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Refining capsaicin-induced pain models: A comprehensive analysis of preclinical practices and their translational potential

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ABSTRACT

Pain remains a significant clinical challenge despite advances in mechanistic understanding. Animal models have been instrumental in advancing our understanding of pain mechanisms, however translating this understanding into positive clinical outcomes has been lacking. Capsaicin, a TRPV1 agonist and active component of chili peppers, is widely used in pre-clinical pain studies to evoke nociceptive responses. This systematic review investigates how capsaicin is currently used as a nociceptive stimulus in preclinical behavioural models of pain. We examine the purpose of capsaicin use, species and sexes of animals tested, methods of administration, dosage, and the types of pain behaviours assessed. Capsaicin-evoked behaviours are primarily used to understand TRPV1 specific pain mechanisms and screen analgesic compounds. We report male rodent studies are the dominant subject for capsaicin-induced behaviours and that within the limited studies involving female rodents, conflating data regarding sex specific effects exists. Very few novel behavioural techniques have been introduced, with studies heavily relying on behavioural measures such as von Frey sensitivity, hindpaw movements and thermal withdrawal latencies. We also report no significant correlation between increasing capsaicin dose and time of observed behavioural sensitivity, suggesting lower doses should be considered. We believe the data collected and reported here is useful to future researchers in both assessment of model relevance and informing experimental design. By analysing current practices and identifying areas for improvement in experimental design, this review aims to inform methodology for future studies and improve animal welfare.

1. Introduction

Everyone will experience acute pain, with a substantial proportion experiencing episodes of chronic pain (Fayaz et al., 2016; Nahin, 2015). Finding an effective treatment is a key focus for clinicians and industry, but innovation is not progressing at a pace required to meet need. One challenge is the limited understanding of how both physical and psychological components contribute to the short and long-term effects of a pain stimulus. Relevant outcome measures are, therefore, difficult to identify. Although our understanding of pain has progressed, few new approaches have been made in the mainstays of animal testing to identify mechanisms of pain and treatment. Reviewing current practice is key for improving animal welfare and the likelihood of identifying a clinically relevant new agent. Capsaicin is a commonly used tool to study pain as it directly activates pain signalling (nociception) in humans and pre-clinical models (Caterina et al., 1997; Fillingim et al., 2009; Hunskaar et al., 1985). This paper will review the usage of capsaicin as a pro-nociceptive stimulus in animal pain models utilising behavioural assessment.

Capsaicin is an alkaloid recognisable as the main pungent ingredient of chilli peppers (Szallasi et al., 1999). It binds transient receptor potential cation channel subfamily V member 1 (TRPV1), a heat-sensitive non-selective cation channel (Caterina et al., 1997; Szallasi et al., 2007). TRPV1 is found on multiple tissue types, including small to medium primary afferent neurons known as nociceptors, responsible for the detection and transmission of noxious stimuli (nociception) to higher

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brain centres, leading to the sensation of pain (Brito et al., 2014; Szallasi et al., 2007). In humans, the application of capsaicin leads to a sensation of burning due to activation of TRPV1-positive nociceptors. Release of neuropeptides (e.g. CGRP and Substance P) cause localised neurogenic inflammation; this is followed by a period of increased sensitivity (sensitisation). Finally, depending on exposure, capsaicin produces long- or short-term loss of sensation known as desensitisation/analgesia. This occurs via multiple mechanisms, ranging from neuropeptide depletion and changes in channel properties, to neurotoxicity (Ilie et al., 2019; Sawynok, 2005). As a result, capsaicin has multiple practical applications ranging from self-defence (pepper spray) to analgesics (Baranidharan et al., 2013; Szallasi et al., 1999; Szallasi et al., 2007).

The dose, application method and age of the subject all contribute to the effects of capsaicin; low doses can produce nociceptor sensitisation without long-term desensitising effects (Holzer, 1991; Zheng et al., 2000). Sensitisation of TRPV1 produces nociceptor sensitivity to several mechanical and thermal (hot and cold) stimuli (Szallasi et al., 1999). In clinical studies, this sensitisation is referred to as either hyperalgesia, an exaggerated painful response to noxious stimuli, or allodynia, a painful response to a previously non-noxious stimulus (Ji et al., 2003; Milligan et al., 2009). Due to sensitisation of spinal dorsal horn neurons, sensitisation also occurs away from the application site and is known as secondary or referred hyperalgesia/allodynia (Kinnman et al., 1995; Laird et al., 2000; Willis, 2002). Pre-clinical studies can observe stimulus-evoked (elicited) or stimulus-independent (spontaneous) responses, used as proxies for clinical mechanical and thermal hyperalgesia/allodynia and spontaneous pain responses respectively (Vierck et al., 2008).

Due to poor translation of pre-clinical results to novel therapeutic agents, it seems prudent to scrutinise the relevance of current pre-clinical models. Examples of promising novel agents identified in pre-clinical models which did not translate to clinical success include neurokinin-1 (NK1) receptor antagonists, sodium channel and gap junction blockers and microglial attenuators (Goadsby et al., 2009; Hill, 2000; Landry et al., 2012; Wallace et al., 2002). Reasons for why these substances failed range from differences between human and murine receptor function and immune cell response, different disease pathologies between clinical and pre-clinical neuropathic pain, and pre-clinical behavioural testing that poorly represents the human pain experience (Hill, 2000; Landry et al., 2012; Wallace et al., 2002). There are examples of successful translation, including N-type voltage-sensitive sodium channel blocker (ziconotide) and capsaicin patches. These compounds work by targeting neural function, blocking neurotransmitter release and ablating neurons, respectively, causing significant side effects which limit usage and, therefore, effectiveness. (Baranidharan et al., 2013; Bowersox et al., 1996; Schmidtko et al., 2010).

Capsaicin is a commonly used tool to study pain due to both the pain response it invokes and the importance of TRPV1 to nociception and pain sensitivity. To assess the clinical relevance of capsaicin research and refine current models, we must understand how capsaicin is currently used. This systematic review will explore the use of preclinical models that utilise capsaicin to examine pro-nociceptive behavioural responses. We report on the purpose of capsaicin use, species and sexes utilised, how capsaicin is used (dose and administration type), and pain types and behaviours assessed. We aim to inform the field on current practice, aiding future experimental design, improving animal welfare and ultimately providing researchers with ability to better assess the suitability of *in vivo* capsaicin use for their research aims.

2. Methods

2.1. Research strategy

The PRISMA statement was used to prepare the following systematic review. Literature searches of PubMed and Embase databases were used to identify all eligible studies. Search terms included "pain," "capsaicin", "behaviour" and related words. Papers used were published in English up until February 2024, these were the only limits of the search. The exact search string can be found in Table 1. Additional eligible studies were identified after manually searching the reference lists of included articles.

2.2. Systematic review publication selection criteria

To determine eligibility; titles, abstracts and/or full text were screened. Studies were eligible for inclusion if they were an original study which used a preclinical *in vivo* model to assess behaviour following application of capsaicin. Studies were excluded if they (i) did not include a vehicle control group in the behavioural assessment (vehicle delivered by the same route of administration as capsaicin, as to rule out effects of localised consequence of injection), (ii) used capsaicin for the purpose of denervation/desensitisation/analgesia; (iii) used capsaicin as a test (i.e. not administered prior to testing), for example, avoidance of drinking capsaicin spiked liquids.

2.3. Systematic review publication data extraction

The following was extracted from each included study: (i) study characteristics (first author, year of publication); (ii) animals used (species, sex, strain, age, weight), (iii) route of capsaicin administration (including anatomical location); (iii) dose of capsaicin used, (iv) behavioural test used (including testing time and duration of observed sensitivity post capsaicin application), (v) purpose of study (hypothesis/stated aim) and (vi) the role of capsaicin in study design. Behavioural consequences of capsaicin administration were assessed. Female and male data was not separated in the analysis due to the low number of studies including females and low number of studies reporting effects of sex on capsaicin response.

2.4. Statistics

Groups of 10 or more studies (administration route + species + behaviour assessed) were analysed for dose-duration relationships by performing nonlinear regressions in GraphPad Prism (Ver 10.4.0).

3. Results

3.1. Systematic review search outcomes

The screening process is described in Fig. 1. The initial search yielded 796 results, 114 of which were duplicates and immediately discarded. Upon examination of abstracts and results, a further 440 were excluded for not meeting entry criteria, including studies that were not preclinical, reported no vehicle control in capsaicin experiments, used alternative TRPV1 agonists, included no behavioural assessment following capsaicin and were not primary research articles. The remaining 240

Table 1
Search terms.

Pubmed	
	capsaicin
AND	allodynia OR hyperalgesia OR assessment, pain[MeSH Terms] OR animal behavior[MeSH Terms]
AND	animal model[MeSH Terms] OR mice OR rat OR primate
Embase	
	capsaicin
AND	allodynia OR hyperalgesia
AND	behaviour OR pain assessment OR von Frey test OR Hargreaves OR licking OR bite
AND	animal model OR mouse OR rat OR primate

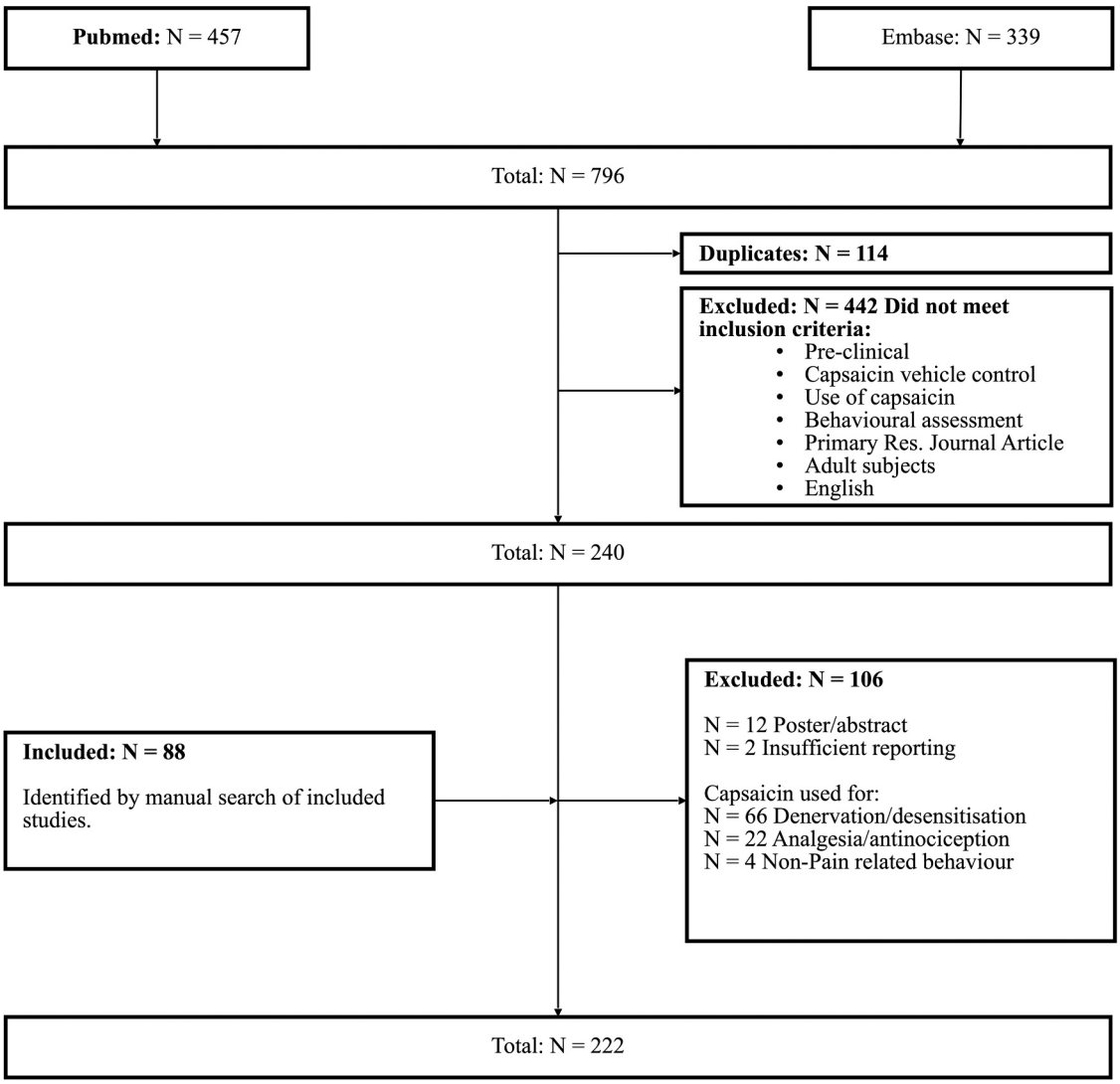

Fig. 1. Search outcomes.

articles underwent full-text review. 88 additional articles were identified based on references, while a further 106 were excluded. Most exclusions were due to primary intentions of capsaicin either being denervation/desensitisation or as an analgesic intervention rather than to induce a pro-nociceptive response. Articles were also excluded if capsaicin was not administered but used as a behavioural test (e.g. capsaicin drinking), insufficient reporting, and not being primary research articles. 222 articles remained and underwent further analysis.

3.2. Paper characteristics

Use of capsaicin in preclinical models to induce nociceptive behaviours increased in the latter half of the 1990's and has remained a constant research interest (Fig. 2B-C). Rodent species are the most common animal model used, accounting for 96 % of all identified studies, followed by primates of the genus *Macaca* (*mulatta & fuscata),* which account for 3.1 % (Fig. 2A). Other species include pigs, guinea pigs and tortoises. It should be noted the use of primates has reduced with only one incidence in the 20 years prior to this review, while the use of non-rodent, non-primate animals is relatively recent (Fig. 2B). Types of behaviours tested has remained consistent (Fig. 2C). Sex is unevenly distributed amongst studies using rodents, with 29 % and 15 % of mouse and rat studies respectively including females. This discrepancy is not present in the smaller number of primate studies. The relative proportion of male and females used in rodent studies has remained constant for the past two decades (Fig. 2D).

3.3. Capsaicin purpose

The majority of capsaicin use in the preclinical literature (59 %) aimed to investigate TRPV1-specific molecular pain mechanisms. 43 papers (19 %) used capsaicin as a pain model for screening potential analgesic compounds. 43 Different compounds were tested of which 7 have been used in clinical trials to investigate potential analgesic effects (Sup.Table 1). From these 7 compounds, three (BCTC, CHF3381 and Ginkgo biloba extract) produced mixed results and three others (maslanic acid, hesperidin methyl chalcone and loperamide) show analgesic effects in clinical trials. Development of new pain models using capsaicin accounted for 13 % of all studies. Fewer studies were conducted to investigate drug/treatment effect mechanism, novel capsaicin delivery (nanoparticle encapsulation), mouse strain characterisation and TRPV1 antagonist discovery.

3.4. Behaviour assessed

Following capsaicin administration, both the spontaneous behaviour and longer-lasting sensitisation were assessed. 44 % of studies assessed spontaneous behaviours only, 40 % assessed elicited behaviours only,

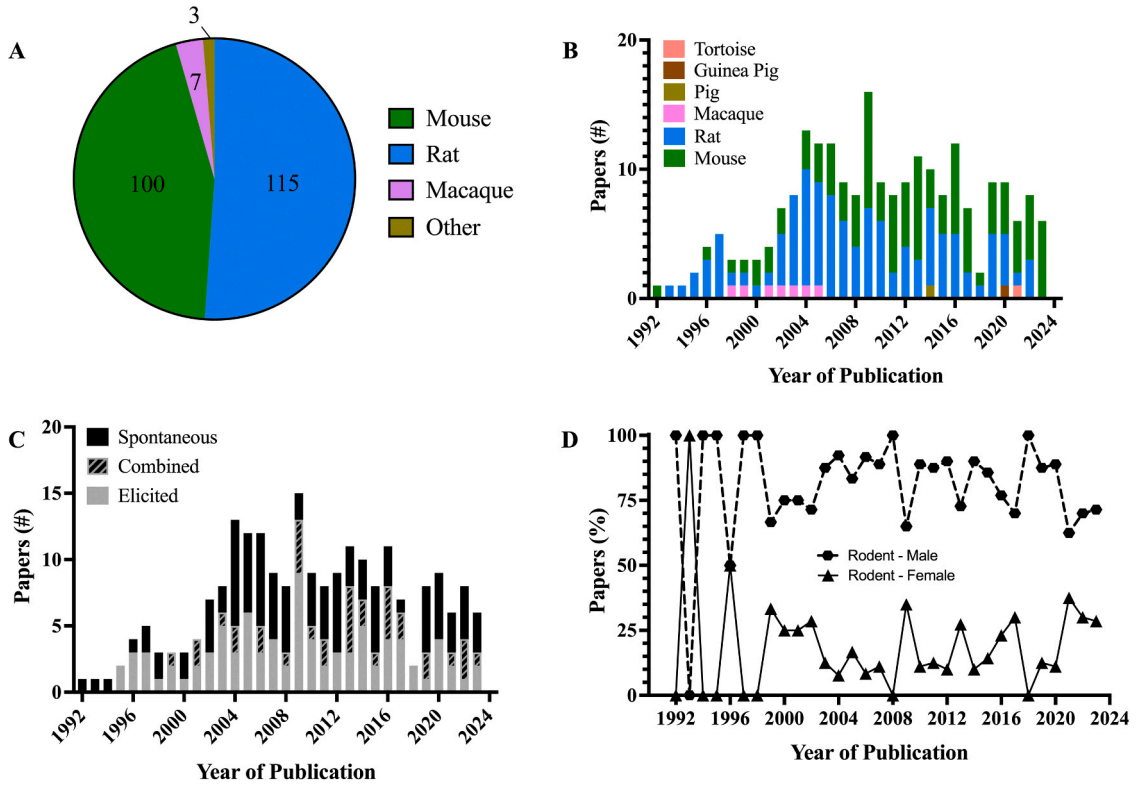

Fig. 2. Paper characteristics. (A) species used, (B) species by year published, (C) behaviours reported by year published, (D) sex percentage (in papers using rodents) by year published.

and 16 % assessed both (Fig. 3A).

3.4.1. *Spontaneous pain*

Spontaneous pain behaviours are more frequently tested in mice, with 75 % of all mice studies reporting spontaneous pain behaviours compared to only 49 % of rat studies (Fig. 3A). 39 different tests were used to quantify spontaneous behaviours (Sup. Table 2). Most tests quantified behaviours by number or duration of behaviours or distance travelled. 62 % Of spontaneous behaviours assessed involved movements of the affected limb, including licking, flinching, biting, retracting, lifting, wiping and grooming (Sup. Table 2). This is followed by spontaneous behaviours associated with visceral pain (24 %), such as adnominal stretching, squashing, and retracting. No new techniques for spontaneous behavioural assessment have been widely adopted in the last decade (Sup Fig. 1).

3.4.2. *Elicited pain*

Rats constitute the most utilised animal to study elicited pain following capsaicin (64 %) (Fig. 3A). 17 different behavioural tests have been used to evaluate elicited pain responses (Sup. Table 2). Commonly used techniques remain those first published before 2003 (Sup Fig. 1). The von Frey test was the first elicited pain test used following capsaicin (1995) and remains the most utilised, comprising 42 % of all tests used. Like spontaneous behaviours, most tests are directed at the affected limb

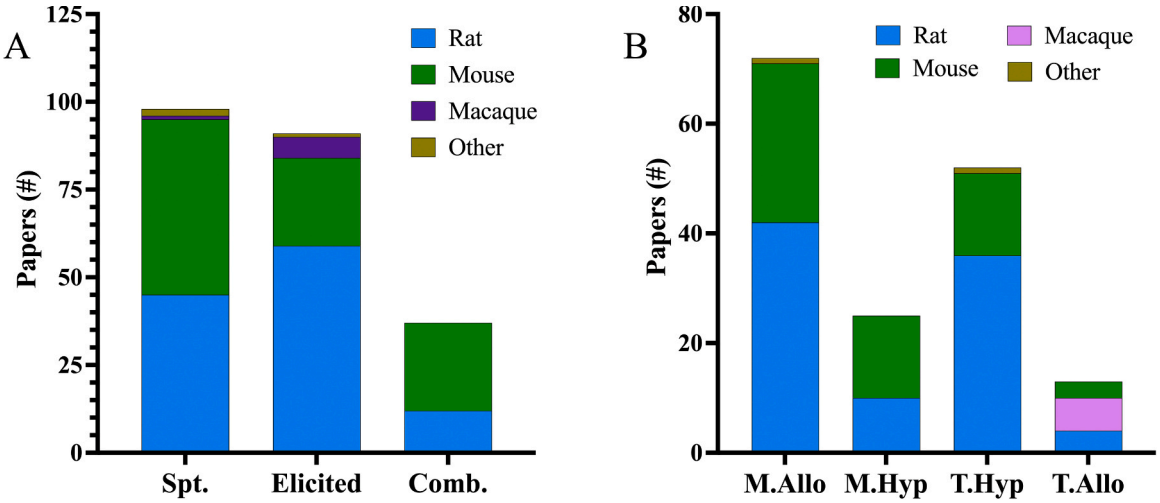

Fig. 3. Pain types observed. (A) Pain type by species, (B) Elicited pain type by species. Comb. = combined, M.Allo = mechanical allodynia, M.Hyp = mechanical hyperalgesia, Spt. = spontaneous, T.Allo = thermal allodynia, T.Hyp = thermal hyperalgesia.

and novel behavioural assessments have not been widely adopted. Mechanical allodynia (51 %) and thermal hyperalgesia (34 %) were the most tested behavioural types (Fig. 3B). The reviewer considers any threshold measurement as testing for allodynia, likewise the use of innocuous stimuli. As a result, allodynia was recorded for tests that met these criteria regardless of what the authors reported. The inconsistency of behavioural reporting is discussed in Section 3.4.3.

3.4.3. Reporting consistency

Reporting of allodynia or hyperalgesia was inconsistent across the articles reviewed. Mechanical threshold showed the greatest reporting inconsistency. Mechanical allodynia is reported in 33 % of threshold testing, with 67 % reporting mechanical hyperalgesia. Five papers reported hyperalgesia in their results while also referring to allodynia in the introduction and/or concluding remarks. Reporting of von Frey data also displayed inconsistency. Thirty articles use a 'best of,' '%,' or 'score' von Frey approach, 5 of which do not report allodynia when using innocuous filaments (innocuous stimulus for von Frey is considered a fibre that elicits lower than one response per trial). Eight articles, six of which were published in the last decade, did not classify elicited responses as either allodynia or hyperalgesia, but rather referred to 'hypersensitivity', 'sensitivity', 'withdrawal threshold,' 'thermal pain threshold' or 'pain threshold pressure'.

3.5. Capsaicin administration

3.5.1. Administration route and location

Route of administration is distinct for inducing peripheral and visceral pain (Sup.Table 3). Intra-colonic administration is the most common method of delivery for visceral pain assessment accounting for 84.2 and 72.4 % of elicited and spontaneous observations, respectively. Peripheral application is more varied. The dominant method is intraplantar, used in 108 studies (Sup. Table 3). Additionally, hind paw administration is also achieved via subcutaneous (dorsal aspect) and topical routes, making it the most targeted area of the studies analysed (53 % of included studies). Outside of the hind paw, the vibrissae pad, lip, tail, and cheek have all been used multiple times via subcutaneous or topical administration (Sup. Table 4). Intraocular administration is the only other method present in greater than 10 studies, used to observe spontaneous blinking almost exclusively in rats (one study in primates) (Sup. Table 3, 4). Despite a wide array of administration routes and sites, it is evident that intraplantar and intra-colonic administration are overwhelmingly used for peripheral and visceral pain, respectively.

3.5.2. Dose and sensitivity

Delivery method, species and associated anatomic location are important contributing factors to doses administered. Low doses, most commonly in the nanogram range, were administered to sensitive highly innervated tissues (e.g. intrathecal, intraocular and intracranial) (Sup.

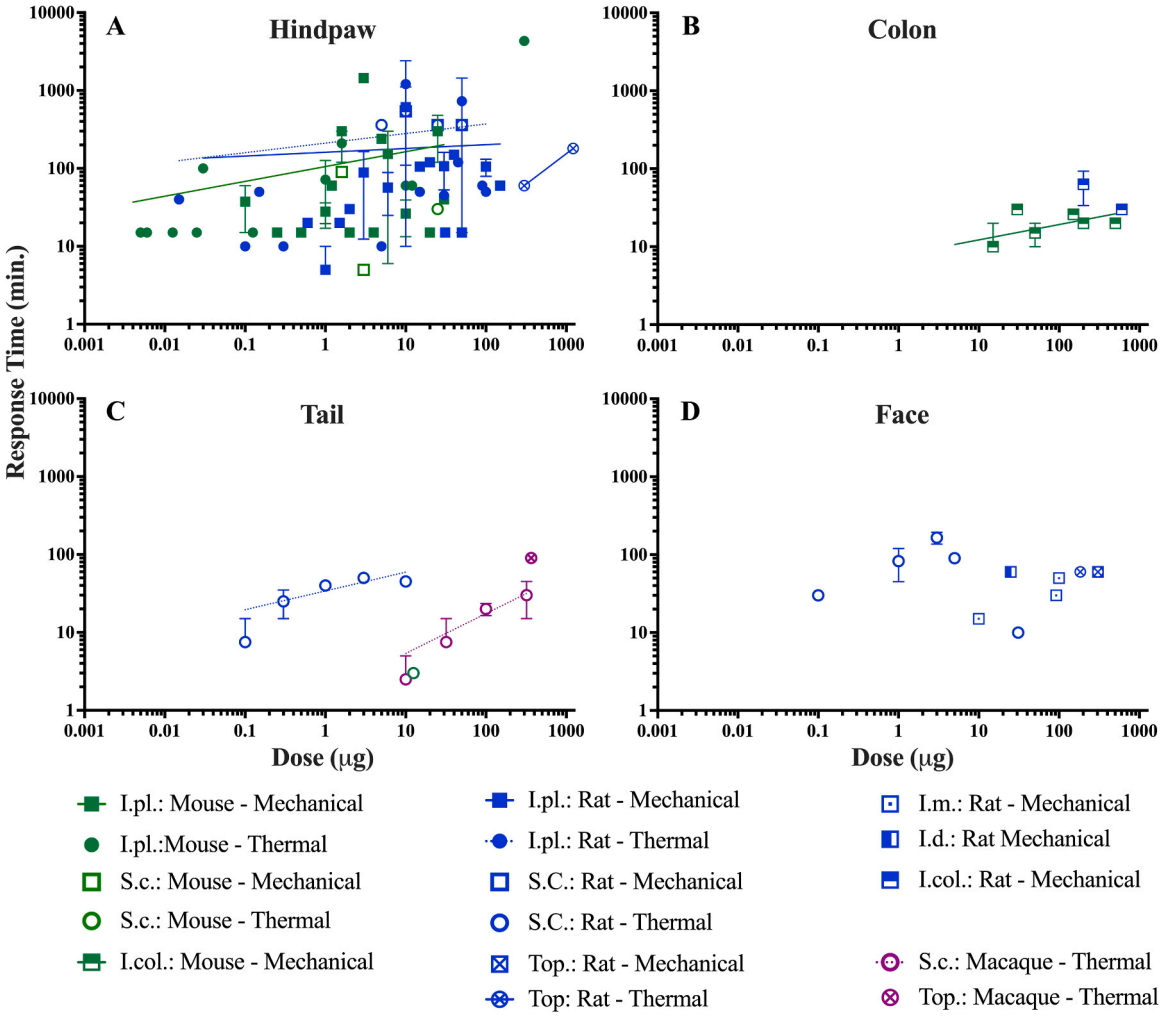

Fig. 4. Increasing capsaicin dose correlates weakly to increased observed time of sensitivity. Capsaicin dose used and duration of response for administration at the hindpaw (A), colon (B), tail (C) and face (D). Correlations were performed if there were greater than 10 species/location/test type combinations.

Fig. 2). Larger tissues including viscera tolerate larger doses, as seen in the case of intra-colonic, intraperitoneal, intramuscular and topical capsaicin application with doses commonly between 100 and 200 micrograms (Sup. Fig. 2). Well established doses in the capsaicin literature include intraplantar doses in both mice (1 μg) and rats (10 μg) (Sup. Table 5). Reporting of topical doses is difficult to quantify as the application of specific volumes is not often stated, instead 'applied liberally' is commonly used. Duration of reporting following capsaicin administration is highly variable. Well established timelines are intracolonic and intraplantar (spontaneous) capsaicin in mice, at 20 and 5 min, respectively (Sup. Table 5). An interesting anomaly is the use of two separate intracolonic administrations spaced two weeks apart, reportedly producing referred mechanical hypersensitivity up to six days following the final administration (Nasir et al., 2016).

There is no strong correlation in the literature between increasing dose and duration of elicited sensitivity. Groups containing greater than 10 studies were analysed for dose-duration correlations (Fig. 4). Mechanical stimulation in mice and rats and thermal stimulation in rats following intraplantar capsaicin showed a positive correlation (0.19 (n = 35), 0.04 (n = 35) and 0.11 (n = 21) respectively) (Fig. 4A). R squared values indicate a poor fit in each case, 0.05, 0.001 and 0.01, respectively (Fig. 4A). A similar pattern is observed in subcutaneous tail administration in rats (n = 12) and Macaques (n = 10) with a positive slope of 0.24 and 0.51 respectively with R squared values of 0.53 and 0.54 respectively (Fig. 4C). Mice receiving intra-colonic capsaicin (n = 18) produced a weak correlation (R squared = 0.19) and positive slope 0.20 (Fig. 4B). It is evident that dramatic increases in dose have little effect on the duration of reported sensitivity. It should be noted that the duration of sensitivity is often dependent on historically chosen timeframes, and behavioural experiments are rarely carried out until the complete reversal of hypersensitivity. From the data presented here it is evident that researchers are using doses above those necessary to produce hypersensitivity duration suitable for their testing timeframes.

3.6. Sex differences

For the past decade, between 60 % and 100 % of rodent studies published each year used male subjects only (Fig. 2D). Of all species, 75 % used male-only subjects, 9 % used female-only, and 13 % used both; 3 % of papers did not report sex. Of the 11 studies that investigated both sexes, 5 did not report sex-independent results, 5 reported no sex-specific effects on capsaicin-induced behaviours, and 2 reported an effect of sex (Table 2). Both studies that reported a sex effect were in rats, one reporting thermal hypersensitivity, the other, spontaneous behaviour. Three other rat studies show no effect of sex on either spontaneous or elicited mechanical behaviours.

4. Discussion

Since the discovery of its receptor, capsaicin has been consistently used to elicit an observable behavioural response (Caterina et al., 1997). Using capsaicin as a nociceptive stimulus is commonly utilised to explore basic physiological questions, providing valuable evidence for the role of TRPV1 in pain signalling. Another utility is the use of a capsaicin pain model for screening novel therapeutic agents. The relevance of capsaicin as a model for drug screening requires further discussion, especially considering the translatability limitations of preclinical pain research. With this review we highlight how capsaicin has been used as a pro-nociceptive stimulus in pre-clinical research, suggest potential areas for refinement and provide a significant dataset to aid experimental design of future studies.

4.1. Species assessed

Rodent models are overwhelmingly used in preclinical pro-nociceptive capsaicin studies (96 %). In studies investigating spontaneous behaviours, this rises to 99 % (1 primate study being the exception). This review included 43 studies which used the capsaicin pain model to screen for potential analgesic compounds (Sup. Fig. 1). No compounds since discovered as analgesic in clinical trials of non-exercise induced pain were identified using rodent models. The only compound (loperamide) from included studies since shown to be an effective analgesic in clinical trials for non-exercise-induced pain (stomatitis pain) was identified in a primate model (Butelman et al., 2004; Jyothi et al., 2021). TRPV1 expression patterns differ between humans and rodents. When comparing TRPV1 expression between the two

Table 2
Sex differences. Studies that used both male and female subjects and reported outcomes of both sexes.

Species	Strain	Author	Behaviour	Admin	Dose (μg)	Time	Male	Female	Reported Significance
Mouse	CD−1	Entrena et al. (2009)	Mech. paw withdrawal lat.	I.pl.	1	15 min.	10.06 (± 0.91) sec.	8.03 (± 0.82) sec.	-
Mouse	B6.129S6NF1^{tm1Fcr}/J	White et al. (2014)	von Frey (thresh.)	I.pl.	0.1	15 min.	0.18 g (EF50)	0.18 g (EF50)	-
Mouse	C57/Bl6	Nasir et al. (2016)	von Frey (thresh.)	I.Co.	50	24 hr.	≈ 1.5 g	≈ 0.5 g	-
Mouse	C57/Bl6	Carey et al. (2016)	Lick, lift, shake, bite / von Frey (thresh.)	I.pl.	1	5 min. / 30 min.	≈ 60 sec / ≈ 7 sec	≈ 60 sec / ≈ 7 sec	NS (P > 0.08 / P > 0.5)
Mouse	C57/Bl6	Ferreira et al. 2021	Bite, lick, shake / von Frey (thresh.)	I.pl.	6	5 min. / 120 min.	≈ 13 sec / ≈ 0.02 g	≈ 11 sec / ≈ 0.02 g	-
Mouse	C57/Bl6	Wang et al. 2022	Flinch, lick (time)	I.pl.	20	10 min.	≈ 35 sec	≈ 25 sec	-
Mouse	C57/Bl6	He et al. 2023	Climb, jump (#); flinch, lick (time)	I.pl.	1.5	5 min.	≈ 60 sec	≈ 50 sec	NS
Rat	Sprague Dawley	Lavand'homme et al. 1999	von Frey (thresh.)	I.pl.	30	2 hr.	64 (± 6.7) sec.	74 (± 5.3) %	NS (P = 0.47)
Rat	F344	Barret et al. 2003	Thermal withdrawal (latency)	SC (tail)	0.1–3.0	75 min	≈ 0 – 300 (AUC)	≈ 100 – 500 (AUC)	P < 0.05 (all concentrations)
Rat	Sprague-Dawley	Lu et al. (2009)	Lick, lift (time)	I.d. (paw)	0.12–15	5 min	≈ 10 – 100 sec	≈ 25 – 110 sec	P < 0.01–0.05 (0.12 – 7.6 μg). P > 0.05 (15 μg)
Rat	Fischer	Ro et al. 2020	Immobility, lick, lift (time)	I.pl.	300	10 min	≈ 475 sec	≈ 425 sec	NS
Rat	Sprague-Dawley	Antoniazzi et al. 2022	Freeze, groom (time), distance (m)	SC (vibrissae pad)	2.5	20 min	≈ 6 sec ≈ 110 sec ≈ 5 m	≈ 5 sec ≈ 160 sec ≈ 6 m	NS

species, the receptor is expressed in different neuron populations (peptidergic vs nonpeptidergic, size) (Kupari et al., 2021; Rostock et al., 2018). Co-expression profiles also vary, for example humans show greater proportion of TrkA/TRPV1 positive neurons compared to mice (Rostock et al., 2018). Together with lack of success identifying novel analgesics, it appears assumptions based on attenuating capsaicin pain in rodents are not particularly helpful for clinical analgesic drug discovery. Non-human primates and pigs offer ready tested options for thermal and mechanical elicited sensitivities, respectively, following capsaicin (Asad et al., 2016; Di Giminiani et al., 2014; Kupers et al., 1997). Other animal species part of the wider pain research domain, includes dogs (synovitis, natural arthritis) and sheep (neuropathic pain) (Brown et al., 2008; Hamilton et al., 2005; Henze et al., 2010; Wilkes et al., 2012). While rodents offer opportunities for mechanistic discoveries, novel alternatives that more closely resemble clinical phenotypes should be investigated if screening for clinically relevant compounds is a study aim.

4.2. Pain type assessed

Two response types are reported following capsaicin; non-elicited (spontaneous) and elicited; the latter comprising allodynia and hyperalgesia from either mechanical or thermal stimuli. 61 % of studies reported spontaneous data, while 57 % reported sensitivity to elicited stimuli.

Due to the direct nociceptor activation, the prevalence of spontaneous behaviours in the capsaicin literature is relatively high when compared to the wider pain community. Between 2000 and 2004, only 10 % of animal studies published in the journal 'Pain' report spontaneous behaviours (Mogil, 2009; Mogil et al., 2004). However, observations of spontaneous behaviour following capsaicin generally occurs within the first 30 min of administration. This suggests behaviours are responses to capsaicin activity at primary afferents which is mechanistically distinct to clinically relevant non-elicited (paroxysmal) pain often experienced by people with chronic pain conditions. For future studies who choose to utilise the capsaicin pain model for analgesic discovery, an appropriate representation of pain comparable and reproducible in clinical setting needs to be considered, including both relevant behaviours and timeframes to observe them.

In the capsaicin literature, 39 different spontaneous behavioural observations were reported (Sup. Table 2). Hindlimb licking and flinching, as well as abdominal stretching/contracting, have been used for decades and remain the most common pain behaviours observed (Sup. Fig. 1). Use of these behaviours for assessing TRPV1 activation is well established, however reduction of these same spontaneous behaviours were used as markers of analgesic efficiency in 67 % of included studies whose aim was to screen novel therapeutics. While these behaviours may be adequate for answering mechanistic questions around TRPV1, introducing novel behavioural cues as reliable indicators of spontaneous pain are needed to improve its relevance in analgesic drug discovery. Facial grimace scores have more recently been used in models with similar duration (formalin test, intraplantar mustard oil) (Langford et al., 2010). Assessment of pain in neonates, another nonverbal population, may also provide possibilities. Several validated pain measures in neonates involve behaviours that can be measured in animals, for example, vocalization, sleep-wake state, facial expressions and breathing patterns (Eriksson et al., 2019; Olsson et al., 2021). This type of approach, observing responses not directly directed towards the application/injury site, is rare in capsaicin literature. Two studies using a capsaicin-induced nonbacterial prostatitis model have used similar eye movement scores (Chuang et al., 2008, 2007). The capsaicin-induced prostatitis model also reports on locomotion, another potential pain score based on automated analysis. In addition to locomotion (type and distance), automated behavioural analysis can pick up grooming and gustation behaviours that change based on pain state. Locomotion (activity) scores have been used in a single drug discovery model, reporting that the novel compound AMG0347 decreases activity in the first 30 s following intrathecal capsaicin (Wu et al., 2008). This result helped confirm the activity of the compounds at TRPV1, but AMG0347 has not yet been used in further pain studies. Activity scores (movement, distance and rest time) have also been used in rat intraplantar capsaicin models exploring central pathways as well as central and peripheral kinase effects, however not in drug discovery studies (Fang et al., 2002; Sun et al., 2007). The use of automated behavioural analysis for these types of general behaviours is becoming more prevalent in pain literature and will hopefully be adopted by capsaicin literature (Brodkin et al., 2014; Roughan et al., 2016). Other non-evoked behaviours used in the pain literature include burrowing, weight-bearing and gait analysis, which offer potential alternatives for investigation in preclinical capsaicin models (Deuis et al., 2017).

Evoked responses in the capsaicin literature heavily lean on static mechanical allodynia and hyperalgesia following von Frey stimulation hours post capsaicin application. However, evoked hypersensitivities reported by pain sufferers occurs via both static and dynamic mechanical stimuli (i.e. clothes brushing against the skin) (Hansson, 2003; Ochoa et al., 1993). The dynamic cotton bud test has been used once in the included studies, testing hind paw responses following intraplantar capsaicin to examine the roles of tachykinin receptors (NK1) (Laird et al., 2001). Air puffs have also been utilised in a single model of orofacial pain (intradermal vibrissae application) to study the role of protein kinase C gamma (PKCγ) expressing interneurons in orofacial sensitivity (Peirs et al., 2016). Therefore, despite a much higher clinical prevalence (20–40 %), only 1 % of studies utilising capsaicin tested a dynamic stimulus (Hansson, 2003). Thermal sensitivities are tested in 29 % of evoked behavioural studies following capsaicin. Although the symptom does present in clinical trials, sensitivity to heat is not reported as a common problem in daily life for neuropathic pain patients (Hansson, 2003; Maier et al., 2010; Staud et al., 2012). Interestingly, three assays tested cold hyperalgesia following capsaicin with mixed results. Dynamic cold plate following topical application of the hind paw revealed cold sensitivity based on the number of jumps (Yalcin et al., 2009). However, standard cold plate tests revealed no capsaicin effect, and cold probes show an increase in thresholds (Honda et al., 2014; Roberson et al., 2013). Increasing use of tests for dynamic mechanical sensitivities seems a ready-made alternative to current practice to better represent clinical symptoms. The longer timepoints tested in these evoked behaviours may also represent an interesting timepoint for spontaneous behavioural analysis since the hypersensitivities are occurring via mechanisms distinct from capsaicin activity at primary afferents.

Very few novel techniques have been introduced into the capsaicin animal model literature. One study, attempting to develop a novel model, introduced operant reward for measuring evoked responses (Rohrs et al., 2015). It should be noted that although they were excluded from analysis here for not having vehicle controls, three other studies were identified which tried to incorporate operant reward while measuring evoked pain responses (Neubert et al., 2008, 2006; Nolan et al., 2011). Interestingly, in some cases pre-clinical operant and condition-placed preference behavioural analysis produces results contradictory to those obtained using standard evoked behavioural testing (Clark, 2016; King et al., 2009). Movement of mice over a thermal gradient surface is a unique technique developed to study vasomotor symptoms using capsaicin, with capsaicin causing the animals to spend longer at cooler temperatures compared to controls (Krull et al., 2017). It should also be noted that sensory loss, hypoalgesia, paraesthesia and dysesthesias are also symptoms of clinical pain that can be assessed using evoked behavioural tests and are not reported here (Hansson, 2003; Maier et al., 2010). Although these are rarely tested for in pre-clinical pain literature, they would have been screened out of our search with articles using capsaicin for desensitisation and/or analgesia (Mogil, 2009).

Lastly, it is prudent to note the inconsistencies in the use of pain-

specific nomenclature within the studies analysed. Clinical symptoms of allodynia and hyperalgesia are used to describe behaviours in animal pain models. However, due to the inability of animals to self-report, it is difficult to define a painful versus non-painful stimulus, leading to researchers describing either allodynia or hyperalgesia employing the same behavioural methodology. We observed a growing number of publications using the terms; 'hypersensitivity', 'sensitivity' and 'reactivity' rather than allodynia and hyperalgesia. In either case, confusion arises from the use of multiple terms to describe the same behavioural phenomenon, and the next step is to agree upon a universally accepted nomenclature for these behaviours. We suggest that hypersensitivity is an appropriate term that would suit the studies using elicited behaviours in this review. While the terms allodynia and hyperalgesia have a place in clinical studies, it may be appropriate to find alternatives in pre-clinical literature to eliminate confusion caused by inconsistent reporting.

4.3. Dose and capsaicin administration

Both species and route of administration influence the capsaicin dose used. Higher doses are typically used in larger animals to produce comparable sensitivities to those observed in smaller animals. Within the same species, higher doses are used for deeper administration (e.g. visceral, intramuscular) compared to the superficial application, such as topical or intraplantar (Sup. Fig. 2). An ethical consideration arising from this data is the use of high doses of capsaicin to elicit pain. This data reveals no significant correlation between increasing dose and observed length of sensitivity (Fig. 4). The strongest correlations were following subcutaneous tail administration in primates ($R^2 = 0.54$). In this instance, doses 3.5–10 times greater were used to elicit sensitivity lasting only an extra 15 min (Fig. 4). This is observed in many routes of application, and it may be useful to consider when making decisions regarding the refinement of methods to improve animal welfare. There were notable exceptions, two studies using intraplantar capsaicin in mice reported sensitivity at 24 and 72 h post-administration, well beyond anything else for the comparable species/administration combination (Chen et al., 2009; La et al., 2017). Due to the low number of studies including females, males and females were not separated for this analysis. For female data, all but two conditions (species, administration, behaviour assessed) had an $n = 3$ or lower. As female inclusion in pain studies increases in the future, it may be pertinent to repeat this analysis with sex effects in mind.

Large or prolonged doses of capsaicin are used to cause denervation and/or analgesia in adult animal studies. A single subcutaneous application can be between 10 and 1000 times greater than the median dose observed here, with multiple applications (Miranda et al., 2015; Saade et al., 2008). Topical application for desensitisation delivers comparable low doses to studies examined here. However, multiple applications over multiple days are administered (Yamaoka et al., 2007). Likewise, intrathecal doses are comparable but administered for extended time periods, up to 24 h (Kamei et al., 2000; Mousseau et al., 1994).

There is mixed evidence on the effect age has on pain sensitivity in pre-clinical models (Scuteri et al., 2020; Shoji et al., 2016; Yezierski, 2012). Importantly, it appears any effects occur in the mid to late life stages. For the studies included here, age was rarely reported in favour of weight. Where ages were reported, they were between 6 and 19 weeks or ambiguous (e.g. adult), consistent with other pre-clinical animal models (Jackson et al., 2017). For these reasons age was not included in the dose/effect correlations. It has been proposed that improving the reporting of age and consistency of age use within models will improve model repeatability and therefore reliability (Jackson et al., 2017). This is particularly relevant for a capsaicin pain model, as age-related reductions in TRPV1 expression have been reported both clinically and in rodents (Oto et al., 2022; Tarun et al., 2005; Wang et al., 2006). Due to this reporting, weight was also considered. Studies which investigate weight and pain outcome focus on obesity as a driver, a factor not relevant to this dataset (Marques Miranda et al., 2021). Further, within included studies weight was consistent within species.

4.4. Sex bias

A greater proportion of chronic pain patients are females, recent evidence suggests there may be mechanistic differences in nociceptive processing between sexes (Fillingim et al., 2009; Mogil, 2012). Despite this, male subjects remain overwhelmingly the most utilised sex in preclinical pain research (Mogil et al., 2005). Capsaicin behavioural studies analysed here are typical of what is found in the wider pain community, with 80 % of studies containing male-only subjects (Mogil et al., 2005). Surprisingly, we observed here that female animals were used in only 23 % of studies which aim to screen for novel analgesics. Of studies that did use both sexes, a large proportion (42 %) did not report sex-independent results. Three papers stated sexes would be evenly distributed and analysed together. Presumably other studies did the same. It is important to note that oestrous cycle stage is relevant to pain outcomes in both clinical capsaicin and chronic pain studies (Fillingim et al., 2009; Gazerani et al., 2005). This variable was not reported in the included capsaicin pre-clinical literature. Evidence now suggests TRPV1 expression changes through the oestrous cycle in rodents; this variable needs to be considered when analysing behavioural outcomes of the capsaicin pain model (Mota-Carrillo et al., 2024).

Of studies which analysed both sexes, five studies reported no sex difference (Carey et al., 2016; Entrena et al., 2009; Lavand'homme et al., 1999; White et al., 2014). Two studies reported a sex difference in response to capsaicin application (Table 2). Barrett et al., 2003 reported male rats require a dose three times greater than females to achieve comparable levels of thermal hypersensitivity following subcutaneous tail administration in rats (Barrett et al., 2003). Gonadectomy altered sensitivity in both males and females by increasing and decreasing capsaicin potency, respectively. The authors took anatomical variance into consideration, finding no difference in tail diameter at the injection site, although report a sex and test location interaction based on testing at different sites along the tail (Barrett et al., 2003). Lu et al., 2009 also report higher sensitivity to capsaicin in female rats at low to medium concentrations (< 15 μg) compared to male rats. This difference is based on nocifensive behaviours in 5 min following intradermal hindpaw capsaicin administration (Lu et al., 2009). The only mouse study to report a response difference across sexes reported a faster resolution to intra-colonic capsaicin-induced mechanical sensitivity. It should be noted that while this difference was noted by the authors, no direct statistical comparison of the sexes was reported (Nasir et al., 2016). Similar contradictory evidence regarding sex effect of capsaicin is also found in the clinical capsaicin literature (Frot et al., 2004; Jensen et al., 2006).

Five studies report sex differences in relation to resolution of capsaicin-induced hypersensitivity. Four concern sex dependant responses to opioids; with select Buprenorphine concentrations and administration routes having enhanced effects in females (Barrett et al., 2003). While others report reduced effectiveness in females by delta opioid receptor agonist (DPDPE), multiple kappa opioid agonists and a morphine/dextromethorphan (NMDA antagonist) combination (Lomas et al., 2007, 2008; Saloman et al., 2011). Conversely, no sex effect was observed following administration of morphine and mixed-action opioids butorphanol and nalbuphine (Barrett et al., 2003; Lomas et al., 2007). Finally, pharmacological inhibition and genetic ablation of PKMζ reduced referred visceral pain in male but not female mice following intracolonic capsaicin (Nasir et al., 2016). Despite most papers reporting no sex effect on capsaicin-induced nociception in preclinical models, mixed results in a small sample size suggest more investigation is required. This outcome is not aided by low levels of female inclusion and poor reporting of sex-independent results in studies that include both sexes.

4.5. Clinical relevance

Only 12 % of compounds (6) tested for antinociceptive properties in the included pre-clinical capsaicin studies are both novel to clinical pain treatment and have since been tested in clinical trials. There are three examples of clinical success, two of which are not used in pathological pain, but to aid recovery and relieve muscle soreness after exercise; maslanic acid and hesperidin methyl chalcone (Luque et a. 2023, Shirai et al., 2023). The third, loperamide, an opioid receptor antagonist repurposed from treating diarrhea, was equivalent to morphine at short term relief of dermal ulcer pain in adults with stomatitis (Jyothi et al., 2021; Nozaki-Taguchi et al., 2008). The remaining three compounds; CHF3381, Ginkgo biloba extract and BCTC have either failed or are yet to show conclusive results. NMDA receptor antagonist CHF3381 attenuates capsaicin induced secondary thermal hyperalgesia on the forearm of healthy male volunteers confirming a clinical relevance to capsaicin pain (Mathiesen et al., 2006). However, as per a release from Versalis in 2010, clinical trials for its use in neuropathic pain appear to have been unsuccessful, and data not published (Mattia et al., 2007). Ginkgo biloba extract (GbE) did not show reduction in pain score, although it was one of many factors tested for in patients with stable angina pectoris (He et al., 2024). BCTC has shown mixed effectiveness in clinical models of acidosis (Heber et al., 2020; Pomonis et al., 2003; Schwarz et al., 2017). Interestingly, BCTC is the only TRPV1 antagonist from included studies investigating novel analgesic compounds to have been tested clinically. Three other TRPV1 antagonists (AMG9810, A-425619, AMG0347) and one TRPV1 interfering protein were tested, showing analgesic efficacy in rodents (Gavva et al., 2005; Honore et al., 2005; Wu et al., 2008; Xiang et al., 2017). Likewise, one TRPV1 agonist (MDR-652) was tested showing analgesic effects in mice, it has not yet been tested clinically (Ann et al., 2020). Many novel analgesics targeting TRPV1 are efficacious in various rodent pain models, however at this stage, high-dose capsaicin patches remain the most common analgesic to target TRPV1 (Arora et al., 2021; Backonja et al., 2008; Rahman et al., 2024).

α/β Amyrin, oprhanin FQ and naringenin were also examined in the included studies and show potential clinical significance. None have been tested in isolation, but all have shown antinociceptive qualities in combination. α/β-amyrin are constituents of the unsaponifiable fraction in fruits of *Olea Europa (olives)*. Applied topically this reduced hand and knee osteoarthritis pain and oedema, while improving mobility over a three-week application period (Gelmini et al., 2016). Oprhanin FQ has been used in combination with nociception to create Cepranapadol, which has been affect at reducing postoperative, caner and chronic low-back pain in clinical trials (Christoph et al., 2017; Eerdekens et al., 2019; Scholz et al., 2018). Naringenin is a component of hop extract which reduced menopausal symptoms which include pain score (Erkkola et al., 2010). It should be noted in this case the pre-clinical capsaicin examination came years after the clinical evidence and that pain was only a component of the testing. Of all 43 compounds tested in the included studies, only one novel compound to treat clinical pain was identified, loperamide.

Given the lack of success overall, it appears that the capsaicin model is not an ideal model for studies screening novel clinically relevant pain therapeutics. Not discussed here are studies that examine the effects of other previously known clinically relevant compounds, including morphine, ketamine, celecoxib, ibuprofen and tetrodotoxin, which all significantly reduce capsaicin-induced hypersensitivities in pre-clinical models (Claudino et al., 2018; González-Cano et al., 2017; Joshi et al., 2006). Despite its poor success rate at identifying novel pain therapeutics, the model may have relevance in studying the mechanism of back-translated clinically relevant therapeutics.

5. Conclusion

Measurement of animal behaviour is crucial to pain research, enabling researchers to answer basic physiological questions, validate model relevance and assess therapeutic options. This systematic review provides insights into how capsaicin has been used to induced behavioural responses in pre-clinical animal models. It is evident that capsaicin dosing needs to be carefully considered to refine the model and improve animal welfare. We observe here that unnecessarily high doses are used relative to the time of sensitivity observed. It is currently unclear if capsaicin has different effects on male and female subjects in pre-clinical models, inclusion of both sexes with inclusion of oestrous cycle stage data is important to future studies. The use of capsaicin to screen for novel therapeutics has had limited success. Any future use for this purpose should consider novel approaches such as inclusion of clinically comparable and relevant behavioural tests (e.g. dynamic mechanical hypersensitivities, non-evoked behavioural monitoring), alternate testing timeframes and alternative species. This analysis highlights inconsistent reporting of pain type, and animal age and weight, which should be addressed by the field moving forward. Ultimately the data presented can be used to aid experimental design, improve animal welfare, raise awareness to standardise nomenclature and ultimately optimise future use of the pre-clinical capsaicin pain model.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neubiorev.2025.106455.

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