Vortex	Ika works Inc.	IP21
6	Pipettes	Eppendorf	2480129/ 2642173/ H17152F/ I3233OC
7	Peristaltic pump	Ismatec	ISM9401E

8	Luxometer		LM37,90505881
9	HD camera	Imaging source	09020146
10	Spotlight	Briloner leuchten Gmbh	BN153491
11	Light dimmer	Emil Lux Gmbh	311545
12	Hargreaves	Stoelting	72808-390GS
14	Animal scale	Soehnle	65840
15	Von Frey monofilament kit	Ugo Basile	
16	External calibrated thermometer	Greisinger electronics	GMH 3750

The behaviour arenas were produced in the house at the CBR's workshop by Mag. Raphael Holzinger.

The arena used for real-time place avoidance (RTPA) and heat escape threshold (HET) experiments had dimensions 58.5 x 31.5 cm and height 28 cm. Rats were individually tested on the Hargreaves apparatus and each cage had dimensions 21 x 21 cm and height 28 cm. The dark/light box arena used in the place escape/avoidance paradigm had dimensions 52 x 31.5 cm and height 25.5 cm. The separator elliptical arch between the two compartments had horizontal diameter 28 cm on the level of the mesh and apex of the arch 12.5 cm. The holes in the metal mesh floor were 0.5 x 1 cm parallelograms.

2.2. Methods

2.2.1. Animal Behaviour

2.2.1.1. Animal Housing

Male Sprague-Dawley rats (200-400 g) were housed in pairs on a 12-h light dark schedule (light cycle: 6 am – 6 pm) under standard conditions (21± 2°C, 55 ± 10% relative humidity) with *ad libitum* access to food and water. Animals were provided by the Institute for Experimental Animal Breeding of the Medical University of Vienna (Himberg, Austria); Janvier Labs (Route du Genest, 53940 Le Genest-Saint-Isle, France); or house-bred at the Centre for Brain Research Vienna. Animals were allowed to acclimatise to the home cages for a minimum of 14 days before experimentation. The experiments were conducted in accordance with guidelines set by the Ethical Committees for the use of laboratory animals at the Medical University of Vienna and the Austrian Ministry for Science and Research (BMWF-2020-0.001.560) and conform to the standards specified by the European Union (directive 2010/63/EU).

2.2.1.2. Inflammatory pain models

Inflammation was induced in deeply anaesthetised rats (5% isoflurane delivered in 5-7 l/min^{-1} oxygen flow) *via* single unilateral intraplantar (i.pl.) injection of 100µL 0.5% carrageenan (CAR) into the left hind paw. Intraplantar injections were performed with gauge 24 needle and the paw cleaned with 70% ethanol. Control rats were shortly anaesthetised with 5% isoflurane. Prior to experiment start the rats were allowed to recover from the anaesthesia in their home cages and were monitored regularly. CAR was prepared in 8.8 mM concentration by mixing powdered CAR and 0.9% saline, then vortexing and sonicating at 45°C until homogenous. All compounds were stored at 4°C short-term.

2.2.1.3. Behavioural paradigms

2.2.1.3.1. Hargreaves test (HG)

The Hargreaves procedure spanned over five consequent days. On the first two days, the animals were habituated to the experimenter for at least 5 minutes per animal and the animals' tails were marked; to the room with the HG apparatus switched on for at least one hour and then to the HG apparatus for 30 minutes. Next, on days 3 and 4, baseline measurements were taken. Thereby, three animals were placed into individual enclosures over a glass floor and allowed to acclimatise for 30 minutes. Then, each animal received focused-beam stimulation with 70 AI intensity of the focused light beam; 3 times to each paw with an inter-trial interval of at least 5 minutes between two measurements from the same rat; alternating between left and right paw for a total of six stimulations. The last day started with injection of CAR into the left paw of the animals and after 3 hours, the test procedure was repeated as during the baseline sessions.

2.2.1.3.2. The punctate heat probe (PHP)

The punctate heat probe (PHP) is an electrical device with standard power supply requirements (input: 100-240V ~50/60Hz 1A; output: 12V ⎓ 3A). The PHP prototype was designed and manufactured in collaboration with IST Austria in Klosterneuburg. The PHP consists of four separate integral parts: a standard EU plug that connects to an AC/DC adaptor; the temperature controller box; and the copper punctate heat probe itself. The purpose of the PHP is to measure thermal pain responses by delivering a precise and accurate thermal heat stimulus to the animal's hind paw; in particular, the PHP was designed to deliver heat stimuli that could reliably induce escape behaviour in rats (this could not be achieved with the Hargreaves radiant beam, probably due to its gradual slope; the hot-plate was also not considered a good option since the rat can guard the paw and then not be motivated to escape and avoid a heated plate).

The PHP can be set to a fixed temperature. The controller unit is equipped with a digital display reporting the set temperature at the base of the probe (the wider metal part, Figure 3). Following thermal calibration of the probe it was determined that the actual temperature at the tip of the PHP was on average 4.5 degrees lower than the indicated on the dial; however, the size of the difference between the set temperature and the actual temperature is strongly correlated to the set temperature, such that: actual temperature = set temperature x 0.86 + 2.4°C. This means that when one sets the temperature to 30°C, the actual temperature is 28.2°C, and that every 5° change in set temperature produces and actual change of 4.5°C. It takes 20-30 seconds to increase 1°C and 30-50 seconds to decrease 1°C; cooling can be accelerated by gently rubbing the PHP with a dry cloth. In several rare experimental events the temperature shift of the PHP had to alternate over a range of 30 degrees (i.e., from 55 to 25 displayed units). This was observed in animals that had received CAR in the left paw during the CAR-induced hypersensitivity tests. To assess the ability of the PHP to achieve this change, an empirical calibration was conducted. Thereby, the time required for cooling from 55 to 25 control panel displayed units was 8 minutes. As the time between stimulations was 2.5 minutes, the PHP was cooled by rubbing with cold hands and in rare occasions by wrapping in a plastic glove and submerging in cold water. The use of dry ice for cooling of the probe was also possible but not applied in this project. Heating of the PHP over large temperature ranges was achieved in less than 2 minutes due to the logarithmic nature of heat transfer.

Figure 3. The contact thermode and the temperature controller unit of the punctate heat probe. The upper panel depicts three foreshortenings of the punctate heat probe. The bottom panel depicts the display of the control unit, the temperature adjustment buttons, and the inlet for the PHP. The temperature can be adjusted by using the three buttons underneath the display. Pressing and holding the leftmost button (Δ1) enters control mode. The set temperature and the °C symbol start alternating on the screen. The two buttons to the right can then be used to set the required temperature. The left button (arrow down) reduces the temperature and the right increases it. The range used for behavioural experiments was between 30 and 70°C (as reported by the monitor, i.e., actual temperatures of 28.2-62.6°C). Once the required temperature has been set, all buttons can be released. The External/Internal switch should always be kept to the 'Internal' side as this defines that the relative temperature (set on the display) should be measured at the contact thermode and not inside the control box.

To use the PHP in behavioural experiments the behaviour arena must be placed on a mesh grid floor. The holes of the mesh were 5 x 10 mm parallelograms and allow for the tip of the probe to reach the paw of the tested animal. The illumination of the arena was specific for different types of experiments and the sessions were recorded with the overhead camera, linked to the Viewer software (Biobserve Gmbh.; version 3.0.1.448, Germany). The PHP has been used in real-time place avoidance tests (RTPA), place escape/avoidance paradigm (PEAP), and determination of heat escape threshold (HET) tests. Each experiment was planned differently but the practical use of the probe was always identical. First and foremost, the experimental animals must be as calm as possible when placed in the behaviour arena. Usually, the animals become agitated by the approach of the experimenter's hand and the probe. Therefore, the approach must start 4-5 seconds prior to stimulation. The hand must be as steady as possible at all times. It is advised to wait for the animal to calm down on all four paws not exploring the arena or rearing. The application of the stimulus must be as gentle as possible (only slightly touching the skin) and without applying pressure (as e.g., with vF filaments). The stimulus is applied for 5 seconds and then the probe is slowly and gently retracted. Escape behaviour is determined by relocation of all four paws within the 5 seconds of stimulation, and after a minimal stimulation time of 1 s. Note that often the animal can be startled by the approaching probe and move prior to stimulation. Sometimes the animal became scared by the sound of the probe touching the edges of the rhomboid holes of the arena's metal mesh floor. This touch and/or rubbing was avoided. The latter two cases excluded recording escape behaviour; the experimenter then waited until the animal calmed down and only then resumed measurement. The PHP usually remained clean after experiments and was only cleaned with a dry paper towel as liquids could cause corrosion and copper could form patina which could change the conductivity of the metal. The mesh floor and the arena was cleaned by removing faeces and urine, then cleaned with neutral smell wipes.

2.2.1.3.3. Place escape/avoidance paradigm (PEAP)

The PEAP was conducted in a behavioural arena with two compartments and a metal mesh floor; one compartment was covered and dark, while the other compartment had an open roof and was illuminated from above (light intensity of 400-420 Lux). The dark chamber was paired with noxious stimulation (stimulation of the i.pl. CAR-injected paw). In case this stimulation was aversive to the animals this led to a preference shift from the dark to the light chamber over time. During habituation, each animal was habituated to the test room as well as to handling by the experimenter. Fuchs et al. emphasize, that it is important the animals are habituated well to the test room, for 1-1.5 hours every day before handling (Labuda & Fuchs, 2000). Animals were taken out of the cage and shortly carried from one room to the other, and trained to be taken up and trained for their placement into the test chamber. Each animal was tested for 30 min; therefore, the treatment of consecutive rats (CAR/isoflurane) was spaced by at least 40 min. Then, during testing, in the light side of the chamber, the right (uninjected) paw and in the dark chamber the left (CAR-injected) paw was stimulated with a 60g von Frey filament (inter-stimulation interval of 15 seconds). Stimulation started directly after the animal was placed into the chamber, and lasted for 30 min (according to stopwatch). PEAP was also conducted with the use of the heated probe. This experiment was identical to the original PEAP, except that stimulation with the vF monofilament was replaced by application of the PHP set at 41.1°C directly and locally to the plantar surface of the rats' paws.

2.2.1.3.4. Real-time place avoidance (RTPA)

The RTPA test is a variation of PEAP which omits the use of a dark compartment and wherein application of painful stimulus is paired with one chamber while the other chamber remains neutral. The same arena used for PEAP was divided into two equally sized chambers with distinct olfactory and visual cues, namely, the left chamber was cleaned with 30% ethanol and had circular pattern on the walls; the right chamber was scented with 1% acetic acid and had striped pattern on the walls. The walls of the arena were cleaned with neutral scented wipes. One day before test commencement the animals were habituated to the handler for minimum of five minutes and for at least an hour to the behaviour suite. On the next day, baseline sessions were recorded; whereby, the animals were allowed to freely explore the arena for 5 minutes. The next 15 minutes of the test were with stimulation every 30 seconds, as follows: stimulation of the left paw was always in the chamber occupied by the rat at the end of the first 5 minutes of free exploration of the arena, the contralateral paw was stimulated exclusively in the opposite chamber. On the subsequent test day, the animals were injected with an inflammatory agent (CAR) and three hours later, the test commenced; the chamber in which the rat was at the end of the first 5 minutes was assigned as 'pain chamber' and there the left paw of the animal was stimulated either with the heat probe set at 45.4°C, the other chamber was 'pain free' and the right paw was stimulated. In tests with application of intraperitoneal morphine (see below), the animals were injected 30 minutes before test commencement. The data was recorded with the Viewer software and the development of aversion to the 'pain chamber' was evaluated.

2.2.1.3.5. Heat escape threshold (HET)

HET tests were performed a behaviour arena with identical dimensions as for RTPA, without olfactory or visual cues (i.e., the walls and floor were cleaned with neutral wipes and the walls were blank and white). One day before test commencement the animals were habituated to the handler for minimum of five minutes and for at least an hour to the behaviour suite. The animals' tails were marked for identification purposes. On the next day, baseline sessions were recorded; whereby, the animals were again habituated to the test room for at least an hour. Then, the animals were allowed to habituate to the arena for 5 minutes. The last 25 minutes of the session involved application of stimuli to the animals' hind paws. The stimulation pattern was the following: each paw was stimulated at least 5 times; left and right paws were alternated with 2.5 minutes interval (i.e., inter-ipsilateral stimulation interval 5 minutes). The first stimulation of each paw was with 41.1°C. Then the animal behaviour was observed and evaluated according to the scoring rubric. If escape behaviour was observed, the stimulation temperature was decreased by one step (4.5°C) and if no escape behaviour was observed the temperature was increased by one step. The rats were stimulated until escape behaviour was detected. On the test day, inflammation was induced with unilateral intraplantar (CAR) injection and three hours later, the animals were tested in the same way as baseline session.Experimenters with longer experience can learn to differentiate escape from exploratory behaviour. Exploratory behaviour is determined by onset late or after the stimulation with the probe. Usually, the rat sniffs and bristles with its whiskers. Escape behaviour is typically preceded by freezing. Withdrawal of the stimulated paw excludes additional stimulation and is considered an end of a stimulation round. Repetition of a stimulus can take place if exploratory behaviour was observed or escape behaviour was triggered by auditory or visual cue. Rats were sometimes distracted by change of smells; therefore, the environment in the experimental suite must be maintained stable at all times. Rats also often groom in the behaviour arena which significantly reduces their sensitivity to the PHP; therefore, the rat must not groom during or shortly before stimulation.

2.2.2. Data Analysis

2.2.2.1. Calibration of tools for mechanical and thermal stimulation

HG tests required the evaluation of the specific intensity (AI) of the focused infrared beam at which the paw withdrawal latencies (PWL) of naïve rats were within 12-15 seconds. That allowed for the detection of hypersensitivity or analgesia. Therefore, calibration tests were performed with naïve animals by applying thermal stimulation at increasing intensity until the required PWL was achieved. The stimulus application intervals were the same as the experimental (2.5 minutes alternating between paws). HET stimulation was applied according to the simplified up/down method (SUDO) described by Bonin *et al.*; only vF monofilament stimulation was replaced by thermal stimulation with the PHP (Bonin et al., 2014; Chaplan et al., 1994; Dixon, 1965, 1980). The SUDO involves the application of a series of stimuli with equally spaced doses at the appropriate scale. For SUDO, thermal stimulation was applied five times. Thermal sensitivity was determined by simply employing a constant adjustment factor (±4.6), depending on the last observed response. If withdrawal response was observed, the adjustment factor was negative and vice versa.

The punctate heat probe (PHP) was produced as a prototype by the IST Austria. The actual temperature of the PHP was empirically calibrated by measuring with an external calibrated device connected to the heat probe via aluminium block and thermal hyper-conductive paste. The temperature of the probe was tested with a super-conductive paste and a thermal voltage converter. Subsequently the thermal time constant (τ) was calculated in order to find out the time necessary for the probe to increase/decrease temperature.

Heat transfer of solid bodies was analysed with lumped system analysis. Thereby, the heat exchange at any given time is proportional to the temperature difference between the body and the ambient, numerically expressed in the following formula:

$$F = hA_s(T(t) - T_a);$$

Where h represents the heat transfer coefficient; A_s – the surface area; $T(t)$ – body temperature at time t; and T_a the constant ambient temperature. When heat is lost to the ambient, the heat transfer is expressed with the formula:

$$-F = \rho c_p V \frac{dT}{dt};$$

Where ρ represents the object's density, c_p is the specific heat and V – the body volume. Then, equating these two equations into:

$$\rho c_p V \frac{dT}{dt} = -hA_s(T(t) - T_a).$$

Another form of the above equation is:

$$\frac{dT}{dt} + \frac{1}{\tau}T = \frac{1}{\tau}T_a;$$

Or (Bahrami, 2011; Dzialowski & O'Connor, 2001; Lewis et al., 2004):

$$\tau = \frac{\rho c_p V}{hA_s} \ .$$

2.2.2.2. Statistical analysis

The raw data from behavioural experiments was statistically analysed in GraphPad Prism (Graphpad Software, Inc.; version 6.01).

PEAP data was grouped in CAR-treated and naïve/control animals. The test was 30 minutes in total and divided into six bins of 5 minutes each. The time spent in the light chamber was manually scored and expressed as a percentage of the total time for each bin. The data was grouped into columns representing the control and test animals. The percentage of time spent by each animal during the corresponding bin was entered in rows. Then two-way repeated measure ANOVA test was performed where the mean of each row was compared with the corresponding value in the neighbouring column. ANOVA multiple comparisons were corrected with Sidak's multiple comparisons post-hoc test for each bin. Thereby, the confidence intervals were computed and significance values iterated.

CAR-induced RTPA was evaluated by calculating the percentage of time spent in the 'pain' chamber by each animal during baseline and test sessions. The data was grouped into columns representing the baseline and test sessions. The percentage of time spent by each animal during the corresponding session was entered in rows. Every row contained the doses of morphine and saline control. Then two-way repeated measure ANOVA test was performed where the mean of each row was compared with the corresponding value in the neighbouring column. TW-ANOVA tested the source of variation within the test groups (control and test), between sessions (baseline and test), and their interaction. ANOVA multiple comparisons were corrected with Sidak's multiple comparisons post-hoc test for each dose of morphine or saline. Thereby, the confidence intervals were computed and significance values iterated.

HET data was analysed using a two-way repeated measure ANOVA test, with Sidak's multiple comparisons test. The data was grouped into columns representing the baseline and test sessions. The HET of each animal during the corresponding session was entered in rows. Every row contained the doses of morphine and saline control. Then two-way repeated measure ANOVA test was performed where the mean of each row was compared with the corresponding value in the neighbouring column. TW-ANOVA tested the source of variation within the test groups (control and test), between sessions (baseline and test), and their interaction. ANOVA multiple comparisons were corrected with Sidak's multiple comparisons post-hoc test for each dose of morphine or saline. Thereby, the confidence intervals were computed and significance values iterated.

HG data comprised a time course of two baseline sessions and a test session. Each dose of morphine formed a column with subcolumns for each test animal. The animals' PWLs were entered into rows that represented the specific session. Then the two-way repeated measures ANOVA test was performed, thereby, comparing the mean of every row within each column. ANOVA multiple comparisons were corrected with Sidak's multiple comparisons post-hoc test for each dose of morphine or saline. Thereby, the confidence intervals were computed and significance values iterated. All data are presented as mean ± standard error of the mean (SEM). Each comparison was reported as multiplicity adjusted value and the family-wise significance and confidence interval was set to 0.05 (95% confidence interval).

3. Results

3.1. Hyperalgesia and pain aversion were induced and detected in a rat carrageenan pain model

Inflammatory hyperalgesia was induced in male Sprague-Dawley and the animals were subsequently tested in three different paradigms that report behavioural reactions to pain. At first, the PEAP paradigm was reproduced as reported by the LaBuda group ((Labuda & Fuchs, 2000)). Thereby, the dark chamber was paired with stimulation of the affected paw and the time spent in the opposite, light chamber was recorded and side preference was statistically inferred. The unaffected paw was stimulated in the light chamber. All stimulations were applied with a 60 grammes von Frey monofilament. As in the original publication by LaBuda *et* al., the animals treated with CAR, developed a robust aversion to the dark chamber, past the ten-minute mark of the experiment (Figure 4, A). Control rats spent on average 21.43% of the total time in the light chamber compared to the pain model who spent on average 53.5% of the total time in the light chamber (Figure 4, B).

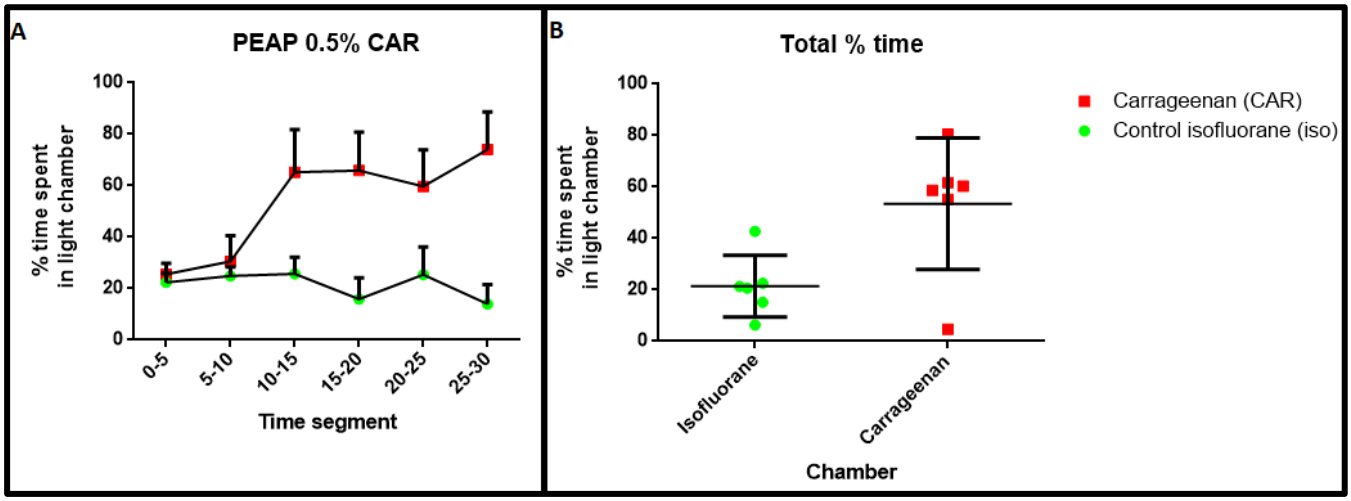

Figure 4. PEAP was established in a rat CAR pain model. A) The 30-minute timeline of the experiment, divided into six bins, each of 5 minutes in duration (x-axis); the marks represent the percentage of time spent in the light chamber (red square – CAR-treated and green dot – the control group that received 1-minute isoflurane anaesthesia). Significant difference in the time spent in the light chamber by pain model rats (13.95 ± 2.105) and control rats (74.06 ± 8.279) was detected in the last 5-minute segment (TW-ANOVA, interaction p = 0.001; time p = 0.0169; groups p = 0.0193; Sidak MC test p = 0.001 for the 25-30 segment, p = 0.0087 for 15-20, no significant difference was detected in the other bins), N=6. B) Percentage of total time, spent in the light chamber during the 30-minute session. The percentage of time spent in the light chamber by animals treated with CAR intraplantarly (53.5 ± 10.43) was significantly higher than control animals (21.43 ± 4.915) two-tailed unpaired t-test p = 0.0194, N=6.

3.2. Implementation of the PHP in pain aversion tests

CAR-treated animals spent, on average, significantly longer time in the illuminated chamber, compared to the control animals who retained a preference to the dark chamber, 58.73% versus 28.44%, respectively

(Figure 5, B). The timeline of the PEAP experiment with the punctate heat probe also reported aversion to the 'pain' chamber over the course of the experiment (Figure 5, A).

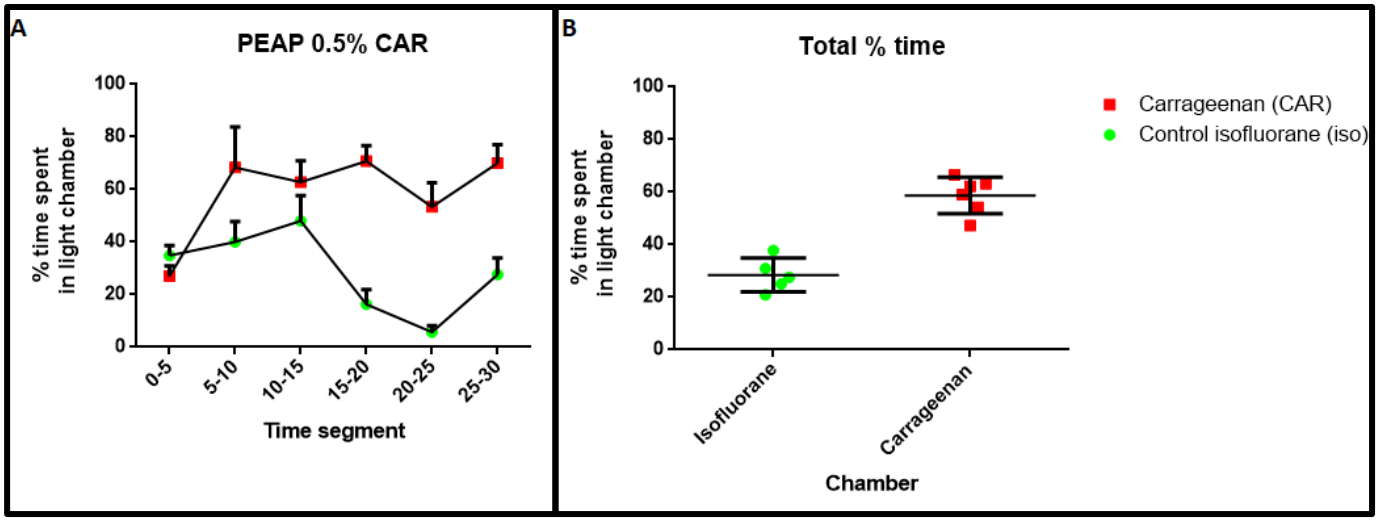

Figure 5. PEAP using the punctate heat probe. A) The 30-minute timeline of the experiment, divided into six bins, each of 5 minutes in duration (x-axis); the marks represent the percentage of time spent in the light chamber (red square – CAR-treated and green dot – the control group that received 1-minute isoflurane anaesthesia. Significant difference in the time spent in the light chamber by pain model rats (27.61 ± 2.105) and control rats (70.06 ± 3.841) was detected in the last 5-minute segment (TW-ANOVA, interaction $p = 0.0028$; time $p = 0.0045$; groups $p < 0.0001$; Sidak's MC test $p < 0.0018$ for the 15-30 segment, no significant difference was detected in the other bins), N=6. B) Percentage of total time (30 min) spent in the light chamber. Control rats spent significantly less time in the light chamber (28.76 ± 6.385), compared to rats treated with CAR (58.73 ± 6.875), two-tailed unpaired t-test $p < 0.0001$, N=6.

CAR-induced hypersensitivity was also evaluated using a method similar to PEAP that excluded the conflicting cues to rodents' innate fear of bright, open spaces. The modified RTPA was used to correlate decrease in time spent in the 'pain' chamber and pain aversion to the CAR-induced inflammation. Indeed, there was no preference or avoidance, detected in the control group of animals; while all of the CAR-treated animals spent less time in the 'pain'-paired chamber (Figure 6).

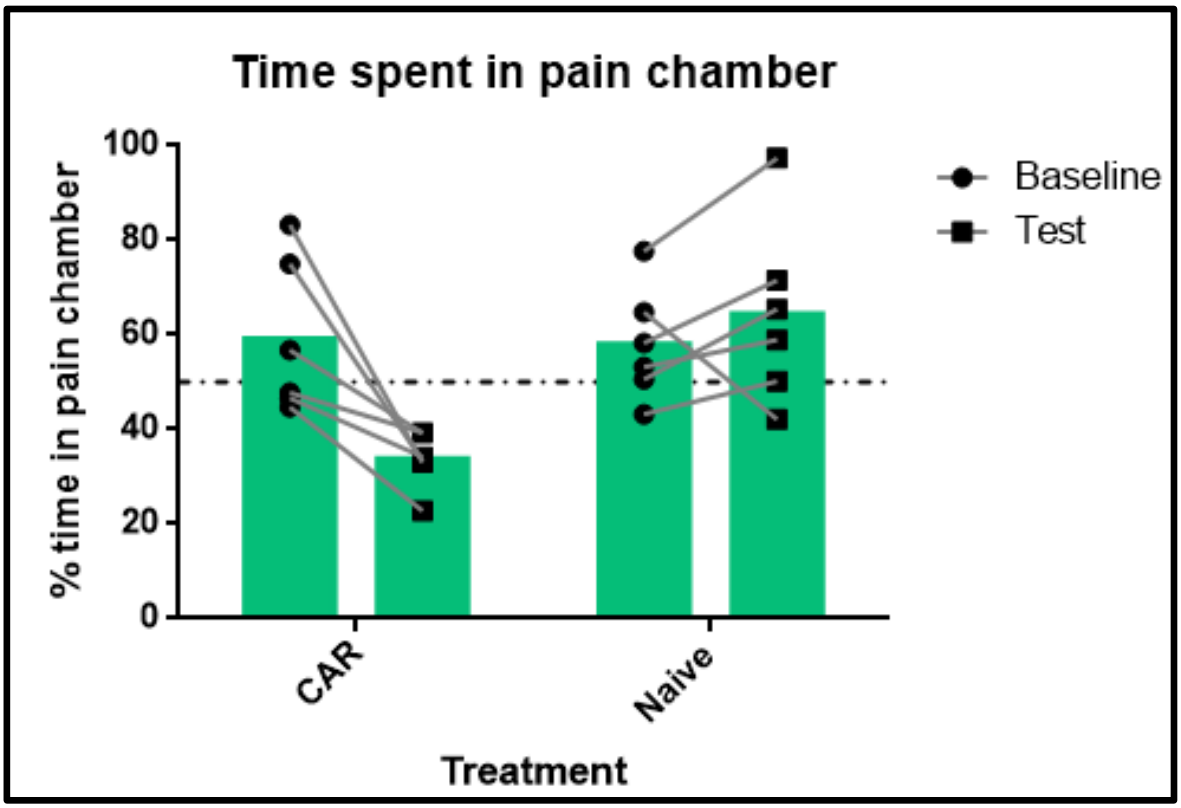

Figure 6. Intraplantar CAR injection induced real-time place avoidance (RTPA) in behaving rats. CAR-injected rats spent significantly less time in the pain-associated chamber during test session (33.653 ± 2.465), compared to baseline session (59.035 ± 6.675). TW-ANOVA found no significant variation within the groups caused by treatment or session (treatment p = 0.0572, session p = 0.0655) but significant source of variation was found when the interaction of the two factors was tested p = 0.0064, N=6; Sidak's MC post-hoc test found significant difference between the baseline and test sessions in the CAR-treated group, p = 0.006. The naïve rats spent similar time in the pain-associated chamber during test session (64.277 ± 7.892) and baseline session (57.966 ± 4.924), p = 0.5853 (N=6).

To augment the PEAP paradigm, the experimental design was adjusted so that specific temperatures that elicited aversive behaviour were summated in a similar fashion to the SUDO method. Ultimately, the HET paradigm provided an escape threshold value that was interpreted as aversion to pain. HETs were compared between the baseline and test sessions and significant aversion was observed in the group that received CAR intraplantarly. Furthermore, the control animals escaped at similar temperatures, regardless of test session or stimulated paw (Figure 7).

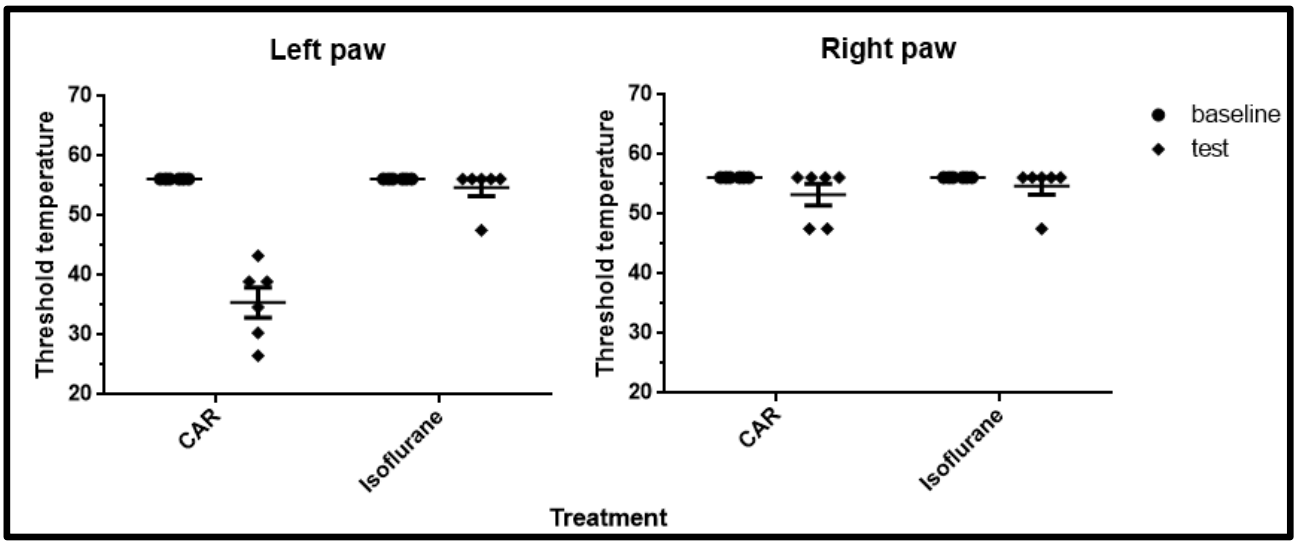

Figure 7. Heat escape threshold (HET) was reduced in a CAR pain model. The left panel compares the left paw HET values of CAR-injected and naïve control rats. The TW-ANOVA test found significant variation in the test groups caused both by the treatment (CAR) and by session (baseline/test), and the interaction of the two factors ($p < 0.0001$) Sidak's multiple comparisons test showed that for the CAR group ($n = 6$), rats escaped at lower temperatures (34.442 ± 2.530 SEM) compared to the baseline session (56.15, $p < 0.0001$); in contrast, in the control group ($n = 6$) there was no difference between baseline (56.15) and test values (54.717 ± 1.433 $p = 0.7438$, $n=6$). Right panel, contralateral paw: TW-ANOVA could not detect significant variation caused by the treatment or session ($p > 0.1739$), Sidak's multiple comparisons test showed that the escape threshold of the right paw remained stable regardless of treatment; in CAR group ($N = 6$), rats escaped at similar temperatures (56.15 ± 1.813), compared to the baseline session (56.15, $p = 0.3401$); similarly, in the control group ($N = 6$) there was no difference between baseline (56.15) and test values (54.717 ± 1.433, $p > 0.9999$, $n=6$).

During the test session of the HET experiment (Figure 7), several CAR-treated animals escaped at temperatures below the expected physiologic values for the plantar surface of the left hind paw. This was sufficient motivation to conduct several control experiments which investigated the effect of mechanical stimulation with the heat probe and for potential learning and association of the aversive stimuli with the heated probe. Overall, escape behaviour following mechanical stimulation was elicited in less than 4% of trials (9/240), and there was no significant difference between CAR-treated animals and controls, or between sessions (Figure 8).

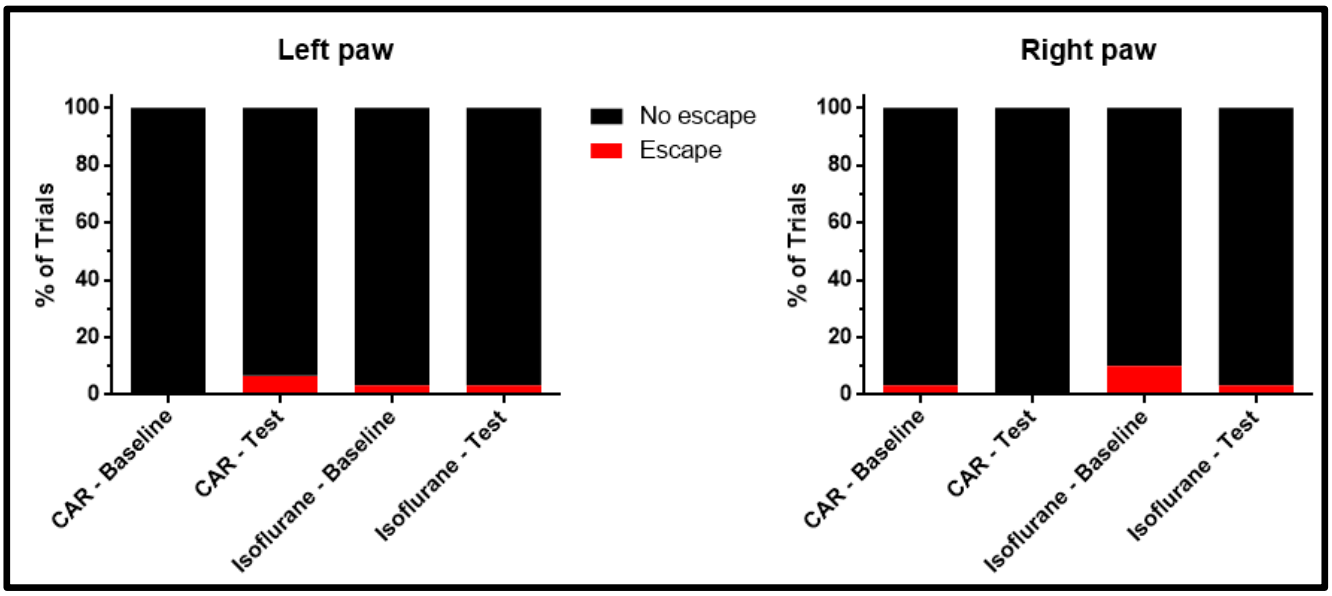

Figure 8. Escape probability following mechanical stimulation with the punctate heat probe. For each condition and paw, the % of trials in which mechanical stimulation with the probe elicited escape is shown in red. Overall, when the probe was applied at room temperature, escape was observed in 3.75% of trials.

Consequently, an experiment with application of heat in reversed increments was designed and conducted; thereby, stimulation commenced at 23.9°C followed by five stimuli as in the standard HET paradigm. The starting temperature and subsequent application pattern did not affect the HET of rats treated with CAR (Figure 9).

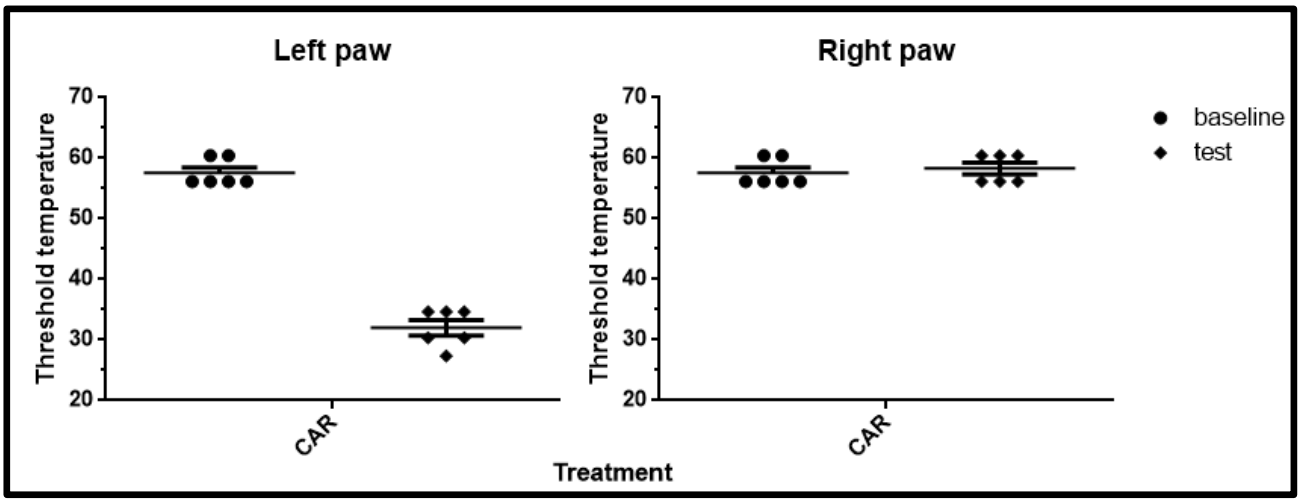

Figure 9. Reverse-increment stimulation reduced the heat escape threshold (HET) in a CAR pain model. Left panel compares the HET values of CAR-injected rats between sessions. The escape temperature during baseline session (57.583 ± 0.907 SEM) and test session (32 ± 1.267), was found significantly different (paired t-test $p < 0.0001$), N=6. Right panel compares the HET values of CAR-injected rats between sessions. The escape temperature during baseline session (57.583 ± 0.907) and test session (58.3 ± 0.962), was not found significantly different (paired t-test $p = 0.6109$), N=6.

3.3. Investigation into morphine's differential effect upon the sensory and emotional aspects of pain

Opioids and specifically morphine have been reported to exhibit dichotomous effect upon the pain experience. Namely, reports have observed that the effect of morphine upon the aversive aspect of pain is superior compared to the sensory. This was concluded following dose-response behavioural experiments. Thereby, alleviation of pain aversion was achieved at doses that did not affect the sensory-motor aspect of the pain experience or activate the mesolimbic pathway (van der Kam, Vry, et al., 2008). To confirm this observation and verify the applicability of the punctate heat probe in experiments investigating anti-nociceptive and analgesic properties of substances a series of experiments were conducted.

At first, the effect of morphine upon the nocifensive-reflexive reactions of animals was evaluated with the Hargreaves test. During HG tests, the animals were heavily habituated to the experimenter and the test apparatus. Baseline recordings were conducted to ensure PWL stability at the set light intensity parameters. On the test day the pain model was induced prior to application of morphine. Notably, intraplantar injections were applied under light isoflurane anaesthesia while intraperitoneal injections of morphine were in awake animals and performed by the same experimenter with a gauge 30 needle. Thereby, a cut-off dose of morphine was elucidated; at which rat reflexes were attenuated. Namely, systemic application of 1 mg/kg morphine had little effect on the paw withdrawal latencies; while, at higher dose (3mg/kg) PWLs were significantly reduced and identical to the contralateral, unaffected paw (Figure 10).

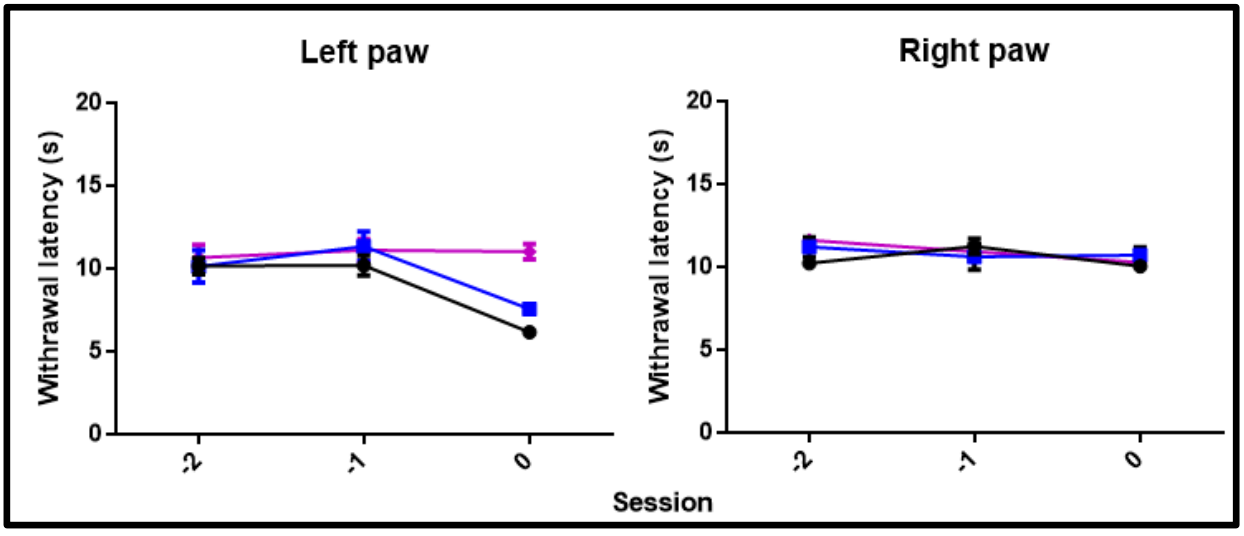

Figure 10. Differential effect of morphine upon CAR-induced reflexive hypersensitivity. Left panel depicts the experimental time course of the left paw of the three test groups. TW-ANOVA reported significant variation within the treatment groups, the test session, or their interaction $p < 0.0173$, $n = 6$. Sidak's MC test revealed that animals receiving 3 mg/kg morphine (purple line and symbol) on the test day had withdrawal latencies (11.082 ± 0.298), identical to the baseline sessions (10.598 ± 0.521, $p = 0.9954$. The group treated with 1 mg/kg morphine (blue line and symbol) had significantly lower withdrawal latencies during test session (7.648 ± 0.108), compared to the baseline sessions (10.395 ± 0.649, $p < 0.0001$), $N = 6$. The withdrawal latencies of the control group (black line and symbol) were significantly different between the test (6.057 ± 0.154) and baseline (10.342 ± 0.391, $p < 0.0001$) sessions, $n = 6$. The right panel depicts the time course of the right paw of all animals (same colour scheme). The withdrawal latencies remained stable regardless of treatment or session.

Subsequently, the effect of morphine was tested in the pain aversion-assessing paradigms: RTPA and HET. RTPA tests reported a significant reduction of time spent in the pain chamber following induction of the CAR inflammatory pain model, irrespectively of whether injected with saline, 0.1, or 0.5 mg/kg morphine intraperitoneally before the test. The effect of morphine upon pain aversion was also tested at 1 and 3 mg/kg and was able to restore the time spent in the 'pain' chamber to percentages similar to the naïve baseline (Figure 11). Thus, the 1 mg/kg dose was ineffective to reverse hypersensitivity (HG test) and able to alleviate pain aversion (Figure 11).

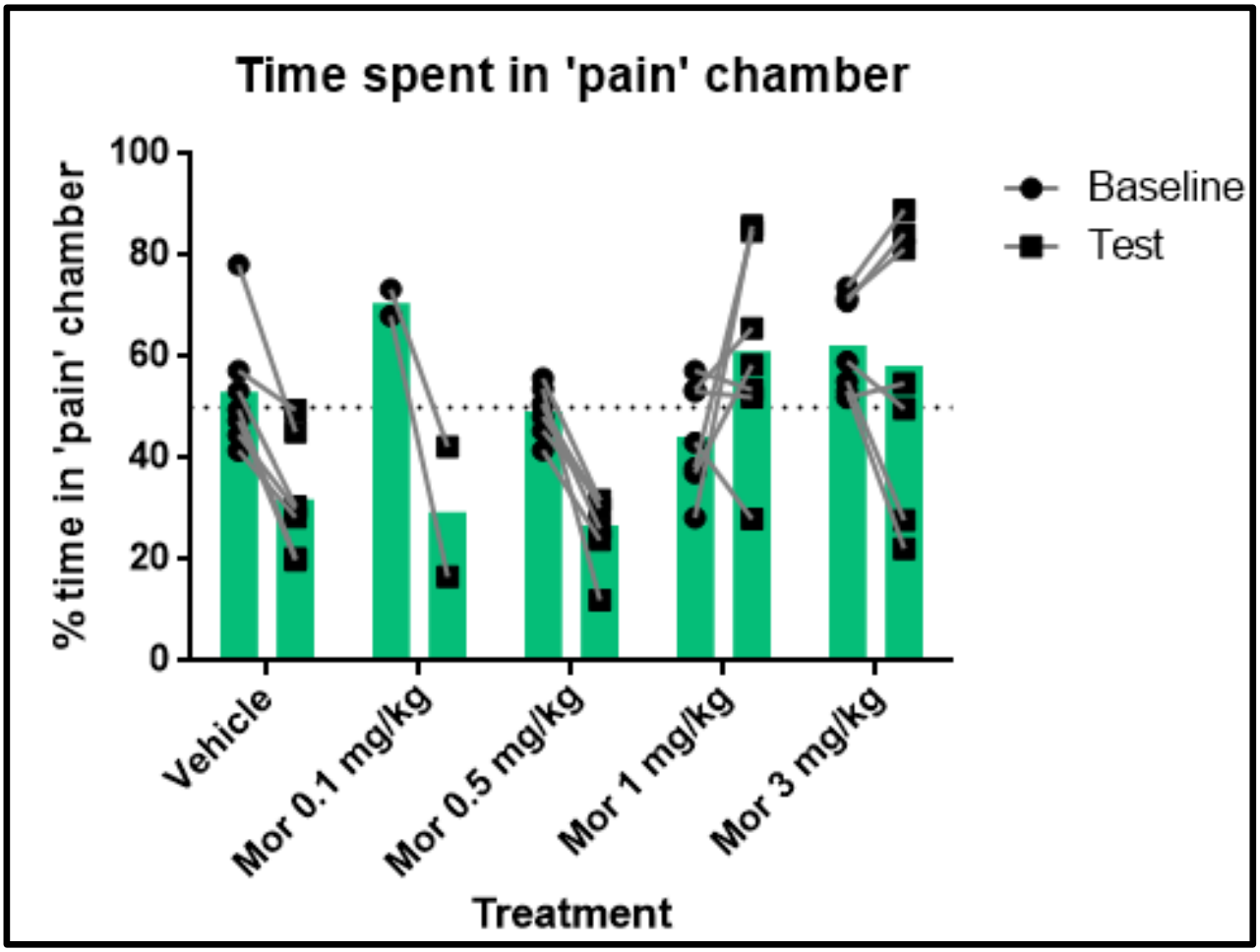

Figure 11. Morphine administered systemically counteracted RTPA in CAR-treated rats. TW-ANOVA found significant variation within the groups, between session, and their interaction ($p < 0.0124$). Post-hoc Sidak's MC tests revealed that rats which received vehicle alone spent significantly less time in the pain associated chamber during the test session (31.979 ± 4.324), compared to baseline session (53.123 ± 4.619), $p = 0.0202$ (N=7). Rats which received 0.1 mg/kg morphine spent significantly less time in the pain-paired chamber between test session (29.433 ± 12.833) and baseline session (70.733 ± 2.6) $p = 0.0145$ (N=2). Rats which received 0.5 mg/kg morphine spent significantly less time in the pain-paired chamber between test session (26.762 ± 2.726) and baseline session (47.429 ± 1.829) $p = 0.0116$ (N=7). Rats which received 1 mg/kg morphine spent identical time in the pain-paired chamber between test session (61.209 ± 7.609) and baseline session (44.382 ± 4.024) $p = 0.0901$ (N=7). Animals which received 3 mg/kg morphine also spent identical time in the pain-paired chamber between test session (58.467 ± 10.312) and baseline session (62.278 ± 3.601) $p = 0.9860$ N=7.

To examine the effect of morphine on pain aversion in the HET paradigm, four doses were used in a rat CAR pain model. Application of 1 and 3 mg/kg morphine intraperitoneally prior to test commencement normalised the HETs to baseline levels. The lower doses: 0.1 and 0.5 mg/kg could not rescue the HET and the CAR-injected animals escaped at significantly lower temperatures compared to baseline sessions and the contralateral paw (Figure 12). Thus, the 1 mg/kg dose was ineffective to reverse hypersensitivity (HG test), but able to alleviate pain aversion.

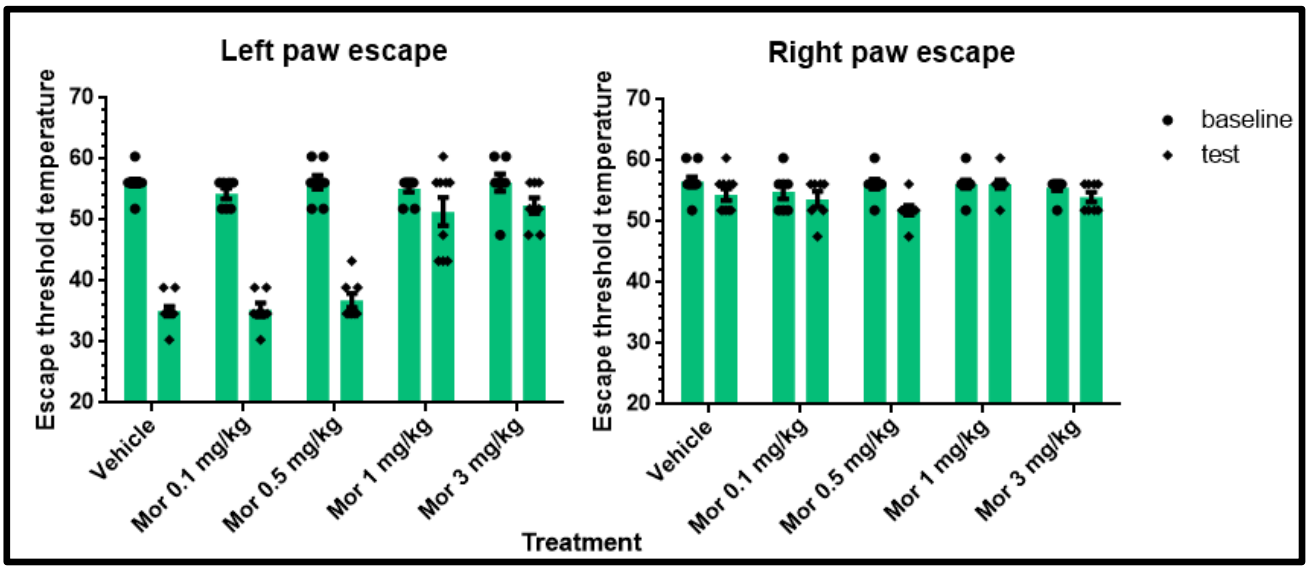

Figure 12. Effect of morphine on escape responses elicited by the punctate heat probe. Pain aversion was tested in control (vehicle) and morphine-treated rats. HET was assessed for the CAR-treated ipsilateral paw (left panel) and the unaffected contralateral paw (right panel). Four doses of morphine were used (x-axis) and differential effect was observed with concentration cut-off at 1 mg/kg. Left panel: TW-ANOVA testing found significant variation within the treatment groups, between sessions, and by their interaction ($p < 0.0001$). Post-hoc Sidak's MC tests revealed that animals receiving saline escaped at significantly lower temperatures following inraplantar CAR (35.08 ± 0.772) compared to baseline (56.15 ± 0.641), $p < 0.0001$, n=10). Animals that received 0.1 mg/kg morphine escaped at significantly lower temperatures following inraplantar CAR (35.264 ± 1.122) compared to baseline (54.307 ± 0.869, $p < 0.0001$, n=7). Animals that received 0.5 mg/kg morphine escaped at significantly lower temperatures following inraplantar CAR (μ=36.8 ± 1.149) compared to baseline (56.15 ± 1.149, $p < 0.0001$, n=8). Animals that received 1mg/kg morphine escaped at identical temperatures following inraplantar CAR (51.372 ± 2.316) compared to baseline (55.194 ± 0.632, $p = 0.1075$, n=9). Animals that received 5 mg/kg morphine escaped at identical temperatures following inraplantar CAR (52.388 ± 1.269) compared to baseline (56.15 ± 1.408, $p < 0.1559$, n=8). Right paw HETs were identical, irrespective of treatment or session ($p = 0.2866$).

The HET paradigm was used successfully to measure inflammatory hypersensitivity in behaving rats. It was also used to evaluate the anti-nociceptive and anti-aversive effect of morphine. Finally, the HET paradigm with the punctate heat probe was tested with a different pain model; namely, opioid-induced hyperalgesia (OIH) rat model. Thereby, HETs to the punctate heat probe were collected over a time course of seven days and compared to control animals which were perfused with Ringer's solution vehicle alone. The pro-aversive effect of remifentanil-induced hyperalgesia was persistent over the course of the experiment (Figure 13).

Figure 13. Heat escape threshold (HET) was reduced in an OIH pain model. During baseline sessions (-1), the rats from the control group escaped at identical temperatures (55.433 ± 0.453) to test animals (34.442 ± 2.530, Sidak's multiple comparisons p= 0.9463, n=6). During baseline sessions (0), the rats from the control group escaped at significantly higher temperatures (54.717 ± 1.063) to test animals (35.367 ± 0.453, p < 0,0001, n=6). One day after surgery (1), the rats from the control group escaped at significantly higher temperatures (52.567 ± 0.717) to test animals (34.65 ± 0.555, p < 0,0001, n=6). At day 3, the rats from the control group escaped at significantly higher temperatures (53.283 ± 1.433) to test animals (34.65, p < 0,0001, n=6). One week after OIH induction, the rats from the control group escaped at significantly higher temperatures (53.642 ± 1.611) to test animals (35.367 ± 0.717, p < 0,0001, n=6).

The animals that underwent surgery to induce OIH had surgical wound in the left, anterior cervical area which was closed with stitches. Some of the animals had opened the stitches but this did not influence their general wellbeing (e.g., feeding, grooming, socialisation with the home-cage-paired animal). The surgical wounds have closed and/or formed scab within 4-5 days into the experiment.

4. Discussion

The paramount goal of preclinical pain research is successfully managing, treating and ultimately alleviating pain. Clinical translation and implementation of research focussing on chronic pain has been facing decades of contradictory or unsuccessful medical solutions. This has been largely attributed to the discrepancies between contemporary preclinical rodent models and the implementation of observed insights into human pain. Critique has also been ascribed to redundancies between molecular mediators and pathways that underscore the effects of advanced therapeutics (Tappe-Theodor & Kuner, 2014). Notwithstanding, the foremost limitation is rooted in the existing deficiencies of modelling pain in rodent models and extrapolating quantifiable, indirect measures of painful states to tackle the full breadth of spontaneous pain experience. Indeed, the compound character of pain in humans is a premise for readout misrepresentation in rodent models that rely mainly on measuring stimulus-evoked nocifensive-reflexive reactions in the classical tests dominating basic research for decades: von Frey (Frey, 1896), tail flick (D'Amour & Smith, 1941), hotplate (O'Callaghan, 1975), and Hargreaves test (K. Hargreaves et al., 1988). Commonly, these tests inquire the ability of substances to attenuate responses to stimuli applied to normal, non-pathological tissues (e.g., integumentary). That contradicts the main goal of the clinician – to reduce pain morbidity while conserving normal responses to acute noxious stimuli. Thus, an ideal analgesic agent would target exclusively pathological pain, while pain-evoked responses remain intact (Tappe-Theodor et al., 2019). The inaptness of such tests has therefore limited drug development and basic research (for clinical applications in chronic pain *cf.* (Mao, 2012; Mogil et al., 2010)). Pain perceptions in humans are standardly analysed with questionnaires that provide detailed information about the quality and incidence of painful episodes. Rodent experimentation relies solely on surrogate readouts that report aspects of the animals' wellbeing. Recently, there has been an incentive to develop paradigms that analyse the emotional, cognitive, and affective factor of pain perception in rodents. Such tests strived to integrate spontaneous and normal behaviour into the test process, for instance: escape/avoidance (Labuda & Fuchs, 2000); place preference (King et al., 2009); facial expression analysis (Langford et al., 2010); home-cage monitoring (Urban et al., 2011); voluntary wheel running (E. J. Cobos et al., 2012); and burrowing (Andrews et al., 2012). Another aspect that impacts rodent experimentation is the choice of appropriate pain model. This has allowed distancing from reflexive responses in healthy rodents and investigating chronic conditions like inflammatory or neuropathic pain, trigeminal neuralgia, phantom limb pain, chronic back pain, and migraine.

4.1. Application of the PHP in thermal assays of pain aversion in rats

Some of the major caveats of preclinical nociception research in rodents stem from the inability to robustly and clearly distinguish between the sensory and affective components of pain (van der Kam, Vry, et al., 2008). Among the most commonly used techniques to measure pain aversion in rats are the ultrasonic vocalisations (e.g., following intraplantar injection of formalin) (Oliveira & Barros, 2006). Yet, such studies frequently face difficulties to correlate vocalisation and nociception and have been subjected to criticism

(Jourdan et al., 2002; Wallace et al., 2005). Alternatively, the tail shock-induced vocalisation can also serve to measure pain aversion in rats but still fails to measure pain processing (i.e., vocalisation involves circuits not involved in pain processing) or memory (Borszcz, 2006; Nandigama & Borszcz, 2003). PEAP is another method to measure pain aversion, but the intensity of stimulation-induced pain relies to a great extent upon the experimenter (Bove, 2006). Furthermore, stimulation or the effect of a certain compound may influence ambulation and the experimental setup is limited to a single session. Moreover, the animals face conflicting cues; namely, the aversive noxious stimulus in the dark chamber, and the aversive environment (illuminated chamber). This conflict can be affected by anxiolytic or anxiogenic states and perturb the experimental readout.

To address this criticism and avoid the confounding factors of these tests, the punctate heat probe was designed and used to directly modulate pain aversive behaviour in a CAR-induced inflammatory pain model. Thereby, the punctate heat probe was able to successfully identify the animals that experienced CAR-induced hypersensitivity. CAR inflammatory pain model was chosen as it has been previously reported as short-lasting (K. Hargreaves et al., 1988) and has been used in CPA paradigms in rat to assess the affective component of nociception in rats (Tzschentke, 1998; van der Kam, De Vry, et al., 2008). Intraplantar injection of carrageenan has been shown to produce intense inflammation in rats and this model has been standardly used to test putative anti-inflammatory drugs' effect. The carrageenan-induced inflammatory model was originally designed as an assay for anti-inflammatory drugs (C. A. Winter et al., 1962). Thereby, it has been shown that intraplantar injection of carrageenan was able to produce oedema and intense inflammation in rats injected in the hind paw. Subsequent research associated hind-paw inflammation with behavioural hyperalgesia and characterized its features using the standard Hargreaves apparatus and testing paradigm (Hylden et al., 1989; Iadarola et al., 1988).

The present utilisation of the punctate heat probe in two pain models aspired to incorporate several aspects of existing paradigms and refine a balanced method that simultaneously provided a clear and readily quantifiable readout while pertaining rodents' natural behaviour with least disruption, strain, and variability and with clearly defined endpoints. Explicitly, several major drawbacks of existing paradigms were addressed during the design of the HET and RTPA paradigms. Namely, the leading PEAP relies on the establishment of short-term memory of painful events that occur exclusively in the dark compartment of the two-chamber behavioural arena. The use of the dark compartment introduces an additional conflicting and stressful cognitive process for the rats; namely, overriding the natural instinct that promotes fear of bright and open spaces. During RTPA, the animals were allowed to freely explore the behaviour arena for five minutes prior to test commencement. Subsequently, the chamber occupied by the rat at the end of this period was paired with painful stimulation. Thereby, RTPA eliminated the confounding factor of predetermining the aversive environment and reduced the conflicting cues that the experimental animals face during the test. Additionally, the standard PEAP requires extensive habituation before testing; only one day of habituation was required for RTPA (4 vs. 1 day, respectively). Furthermore, RTPA incorporated an additional baseline measurement which allowed for comparison between baseline and test sessions; while, PEAP relied solely on comparison between test and control rats during the test session. Another central testing feature was the introduction of the PHP as a stimulation tool. This device tackled two long-standing deficiencies of traditional paradigms. Namely, the application of mechanical stimulation with the vF monofilaments which is often highly variable due to variation of the experimenter stimulus application

pattern, wearing of the material and loss of tensile strength, and dependence on environmental conditions (i.e., humidity). Moreover, application of focused infrared light during testing on the HG apparatus does not provide a clear and readily quantifiable readout of the exact stimulation temperature; it relies on the time rats take to withdrawal the stimulated paw, which is a relative measure following application of a variable stimulus. Another caveat of HG testing is the floor of the test behaviour arena. The transparent Plexiglas surface can often be bedraggled following micturition, and the protocol necessitates cleaning of the surface during experimentation. This can first change the beam intensity and second agitate the experimental animal. Experimentation with the PHP allows for the application of a calibrated thermal stimulus and excludes the occurrence of anxiogenic events. In fact, the standard HG protocol requires extensive habituation of the animals to the apparatus and the experimenter; additionally, it mandates the recording of two baseline sessions. Both RTPA and HET, require one day of habituation, one baseline day, and the test takes only one day.

The RTPA paradigm was used to infer aversion to thermal stimulation of the affected paw by reporting the time spent in the 'pain'-paired chamber. This experiment was inspired by the existing PEAP paradigm (Labuda & Fuchs, 2000), but excluded the inherent preference of rodents to dark, closed spaces. Namely, the standard PEAP reports baseline occupation ratio between the bright and light chamber, respectively as 2:8 (60 grammes von Frey monofilament stimulation) and 3:7 (PHP stimulation), favouring the dark chamber (Figure 4, B and 5, B, respectively). This strong initial preference was eliminated in the modified PEAP: RTPA; when no initial preference to either compartment could be detected (Figure 6). RTPA data can sufficiently be reported as the summated percentage of time spent in each chamber over the course of the experiment. As previously stated, the use of von Frey monofilaments has been associated with intra-subject and inter-subject variability; also, with variation within and between laboratories and the kinetics of stimulus application often involve stochastic factors, such as buckling and deflection to neighbouring innervation territories. Therefore, PEAP was conducted with the PHP contact thermode and circumvented this drawback.

Nevertheless, reporting the percentage of time in each of the chambers of the behaviour arena can be influenced by a number of factors, including: auditory and visual stressors, olfactory cues, architectural specificities of the experimental suite, exploratory behaviour, or establishment of a preferred occupation area. The HET paradigm was inspired by the SUDO method used to measure tactile hypersensitivity with von Frey monofilaments. Thus, the HET paradigm analysed time-restricted events of thermal stimulation over a time course with alterations of the stimulation directly defined by the behaviour of the experimental rat. This reduced the variation of the datapoints of HET, compared to RTPA. The HET paradigm was also successful to identify aversion to thermal pain and additionally, provided a threshold temperature value that invoked the aversive reaction (Figure 7). Conversely, the homeostatic temperature of either of rat's, hind paws lies within the range of 24.795 ± 0.805°C (Całkosiński et al., 2015). Both an infrared thermographic study and experimentation with contact-type thermometer reported that CAR-induced inflammation significantly increased the temperature of the plantar surface or rats' paws (Całkosiński et al., 2015; Shirota et al., 1984). Shirota *et* al. reported the homeostatic temperature of an unaffected paw as 27°C and the temperature of an inflamed paw at 30°C, 3 hours post-injection and at 32°C, 5 hours after CAR injection (which was also the highest detected plantar surface temperature) (Shirota et al., 1984). Notwithstanding, our HET data provided escape temperature values close to the homeostatic range of rats

(Figure 7). This motivated the coordination of two control experiments that tested for learned behaviour or reaction to the mechanical stimulation with the PHP. The PHP was used without thermal charge to investigate the mechanical effect of the thermode and escape behaviour was observed in 3.75% of the stimulation instances, irrespective of session, treatment, or paw lateralisation (Figure 8). Consistently to these findings, a pharmacological investigation from 1998, reported paw guarding behaviour in rats treated with CAR intraplantarly, at temperatures in the range of 25°C (Dirig et al., 1998). Subsequently, the possibility for development of associative learned behaviour was tested with an experiment that applied heat stimulation in increments in reverse order; i.e., starting from lower temperature and gradually increasing. Thereby, the previously acquired HETs were reproduced in similar ranges to the standard HET (Figure 9). Overall, the control tests revealed that the mechanical effect of the thermode had negligible effect upon escape behaviour and this behaviour was not a product of associative learning of paw stimulation and aversion to pain.

4.2. Differential effect of morphine upon rat thermal pain aversion

Despite the growing understanding of the neuronal circuitry and processing underlying the sensory component of pain, its affective faculty has only recently become subject to specific and focused research. This has led to the elucidation of neural systems responsible for the processing of the unpleasant modality of pain (Gao et al., 2004; Johansen et al., 2001; Tanimoto et al., 2003). As a composite of affective and sensory modalities, pain perception is also differentially modulated by chemical compounds; most prominently by opiates. Several lines of evidence have reported that morphine exerts differential effects spinally and supraspinally, and on the sensory and affective components of pain (Dickenson, 1995; Sinclair et al., 1988; Stamford, 1995). Interestingly, a peripheral analgesic effect of morphine has also been reported by Hargreaves et al.; using several inflammatory models (K. M. Hargreaves & Joris, 1993; Wenk et al., 2006), (for review of the topic *cf.* (Stein et al., 2009)). To ascertain whether escape behaviour following stimulation with the PHP contact thermode required supraspinal regulation an experiment was designed where a limit application dose was determined. Application of doses higher than the threshold had an effect on the reflexive-nocifensive responses of rats tested in the Hargreaves apparatus; while application of morphine with threshold concentration affected escape behaviour with putatively supraspinal regulation. Thereby, rat's paw withdrawal latencies (PWL) were significantly reduced in animals treated with 1 mg/kg morphine or saline; while 3 mg/kg morphine was able to restore the PWL to baseline values and the values of the unaffected contralateral paw (Figure 10). Correspondently, five doses of morphine were tested in the HET and RTPA paradigms. During RTPA tests, the rats' aversion to pain was estimated based on the fraction of time spent in the 'pain'-paired chamber. As all animals were injected with CAR and were experiencing tissue inflammation and hind paw pain; only the animals affected by the application of intraperitoneal morphine had similar percentage of time spent in each respective chamber. Stimulation with the PHP was able to produce aversion in rats that received morphine at sub-threshold concentration. Indeed, the rats injected with morphine at doses of 0.5 mg/kg or lower developed in real time a strong aversion to the 'pain'-paired chamber. Aversion was counteracted by doses of morphine of 1 mg/kg or higher, rats receiving these doses spent similar duration of time in each compartment of the behaviour arena (Figure 11). This observation

was similar to previous publication that compared differential effect of morphine on reflexive responses (evaluated by the Randall-Selitto paw pressure test) and the development of preference towards one compartment in the conditioned place preference (CPP) paradigm (van der Kam, De Vry, et al., 2008).

To further test the hypothesis of differential effect of morphine upon thermal pain aversion in an inflammatory pain model, the HET paradigm was used to compute the thermal thresholds that elicited escape behaviour in rats. Similarly, to the RTPA data, animals injected intraperitoneally with doses of morphine of 1 mg/kg or higher, escaped at temperatures similar to the escape temperatures required to promote escape behaviour in naïve animals or following stimulation of the contralateral, unaffected hind paw. Conversely, the animals that received morphine at doses of 0.5 mg/kg or lower, escaped at significantly lower temperatures, that were similar to the escape HETs obtained in rats who did not receive morphine (Figure 12). It is important to note that the HET and RTPA paradigms compare the test rats with the naïve control rats; and also, with the baseline values of the same animals obtained prior to testing. This experimental design, unlike the PEAP, allows for superior statistical power and reduced the number of experimental animals sacrificed. In combination with the reduced animal suffering caused by the pain model used; namely, the short-lasting and recoverable effect of CAR-induced inflammation; the HET and RTPA paradigms fulfil the three principles for humane experimental techniques: replacement, reduction, and refinement (Tannenbaum & Bennett, 2015). Of these principles, animal numbers were reduced by introducing a baseline session in the experimental design that allows for comparison between and within test groups; the second principle was addressed by refining the experiments so that experimentation times were reduced to three days in total and reducing stress levels induced during the transition from the dark to light chambers during RTPA and excluding the required cleaning during HG tests. Another conceptual advantage of the HET and RTPA paradigms arises from their duration. The whole experiment, including habituation, baseline and test sessions spans over three consecutive days. As recent reports have suggested a certain interplay between initial aversive behaviour followed by a later onset increase of anxiety and reduced aversive behaviour responses (Wu et al., 2017); animal behaviour experimentation should favour shorter-lasting experiments with concise design. Conversely, conditioning paradigms involve long and cumbersome habituation, baseline, and/or test schedules which might influence the outcome, consistency, and reliability of the experimental results.

To investigate the applicability of the PHP and the HET paradigm in thermal pain aversion experimentation; the opioid-induced hyperalgesia (OIH) pain model was induced and implemented in rats. A previous publication from our lab has reported persistent hypersensitivity in a rat OIH model. Thereby, pain was prolonged (i.e., persistent over seven consecutive days) and inferred from lowered hind paw response thresholds to von Frey monofilaments stimulation (Heinl et al., 2011). Similarly to the long-lasting elevation of mechanical sensitivity observed in the original publication (Figure 7 from (Heinl et al., 2011)); there was an increase in thermal sensitivity, following stimulation with the PHP (Figure 13). The OIH pain model affected both hind paws of the experimental rats and was used with the HET paradigm. Thereby, HET was able to detect hypersensitivity and lowered escape thresholds. The RTPA paradigm was found restricting in that it required one affected and one unaffected hind paw to generate aversion to the ‘pain’-paired chamber. Therefore, the HET paradigm was found more suitable for pain models that affect the whole body or both hind paws of experimental animals (e.g., LPS-induced inflammation).

4.3. Future outlook

Pain sensation is a complex process that integrates sensory-discriminative and affective-motivational modalities. This dichotomous character underpins distinct pathways and neural networks. Current preclinical pain models and test paradigms successfully inquire states of nociception; yet, they rarely permit for fine distinction between the sensory and affective aspects of pain. This caveat becomes critical in basic research, and translation and development of analgesic compounds. Indeed, a growing literature database report that pain aversion is selectively attenuated by lower doses of standardly used compounds. Most strikingly, morphine was 56-fold more potent in reversing locomotion suppression in mice injected intraperitoneally with acetic acid than reversing writhing caused by acetic acid (E. Cobos & Portillo-Salido, 2013). This strongly suggests that morphine and opioids may have a selective and strikingly stronger effect upon the emotional, affective, and/or cognitive parameters of pain. Nevertheless, such tests are biased by the sequence of experimental steps (i.e., writhing precedes locomotion and morphine affects both). Most importantly, tests that putatively measure pain aversion are severely prone to external influences, involve cumbersome protocols, and provide highly variable results. In an attempt to answer the need for reliable and robust techniques to measure affective pain in behaving rats, the punctate heat probe prototype was tested in several behavioural paradigms. The PHP is readily available for implementation in more sophisticated experiments that shed light upon the neuronal mechanisms that underscore pain aversion. Foremost, the PHP can be used in experiments that involve the application of a substance locally in a brain or spinal structure via microinjection (Deyama et al., 2007; Navratilova et al., 2020; Ren et al., 1992; Saadé & Jabbur, 2008). Such preliminary pilot tests were recently conducted at our lab. Tests with local application of *E. coli* lipopolysaccharide (LPS) into the PBN of behaving rats, revealed lowered heat escape thresholds. Moreover, microinjection of CNQX-AP5 (selective AMPA/kainite receptor antagonist), into the PBN of behaving rats revealed normalisation of HET values to pre-CAR-induced, inflammatory, hind paw, pain model. Future work should complete the experimental groups to reach statistical power and integrate histological examination of the microinjected lPBN tissue. Furthermore, tests with local application of opioids or their agonists and antagonists to the lPBN and comparison to systemic application data could confirm this brain area's involvement in states of pain aversion. Moreover, optical methods for selective neuronal modulation can be used in pain aversion tests with the PHP. Optogenetics is a revolutionary technique that employs engineered ion channels (opsins) that allow for rapid activation or inactivation of selected cell populations by using focused light (Harding et al., 2020). Opsins integrate into specific tissues via viral transduction, transgenic animal lines, or combinations of the two. Opsin ion channels are activated by light at specific wavelength (e.g., 488 nm blue light) and permit ion influx or efflux from the cell (Harding et al., 2020). Optogenetics can be used for peripheral stimulation (e.g., stimulation of hind a paw); central stimulation of the spinal cord or of descending pathways from the brain; or also for *in-vitro* photostimulation (Harding et al., 2020). Additionally, future experimental planning considerations should take into account the sex and age differences in rat responsiveness to thermal pain (Samir et al., 2017; Vierck et al., 2008). The PHP is a versatile device that can be used in a range of basic research experiments and pre-clinical screens for analgesic properties of therapeutic compounds.

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6. List of Figures

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8. List of Abbreviations

PNS	Peripheral nervous system
CMH	C-fibre mechano-heat sensitive nociceptors
AMH	A-fibre mechano-heat sensitive nociceptors
MIA	Mechanically insensitive afferents
MSA	Mechanically sensitive afferents
QC	Quick C
SC	Slow C
CNS	Central nervous system
STT	Spinothalamic tract
SHT	Spinohypothalamic tract
PBN	Parabrachial complex/ Parabrachial nucleus
mPBN	Medial PBN
lPBN	Lateral PBN
CGRP	Calcitonin gene-related peptide
NK1	Neurokinin 1
VAS	Visual analogue scale
MED	Minimal effective dose
R-S	Randall-Selitto test
TRPV-1	Transient receptor potential vanilloid nociceptor 1
CFA	Complete Freund's adjuvant
CAR	Carrageenan
CCI	Chronic constriction injury
SNL	Spinal nerve ligation
PSL	Partial sciatic nerve ligation
CPP	Conditioned place preference
CPA	Conditioned place aversion
PEAP	Place escape/avoidance paradigm
RTPA	Real-time place avoidance
HET	Heat escape threshold
CBR	Centre for Brain Research, Vienna
IST	Institute of Science and Technology, Vienna
i.pl.	Intraplantar
i.p.	Intraperitoneal
SUDO	Simplified up-down method
PHP	Punctate heat probe
vF	Von Frey
ANOVA	Analysis of variation
SEM	Standard error of the mean
PWL	Paw withdrawal latencies
OIH	Opioid-induced hyperalgesia

9. Supplementary Material

Deutsche Zusammenfassung

Der aversive Aspekt von Schmerzen stellt eine große Belastung für Schmerzpatienten dar. Dies hat die Entwicklung von Assays zur Beurteilung der Schmerzaversion in präklinischen Studien mit Nagern vorangetrieben. Bei Mäusen wird das durch eine heiße Platte hervorgerufene Sprungverhalten häufig als Indikator für die Schmerzaversion verwendet. Adulte Ratten zeigen diese Art von Verhalten jedoch nicht, und es fehlen gut etablierte Ersatzmethoden für den Test der Schmerzaversion bei Ratten. Hier berichten wir über zwei Paradigmen, die zur Untersuchung der thermischen Schmerzaversion bei Ratten unter Verwendung einer punktförmigen Wärmesonde entwickelt wurden: Echtzeit-Ortsvermeidung (heat-RTPA) und Wärmefluchtschwelle (HET). Wir zeigen, dass diese Paradigmen eine erhöhte Wärmevermeidung im intraplantären Carrageenan (CAR)-Modell für entzündliche Schmerzen nachweisen können. In Übereinstimmung mit früheren Daten, die eine erhöhte Wirksamkeit von Morphin auf die aversive im Vergleich zur sensomotorischen Komponente des Schmerzes zeigen, fanden wir heraus, dass Morphin die Hitzeflucht/-vermeidung bei einer geringeren Dosis umkehrte, verglichen mit der Dosis, die für die Umkehrung der reduzierten Latenzzeiten des Pfötchenentzugs im Hargreaves-Test erforderlich ist. Schließlich konnte mit dem HET-Paradigma eine erhöhte Wärmeaversion im Opioid-induzierten Hypersensitivitätsmodell (OIH) nachgewiesen werden, was seinen Vorteil im Kontext von generalisierten Schmerzmodellen demonstriert.

Um Entzündungsschmerz zu induzieren, wurden Ratten kurz mit Isofluran betäubt und mit 100 µL 0,5% CAR in die plantare Oberfläche der linken Hinterpfote injiziert; Kontrollratten wurden betäubt, aber nicht injiziert. OIH wurde durch i.v. Infusion von Remifentanil (Bolus 30µg/kg + 450 µg/kg/h) in die Jugularvene unter ~1 Std. Isofluran-Narkose induziert; die Kontrollratten waren narkotisiert und erhielten eine Vehikel-Infusion. Die Hitze-RTPA- und HET-Tests fanden in einer Verhaltensarena mit einem Netzboden statt. Im Wärme-RTPA-Paradigma wurde die Wärmesonde auf 41,1°C eingestellt und die Ratte durfte eine Arena mit zwei Kammern erkunden. Eine Kammer war mit der Stimulation der CAR-betroffenen Pfote gepaart ("Schmerzkammer"), während die andere Kammer mit der Stimulation der kontralateralen Pfote gepaart war. Wir berechneten den Vermeidungs-Score, indem wir die in der Schmerzkammer verbrachte Zeit in einer Baseline-Sitzung (kein Schmerz) mit einer Test-Sitzung (nach CAR-Injektion) verglichen. Beim HET-Test begann die Stimulation bei 41,1 °C, und die Temperatur wurde pro Versuch um 4,6 °C gesenkt oder erhöht, je nachdem, ob die Ratte mit Flucht reagierte oder nicht; wir berechneten die "Fluchtschwelle" nach der vereinfachten Up-Down-Methode, die von Bonin et al. (2014) entwickelt wurde. Beim Hargreaves-Test wurde ein Wärmestrahl auf die Pfote gerichtet, und die Latenzzeit für den Pfotenentzug wurde über zwei Baseline-Sitzungen bestimmt, gefolgt von einer Testsitzung nach CAR-Injektion. In einigen Fällen wurde Morphin (0,5-3 mg/kg) intraperitoneal injiziert, um seine Auswirkungen auf die Hitzeaversion (Hitze-RTPA und HET) und den durch Hitze hervorgerufenen Pfotenentzug (Hargreaves-Test) zu beurteilen.

Im Hitze-RTPA-Test verbrachten CAR-behandelte Ratten signifikant weniger Zeit in der Schmerzkammer, was darauf hindeutet, dass sie die Hitzestimulation der CAR-behandelten Pfote als aversiv erlebten; Kontrollratten verbrachten ähnliche Zeit in den beiden Kammern der Verhaltensarena. Im HET-Test führte die Stimulation der CAR-behandelten Pfote bei signifikant niedrigeren Temperaturen zur Flucht im Vergleich zur unbehandelten Pfote und im Vergleich zu nicht injizierten Kontrollratten. Im Hargreaves-Test induzierte die Stimulation der CAR-behandelten Pfote mit Strahlungswärme den Rückzug bei niedrigeren Latenzen, wie zuvor berichtet. Morphin war in einer Dosis von 1 mg/kg wirksam bei der Verringerung der Hitzeaversion, wie sie durch das Hitze-RTPA- und HET-Paradigma nachgewiesen wurde, jedoch unwirksam bei der Verringerung der CAR-induzierten Hypersensibilität, wie sie im Hargreaves-Test gemessen wurde. Schließlich berichten wir, dass Ratten mit OIH im Vergleich zu Kontrollratten im HET-Paradigma beidseitig niedrigere Fluchtschwellen aufwiesen.

Die vorliegende Arbeit berichtet über zwei neuartige Methoden zur Bewertung der Hitze-Schmerz-Aversion bei Ratten: Hitze-RTPA und HET. Die mit beiden Tests gemessene Hitze-Aversion wurde durch ein entzündliches Schmerzmodell erhöht. Darüber hinaus konnte der HET verwendet werden, um die Schmerzaversion im OIH-Modell für generalisierten Schmerz zu erfassen. Bei CAR-injizierten Ratten konnte eine Dosis von 1 mg/kg Morphin das Flucht-/Vermeidungsverhalten, gemessen im Hitze-RTPA- und HET-Test, blockieren, während der reflexive Pfotenentzug im Hargreaves-Test unbeeinflusst blieb. Dies steht im Einklang mit klinischen und präklinischen Studien, die eine stärkere Wirksamkeit von Morphin bei der Behandlung der aversiven Aspekte von Schmerzen im Vergleich zum sensomotorischen Aspekt berichten.