Learned analgesic cue association and placebo analgesia in rats: an empirical assessment of an animal model and meta-analysis.

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Care and use of animals

All experimental work in this thesis adhered to the guidelines on ethical use of animals maintained by the Australian code of practice for the care and use of animals for scientific purposes. Male and female Sprague Dawley rats were used and purchased from the Animal Resource Centre in Perth, WA. All experimental procedures were approved by the Animal Ethics Committee at the University of Sydney under protocol 1186 titled “Placebo analgesics in laboratory animals”. Every effort was made to minimise potential suffering and reduce the number of animals used.

Originality statement

This is to certify that the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Rosie Swanton

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Abstract

Research into the underlying neuro-mechanisms of placebo analgesia have been limited by unreliable animal models of the effect. Attempts to replicate human placebo testing paradigms in animals have had mixed outcomes, and the few reported successful models of analgesic placebo in animals are difficult to replicate and cannot therefore be reliably utilised to investigate the neurobiological mechanisms responsible for the effect. The current thesis attempted to replicate a non-drug-based rodent model of conditioned placebo analgesia that was recently published in the literature, and then attempted to alter the paradigm to make it more practical and potentially useful. No evidence for placebo analgesia was observed in either the replication or the amended experiments. The second part of the current thesis consisted of a meta-analysis of existing rodent placebo analgesia literature to measure the size of the effect, and to determine the factors that mediate the effect in rodents. The meta-analysis demonstrated a moderate effect size of placebo analgesia in rodents. Cue type was found to significantly moderate the effect size of placebo analgesia in rodents, and specific context cue chambers were most strongly related to outcomes of placebo analgesia. These results are significant as they demonstrate that the placebo effect is not purely observable in animals capable of higher cognitive functioning like humans and that continued research into establishing a reliable animal model would be valuable. Future investigations should aim to include specific context cue chambers, as well as olfactory and taste cues, when developing conditioned placebo analgesia in rodents. There is a need to methodically address the remaining boundary conditions that lead to placebo analgesia in rodents so that a standardized model can be developed, and to better understand the delineating factors that lead to placebo analgesia versus nocebo hyperalgesia.

Chapter 1 - An introduction to the placebo effect

What is a placebo effect?

A placebo is a neutral substance, object, or process that on its own has no relevant active pharmacological or physiological properties. Under the right circumstances, a placebo is able to mimic an active treatment and produce a beneficial change in psychology, physiology, and treatment outcomes (Gertsch, 2018; Stewart-Williams & Podd, 2004). In research, placebos have been treated as nuisance factors in drug trials (Benedetti, 2012), and were for a long time considered a subjective non biological occurrence (Luana Colloca (2018b)). Historically, the name placebo was given to fake mourners at funerals, to sycophants, and to medicines that had no known benefit but were given to patients to please them (Thompson, 2000). In short, a placebo has historically been viewed as something that is not genuine.

The idea of the placebo as an illusory effect has until relatively recently pervaded scientific research and resulted in research trials discounting potentially helpful (or at the very least insightful) results because they were 'nothing more than a placebo'(Wampold, Minami, Tierney, Baskin, & Bhati, 2005). When studied with the inclusion of a no-treatment arm, placebos are clearly distinguished from other non-treatment reasons for symptom improvement such as spontaneous recovery, rest, or regression to the mean (Davis, 2002; Vase & Wartolowska, 2019). While the importance of the placebo in modern medicine was recognized by the mid-1900s (Beecher, 1955; Shapiro, 1960), it was not until the latter part of the century that researchers began to see the placebo as the intricate, complex, and therapeutically relevant combination of biology, psychology, and sociology that it is.

The *placebo* as an agent needs to be understood separately to the *placebo response.* The placebo response is the positive treatment outcome that is attributable to the placebo substance,

object, or process administered. It is the combination of the placebo and the placebo response that forms the *placebo effect*. Placebo effects can be very specific and can take the form of a variety of outcomes – the same placebo agent that is given to one person as a stimulant and another as a depressant can induce inverse placebo responses and act as a stimulant in the first, and a depressant in the second person (De La Fuente-Fernandez & Stoessl, 2002). The placebo effect should be understood as a process that induces *positive* treatment outcome. That is, it results in an improvement in symptoms or an increase in beneficial effects as opposed to a negative outcome, where there is a worsening of symptoms or an increase in detrimental effects.

In contrast, *nocebo* responses are negative outcomes not attributable to the active treatment being delivered. In a nocebo response psychological or physiological detrimental effects or worsening of symptoms is brought on by factors related to treatment, not by the actual treatment itself. Where a suggestion of pain relief with the administration of an inert medication may result in the positive *placebo* response of pain reduction, suggestion of more pain may result in the negative *nocebo* response of increased pain. Similarly, previous positive experiences with a treatment can lead to improved treatment outcomes the second time it is delivered, but negative experiences with a treatment may reduce effectiveness when delivered again (Luana Colloca, Petrovic, Wager, Ingvar, & Benedetti, 2010). While both placebo and nocebo effects have significant clinical relevance (Luana Colloca, Sigaudo, & Benedetti, 2008) the current paper will focus on the conditions and processes underlying the placebo effect, while recognizing that placebo exists within a context that can include nocebo responses.

Examples of the placebo effect in humans

Placebo effects occur across a range of physiological systems, symptoms, disorders, and pathologies (Geuter, Koban, & Wager, 2017). People living with Parkinson's disease have

reported improvements in motor functions after placebo drug treatment (Goetz, Leurgans, Raman, & Stebbins, 2000) and placebo surgery (Freeman et al., 1999). The heart rate of healthy people can be altered via placebo processes (Pollo, Vighetti, Rainero, & Benedetti, 2003), and respiratory distress can be induced by placebo agents (Benedetti, Amanzio, Baldi, Casadio, & Maggi, 1999). Placebo bronchodilators have been shown to reduce asthma symptoms (Kemeny et al., 2007) and in auto-immune disease, placebo effects can induce immunosuppression and can assist in reducing the dose of immunosuppressant drugs required to achieve the same result (Kirchhof et al., 2018).

Placebo effects can arise from many types of triggers. For instance, placebo effects can be triggered by the context of a hospital or medical center, by the attitude of the treating professional, or the colour of the pill being administered (De Craen, Roos, De Vries, & Kleijnen, 1996). Many stimuli or contextual cues associated with treatment can become associated with and then mimic the effects of the treatment (Gryll & Katahn, 1978).

Similarly, placebo effects can produce many different outcomes across a range of physiological and psychological processes, medical conditions, and treatment modalities. For instance, placebo immune responses have been demonstrated in humans and animals where placebos can either stimulate or suppress immune parameters. Otherwise neutral cues, such as a taste, paired with delivery of an immunomodulatory drug, can then produce a placebo immune response identical to the effect of the immunomodulatory drug (Albring et al., 2012; Kirchhof et al., 2018; Lysle, Cunnick, Kucinski, Fowler, & Rabin, 1991; Schedlowski & Pacheco-López, 2010; Vits et al., 2011). In high altitude environments, providing two exposures to oxygen treatment later resulted in a reduction in high-altitude sickness symptoms in the same subjects when given sham oxygen treatment with the mask and oxygen tank acting as cues (Benedetti,

Barbiani, & Camerone, 2018; Benedetti, Durando, Giudetti, Pampallona, & Vighetti, 2015). Patient perceptions of treatment can also impact outcomes: post-surgical drugs that are administered in the presence of a treating physician have better outcomes than when the same drug at the same dose is administered surreptitiously without a physician present (Enck, Bingel, Schedlowski, & Rief, 2013) and at 6 month post-surgery, treatments based purely on building positive expectations relating to treatment have been associated with lower disability scores at 6 month follow up (Doering, Glombiewski, & Rief, 2018).

Individual differences in placebo responses have been shown to have significant variability across time and between studies (Kaptchuk et al., 2008) and it is still not completely clear if responding varies by person, or by trial. Do placebo responses occur in 50% of people, or 50% of the time? (Kaptchuk et al., 2008). One study did attempt to clarify this by re-creating the same placebo environment 8 days apart and found that there was a high correlation between placebo response in the first instance and the second (Whalley, Hyland, & Kirsch, 2008). As with placebo research more broadly, however, findings related to the reproducibility of the effect are confounded by the lack of natural history control groups (Hróbjartsson, Kaptchuk, & Miller, 2011). Treatment history, patients' understanding of treatment efficacy, and the treatment environment are all external factors that are known to influence the magnitude of the placebo effect (Geuter et al., 2017). Internally, personal attributes, personality traits and motivational states are also thought to account for the substantial individual difference in placebo responding that has been documented in the literature (Scott et al., 2007; Stewart-Williams & Podd, 2004). In particular, a disposition that leans toward optimism or hopefulness has long been associated with higher rates of placebo responding (Morton, Watson, El-Deredy, & Jones, 2009). Overall, the recent focus on the placebo as a clinically relevant area of research has furthered the

communal understanding of the effect and highlighted the complex and layered conditions under which it can occur.

As demonstrated above, the placebo effect occurs across a wide array of symptoms and bodily systems. Perhaps the most investigated branch of the placebo effect is the application of placebo to pain (Luana Colloca, 2018b; Vase & Wartolowska, 2019). Unlike many illnesses that affect the periphery organs and structures, pain conditions are an intricate mix of peripheral and central nervous system processes, and are impacted greatly by emotional, social, and cognitive states (Wager & Atlas, 2015). The remainder of this thesis will focus on the placebo effect in the context of placebo analgesia.

Placebo analgesia

What is placebo analgesia?

Placebo analgesia can be defined as a positive change in pain symptoms attributable to the treatment experience and psychosocial cues rather than the actual substance, object or process being administered (Luana Colloca, 2018b). During world war II, surgeon Henry Beecher reported seeing patients from the battle field who had suffered immense trauma, but felt little pain (Best & Neuhauser, 2010). His observation that the intensity of the wound did not always correlate with the perception of pain led him to investigate whether psychological suggestion could influence the way pain was experienced in other settings, such as a post-operative ward (Lasagna, Mosteller, von Felsinger, & Beecher, 1954). The findings from this work showed that when people in significant pain were administered multiple doses of morphine, and then given saline under the guise that it was another dose of morphine, a significant proportion (over 50%) of them experienced pain relief at a level similar to that given by the morphine (Lasagna et al., 1954).

Since these early investigations, research into placebo analgesia has been consistent and in the late 1990s and early 2000s the potential therapeutic benefit of placebo treatments started to gain significant attention. In the decade 1991 – 2000 more than 400 published papers referenced the term "placebo analgesia", in 2001 – 2010 there were just under 3000 publications, and from 2011-2020 there were close to 5000.

Examples of placebo analgesia

Placebo analgesia has been reported in a diverse range of pain conditions in the clinical setting. In migraine patients presenting to the emergency department, those who were given an hour long placebo drug treatment reported pain relief comparable to administration of active analgesics (Bigal, Bigal, Bordini, & Speciali, 2001). In a surgical lung cancer cohort (Pollo et al., 2001), patients were administered either buprenorphine or a saline placebo in a double blinded or deceptive design. Those who were given a deceptive placebo (i.e. they believed it was a pain killer) requested the least amount of pain killer top ups, indicating they had less need for pain relief, and had statistically similar subjective pain ratings to the drug treatment group across the three day experimental period (Pollo et al., 2001). In chronic back pain, treatment with an active lidocaine patch resulted in reductions in pain comparable to those treated with a placebo patch, and treatment with any patch resulted in reduced pain after 2 weeks compared with no-treatment groups (Hashmi et al., 2012). A recent review by Castelnuovo and colleagues (2018) further identified fibromyalgia, pain related to HIV, and neuropathic pain as being capable of placebo manipulation.

In experimental settings, placebo analgesia is commonly induced using conditioning with acute pain models such as electric shock, thermal pain, or compression. Yeung et al (2014) gave participants a baseline electric shock, which they reported to be painful. They then conditioned

the participants to associate a pretend Transcutaneous Electrical Nerve (TENs) on their arms with a reduction in pain. This was achieved by pairing 'activation' of the device (i.e., the participants were told that the device was active when a light was on) with surreptitious reductions in the shock intensity during these trials. On other trials when the TENs machine was not thought to be active, the participants received the full shock intensity. The reduction in pain was attributed to the TENs machine. During test trials, the participants rated the pain intensity of the electric shocks on trials with or without the active TENs device, but with the same shock intensity on all trials. Participants reported that their pain was significantly less when the pretend TENs device was on (Yeung, Colagiuri, Lovibond, & Colloca, 2014). Thermal pain was used in a similar experimental design where participants were given two different creams and told that one was an analgesic and the other was not (Schafer, Colloca, & Wager, 2015). An association was developed between the 'analgesic' cream and reduced pain by surreptitiously reducing the temperature when the placebo cream was applied. Both control and placebo creams were presented with the same high temperature at test, and those in the placebo cream group reported less pain. The analgesic placebo persisted even after it had been revealed that the cream was a placebo, indicating that the analgesia resulting from the conditioning trials was not reliant on participants believing the cream was an analgesic.

Experimental models can also bring about placebo analgesia via verbal instructions on their own without repeated pairings with a cue. Using a pain model based on irritable bowel syndrome Price and colleagues (2007) demonstrated that placebo analgesia could be induced in visceral pain simulations when participants were simply informed that the rectal balloon used to generate visceral pain was coated in an analgesic jelly. Those who did not receive this instruction

reported higher pain levels, and showed increased activity in pain regions in their brain compared to the placebo group (Price et al., 2007).

These examples highlight that placebo analgesia occurs across both clinical and experimental environments and is observed in both acute and chronic pain conditions. They also show that placebo responses can be established via different methodologies and processes, and that the strength of the response varies across all of these domains.

Magnitude of the placebo analgesic effect?

The broad reach of the placebo analgesic effect suggests its clinical significance could be high, but variability of published results indicate that efficacy varies according to the population. While inconsistent outcomes can be difficult to interpret, the potential benefit of placebo treatments being used in pain management is extremely high and therefore persistence in this field of research is vital. The inadequacy of pain management, particularly for chronic pain conditions, is a major contributor to the worldwide burden of disease (Rice, Smith, & Blyth, 2016). In a recent large scale world view report (Vos et al., 2015), seven of the top ten most common chronic health conditions were related to pain, and pain itself was identified as *"clearly the most important current and future cause of morbidity and disability across the world"*. (Rice et al, 2016, pg 792). Treatment options are minimal for chronic pain. The side effects that accompany available pain medications are often severe and debilitating themselves, and international research into the opioid crisis clearly shows that the current reliance on opioids for pain management is unsustainable (Volkow & McLellan, 2016). Harnessing the placebo analgesic response to limit the need for pain pharmacotherapy has the potential to reduce not only the immense burden caused by pain, but also the burden of managing the consequences of over-reliance on pharmaceuticals.

In people, the magnitude of placebo analgesia varies substantially across studies. Published meta analyses show how varied results can be: A 2001 paper (Hróbjartsson & Gøtzsche, 2001) reported a very low overall effect of placebo agents on the pain experience when compared to no treatment controls, and a 2004 follow up analysis (Hróbjartsson & Gøtzsche, 2004) supported these findings. In 2002, it was reported that higher effect sizes were observed when the placebo analgesic effect was the focus of the study, rather that when it was included merely as a control condition as would be found in most clinical trials (Vase, Riley III, & Price, 2002). A recent meta-analysis (Forsberg, Martinussen, & Flaten, 2017) tried to understand this variability by investigating factors associated with placebo analgesia. They found higher effect sizes for placebo analgesia in patient populations with clinical pain compared to healthy people who were administered pain in the experimental environment, suggesting that when there is a stronger incentive for the placebo to work, it does (Forsberg et al., 2017). Finally, Castelnuovo and colleagues (2018) meta-analysed type of pain as a factor contributing to the placebo analgesic response and found moderate effects for fibromyalgia, migraine, and pain associated with HIV, but weak effects in central neuropathic pain (Castelnuovo et al., 2018), signifying that placebo responses are not consistent across all pain experiences.

Further investigation into the mitigating factors involving placebo analgesia and the underlying mechanisms needs to be pursued so that harnessing the effect for therapeutic benefit can be made possible.

Causes of placebo analgesia

Theories for the development of placebo analgesia are primarily divided into two branches: the conditioned theory, and the expectancy theory. There is significant evidence

underpinning both theories, and in fact much of the literature suggests that *both* constructs are integral to the development of placebo analgesic effect (Schafer, Geuter, & Wager, 2018; Stewart-Williams & Podd, 2004).

By definition, placebo interventions do not have direct physiological effects on the body. It is the surrounding social, cultural and physical cues, verbal and written suggestions, and history with a given treatment that induce the positive outcomes received from placebo treatment (Wager & Atlas, 2015). These factors, whether direct or indirect, have emotional and cognitive meaning and are interpreted by the brain as signals for recovery, health, and benefit (Luana Colloca, 2018a). Signals are meaningless without knowing what they are signifying, thus in the placebo context knowledge of signals must be imparted before treatment begins. This can be done by repeated prior exposure to the signal and the outcome it precedes (conditioning) or simply by explaining the benefit the signal will provide (expectation).

Conditioned theory of placebo analgesia

The conditioned theory of placebo analgesia proposes that the effect is the result of Pavlovian conditioning (Siegel, 2002). That is, the effect is a learned physiological response developed over numerous trials pairing a neutral cue with a biologically relevant stimulus and response. Conditioned placebo analgesia occurs when an unconditioned response (UR - lessened pain experience e.g. relief from headache) following an unconditioned stimulus (US – active analgesic e.g. paracetamol), is paired with a conditioned stimulus (CS – the cue e.g. a white pill). Repeated exposure to the CS in the presence of both the US and the UR builds an association so that when the CS is presented alone, a *conditioned* response (CR – lessened pain experience e.g. relief from headache) occurs, even though the US is no longer present (Luana Colloca & Miller,

2011). In the context of the analgesic placebo, the placebo agent is the CS, and the placebo response is the CR (Stewart-Williams & Podd, 2004).

In the clinical setting, the CS can be represented by almost anything associated with the treatment environment. The smell of the hospital, characteristics of the clinician, the injection procedure, and the shape, taste, and look of the medication being delivered are all inert factors that can act as a CS. Across the lifespan, patients experience multiple trials pairing these CSs with the UR, and thus they develop an ability to alter treatment outcomes (Montgomery & Kirsch, 1997). Experimentally pairing an active drug (e.g. paracetamol) with a direct or indirect cue has been shown to elicit an analgesic response at test when the cue is presented, and saline or other inert substance is delivered. Bendetti et al (2003) demonstrated this in humans by administering healthy participants ketorolac (a pain killer, the US) via injection in a designated room (the CSs), while they underwent the painful tourniquet technique across two trials on different days. Receiving the drug significantly increased their pain tolerance (UR) compared to baseline. At the test on day 3 participants were told they were receiving the drug for a third time but were instead administered saline via the same process (CS), and this resulted in higher than baseline pain tolerance (CR) i.e. placebo analgesia (Benedetti et al., 2003).

Importantly in classical drug conditioning, the CR is not always equivalent to the drug effect, and this is demonstrated in some studies of animal opioid drug conditioning (Siegel, Hinson, & Krank, 1978). Unlike the model for development of placebo analgesia that has been discussed so far, studies using opioid analgesics often result in the opposite effect – a conditioned *nocebo* response that results in drug tolerance and/or hyperalgesia. In this instance, the CS, US and UR remain the same, but presentation of the cue at test results in a *compensatory* CR occurring and instead of *hypo*algesia, *hyper*algesia is observed. Eikelboom and Stewart

(1982) discuss this as the difference between the unconditioned effect of the drug (direct analgesia), which is not mediated by the central nervous system, and the compensatory unconditioned response (hyperalgesia) to the drug effect, which *is* controlled by the central nervous system. As the compensatory response is mediated by the subject's central nervous system, they argue that it is susceptible to conditioning. As a result, cues associated with opioid analgesics can come to elicit the compensatory response and reduce the analgesic effectiveness of the drugs producing conditioned tolerance.

Eikelboom and Stewart (1982) posit that the conditioned compensatory response occurs only when the drug effect acts as unconditioned stimulus and not as the unconditioned response. That is, when the drug effect acts as an afferent input to the central nervous system (unconditioned stimulus), which in an attempt to maintain homeostasis generates an efferent response to the drug input (unconditioned response). If the drug merely has a peripheral effect (an example given is antacids), then the conditioned response will mimic the drug effect because no compensation is occurring and thus cannot be conditioned. This theory helps to explain the difference in outcomes reported across drug types, but doesn't fully explain why some conditioning studies using opioids report placebo analgesia(Lasagna et al., 1954), and others report conditioned drug tolerance (Siegel, 1975a)

Behavioural conditioning models that exclude an active pain reliever are another way that placebo analgesia can be induced via conditioning and can help avoid the problem of compensatory responses. Instead of using an active substance during the conditioning phase, the CS is paired with a reduction in noxious stimulus and at test, the CS alone can induce placebo analgesia. Colloca and Benedetti (2011) demonstrated the effectiveness of this design in their study in humans which paired a red-light cue with a painful shock and a green-light cue with a

sham ‘protective’ electrode placed on their finger and a reduced shock intensity. The distinction between shock intensities was not disclosed to the subjects and they believed the reduced pain was caused by the sham electrode. At test, the cues were presented with the same intensity of shock. Immediately following conditioning trials, and 1 week later, those in the paired placebo group showed increased tolerance to pain after the green light cue, but those in the unpaired group did not. Similarly, Yeung et al (2014) conditioned participants to associate a pretend Transcutaneous Electrical Nerve (TENs) on their arms with a reduction in pain from electric shock. False ‘activation’ of the TENs device was paired with surreptitious reductions in shock intensity during these trials. On other trials the TENs machine was not thought to be ‘off’ and the participants received the full shock intensity. During test trials, the participants rated the pain intensity of the electric shocks on trials with or without the active TENs device, but with the same shock intensity on all trials. Participants reported that their pain was significantly less when the pretend TENs device was on.

Behavioural models of conditioning can lead to the development of expectations beyond a simple learned association. Unlike pavlovian models that build associative physiological learning based on contiguity (i.e. at the cue, there is a simultaneous drug effect) behavioural conditioning models provide the subject with *information* about future events i.e. when the cue appears, I will not experience as much pain (Rescorla, 1988). Rather than learning a simple association, the subject learns what the cue means in context for *them* and develops an expectation based on this without the confounder of a physiological drug effect serving as an additional US. In the case of placebo analgesia, the expectation of the US (pain relief) becomes the UR, and the CR is the pain relief that follows (Montgomery & Kirsch, 1997). Table 1 details the shift in conditioning components between these two approaches.

Table 1 *Difference between placebo responses brought on by drug paired conditioning and behavioural conditioning*

	Conditioned stimulus (CS)	**Unconditioned stimulus (US)**	**Unconditioned response (UR)**	**Conditioned response (CR)**
Drug paired conditioning	White pills	Physiological effect of drug (e.g. analgesia)	Pain relief	1. Compensatory (hyperalgesia) 2. Complimentary (hypoalgesia – placebo analgesia)
Behavioural conditioning	Green light	Reduction in pain	Anticipation of reduction in pain	Hypoalgesia – placebo analgesia

Conditioned expectancy is one method of developing an expectation – if a cue is paired with a stimulus enough times it builds an expectation in the subject. Seemingly small cues can result in large conditioned expectations and thus large changes in treatment outcomes (Geuter et al., 2017), for example changing the name of a medication brand that is well known to a person can result in a different expectations about efficacy and thus a different treatment outcome (Whalley et al., 2008).

Expectancy theory of placebo analgesia

Contrasting with conditioning theories which propose that placebo analgesia occurs because of prior exposure to drug or cue effects, the expectancy theory posits that a placebo response occurs because an internal prediction about the outcome triggers a physiological response in line with that prediction. In short, a placebo analgesic creates analgesia because the patients expect that it will provide relief (Stewart-Williams & Podd, 2004).

The expectancy theory does not require previous exposure to a treatment, and instead conceives that the effect is dependent on the beliefs the subject has toward the treatment being

delivered, regardless of how they were formed. In animals, expectation from conditioning is common, in humans there are many other ways of producing an expectation. Expectations can be influenced by contextual and situational features that hold meaning related to a treatment. A patient's relationship with their doctor, cultural or religious beliefs, and paraphernalia related to treatment delivery (latex gloves, syringes for example) can all be considered informational signals that build an expectation about treatment outcomes (Stewart-Williams & Podd, 2004). Even without any prior personal exposure to these signals they can hold meaning because of social and observational learning (Luana Colloca & Benedetti, 2009).

Perhaps the strongest and most researched method of expectation formation is verbal or written instruction. Being explicitly told that something will benefit you, especially when it is a person of authority telling you, is a strong factor in expectancy development and has been experimentally used to induce placebo responses independent of associative conditioning (Geers, Wellman, Fowler, Helfer, & France, 2010; Montgomery & Kirsch, 1997). In one study using the tourniquet method of pain induction, patients experienced pain relief in a single session of being given a saline solution alongside the instruction that it would reduce pain significantly (Benedetti et al., 2003). In the same study, patients who were told that the drug would increase pain experienced a nocebo response and reported higher ratings of pain than they had at baseline under the same conditions. Self-reported expectations in patients have also been shown to predict treatment outcomes in meta-analysed data sets (Vase & Wartolowska, 2019). These examples all demonstrate that a patient's expectation of a treatment is directly linked to the outcome they experience, regardless of the actual treatment they receive.

More than just conditioning or expectation?

While conditioning and expectation are often spoken about as competing explanations for placebo analgesia (Kirsch et al., 2014), neither can account for the full extent of the effect on its own. A third line of thinking suggests that conditioning and expectancy both play important but distinct roles in the development of a placebo response (Finniss & Benedetti, 2005; Stewart-Williams & Podd, 2004). In studies that involve both conditioning and verbally induced expectation, placebo responding can be reduced but not reversed by revealing that the active agent is in fact inert (Schafer et al., 2015), indicating that there is an additive effect of the two processes. The additive effect of conditioning and expectation was examined in a 2018 review (Coleshill, Sharpe, Colloca, Zachariae, & Colagiuri, 2018) who found evidence for additivity in only 3 of 7 studies assessed. They suggest that additivity in placebo analgesia could be dependent on the analgesic being administered, on dose, and on duration of treatment.

This idea of expectancy and conditioning playing distinct yet equally important roles was further explored by Schafer, Geuter and Wager (2018) who propose that the placebo is a dual process effect. Their theoretical proposition is that placebo is developed by both long-term and short-term learning that leads to a schema for treatment outcomes – e.g. pain relief – being developed. Expectancy and conditioning both contribute to the development of this schema, and it is the separate yet combined effect that can explain variability in the development of placebo analgesia across different studies and populations (Schafer et al., 2018). This theory is supported further by Colloca and Miller (2011) and Geuter, Koban, and Wager (2017) who suggest that a concept-based understanding of the outcome being manipulated (e.g. pain experience) and the things that can lead to a shift in this outcome (e.g. cues and signals) is developed using a complex combination of learned associations, suggestion, and unconscious cues. Prior perception, predictive pathways, and sensory modulation (Geuter et al., 2017) as well as social

learning and evolutionary prioritisation (Luana Colloca & Miller, 2011) all contribute to the eventual downstream effect that is placebo analgesia.

Exactly how these processes lead to the development of placebo analgesia is unclear, and the distinct role they play in different parts of pain modulation and placebo is the focus of current research. One way that research is moving forward in the space is via the use of animal models that allow for control of the complex and highly variable social and environmental factors outlined above.

Animal models of placebo analgesia

Why develop an animal model?

Research into placebo analgesia to date has largely been in human cohorts, despite the numerous advantages of studying neurobiological processes of pain in animals (Keller, Akintola, & Colloca, 2018), including practical considerations such as ease of obtaining and housing subjects, as well as the ability to control relevant variables such as exposure to pain and pain experiences; genetic factors; and the environment.

Mammals have numerous neurobiological mechanisms for modulating the pain experience, and these systems are homologous between humans and laboratory rats. Specifically, a descending endogenous analgesic system from the brain to the spinal cord has been identified in studies of pain modulation in laboratory rats, comprising of the midbrain periaqueductal grey (PAG) and rostro ventral medulla (RVM), which sends projections down to the spinal cord that inhibit the transmission of nociceptive information from the body to the brain. This system has been extensively studied in laboratory animals and has been shown to mediate the analgesic effects of opioid analgesics and stress (Basbaum & Fields, 1984; Mogil, Davis, & Derbyshire, 2010). Modern imaging from PET and fMRI in humans shows that the same brain areas are

activated during placebo analgesic responses (Wager & Atlas, 2015). Therefore, we have an opportunity to complement human imaging studies by comprehensively studying the structures and pathways that are implicated in the effect. And unlike using human subjects, rodent studies allow us to target specific brain regions using more invasive methodologies. In the current research climate where new brain regions are added to the list of areas involved in the placebo effect at a rapid rate (Amanzio, Benedetti, Porro, Palermo, & Cauda, 2013), the ability to specifically isolate and investigate particular areas would add invaluable knowledge to the neurobiological theory of placebo analgesia.

What do we know about animal models of placebo analgesia?

After significant research into morphine tolerance and resulting hyperalgesia by Siegel and colleagues in the 1970s and 80s (Krank, Hinson, & Siegel, 1981; Siegel, 1976; Siegel, 1979) Kehoe (1989) demonstrated that an olfactory cue previously paired with morphine could induce *hypo*algesia in ten-day old rat pups and concluded that the reasons for obtaining placebo analgesia and not morphine tolerance could be related to number of trials or age of the animal. Bevins et al (1995) investigated the role of trial count in morphine conditioning and placebo analgesia in rats and found that 6 conditioning trials resulted in placebo analgesia, and 3 or 1 trials resulted in no change to pain response (Bevins et al., 1995).

Cue type has also been investigated in relation to placebo analgesia in rodents. Miller and colleagues (1990) tested the hypothesis that tolerance and hyperalgesia resulted from pairing morphine with external environmental cues, and that analgesia related conditioned responses would be better paired with gustatory cues, similar to taste aversion conditioning (Miller, Kelly, Neisewander, McCoy, & Bardo, 1990). They found that analgesic responses were observed after taste CS exhibition. Bardo and Valone (1994) also found that a taste cue paired with morphine

elicited placebo analgesia after a conditioning period, and that placebo analgesia was strongest when conditioned with a 30 mg/kg dose of morphine.

Two additional papers (Randall, Kraemer, Valone, & Bardo, 1993; Valone, Randall, Kraemer, & Bardo, 1998) looked more closely at dose response and placebo analgesia. Valone and colleagues'(1998) results contradicted previous work (Bardo & Valone, 1994) and found significant placebo analgesia in rats conditioned with 10 mg/kg but not in those conditioned with 3 mg/kg or 30 mg/kg. Randall et al (1993) also found conditioned placebo analgesia when an odor cue was paired with 10 mg/kg of morphine.

Following a ten-year gap in the literature, non-morphine based conditioning models for placebo analgesia began to emerge. First Bryant et al (2009) reported placebo analgesia in mice after a single conditioning trial pairing fentanyl and an environmental cue. Guo, Wang and Luo (2010) demonstrated placebo analgesia in morphine and aspirin conditioning models that utilized specific context cue chambers with visual and tactile cues over 4 conditioning trials. They determined that there was a non-opioid element to placebo analgesia by showing that naloxone blocked the morphine conditioned response, but not the aspirin (J. Guo, Wang, & Luo, 2010). The same group presented similar morphine based findings of placebo analgesia compared to saline controls in two subsequent publications (J. Y. Guo et al., 2011; Zhang, Zhang, Wang, & Guo, 2013). In a non-published study, Jeon (2013) found no evidence of placebo analgesia when attempting to replicate these findings using a similar model to that outlined in Guo et al (2010; 2011) and Zhang et al (2013).

In the only paradigm to involve emotional motivation when investigating placebo analgesia in rodents, Nolan and colleagues (2012) designed a pain model that required hairless Sprague Dawley rats to push their bare snouts through a hot metal barrier to receive a food

reward. Their study design involved two pairings of morphine (1 mg/kg) with a tactile cue, and then delivery of a saline injection and the cue at test. Their findings demonstrate a different *distribution* of responding between conditioned and control groups, but no significant difference to show evidence of placebo analgesia in the paired group.

Nerve ligation was used by 3 published studies to assess rodent placebo analgesia in chronic rather than acute pain models (Akintola et al., 2019; McNabb, White, Harris, & Fuchs, 2014; Zeng et al., 2018). In this model, rats have a nerve surgically ligated by a thread causing allodynia and hyperalgesia in the areas this nerve innervates. McNabb and colleagues (2014) trialed nerve ligation as a method of inducing placebo analgesia in three experiments that used morphine, gabapentin, and loperamide in a 4 trial conditioning model. None of their experiments demonstrated placebo analgesia, and unlike Nolan et al (2012) they found no significant differences in response distribution. Akintola and colleagues (2019) used an orofacial nerve ligation pain model and paired fentanyl with a multi-faceted cue that included visual, audio, tactile, taste and olfactory elements but found no evidence of placebo analgesia. Both groups suggest that the nerve ligation model may not be appropriate for placebo analgesia as it a measure of sensory/reflexive pain and is not sensitive to cognitive control required for a placebo response. Contiguity, number of trials (McNabb et al., 2014) and interrupted learning from too many cues (Akintola et al., 2019) were also potential contributors to the null-results.

An additional unpublished study by Boorman (2018) also used nerve ligation. This study found no overall results of placebo analgesia in a 7-trial conditioning paradigm pairing morphine and a specific context cue chamber with visual, tactile and olfactory cues. They did, however, observe a different pattern of distributions of results, suggesting there were high and low placebo responders in the conditioned sample. A single recent study (Zeng et al., 2018) reported

successful induction of placebo analgesia using L5/L6 spinal nerve ligation. Their conditioning model paired the experimental environment with an injection of gabapentin over 4 trials.

Finally, two papers (Lee et al., 2015; Xu et al., 2018) employed non-drug behavioural conditioning paradigms to develop placebo analgesia in rodents. Instead of pairing a CS with a drug injection, the studies paired a specific context cue chamber with a high heat pain (50 degrees Celsius), and another context cue with a reduction in heat pain (45 degrees Celsius) across an 11 day conditioning period. At test, the low pain cue was presented with the high heat stimulus and both studies reported placebo analgesia in the conditioned group when compared to the control.

What does all this mean?

While there is evidence that placebo analgesia occurs in rodents, results are mixed, and several unpublished and published studies report null-results. In their 2018 review, Keller and colleagues comprehensively summarized the literature and concluded that more research was needed to establish a valid and reliable model. They also discussed a need for more robust analytical and methodological reporting in animal literature. Type 1 errors were not managed adequately in many of the analytic strategies, and often only select control groups were included in result analysis (Keller et al., 2018).

Current aims

The current thesis aims to investigate placebo analgesia in animal models to try and assess if this is a valid and reliable way of studying the effect. The first aim was to replicate and validate a recent study of placebo analgesia in rodents in the local environment.

To properly translate animal research into human samples appropriate models that can be compared and contrasted need to be used. This study will attempt to replicate the animal model

of placebo analgesia presented by Lee and colleagues (2015), which utilises a behavioural conditioning model. The model mirrors work done by Yeung et al (2014) and Colloca and Benedetti (2011) where a cue is presented to signal a high pain experience, and a separate cue is given alongside a placebo agent and surreptitiously reduced noxious stimuli. The use of a behavioural model is ideal because it removes the problem of tolerance, and also reduces stress for the animal as no injections need to be administered. As all methods will be followed exactly, it is hypothesised that results will mirror Lee et al and rats in the paired conditioning group will exhibit higher pain thresholds on the high heat hotplate after the low pain cue compared to the unpaired controls.

Chapter 2 – Experiment methods and results

Experiment 1

Research demonstrates that it is possible to establish an analgesic placebo effect in rodents using a behavioural conditioning paradigm without the inclusion of active drug substances such as morphine, and that the pain experience can be modulated by expectation or Pavlovian conditioning (Lee et al., 2015; Xu et al., 2018). When studying endogenous pain mechanisms, the use of active substances can introduce confounding factors such as the inability to observe certain behaviours due to lethargy or immobility caused by the drug; issues to do with withdrawal and tolerance; interrupted learning caused by drug induced cognitive impairments; and finally added stress and discomfort for the animals related to the administration of injections (Lee et al., 2015).

Recent studies in humans have also been able to demonstrate a placebo analgesia effect using only a behavioural conditioning paradigm (Yeung et al., 2014). Yeung et al (2014) gave participants a baseline electric shock, which they reported to be painful. They then conditioned the participants to associate a pretend Transcutaneous Electrical Nerve (TENs) on their arms with a reduction in pain. This was achieved by pairing 'activation' of the device (i.e., the participants were told that the device was active when a light was on) with surreptitious reductions in the shock intensity during these trials. On other trials when the TENs machine was not thought to be active, the participants received the full shock intensity. The reduction in pain was attributed to the TENs machine. During test trials, the participants rated the pain intensity of the electric shocks on trials with or without the active TENs device, but with the same shock intensity on all trials. Participants reported that their pain was significantly less when the pretend TENs device was on. Thus, the activation light on the TENs device appeared to act as a conditioned stimulus (CS) for a conditioned placebo

response. Mirroring these findings in an animal model would give us a direct pathway to studying the underlying mechanisms involved in endogenous pain modulation.

Lee et al (2015) developed a protocol to condition placebo analgesia in laboratory rats using a similar design to Yueng et al (2014) in which they paired a cue with a reduction in intensity of a painful stimulus. They used an 11 day conditioning protocol that utilised conditioned place preference (CPP) boxes. The CPP box has 2 distinct chambers that are separated by a smaller corridor/chamber, all of which can be cut off from each other with retractable doors. Rats were placed into one compartment (placebo context, pCX) for 15 minutes, and then put onto a hotplate set at 45°C (low pain). In a subsequent trial, rats were placed into the other compartment (control context, cCX) for 15 minutes and then put onto the hotplate set at 50°C (high pain), and this was repeated daily for the duration of the experiment. At test, rats in the paired conditioning group were able to withstand the pain of the hotplate for longer after experiencing pCX and then being placed onto the high pain hotplate (50°), compared to being placed in cCX and then experiencing the 50° hotplate. Their results suggest that the pCX gave the rats in the paired group a conditioned expectation of low pain, and this expectation elicited a placebo analgesia response. Table 2 outlines the parallels between Lee et al's animal model and Yeung et al's human model of placebo analgesia.

Table 2 *Comparison of the human (Yeung et al., 2014) and animal (Lee et al., 2015) models of behaviourally conditioned placebo analgesia.*

Subject	Pain stimulus	Low pain cue	Reduced pain stimulus
Human	Electric shock	TENs machine	Shock reduced
Rat	High heat hotplate	pCX	Temperature reduced

Xu and colleagues (2018) reported very similar findings in two independent groups of adult male rats. Their model used the same design as Lee et al (2015) but included an extra

visual cue in the form of a different light levels in each of the CPP chambers (chamber 1 was dimmed, chamber 2 was bright). As with Lee et al (2015), they found at test that rats in the paired group could withstand the heat pain for longer after the low pain cue compared to those in the unpaired group.

Lee et al (2015) use the CPP test to demonstrate that a learned association between the cues and the pain stimulus has formed, independent of placebo analgesia being observed. This is important as it demonstrates that the basic conditioning process on which placebo analgesia is based has occurred. At its core, a CPP test shows that an animal has developed a preference for one side of the chamber over the other. Traditionally, this would be done by associating one side with a shock or other aversive stimulus. The animal develops an aversion to the CS+ and a preference for the CS-. The same basic principle is present here – if rats show a preference for the low pain paired room (pCX), there is evidence that they have learned this cue is paired with lower pain and thus developed a preference for it.

The aim of experiment 1 was to replicate the rodent placebo analgesia model used by Lee et al (2015) under local conditions and with our equipment. We hypothesized that rats in the paired conditioning group would spend more time in pCX after conditioning than cCX in the CPP test, but this difference would not be observed in the unpaired conditioning group. For the HPT, after conditioning, it was hypothesized that rats in the paired group would demonstrate a significantly longer hind paw withdrawal latency (HPWL) on a 50° hotplate after the pCX than those in the unpaired control group.

Method

Animals

Female Sprague Dawley rats aged 10-12 weeks (N=31) that weighed 220-240 grams were obtained from the Animal Resource Centre in Perth, WA. Animals were housed in groups of 4 in hanging polycarbonate air flow cages. They were in a limited access room with a reverse 12:12 dark/light cycle (lights on 10AM – 10 PM) and were provided standard chow and sterilised water ad libitum. Cages were cleaned 3 times per week by the University of Sydney's Laboratory Animal Staff. The University of Sydney Ethics Committee approved the research.

Apparatus

Hot plate test apparatus

The hot plate test was administered on an Ugobasile Hot/Cold Plate NG with a range of -5°C - 65°C. The centre metal plate (20CM in diameter) was contained by a 25cm tall plexiglass cylinder. The hotplate was kept adjacent to the CPP boxes to minimise handling and movement for the animal between the CPP and the hotplate. Next to the hotplate apparatus, a video camera was set up to record the conditioning trials and hot plate tests.

Conditioned place preference box

Conditioned Place Preferences (CPP) boxes were custom built. Each chamber was 300mm long, 200mm wide and 200 mm deep. A central chamber separated the two boxes and measured 150mm by 200mm by 200mm. pCX had high contrast black and white stripes on the walls and a metal bar floor. cCX had black walls with white spray paint overlay and had a mesh metal floor. Each chamber had a red Perspex lid that lifted open from a hinge, and guillotine doors that could be locked or open separated each room from the centre chamber. The design of the boxes is outlined in Figure 1. Above each individual CPP box, an infrared

camera was set up to film the rats as they moved freely throughout the chambers in the CPP test.

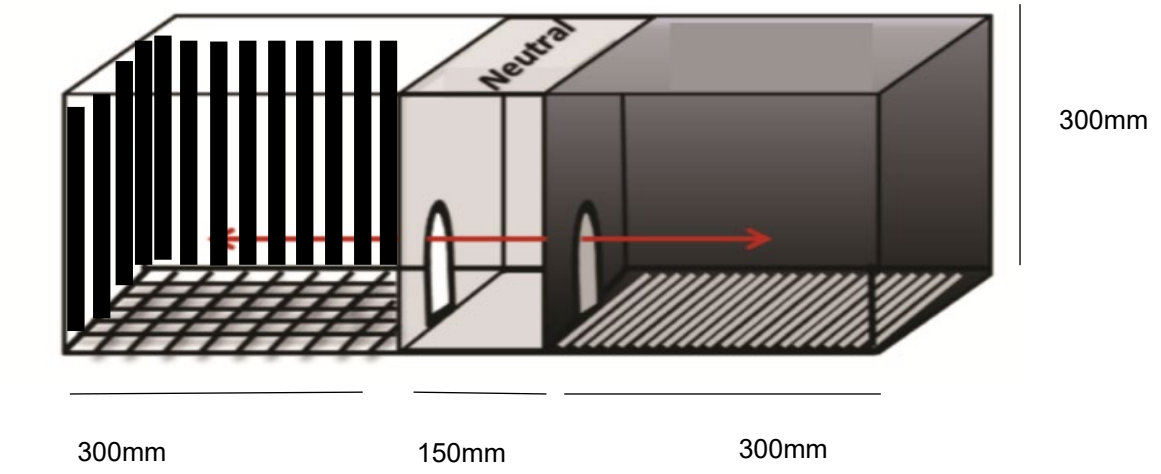


Figure 1 Basic design of the conditioned place preference (CPP) chambers.

Experimental environment

All tests and conditioning were conducted in a separate room to the housing room, and animals were taken into the room one box at a time. They were transported using a trolley and were covered by a large sheet when being transported to minimise stress. In the experimental room, they were taken straight from their home cage, into the test apparatus, and then afterward immediately returned to the home cage. Rats were not left in the home cage in the experimental room for extended periods of time, and experiments were timed so that all animals had the same exposure time to all apparatus, and the same amount of time in their home cage in the experimental room.

The room in which all experiments were conducted was dimly lit with 1 desk lamp sitting on the floor to keep light in the room to a minimum. Door frames were cushioned to avoid door slamming, and the room was silent during all conditioning and tests. Figure 2 outlines the room layout for experiment 1.

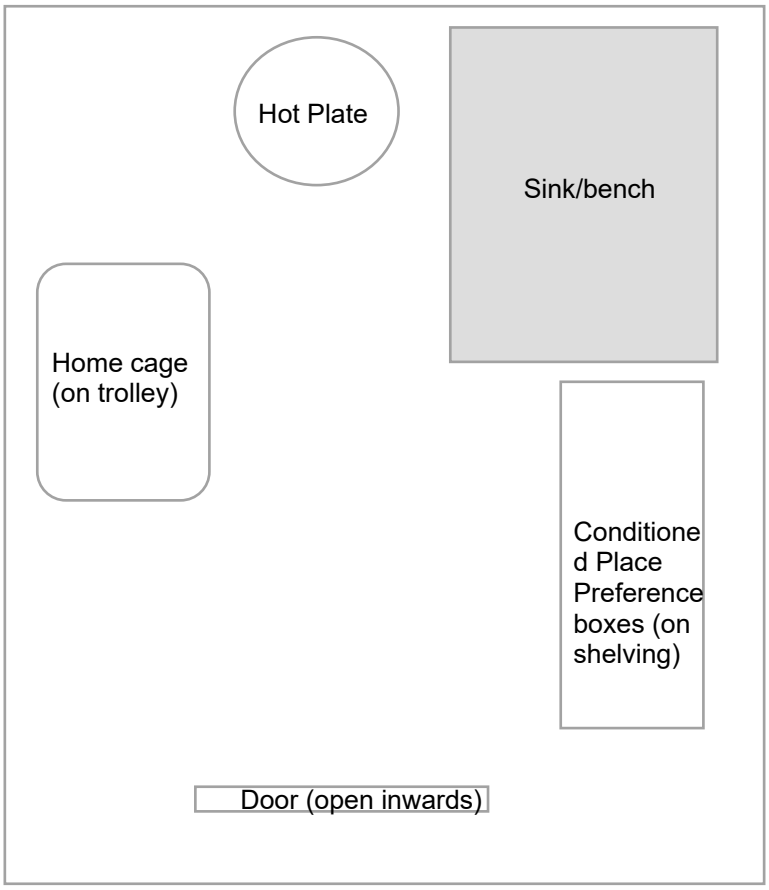


Figure 2 the layout of the room for experiment 1.

Grouping

Before grouping, animals were assessed against the inclusion and exclusion criteria to ensure similar baseline characteristics across groups.

Animals were randomly assigned by box to either the paired group (n = 16) or the unpaired group (n = 16). Randomization was done by importing box numbers into an online randomization program (https://www.random.org/). The random list was split in half, with

the first half being assigned placebo and the second half assigned control. Table 3 outlines the groups and schedule for conditioning.

Age, weight, and gender were all consistent across the animals and so distribution of these traits across groups was considered to be even.

Table 3 *Conditioning groups and schedule. Animals in the placebo group were conditioned using 6 x pairings of the CS (pCX or 2) and the US (low (45°) or high (50°) temperature).*

Group (n)	Conditioning Pairings (CS+US)	Number of Pairings
Placebo (16)	PCX of CPP paired with 45°	6
	CCX of CPP paired with 50°	6
Control (15)	PCX of CPP paired with 45°	3
	CCX of CPP paired with 50°	3
	PCX of CPP paired with 50°	3
	CCX of CPP paired with 45°	3

Acclimatisation, handling, and habituation.

Prior to the experimental phase, animals acclimatized to the laboratory for at least 4 days after arrival in the lab. Following this, they were each handled for 5 minutes daily for 5 days in total by the primary experimenter (RS), so that they could adjust to the handler's presence and scent. The same experimenter handled the rats at each stage of the procedure, and wore the same attire (lab gown, breathing mask, and gloves) throughout. Animals were weighed daily throughout the experimental period to ensure they were not being adversely affected by the process.

Before any of the tests or trials began, each rat was given a 1 x 15-minute habituation session in both the hotplate chamber (set to 25 degrees C) and the CPP box (guillotine doors opened, so they could roam freely throughout the compartments).

Heat pain sensitivity test

On day 1 of the experimental period, the heat pain sensitivity test was conducted to assess rats against the hyper/hypo sensitive exclusion criteria. The same criteria as Lee et al (2015) were used and animals were excluded if they had a HPWL of longer than 20 seconds on the 50°C hotplate (hyposensitive) or less than 50 seconds on the 45° hotplate (hypersensitive). The heat pain sensitivity test was conducted over two sessions, one at 10 AM and the other at 5 PM, in a counterbalanced order. The hind paw withdrawal latency (HPWL; the time until rats lift their hind paw and lick it) was used as a measure of pain tolerance on the hot plate test (HPT). In one session rats were exposed to the hotplate at 45 degrees and their HPWL was measured, in the other session they were exposed to 50 degrees and their HPWL was measured. As soon as HPWL was observed they were removed from the hotplate to limit stress and learning, and no rat was left on the hotplate for longer than 60 seconds. Rats were then immediately returned to their home cage.

Conditioned place preference tests

Two tests for place preference in the CPP were conducted, one before (day 2) and one following (day 10) the conditioning sessions. In these tests, rats were placed one at a time into the centre chamber of the CPP boxes and guillotine doors were opened so they could roam freely between the compartments. The rats were left alone in the boxes for 15 minutes and their movements were recorded using infrared cameras.

Recorded video was then analysed using custom *Labview* software that counted the proportion of time spent in each of the three compartments.

Measurement of placebo analgesia using the Hot Plate Test

Two hot plate tests were conducted, one before (day 3) and one following (day 11) the conditioning sessions. The HPT was administered once at 10AM and again at 5 PM, in a counterbalanced order. In one session, rats were placed in cCX (guillotine doors closed) for

15 minutes and then immediately moved onto the 50 degree hotplate, and in the other session they were placed into pCX (guillotine doors closed) for 15 minutes and then immediately moved to the 50 degree hotplate. Their HPWL was recorded for each session.

Conditioning sessions

Conditioning happened across 2 sessions per day for 6 days (12 sessions in total). Figure 3 gives an overview of the conditioning process. Guillotine doors in the CPP were closed during the conditioning sessions. In one session, rats in the paired group were put into pCX for 15 minutes, and then placed immediately onto the hotplate set to 45 degrees for 60 seconds. In the other session, paired group rats were placed into cCX for 15 minutes, and then placed onto the hotplate set to 50 degrees for 60 seconds. Rats in the control group were given one of four different pairings – pCX and 50 degrees, pCX and 45 degrees, cCX and 45 degrees, and cCX and 50 degrees. Each trial included 15 minutes in the chamber, and 1 minute on the hotplate. Control rats experienced 3 sets of each pairing across the 12 sessions. In each session across the groups, the HPWL on the hot plate was measured and recorded and rats remained on the hotplate until 60 seconds had elapsed. Rats were not kept on the hotplate for longer than 1 minute at any stage. One session started at 10 AM (when lights turned off) and the other at 5 PM, and the order was counterbalanced. All sessions were video recorded.

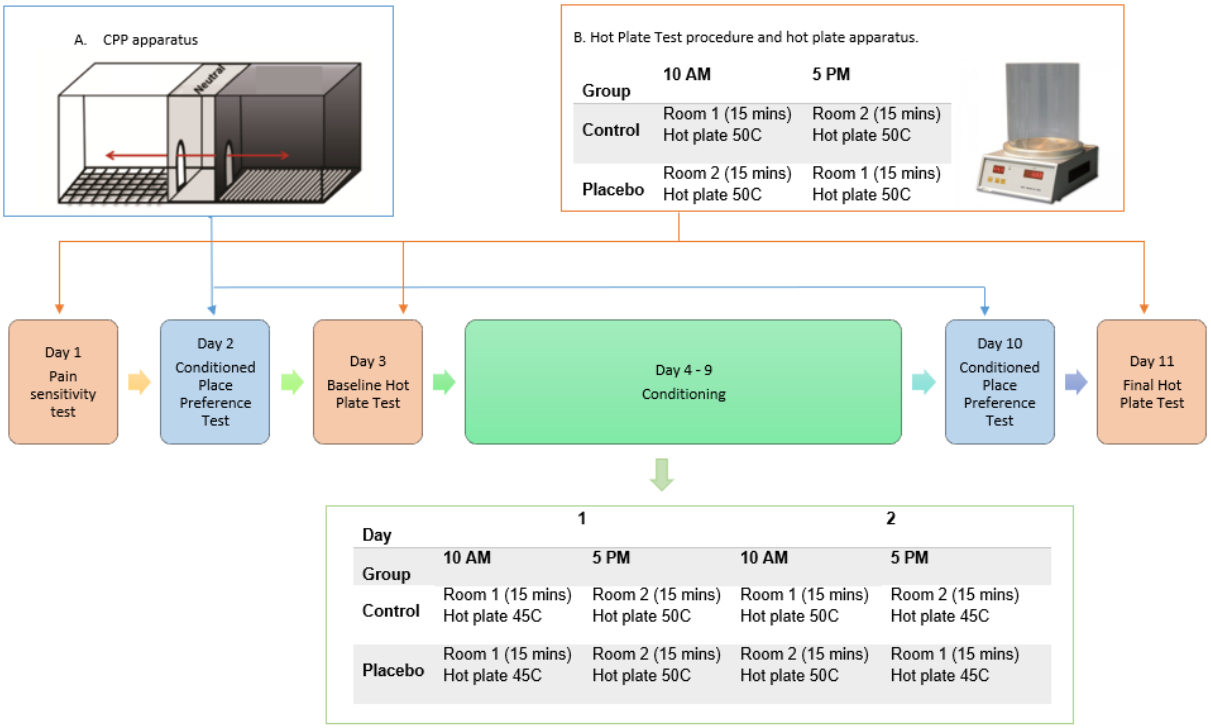


Figure 3 The experimental procedure and test apparatus detail. The 11 day process involved a pain sensitivity test (day 1) 2 x CPP tests (days 2 and 10), 2 x hot plate tests (days 3 and 11), and 6 days of conditioning (days 4 – 9). Room 1 = pCX and Room 2 = cCX.

Statistical analysis

The dependent variable for the CPP was the amount of time spend in pCX and cCX at test, and cue learning was thought to have occurred if the paired group rats spent significantly more time in the pCX after conditioning than the cCX. The dependent variable for HPT was the amount of time it took for rats to show the HPWL on the 50° hot plate at test. Placebo was considered to be present if the HPWL on the 50° hotplate after a low pain cue was significantly longer for rats in the paired conditioning group compared to those in the control group, after conditioning. Animals were excluded if they were hypersensitive (had a HPWL at 45°C of < 50 seconds), were hyposensitive (had a HPWL at 50°C of > 20 seconds), spent more than a third of the time in the centre chamber in the initial CPP test, or had malformed feet.

The data was analysed in three ways for both the CPP and the HPT results. First, mean scores from the CPP and the HPT were analysed using a 2x2x2 factorial ANOVA to partition observed variance into within-subjects main effects of room (pCX vs cCX) and time (pre vs post conditioning), a between groups main effect of conditioning type (control vs placebo), and their interactions for both dependent variables (CPP and HPWL).

Second, we transformed the dependent variables into the same coefficients used by Lee et al (2015). Placebo analgesia was defined as an *increase* in the HPT coefficient after conditioning. Learning the difference in the CPP boxes was defined as an *increase* in the CPP coefficient after conditioning. The coefficient measured percentage changes in the CPP test and the HPT before and after conditioning. The CPP coefficient was calculated as: (Time spent in pCX- Time spent in cCX)/(Time spent in pCX + Time spent in cCX) x 100. The HPT coefficient was calculated as: (HPWL at 50° after pCX – HPWL at 50° after cCX)/(HPWL at 50° after pCX + HPWL at 50° after cCX) x 100. The coefficients were analysed using a 2x2 factorial anova to partition variance into a within subjects' main effect

of time (pre- vs post-conditioning), a between subjects main effect of conditioning type (control vs placebo), and their interactions.

Finally, we replicated Lee et al's (2015) analytical procedure of conducting T-tests on coefficient data. They conducted 4 t-tests comparing pre and post coefficient scores for the CPP test within groups, and pre and post coefficient scores for the HPT test within groups.

All analyses were conducted with SPSS version 25, and alpha was maintained at $p < 0.05$ for statistical significance.

Results

Heat Pain Sensitivity Test

The mean HPWL to the 50-degree hotplate was 10.22 seconds ± 3.4. Rats who withstood the 50-degree hotplate for longer than 20 seconds (n = 4) were excluded. No rats exhibited the HPWL on the 45-degree hotplate.

ANOVA of raw scores

Preference for pCX or cCX before and after conditioning can be seen in Figure 4. There were no differences in time spent in the pCX or cCX between the groups before or after conditioning. This is supported by statistical analysis in which a 2x2x2 ANOVA did not show any significant main effects of room, time or conditioning, or any interactions (all Fs <2.03, ps > 0.05).

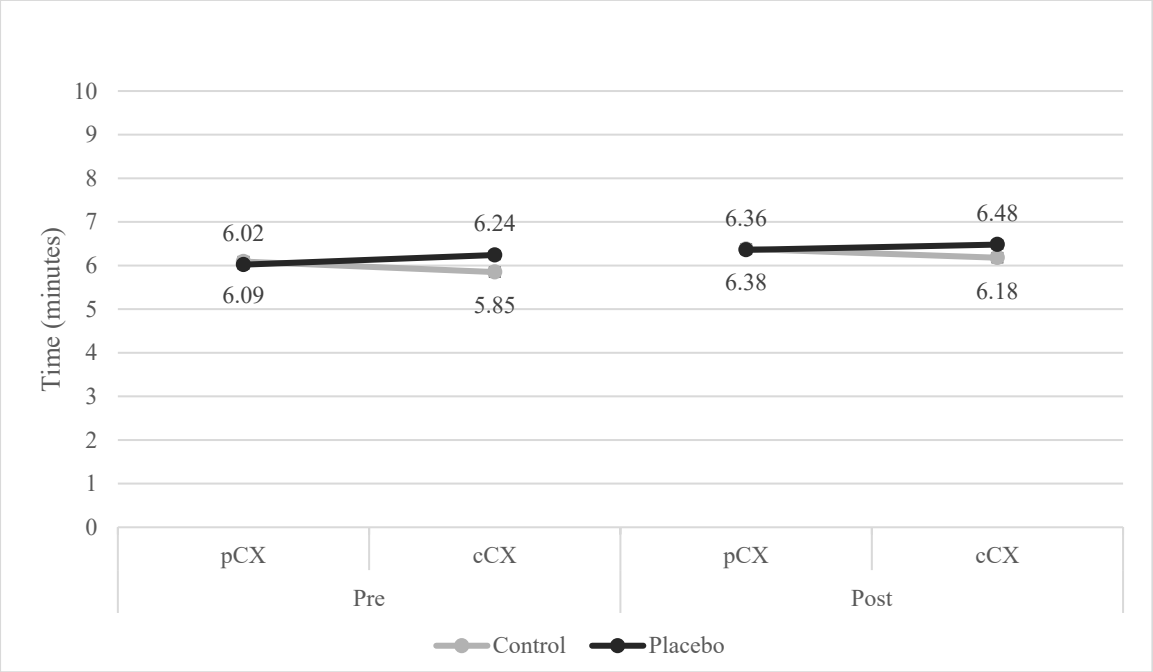


Figure 4 Mean time (minutes) spent in pCX and cCX in the control group and placebo group during the Conditioned Place Preference test, before and after conditioning in experiment 1.

The time (seconds) until rats demonstrated HPWL after pCX and cCX, before and after conditioning, can be seen in Figure 5. There were no differences in the time until HPWL after pCX and cCX between the groups before or after conditioning. There was an overall effect of time ($F_{(1,29)} = 10.47$, p=0.003) as all animal reduced their responding across trials. There were no significant other effects or interactions as supported by statistical analysis in which a 2x2x2 ANOVA did not show any significant main effects of room, time or conditioning, or any interactions (all Fs < 2.90, ps > 0.05).

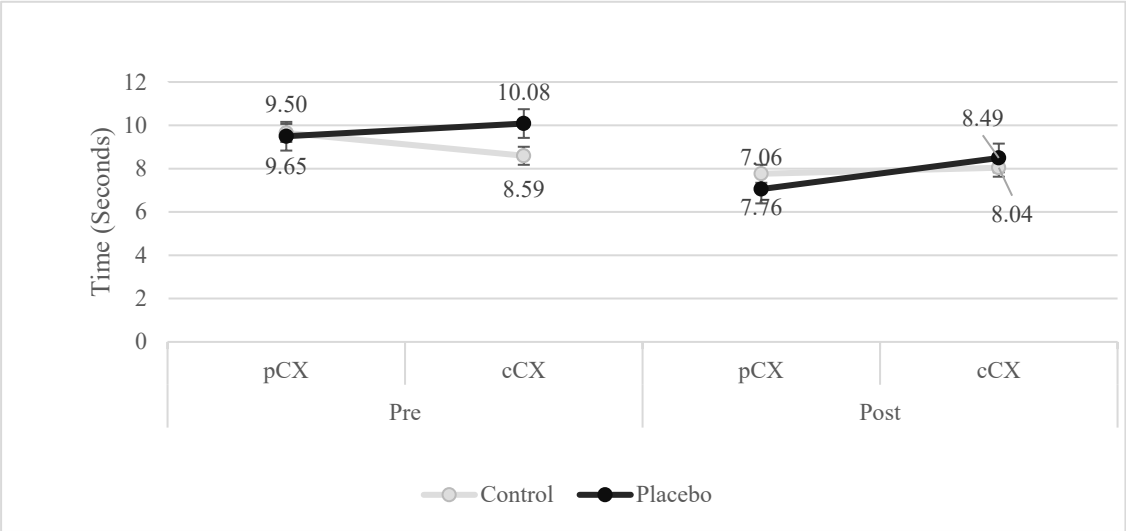


Figure 5 Mean time (seconds) until the HPWL was observed in the Hot Plate Test in the Control group and Placebo group, before and after conditioning in experiment 1.

ANOVA of transformed data

Figure 6 shows the CPP coefficients used by Lee et al (2015) and depicts the change in CPP coefficients after conditioning. Analysis of coefficient scores revealed there was no significant differences in time spent in pCX or cCX in the control or placebo group, before or after conditioning (all *Fs* < 1; Figure 6).

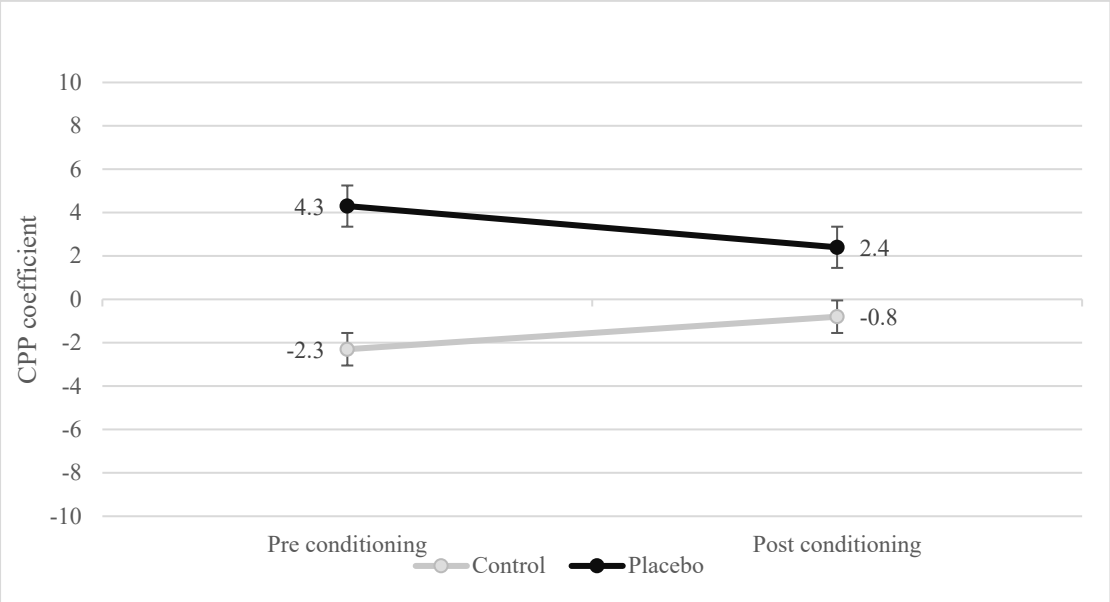


Figure 6 Mean Conditioned Place Preference coefficient values in control and placebo groups, before and after conditioning in experiment 1. The CPP coefficient = (Time spent in pCX – Time spent in cCX) / (Time spent in pCX + Time spent in cCX) x 100.

Figure 7 shows the HPWL coefficient across groups before and after conditioning. The coefficient decreased in both groups after conditioning, but there was no difference between the groups. This is supported by statistical analysis in which 2x2x2 ANOVAs confirmed a significant main effect of time ($F_{(1,29)} = 10.47$, $P < 0.05$) and no significant effects of room or conditioning or interactions between the main effects (all Fs < 1).

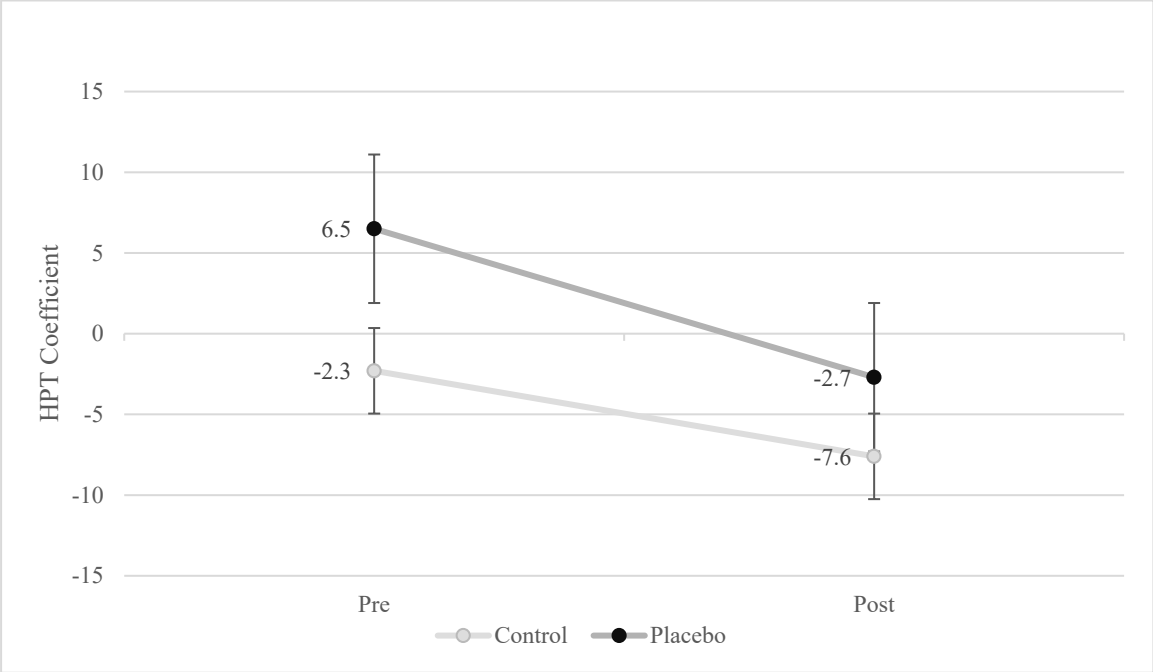


Figure 7 Mean Hot Plate Test coefficient values in control and placebo groups, before and after conditioning in experiment 1. The CPP coefficient = (Time spent in pCX – Time spent in cCX) / (Time spent in pCX + Time spent in cCX) x 100.

T tests of transformed scores

Table 4 shows the coefficients scores used by Lee et al and gives the corresponding t scores used to compare the pre and post conditioning coefficient scores for both the CPP and the HPT in both groups. There were no significant differences within or between the groups.

Table 4 *The analytical procedure used by Lee et al (2015) utilized t-tests for within and between group significance testing, here the current results for CPP (a) and HPT (b) and corresponding t scores are presented.*

(a) Conditioned place preference coefficients and t scores

	Control	**Placebo**	**T score (unpaired)**
CPP Pre conditioning coefficient	2.33, ± 16.33	-2.25, ± 20.83	$t(29) = 0.34$, $p > 0.05$
CPP Post conditioning coefficient	1.12, ± 18.26	-0.76, ±5.38	$t(29) = 0.34$, $p > 0.05$
T score (paired)	$t(16) = -0.27$, $p > 0.05$	$t(15) = 0.17$, $p > 0.05$	

(b) Hot plate test coefficients and t scores

	Control	**Placebo**	**T score (unpaired)**
HPT Pre conditioning coefficient	6.48, ± 14.97	-2.30, ± 18.92	$t(29) = 1.43$, P>0.05
HPT Post conditioning coefficient	-2.70 ± 14.17	-2.70 ± 14.17	$t(29) = 1.01$, P>0.05
T score (paired)	$t(14) = 1.64$, P>0.05	$t(15) = 0.94$, P>0.05	

Discussion

The aim of Experiment 1 was to replicate the findings of Lee et al (2015), who found that rats exposed to an 11-day conditioning paradigm demonstrated a placebo analgesia effect. The hypotheses for experiment 1 were drawn from Lee et al's (2015) findings and predicted that for the Conditioned Place Preference (CPP) test, rats in the paired conditioning group would spend more time in pCX (Placebo low pain context, pCX) after conditioning than cCX (control high pain context, cCX), but this difference would not be noted in the unpaired conditioning group. For the Hot Plate Test (HPT), it was predicated that rats in the paired group would spend significantly more time on the 50° hot plate after pCX than those in the control group, and this would be taken to represent placebo analgesia.

The findings from experiment 1 do not support these hypotheses, nor do they reflect the findings of Lee et al (2015).

Before conditioning, there was no difference between the 2 groups in the CPP or the HPT. Neither group showed a significant preference for pCX compared to cCX. After conditioning, the same trend was found and there was no preference for either room in either of the groups. The same outcome was found to be true whether looking at coefficient data or mean time data. In the HPT after conditioning there was a reduction in HPWL across both groups, but there were no differences in the HPWL on the 50° hotplate after pCX compared to cCX. This suggests that in the current replication study, there was no indication of placebo analgesia.

The lack of results in the CPP suggests that there may be a problem with learning, as rats did not appear to make the association with the pCX and low pain, and cCX and high pain. If they had, we would have expected the paired rats to show a clear preference to the placebo context after conditioning. Without learning the distinction between the cues,

conditioned expectation did not develop, and placebo analgesia could not occur. There are several reasons that this interruption to learning could have happened.

First, it is possible that the chambers within the CPP boxes were not distinct enough to enable learning. The two chambers are the same size and included both visual and tactile cues. The current study used albino Sprague Dawley rats who are known to have significant vision impairment (Prusky, Harker, Douglas, & Whishaw, 2002), which may render the visual cues ineffective in the CPP, leaving the tactile cues the only salient cues. This argument is countered, however, by previous studies that have been run in the same boxes in the same lab reporting significant findings.

Learning may also have been interrupted because the handling of animals between the holding room, the CPP, and the HPT was more challenging in the current experiment than in the work done by Lee et al (2015). Their protocol suggests that the CPP and HPT were in the same room but does not give detail beyond this. The local replication may have had the boxes further apart, and thus learning was interrupted by a more stressful handling experience between the CPP and the HPT. Stress could also have been a factor because during conditioning the animals remained on the hotplate for a full 60 seconds, regardless of the time to HPWL. For the 45-degree hotplate, this did not matter, but for the 50 degree hotplate this could have resulted in significant stress for the animals. Stress can lead to impairments in learning (K. B. Baker & Kim, 2002), and thus it is recommended that this time be reduced in future research.

Experiment 1 utilised a convenience sample of female Sprague Dawley rats instead of male, as used in Lee et al (2015). Sex differences are known to mediate pain experiences in rats, especially in regards to thermal pain (Vierck, Acosta-Rua, Rossi, & Neubert, 2008), and this could be one reason the current results do not mirror those found in Lee et al (2015). It is recommended that future studies continue to work with male rats until there is a reliable

model that can be re-assessed with female subjects; and ensure that context-based cues are distinct enough for rodents to perceive the differences.

Finally, the main experimenter was new to animal experimentation and the processes (such as handling and administering the tests) that we assume to be consistent across different personnel may be more variable than is thought. Slight changes in movement, the stress levels of the experimenter, and experience in working with animals have all been shown to impact experimental outcomes (Bohlen et al., 2014). Additionally, rats handled predominantly by a male experimenter were shown to have increased stress hormones when compared to those being handled by a female experimenter (Sorge et al., 2014). While this doesn't explain the current result discrepancy, as the main experimenter is female, it does build on the evidence that differences in experimenter characteristics could significantly contribute to study outcomes.

Critically, the key first step of *learning* a distinction between cues and thus developing an expectation for a reduction in pain did not occur in the current experiment, as evidenced by the null results in both CPP and HPT. Without learned associations the expectation of reduced pain cannot be developed, and thus there is no basis for the development of placebo analgesia (Keller et al., 2018; Kirsch, 2018; Stewart-Williams & Podd, 2004).

Experiment 2

Experiment two was a second attempt to replicate Lee et al (2015). In this study, several minor differences between the protocol of Experiment 1 and Lee et al (2015) that might have affected the ability of the animals to associate the conditioning contexts with reductions in pain were amended. Experiment 2 used male sprague dawley rats as were used by Lee at al (2015) instead of female rats; the main experimenter/handler underwent extra training in rodent handling to reduce potential stress placed on the animal subjects; the CPP boxes were modified to allow the rats to discriminate between them more easily; and the distance between the CPP boxes and the hot plate was reduced, to minimise time between the cue and the stimulus.

In addition, there is the possibility that stress caused by spending 60 seconds on the 50 °C hotplate could have interfered with learning in the animal subjects. As the HPWL was exhibited by all animals in under 30 seconds, this was set as the maximum amount of time animals would remain on the hotplate for experiment two.

As with Experiment 1, it was hypothesized that for the CPP test, rats in the paired conditioning group would spend more time in pCX after conditioning than cCX, but this difference would not be observed in the unpaired conditioning group. For the HPT, after conditioning, rats in the paired group would spend significantly more time on the hot plate after the low pain cue than those in the control group, and this would be taken to represent placebo analgesia.

Method

Animals

Male Sprague Dawley rats aged 10-12 weeks (N=32) that weighed 220-240 grams were obtained from the Animal Resource Centre in Perth, WA. Animals were housed and cared for in the same manner as in experiment 1.

Apparatus

Apparatus from experiment 1 were all reused for experiment 2. Visual cues in the CPP boxes were re-painted and the distance between the CPP boxes and the hot plate was reduced, to minimise time between the cue and the stimulus (Figure 8).

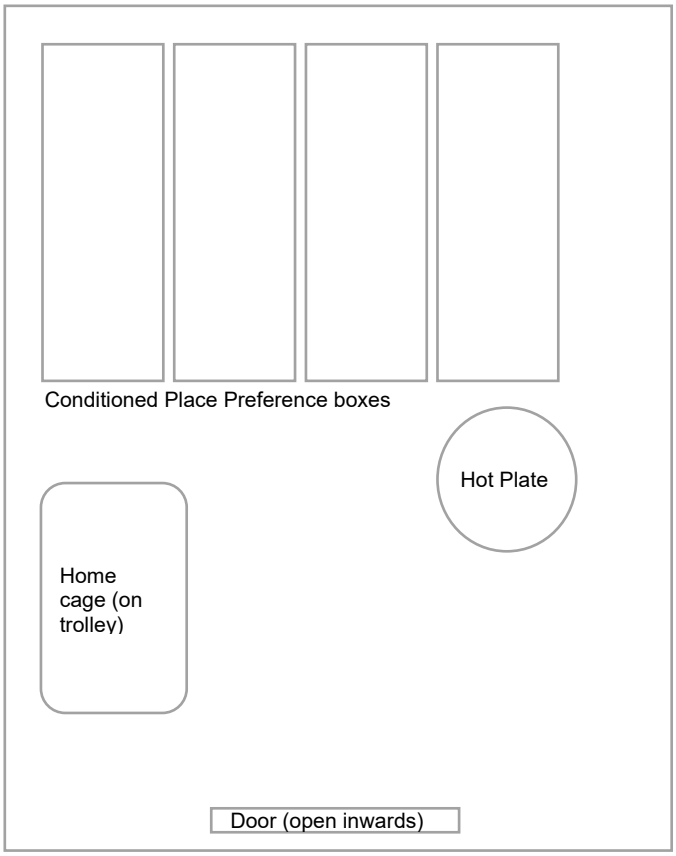


Figure 8 Layout of the experimental room for experiment 2.

Group allocation

Animals were randomly assigned by box to either the paired group (n = 16) or the unpaired group (n = 16). Randomization was done by importing box numbers into an online randomization program (https://www.random.org/). The random list was split in half, with the first half being assigned placebo and the second half assigned control. Table 3 in experiment 1 outlines the groups and schedule for conditioning.

Age, weight, and gender were all consistent across the animals and so distribution of these traits across groups was considered to be even.

Experimental design

Experimental design was the same as Experiment 1 (see Figure 3), except that animals were only left on the hotplate for a maximum of 30 seconds at a time.

Statistical analysis

The same analysis for both CPP and HPT from Experiment 1 was used for Experiment 2.

Results

Heat pain sensitivity test

The mean HPWL to the 50-degree hotplate was 14.89 ± 7.09 seconds. Rats who withstood the 50-degree hotplate for longer than 20 seconds (*n = 5*) were excluded. No rats exhibited the HPWL on the 45-degree hotplate.

ANOVA for raw scores

Preference for pCX or cCX in the CPP before and after conditioning can be seen in Figure 9. Within groups there was a significant effect of time ($F_{(1,30)} = 10.87$, p=0.003) and a significant interaction between time and room ($F_{(1,30)} = 7.03$, p=0.013). There was no effect of group, room on its own, or any interaction between any of the other factors (all Fs <1.35).

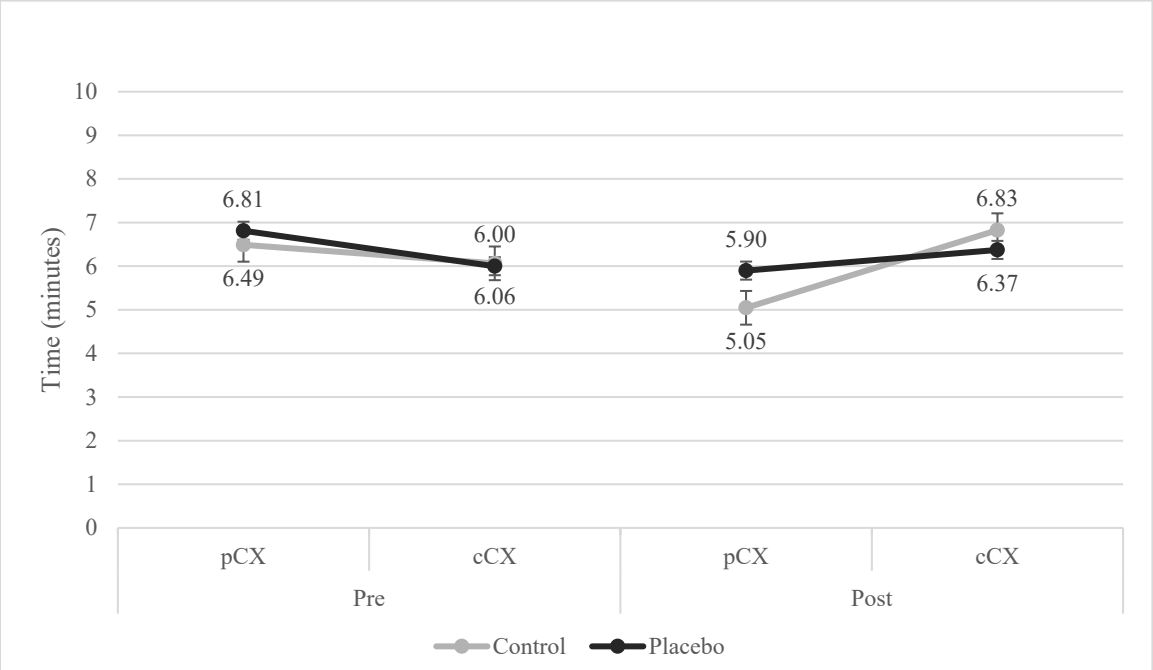


Figure 9 Mean time (minutes) spent in pCX and cCX in the control group and placebo group during the Conditioned Place Preference test, before and after conditioning in experiment 2.

Figure 10 presents mean HPT scores across groups before and after conditioning and shows that rats in all groups reduced their HWPL over time. Statistical analysis confirmed that the effect of time was significant ($F_{(1,30)} = 31.14$, $P < 0.05$) . While there was an observable increase in time on hot plate after cCX in the paired group, analysis revealed there was no significant effect of room or conditioning (both *F*s < 1) on HPWL.

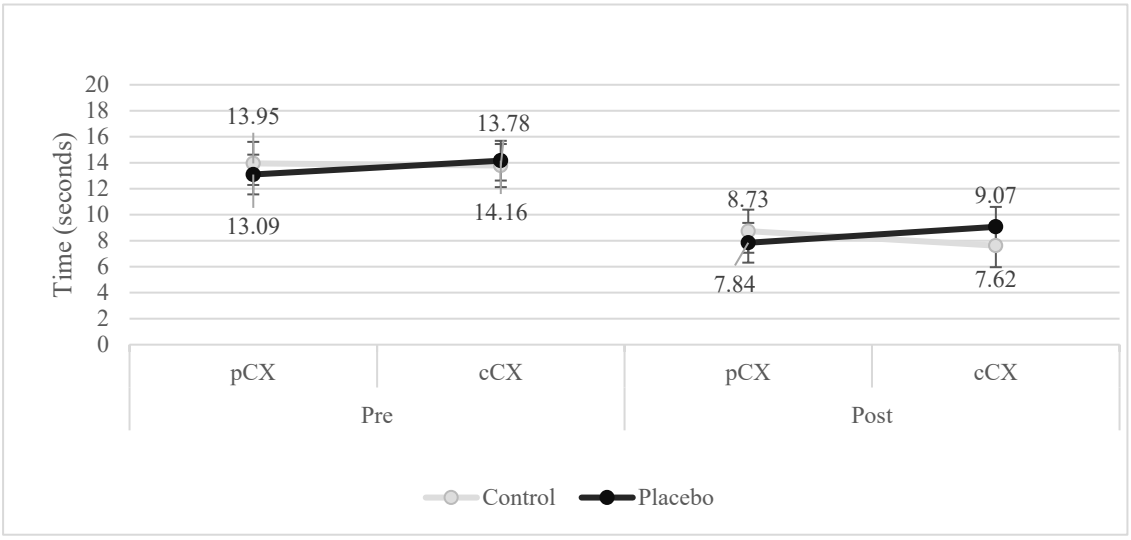


Figure 10 Mean time (seconds) until the HPWL was observed in the Hot Plate Test in the Control group and Placebo group, before and after conditioning in experiment 2.

ANOVA of transformed data

Figure 11 shows the CPP coefficients used by Lee et al (2015) and depicts the change in CPP coefficients after conditioning. A 2x2x2 ANOVAs analysis of coefficient scores revealed there was a significant effect of time ($F_{(1,30)} = 7.68$, $P < 0.05$) but there were no significant differences in time spent in pCX or cCX in the control or placebo group, before or after conditioning (all *Fs* < 1).

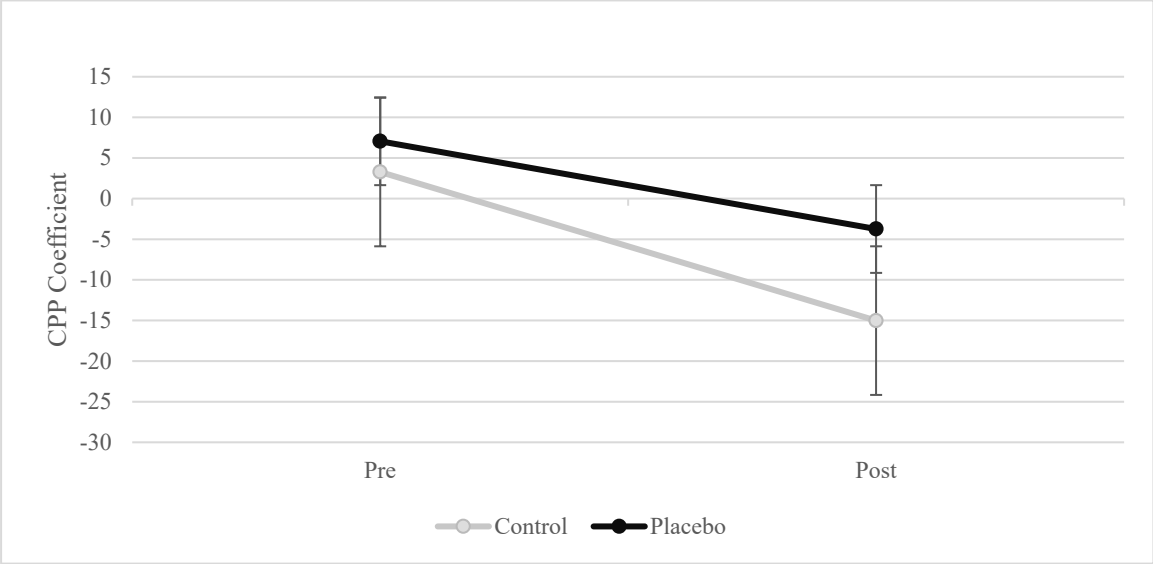


Figure 11 Mean Conditioned Place Preference coefficient values reveal preference for room cues in control and placebo groups, before and after conditioning in experiment 2. The CPP coefficient = (Time spent in pCX – Time spent in cCX) / (Time spent in pCX + Time spent in cCX) x 100.

Figure 12 presents the coefficient for HPT in both placebo and control groups, before and after conditioning. A 2x2x2 ANOVA of HPT coefficient scores across the groups revealed there was no significant differences in time until HPWL after pCX or cCX in the control or placebo group, before or after conditioning (all *Fs* < 2; Figure 12).

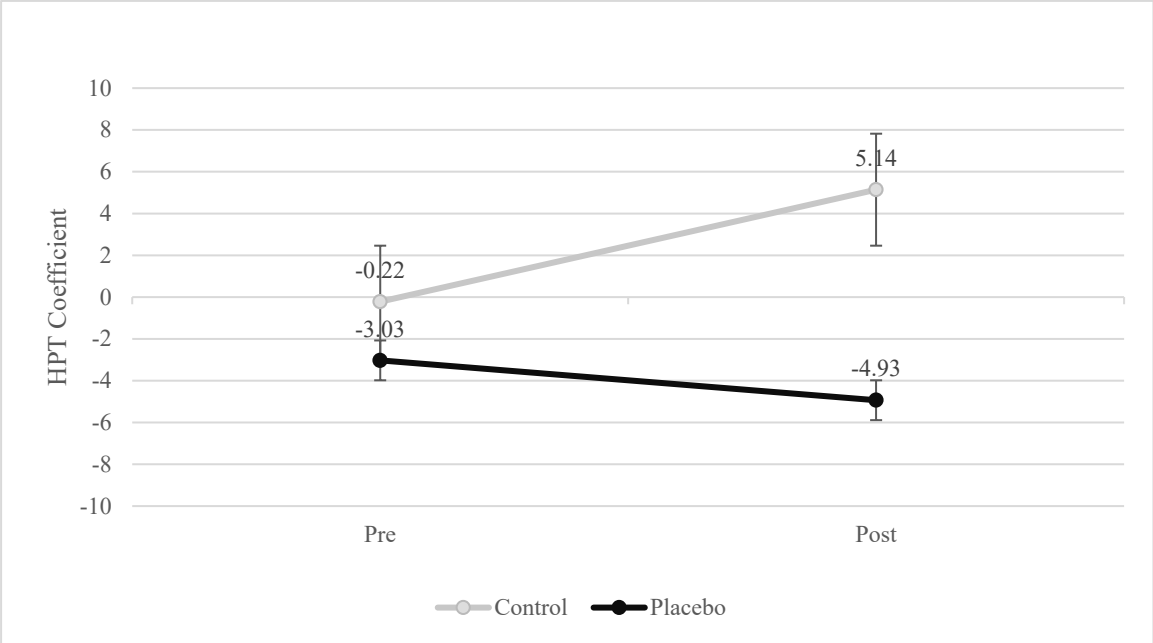


Figure 12 Mean Hot Plate Test coefficient values in control and placebo groups, before and after conditioning in experiment 2. The CPP coefficient = (Time spent in pCX – Time spent in cCX) / (Time spent in pCX + Time spent in cCX) x 100.

Coefficient t-tests

The coefficients scores used by Lee et al are presented in Table 5, along with the corresponding t scores used to compare the pre and post conditioning coefficient scores for both the CPP and the HPT in both groups. There was a significant difference within the control group before and after conditioning ($t(16) = 4.25$, $p = 0.001$), and this showed that rats in this group had a preference for cCX after conditioning, but not before. No other t-tests were significant.

Table 5 *The analytical procedure used by Lee et al (2015) utilized t-tests for within and between group significance testing, here the current results for CPP (a) and HPT (b) and corresponding t scores are presented.*

(a) Conditioned place preference coefficients and t scores

	Control	**Placebo**	**T score (unpaired)**
CPP Pre conditioning coefficient	3.27 ± 17.93	7.05 ± 22.77	$t(30) = -0.52$, $p > 0.05$
CPP Post conditioning coefficient	-15.02, *SD*±18.18	-3.74 ± 30.31	$t(30) = -1.28$, $p > 0.05$
T score (paired)	***t*(16) = 4.25, *p* = 0.001**	$t(16) = 1.13$, $p > 0.05$	

(b) Hot plate test coefficients and t scores

	Control	**Placebo**	**T score (unpaired)**
HPT Pre conditioning coefficient	-0.22, ± 23.52	-3.03, ± 18.46	$t(30) = 0.38$, P>0.05
HPT Post conditioning coefficient	5.14 ± 14.69	-4.93 ± 17.52	$t(29) = 1.01$, P>0.05
T score (paired)	$t(15) = -0.77$, P>0.05	$t(15) = 1.76$, P>0.05	

Discussion

Experiment 2 attempted to replicate the findings of Lee et al (2015), who found that rats exposed to an 11-day conditioning paradigm demonstrated a placebo analgesia effect. The hypotheses for experiment 2 were drawn from Lee et al's (2015) findings and predicted that for the Conditioned Place Preference (CPP) test, rats in the paired conditioning group would spend more time in pCX after conditioning than cCX, but this difference would not be observed in the unpaired conditioning group. For the Hot Plate Test (HPT), it was predicated that rats in the paired group would spend significantly more time on the 50°C hot plate after the low pain cue than those in the control group, and this would be taken to represent placebo analgesia.

The findings from experiment 2 do not support these hypotheses, nor do they reflect the findings of Lee et al (2015).

The current study reported similar results to experiment 1, there was no evidence that learning the distinction between the low pain cue (pCX) and high pain cue (cCX) had occurred in the paired group. Rats in the unpaired control group demonstrated a significant change in their room preference – they spent more time in cCX after conditioning than pCX – but this change was not evident in the paired placebo group and did not reflect changes that could be attributed to the conditioning paradigm. Importantly, these changes were only significant in a single t-test of the transformed coefficient data and did not present as significant when looking at the coefficient ANOVA.

This very weak evidence for a shift in preference in the control group only could potentially be explained if we consider the rooms to have some unaccounted for features that rats prefer. If, for example, cCX had less direct light, or a slightly more favourable smell that was unknown to the experimenters, rats may learn during conditioning that this is the favoured room and spend more time there. For the paired placebo rats, this preference could

have been overridden by the learned aversion to the room caused by the conditioning paradigm, which associated cCX with high pain. To fully measure this, we would need to have a naïve sample of rats to compare the paired and unpaired groups to. This could potentially reveal that the subjects learned to distinguish between the two cues, in a way that was not captured in the current experiment.

The lack of evidence for a preference for pCX in the paired placebo group does, however, suggest that learning the association between pCX and low pain and cCX and high pain did not occur.

Learning in rodents is most effective with the use of salient cues that clearly distinguish one set of circumstances from another (Valone et al., 1998). In the current experiment, the cues (CPP boxes) were mainly visual, which are not ideal for the almost blind albino sprague dawley rat (Green, de Tejada, & Glover, 1991). While Lee et al (2015) reported using similar boxes with positive results, images of these were not made available and it was therefore unknown if they are comparable to the ones used in the current experiment. Attempts were made to contact the authors for further information, but there was no response. Rodent placebo analgesia studies that reported positive results utilised olfactory (Kehoe & Blass, 1989; Randall et al., 1993), taste (Bardo & Valone, 1994; Bevins et al., 1995), and high contrast visual cues (Xu et al., 2018; Zhang et al., 2013). Future research into conditioning based placebo analgesia in rats should use strong salient cues that target more than one sensory system to aid in both learning to distinguish between cues and the development of conditioning and the expectation of pain relief.

All groups reduced the HPWL in the post conditioning HPT compared to the pre conditioning HPT, and this was a significant reduction. As in experiment 1, however, there was no significant differences in HPWL between the paired and unpaired groups, before or after conditioning, and thus the placebo analgesia effect was not observed. The same

conclusion from experiment 1 can be drawn here: without the basic associative learning required to develop conditioned expectancy in the rat, placebo analgesia cannot occur.

As in experiment 1, the critical step of learning a distinction between cues and thus developing an expectation for a reduction in pain did not occur in the current experiment, as evidenced by the null results in both CPP and HPT. In order to maximise the opportunity for learning, and thus the expectation of reduced pain, a more robust and multi-layered cue stimulus needs to be utilized.

Experiment 3

Experiments 1 and 2 aimed to replicate the findings of Lee et al (2015) but failed to do so. A key feature of the protocol used by Lee et al (2015) was the manner in which it relied on a procedure used to condition placebo analgesia in people – namely, associating a cue with a reduction in expected pain (Luana Colloca et al., 2010; Yeung et al., 2014). Therefore, Experiment 3 was designed to maintain this key feature of Lee et al's protocol but was modified to reduce handling stress, to increase the salience of the to-be-conditioned placebo cue, and to reduce stress-induced analgesia.

Stress

Stress is known moderator of the pain response in rodents (Madden, Akil, Patrick, & Barchas, 1977) and can effect learning (K. B. Baker & Kim, 2002).Thus, stress needs to be considered when manipulating or measuring the pain experience or trying to develop learned associations. Alterations were made to the third experiment in this series to reduce stress in the animal subjects.

The stress of being repeatedly moved and handled during conditioning is known to have a negative effect on learning (Bohlen et al., 2014), and in Lee et al's (2015) assay animals were moved 4 times at a minimum in each conditioning trial. To remove this as a potential confounder, a custom designed context chamber that fit over the hotplate itself was built for experiment 3. This meant conditioning occurred *on* the hotplate and reduced the number of times the rodents were moved. Helpfully, this change also improved temporal contiguity of the cue and the stimulus, another factor that should improve learning outcomes in rodents (Abrams & Kandel, 1988).

Being familiar with the experimenter/handler also reduces stress in rodents and improves research outcomes (Bohlen et al., 2014). To improve rodent/experimenter

familiarity pre-experiment handling days were increased from 1 session daily for 5 minutes to 2 sessions daily for 5 minutes for 10 days.

Multi-faceted context chamber

Experiments 1 and 2 provided no evidence of discrimination between the contextual cues associated with high pain or low pain. Experiment 3 utilised a design in which a single multi-faceted highly salient cue was paired with pain reduction. A custom-built context chamber which consisted of visual, auditory, olfactory, and taste was constructed.

In addition to creating a stronger cue for pain reduction, we also gave the animals more experience with the 50C hotplate in order to increase the ability of the rats to detect a change in pain intensity on trials when the placebo cue was paired with the 45C hotplate. This relies on a common finding that discrimination learning about a cue that predicts the absence of an expected outcome occurs more rapidly if the signaled non-reinforced trials are less frequent than unsignalled reinforced trials. This involved exposing the rats to repeated sessions of home cage > high pain (50°) hot plate pairings over a number of days. After establishing a baseline rate of responding to the high pain hotplate, less frequent trials were introduced in which the multi-faceted cue chamber box were paired with the low pain hot plate. Unlike in experiments 1 and 2, where animals were put immediately into the conditioning process, experiment 3 first established the experience of pain, then introduced the multi-faceted cue to serve as a learned safety cue.

In addition, repeated exposure to the hot plate before conditioning helped to sensitize rodents to the hot plate and stabilize their HPWL response. The key outcome measure (HPWL) had significant individual variability at baseline in experiments 1 and 2, and the time to HPWL reduced significantly over time in all groups in both experiments. The sensitization period introduced in experiment 3 helps manage this variance by stabilizing the baseline responding to the HPT before grouping and conditioning occurred.

Stress induced analgesia

To attenuate the acquisition of stress induced analgesia caused by repeated exposure to the high pain hotplate in sensitization (Miguez, Laborda, & Miller, 2014), the rats were removed from the 50 degree hotplate no longer than 2 seconds after they exhibited the HPWL in the sensitization period to minimize exposure to pain and reduced stress.

Threshold versus tolerance

There are multiple ways of measuring the pain experience in rodents. While the HPWL is a standard supraspinal measure (Barrot, 2012), it does not give us the same information as other measures such as those of tolerance (Gelfand, 1964). To capture tolerance in the current experiment, total number of paw licks observed in the HPT were used as a secondary dependent variable.

Based on previous evidence of placebo analgesia in rodents (Bevins et al., 1995; J. Guo et al., 2010; Lee et al., 2015; Randall et al., 1993; Zhang et al., 2013), it was expected that in the HPT rats in the placebo/paired group would exhibit a shorter HPWL after the context chamber than after the home cage. It was also expected that they would display less paw licks after the context chamber. We did not expect to see any difference in the unpaired control rats in the HPT in either the HPWL or the paw licks after the context chamber when compared to after the home cage.

Method

Protocol registration

The protocol for this study was registered on the 4th of December 2018 with the AsPredicted database for animal studies under the title "Analgesic placebo in a rat - University of Sydney 2018" reference number: 17431.

Animals

Male Sprague Dawley rats aged 10-12 weeks (N=32) that weighed 220-240 grams were obtained from the Animal Resource Centre in Perth, WA. Animals were housed and cared for in the same manner as in experiments 1 and 2.

Apparatus

Hot plate apparatus

The same hot plate apparatus from experiments 1 & 2 was used in experiment 3

Context box

The context box was a large transparent plastic tub (300 x 150 x 150 mm) that sat upside down on top of the HPT. The lid of the container had a circle hole cut into it so that the hotplate protruded through the lid into the context chamber. It contained visual, auditory and olfactory cues. Figure 13 shows the layout for the context chamber.

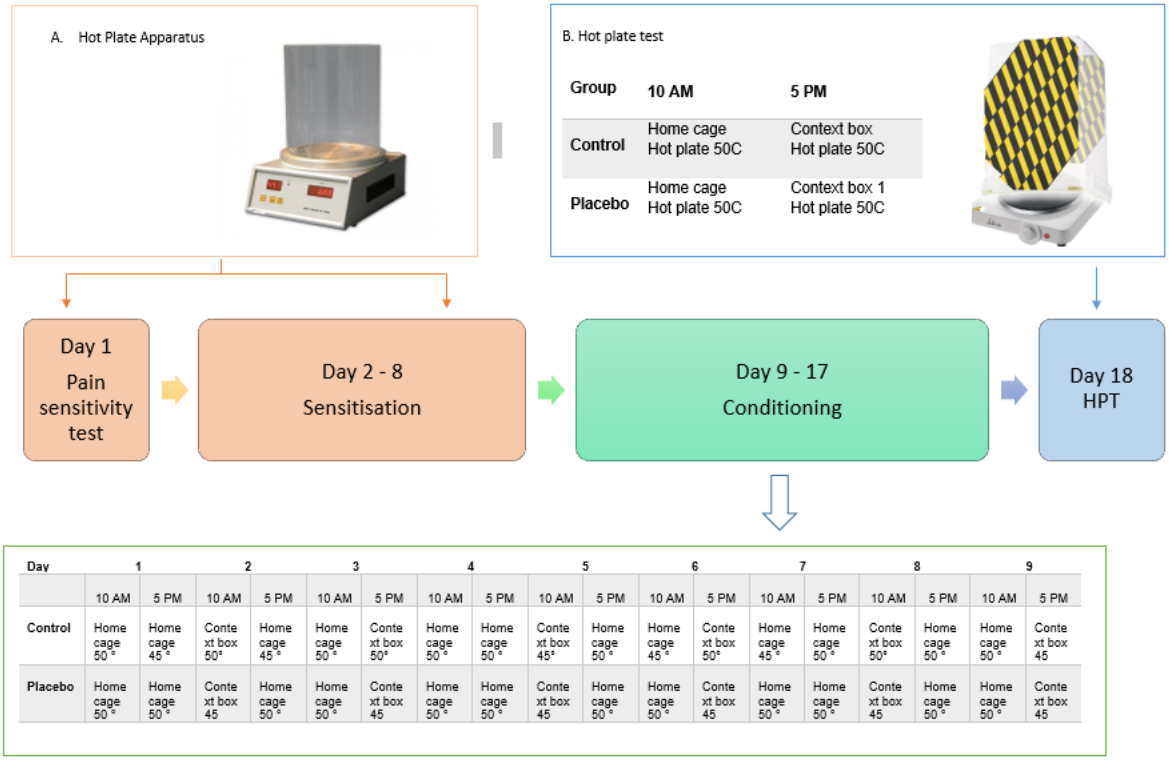
Visual cues were provided by the interior of the tub was covered in "caution" tape (bright yellow and black striped) to create a high contrast pattern, and there was a small window cut out of one side so that rats could be video recorded inside the box. The same small lamp from experiments 1 and 2 dimly illuminated the room in experiment 3. Audio cues were provided by playing "Music for rats - Relaxing music to help your rat calm down and sleep" (https://www.youtube.com/watch?v=AVzCqrEn7kc) via Dell laptop speakers right next to the context box during context trials. Olfactory cues were provided by approximately 3 drops of vanilla essence being placed onto the hot plate and onto a sponge

that sat at the top of the HPT during context trials. After each individual conditioning trial and test the hotplate was wiped and the essence was re-applied. Taste cues were provided by giving rats access to vanilla flavoured water in their home cage for 30 minutes prior to conditioning trials. Vanilla essence (Queen Fine Foods brand) was diluted in water at 1%.

Figure 13 diagrammatic representation of the context chamber from experiment three. Visual cues were included with the 'caution' high contrast tape, audio by a song playing, olfactory by vanilla essence, and taste by vanilla flavoured water (delivered in the home cage prior to the context chamber sessions)..

Procedure

Animals were excluded if they were hypersensitive (had a HPWL at 45° of < 50 seconds), were hyposensitive (had a HPWL at 50° of > 20 seconds) or had malformed feet. An overview of the experimental process for experiment 3 is presented in Figure 14.



Day	1		2		3		4		5		6		7		8		9	
	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM	10 AM	5 PM
Control	Home cage 50°	Home cage 45°	Context box 50°	Home cage 45°	Home cage 50°	Context box 50°	Home cage 50°	Home cage 50°	Context box 45°	Home cage 50°	Home cage 45°	Context box 50°	Home cage 45°	Home cage 50°	Context box 50°	Home cage 50°	Home cage 50°	Context box 45
Placebo	Home cage 50°	Home cage 50°	Context box 45	Home cage 50°	Home cage 50°	Context box 45	Home cage 50°	Home cage 50°	Context box 45	Home cage 50°	Home cage 50°	Context box 45	Home cage 50°	Home cage 50°	Context box 45	Home cage 50°	Home cage 50°	Context box 45

Figure 14 The experimental procedure and test apparatus detail for experiment 3. The 18-day process involved a pain sensitivity test (day 1) 7 x sensitization sessions (days 2 to 8), 9 days of conditioning (days 4 – 9) and 1 day of HPT (day 18).

Grouping

Animals were randomly assigned by box after acclimatization, handling, and sensitization to either the paired group (n = 16) or the unpaired group (n = 16). Randomization was done by importing box numbers into an online randomization program (https://www.random.org/). The random list was split in half, with the first half being assigned placebo and the second half assigned control. Table 6 outlines the groups and schedule for conditioning.

Age, weight, and gender were all consistent across the animals and so distribution of these traits across groups was considered equal.

Table 6 *The conditioning process. Animals in the placebo group were conditioned using 6 x pairings of the low pain CS (context box) and US (low (45°) temperature) and 12 x pairings of the high pain CS (home cage) and US (high (50°) temperature). Control group rats were given a mix of paired and unpaired sessions.*

Group (n)	Conditioning Pairings (CS+US)	Number of Pairings
Placebo (16)	Context box paired with 45°	6
	Home cage paired with 50°	12
Control (16)	Context box paired with 45°	2
	Context box paired with 50°	4
	Home cage paired with 50°	8
	Home cage paired with 45°	4

Acclimatisation, handling, and habituation.

Prior to the experimental period, animals were left to acclimatize for 4 days after arrival in the lab. Following this, they were each handled for 5 minutes twice daily for 10 days in total, so that they could adjust to the handler's presence and scent. The same experimenter handled the rats at each stage of the procedure, and wore the same attire (lab gown, mask, and gloves) throughout. Animals were weighed daily throughout the experimental period, and their coat and paws were examined to ensure they were not being adversely affected by the process.

Before any of the tests or trials began, each rat was given 2 x 15-minute habituation session in the hotplate chamber (set to 25° C), and 1 x 10-minute habituation to the context box.

Heat pain sensitivity test

On day 1 of the experimental period, the heat pain sensitivity tests were conducted to assess rats against the hyper/hypo sensitive exclusion criteria. The tests were conducted over two sessions, one at 10 AM and the other at 5 PM, in a counterbalanced order. In one session rats were exposed to the hotplate at 45° and their HPWL was measured, in the other session they were exposed to 50° and their HPWL was measured. As soon as HPWL was observed they were removed from the hotplate to limit stress and learning. Rats were then immediately returned to their home cage.

Sensitization

After the pain sensitivity test, rats were given daily exposure to the hotplate at 50° for 7 days (10 AM) until the HPWL stabilised (mean HPWL and SD stable for two days straight, with a minimum sensitization period of 3 days) across groups.

Conditioning sessions

Conditioning happened across 2 sessions per day for 9 days (18 sessions in total). In conditioning trials, rats on the 50° hotplate were removed as soon as the HPWL was observed, and the time taken was recorded. All rats were left on the 45° hotplate for 10 seconds. One session started at 10 AM (when lights turned off) and the other at 5 PM, and the order was counterbalanced. Figure 14 depicts the session schedule.

In home cage trial sessions, rats were taken from their home cage and placed immediately onto the hotplate at either 50° (all placebo rats) or 45° (control group, depending on the trial - see Table 6 for the pairing schedule). In context box sessions, 1% vanilla essence water replaced regular water in the home cages for 30 mins prior to the test. Rats

were then taken into the testing room and were transferred from their home cage onto the hotplate that was situated within the context box. For placebo rats, the hot plate was always set at 45° when within the context box and for control rats the hot plate was either 50° or 45° (see Table 6 for the pairing schedule).

Experimental environment

All tests and conditioning were conducted in a separate room to the housing room, and animals were taken into the room one box at a time. They were transported using a trolley and were covered by a large sheet when being transported to minimise stress. In the experimental room, they were taken straight from their home cage, into the test apparatus, and then afterward immediately returned to the home cage. Rats were not left in the home cage in the experimental room beyond the experimental time, and experiments were timed so that all animals had the same exposure time to all apparatus, and the same amount of time in their home cage in the experimental room.

The room itself was dimly lit with 1 desk lamp sitting on the floor to keep light in the room to a minimum. Door frames were cushioned to avoid door slamming, and the room was silent during all conditioning and tests, except when the audio cue for context chamber was being played. Figure 15 outlines the room layout for experiment 3.

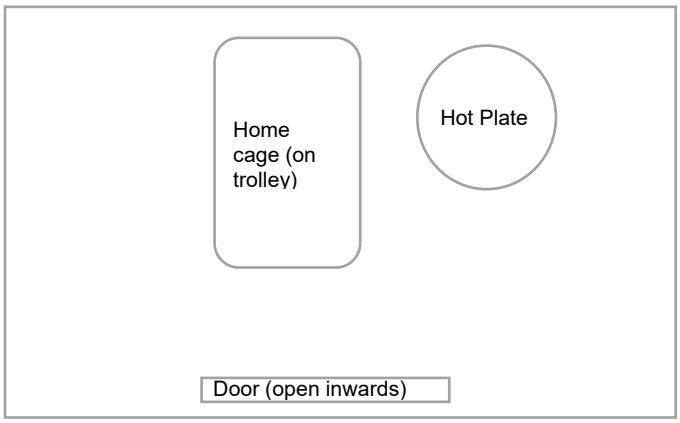


Figure 15 the layout of the room for experiment 3.

Measurement of placebo analgesia using the Hot Plate Test

The HPT for experiment 3 was administered on day 18, once at 10AM and again at 5 PM, in a counterbalanced order. In one session, rats were placed from their home cage onto the 50° hotplate, and in the other session their standard cage water bottles were replaced with vanilla water 30 minutes prior to the test. At test animals were then placed from their home cage onto the 50° hotplate situated within the context box. All rats were kept on the hotplate for 30 seconds in each session and the sessions were video recorded. The HPWL was recorded for each session, as was the number of paw licks across the thirty seconds.

Statistical Analysis

Placebo analgesia was defined as a larger HPWL after the context box than after the home cage. A secondary dependent variable was also assessed, which was mean paw licks across the whole testing session. HPWL and paw licks means from the HPT across groups were analysed using Factorial (2 x 2) ANOVA. The main effects of conditioning (between groups) and context (within groups) were examined.

Statistical analysis was done using SPSS version 25 for Windows and alpha was set at 0.05. Post hoc analysis for both tests were conducted with Scheffe's adjusted critical value.

Results

Heat Pain Sensitivity

The mean HPWL to the 50-degree hotplate was 10.55 seconds ± 3.4. Rats who withstood the 50-degree hotplate for longer than 20 seconds (*n = 1*) were excluded. No rats exhibited the HPWL on the 45-degree hotplate.

HPT (measure of placebo analgesia)

In the post conditioning HPT, there was no significant effect of context or group on the HPWL measure (all *Fs* < 2). Mean HPWLs are shown in Figure 16.

In the post conditioning HPT, there was a significant main effect of context ($F_{(1,29)}$ = 13.55, *p*=0.001) and more paw licks were observed in the context chamber than after the home cage in both groups, but there was no interaction between cue and group (F<1), or effect of group on its own (F<1; Figure 17).

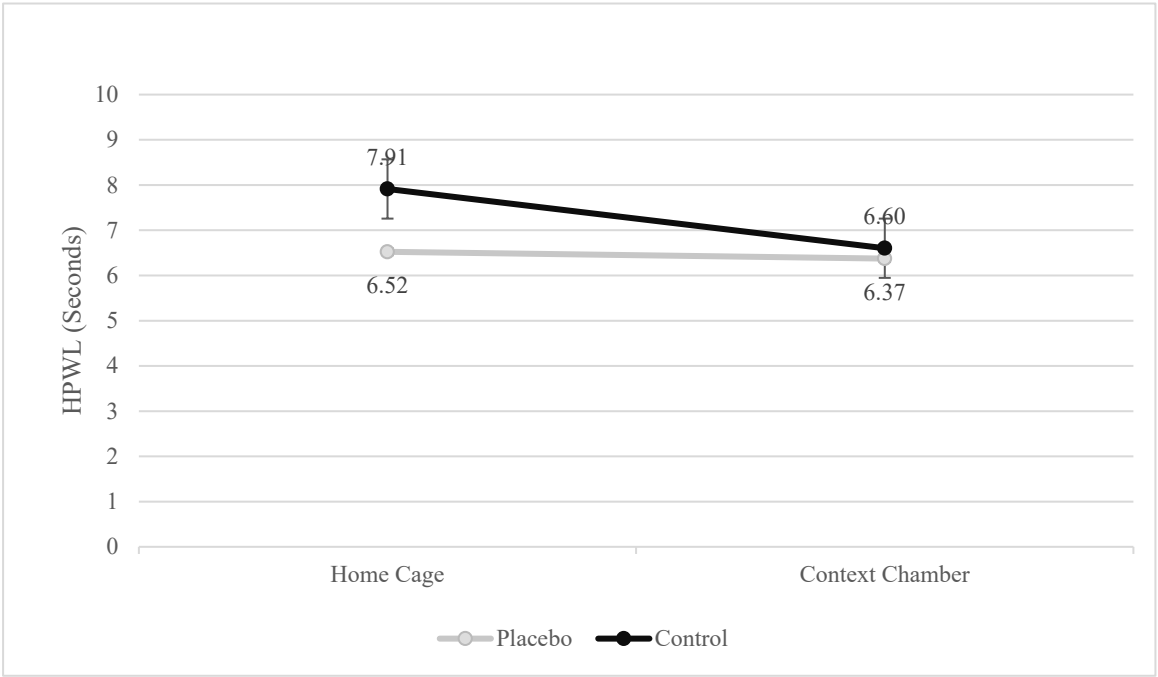


Figure 16 Mean time (seconds) until the HPWL was observed in the Hot Plate Test in the Control group and Placebo group, after the home cage cue and the context chamber cue.

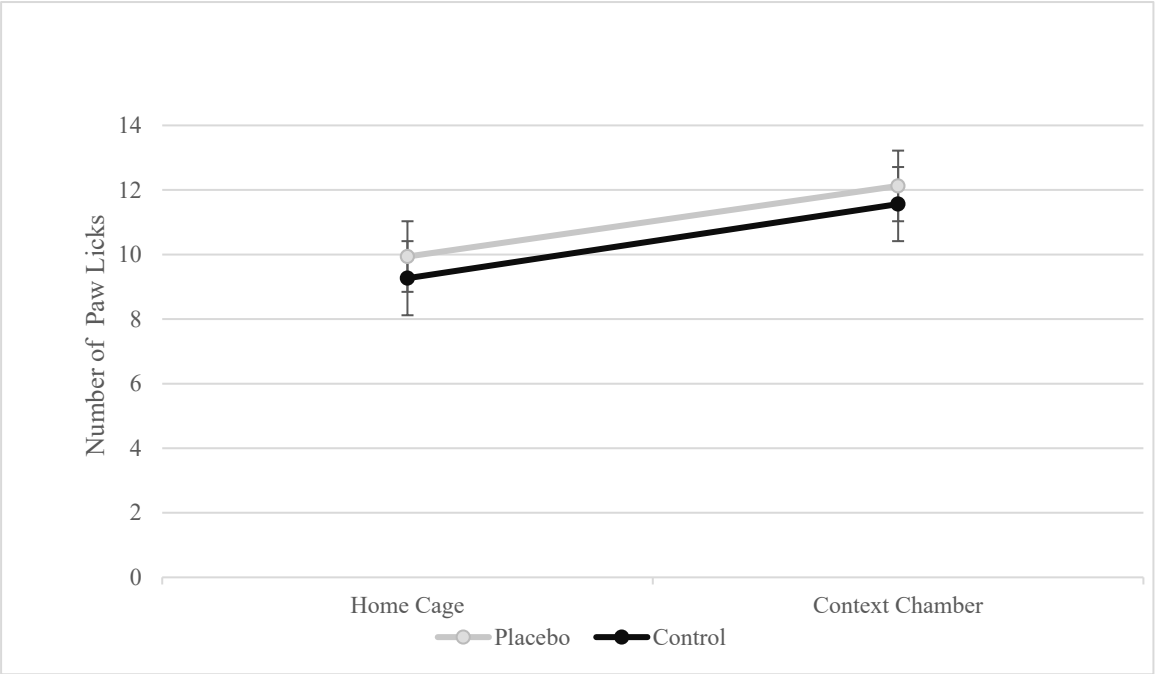


Figure 17 Mean number of paw licks exhibited by the Placebo and the Control group during the 50° HPT after the home cage and the context chamber on day 20.

Discussion

Experiment 3 attempted to establish an analgesic placebo effect in rodents using a novel conditioning paradigm. In the pain test utilized (the Hot Plate Test, HPT), it was predicated that rats in the paired group would spend significantly more time on the 50° hot plate after the low pain context cue than those in the control group, and this would be taken to represent placebo analgesia. It was also predicted that those in the paired group would display fewer paw licks over the 30 second HPT than those in the unpaired group, after exposure to the low pain cue. Neither of these predicted outcomes were observed in the current experiment, and no evidence for placebo analgesia in rodents was found.

It was anticipated the HPWL of the paired group would be longer than the unpaired group because the expectancy of low pain brought about by the context chamber would induce an analgesic placebo (Keller et al., 2018; Kirsch, 2018; Kirsch et al., 2014; Stewart-Williams & Podd, 2004). Instead, no significant differences between the two groups were observed. Additionally, there were no observable differences within the two groups when HPWL after exposure to the context chamber and the home cage were compared.

For the paw lick measurement, findings contradicted previous research and provided no support for the current hypothesis. For both the paired and unpaired groups, rats exhibited more paw licks after the context chamber than after the home cage and this difference was significant. There was no difference between the groups. This suggests that although we attempted to attenuate the onset of conditioned stress induced analgesia by removing animals when they presented the HPWL, repeated exposure to the hot plate at 50° appears to have developed this form of conditioned analgesia in the animals regardless. Unlike previous research using behavioural conditioning to develop placebo analgesia (Lee et al., 2015; Xu et al., 2018) the current experiment has instead conditioned rats to fear the home cage > hotplate pairing and as a result, a stress response has likely developed, causing analgesia (Chance,

1980; Finn et al., 2010; MacLennan, Jackson, & Maier, 1980). The fact that there was no difference between the two groups even though the unpaired group had a third of the exposures to the context chamber > 45°C pairing compared to the paired group suggests there could have been an element in the context chamber (relaxing music, vanilla scent) that was increasing pain behaviours. Systematic exploration of the different cue elements and their effect on pain responses would eliminate this as a contributor to the increased paw lick response.

It is also possible that there was a problem with the experimental design, causing one of three potential issues:

First, learning about the low pain cue did not occur because the ratio of home cage: context chamber pairings was not consistent with previous research. Our conditioning period of 9 days mapped out the same number of exposures to the low pain cue as previous behavioural paradigms, but much more exposure to the high pain cue was administered across the sensitization and conditioning phases combined. Unpaired subjects received only 2 pairings of high pain cue and low pain stimulus and paired received just 6, potentially too few to override the learned association between high pain cue and high pain.

Second, learning about both cues did occur, but the development of conditioned stress induced analgesia reduced pain tolerance so much after the high pain cue that placebo analgesia after the low pain cue was not observable. Having a third, naïve, group would help manage this as a potential confounder.

Third, conditioned analgesia was not observed when measuring the HPWL alone. It is possible that previous research would have presented different results had they included both HPWL and paw lick as their outcome measures.

Keeping the ratio of cue exposures stable and including sensitization in this ratio, introducing a naïve control group, and consistently reporting on at least two outcome measures could help refine future models of animal placebo analgesia.

There was no evidence for a learned association between low and high pain cues and their related stimulus in experiments one and two, possibly because of the low salience and/or distinguishability between the cues. It is therefore possible that the current results would have been observed in the previous experiments, should the cues have been better suited to learning. Perhaps the use of non-acute pain models, such as nerve ligation, where a cue for high pain is not incorporated in the paradigm, would be a better way to try and understand the placebo effect without the confounder of stress induced analgesia.

This does not, however, explain the discrepancy between the current set of results and those presented by previous researchers using similar paradigms (Lee et al., 2015; Xu et al., 2018). The lack of any findings in the HPWL measurement suggests that the behavioural conditioning approach to developing placebo analgesia is inconsistent and difficult to replicate. Without the addition of the paw lick measurement, this would have been a third null results experiment and would strongly imply considerable weaknesses in the behavioural approach to developing placebo analgesia in rodents.

These null results lead to the question of whether an animal model of placebo analgesia is a valid approach to studying the effect. To do this, we need to assess existing literature and determine whether placebo analgesia is actually observable in rodent models, and if so, which models or conditions are most reliably connected to a positive placebo analgesic outcome. Understanding these two things will help build a stronger case for an animal model of placebo analgesia within which the parameters of the effect and its underlying neural mechanisms can be studied effectively.

The next part of this thesis will examine the existing literature to determine if there is any evidence that placebo analgesia does actually occur in rodents. By collating all relevant papers that attempt to find an animal model of placebo analgesia and meta analysing the data we aim to see if there is an overall effect of placebo analgesia in rodents, and if yes, the size of the effect. Secondary assessment will analyse relevant factors identified in the literature to understand which factors moderate the size of the placebo analgesic effect in rodents.

Chapter 3 – meta analysis of placebo analgesia in rodents

Research into placebo analgesia to date has largely been in human cohorts, despite the numerous advantages of studying neurobiological processes of pain in animals (Keller et al., 2018), as well as the ability to control relevant variables such as exposure to pain and pain experiences; genetic factors; and the environment, including practical considerations such as ease of obtaining and housing subjects. This trend toward human subjects (Benedetti, Amanzio, Rosato, & Blanchard, 2011; L. Colloca & Benedetti, 2007; Geuter et al., 2017) is in part due to the history of placebo analgesia knowledge coming from clinical trials (Finniss & Benedetti, 2005), as well as more philosophical questions regarding a non-human animal's ability to experience a placebo effect.

Theories of placebo analgesia postulate that expectancy is an integral part of development of the placebo effect. Expectancy is commonly thought to be a complex cognitive process that non-human animals are incapable of experiencing, and without the ability to develop expectancy it is therefore assumed that non-human animals are incapable of developing the placebo effect. This is unusual because there is evidence that humans can develop placebo analgesia from simple conditioning processes, and that animals are capable of developing expectancies from conditioning. If animals can indeed demonstrate expectancies from conditioning, then it flows that they should also be able to develop placebo analgesia.

There is significant evidence to show that rodents in particular are capable of developing expectancy. As early as 1963, Barnett and colleagues showed that changing the amount of food presented at test changed the behaviour of rats. When food allocations were visibly reduced, rats slowed their movements and approached the reward less enthusiastically, suggesting they were

"disappointed", because they were expecting to find more food (Barnett, 2017). Robinson and Berridge (2013) conditioned rats to press a lever for a food reward until the response was reliable and then paired the lever pressing with a highly aversive infusion of salty water into their mouth. Lever pressing ceased almost immediately. Later, they induced a state of sodium deficiency in the rats and placed them back into the original chamber. In this state, where they had an intense drive for something salty; the rats immediately approached the lever that had been paired with salty water showing that the rats had an expectancy of something salty and not just simply learned the positive or negative value of the stimulus. Finally, Holland (1990) paired a taste conditioned stimulus (CS) with a tone, and later paired the same tone with illness. When presented with the original taste CS, animals showed a dislike for it. This suggests that the animals were 'thinking' of the taste cue when the paired aural cue was presented with the aversive stimulus, and thus developed an aversion to it even though the taste it was never directly presented with the aversive US. The above examples demonstrate that rats are capable of more than simple value learning, and that they are able to develop an expectation based on prior learning (Geuter et al., 2017).

If rats can develop conditioned expectations, one of the theoretical foundations required for placebo analgesia, then it follows that they should theoretically also be able to experience placebo analgesia. Recently, there have been a number of attempts to establish an animal model of placebo analgesia (Akintola et al., 2019; J. Guo et al., 2010; Lee et al., 2015; McNabb et al., 2014; Nolan, Price, Caudle, Murphy, & Neubert, 2012) but the outcomes of these studies have been inconsistent, with some reporting positive outcomes of placebo analgesia, some reporting no results, and others reporting the opposite effect (hyperalgesia). The first aim of the current

review is to assess the existing literature on placebo analgesia in rodent models and determine if there is evidence that animals are capable of developing the effect.

Papers that specifically aim to establish an animal model of placebo analgesia report a wide variety of approaches to doing so. The second aim of this review is to assess which factors, if any, moderate the effect size of placebo analgesia in rodents. The following factors will be considered.

Conditioning type

In human cohorts, it is not uncommon for behaviourally conditioned placebo analgesia to occur (Luana Colloca & Miller, 2011; Yeung et al., 2014). Rodent studies largely use drug conditioning to establish the effect (Akintola et al., 2019; McNabb et al., 2014; Valone et al., 1998), with a small minority reporting behaviourally conditioned approaches (Lee et al., 2015; Xu et al., 2018). In order to establish if behaviourally conditioned approaches are effective in rodent models, and if conditioning type mediates the effect size of placebo analgesia, conditioning type will be examined.

Drug type

Opioids are commonly used in drug conditioned models of placebo analgesia in both humans and animals (Bevins et al., 1995; Bryant et al., 2009; Nolan et al., 2012), but these can act as confounders particularly in animals if they develop tolerance to the drug and compensatory responses occur (Krank et al., 1981). Other non-opioid drugs such as aspirin (J. Guo et al., 2010) and gabapentin (McNabb et al., 2014) have been reported to produce placebo responses, removing the impact of tolerance or compensatory responses. Understanding which, if any, drug types are more closely associated with development of placebo analgesia would be beneficial in helping shape future conditioning protocols.

Cue type

In animal placebo analgesic literature, cue type varies substantially. From injection and drug administration cues only (Bryant et al., 2009; Nolan et al., 2012), to a single specific sensory cue such as olfactory (Randall et al., 1993) to more than 5 specific sensory cues being delivered with the US (Akintola et al., 2019; McNabb et al., 2014), no two protocols are the same in regards to cue type. There is some discussion regarding placebo responses being more reliably linked to particular groups of cues (e.g. gustatory or external cue types) as is seen in conditioned taste aversions (Randall et al., 1993), but no definitive conclusions have been drawn. Analysis of whether cue type mediates placebo response could help in developing a more reliable animal model of placebo analgesia and reduce the number of studies that use non-salient cues that do not lead to development of placebo analgesia in rodents.

Pain test

While most of the animal research into placebo analgesia uses paw withdrawal latency on the hot plate test to measure pain responding, there are a few other methods utilised. These include the nerve ligation and motor withdrawal approach (Akintola et al., 2019; McNabb et al., 2014; Zeng et al., 2018), and a model of irritable bowel syndrome that employs an inflated balloon to mimic bowl expansion (Liu et al., 2017). It is not known if there are changes in placebo responding that are mediated by type of supraspinal pain measurement implemented and understanding this would mean less unnecessary variation in methods across placebo analgesic models.

Dose of morphine

A number of papers have tried to establish if placebo response varies according to dose of morphine, and have had contradicting results (Bardo & Valone, 1994; Randall et al., 1993;

Valone et al., 1998). One study indicated that a higher dose (30 mg/kg) produced a more robust effect (Bardo & Valone, 1994) , and another purported to have better results with just 10 mg/kg (Valone et al., 1998). Across the animal literature, morphine dose used to induce placebo response varies from 1 mg/kg – 30 mg/kg, and significant confounders such as tolerance, motor ability, and drug induced learning deficits could all be mediated if a clear dose/response relationship was established.

Number of conditioning trials

In humans, number of trials is known to correlate with strength and longevity of conditioned placebo analgesic responses (Luana Colloca et al., 2010). Animal literature consistently reports varied numbers of trials included in conditioning paradigms and assessing if there is a relationship between number of trials and placebo responding would allow for more streamlined conditioning protocols to be developed.

Pain present during conditioning

Early drug conditioning research established that cue-associated morphine resulted in hyperalgesia from drug tolerance, not placebo analgesia (Siegel, 1975b). More recent work using almost identical conditioning models have reported opposing results of placebo analgesia (J. Guo et al., 2010; Zhang et al., 2013). Within models specifically looking to develop placebo analgesia in rodents using morphine conditioning, some paradigms expose rodents to the pain of the hotplate during conditioning, while others simply allow the rats to feel the effect of the drug without exposing them directly to the analgesic properties of the drug. It is thought that exposure to pain during conditioning may be the factor that mediates the development of either tolerance and hyperalgesia, or placebo analgesia. Pain presence during conditioning will

therefore be assessed as s factor that potentially mediates development of placebo analgesia in rodents.

Understanding these factors will help to dissect the type of conditions that are ideal for generating the learning and expectation required for placebo analgesia and will reduce the amount of unnecessary human and animal resources being consumed in the endeavour to find a reliable animal model of placebo analgesia.

Methods

Protocol and registration

The protocol for this study was registered on the 9th of August 2019 with the PROSPERO database for systematic reviews and meta-analysis under the title "A meta-analysis of placebo analgesia in rodents" reference number: CRD42019112453.

Selection criteria

To be included in the meta-analysis, studies needed to meet the following criteria: i) Study subjects needed to be laboratory rodents (rats or mice); ii) studies had to attempt to establish placebo analgesia using a cue that is paired with reduced pain either via an active substance (e.g. morphine) or via experiential learning (e.g. a noxious stimulus being surreptitiously reduced after cue presentation); iii) dependent variables needed to be measurements of pain (no other type of placebo response e.g. immune response was included); include a control group (natural history or unpaired); iv) Be published after 1950; v) be written in English.

Furthermore, studies were excluded if they met any of the following criteria: i) involved any animal (including human) other than laboratory rodents; ii) included explicit models of stress

induced analgesia i.e. pairing a cue with an *increase* in pain stimulus; iii) measure non-analgesia placebo (e.g. immune response – note that papers which measured both analgesia and immune response in different cohorts had the analgesia study only included in the review. Studies where the same cohort of rats had both immune (or other) and analgesic placebo responses measured were excluded from the review); iv) interventions that involved placebo conditions without an explicit cue/signal (e.g. pre-clinical trials); v)studies that examined drug conditioning in a broader context than placebo analgesia and; vi) comprised of individual case studies.

Search strategy

Articles were sourced through a systematic search of PubMed (Medline), PsychINFO, EmBase, and Web of Science.

Medical Subject Heading terms that mapped to the 3 main search terms were combined and then exploded to expand the search and source all relevant studies. The main MeSH content areas mapped included: Placebo AND Pain relief AND Animal model. The full list of search terms is available in appendix 1. PRISMA guidelines for conducting and reporting a meta-analysis were followed throughout (Moher, Liberati, Tetzlaff, & Altman, 2009).

Moderators

In addition to assessing the effect size of placebo analgesia in animals, we also sought to understand the factors that moderated this effect size. These included the categorical moderators of conditioning type (drug or behavioural), drug type (opioid or other), cue type (general or specific) and outcome measure used to assess pain (HPT or other). The included continuous moderators were dose of drug – amount of opioid given and number of conditioning trials

Study selection

All titles and abstracts were assessed by the lead author (RS) and those that were clearly unrelated were excluded. A second author (IJ) reviewed 20% of the full study list and exclusions were compared to RS' to ensure consistency. Discrepancies were discussed and compared to the inclusion criteria until a resolution was found. The full text of remaining articles were downloaded and independently assessed by two authors (RS and IJ) for inclusion. Discrepancies were resolved by consensus.

Data extraction

Two authors independently completed data extraction (RS and IJ) using pre designed data coding and extraction sheets. Data extracted included: Authors, year of publication, title, subject animal, comparison group, unconditioned stimulus, conditioned stimulus(CS)/cue type, timing of CS presentation, unconditioned stimulus, pain assay used, drug used, drug dose, presence of pain during conditioning, outcome measure, N and n, means, SD, F, T, and any effect sizes reported.

For papers that did not report means or other essential information in text, data was extracted from graphs using WebPlotDigitizer (https://automeris.io/WebPlotDigitizer/). This is a validated online tool that allows for measurement of graphs (Rohatgi, 2011). Means and SEMs were extracted, and SDs were calculated.

Computing effect sizes

Means and standard deviations for outcome measures were used to calculate the standard mean differences (Hedge's g) between the control group and the intervention (placebo) group. Hedges' g was chosen as it is more appropriate for the small sample sizes typical of animal studies (Vesterinen et al., 2014). It can be interpreted in a similar way to Cohen's d (a small effect < 0.03, medium >0.03 <0.08, and a large effect > 0.08).

Independence of results

For studies that had multiple outcome measures (e.g. HPWL and FP lick) or two comparison groups (e.g. natural history and unpaired control) or one comparison group with multiple intervention groups (e.g. different doses of the same drug all compared to one control) an average effect size was calculated using CMA and they were treated as one study in the overall meta analysis. For moderator analysis, relevant studies were considered independent.

Independent experiments that were reported in the same paper were treated as unique studies so long as they had both a unique control and intervention group and met the previously outlined inclusion criteria.

Heterogeneity

To assess heterogeneity across the studies, both Q and I^2 scores were calculated. When numbers of studies are low, Q (the conventional assessment) does not have enough power to make a clear assessment of heterogeneity (Higgins & Thompson, 2002), and I^2 can help make an assessment instead. I^2 gives an estimate of how much of the between study variability is caused by genuine differences between the studies, and not chance.

Quality assessment

The SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) tool was used to assess the risk of bias. SYRCLE is a validated assessment tool specifically designed for animal studies (Hooijmans et al., 2014). This tool is based on the Cochrane Collaboration Risk of Bias (RoB) tool, and items in the tool address key areas that can influence bias in animal studies (e.g. random allocation to groups, blinding of animal handlers). Items are assessed independently of each other, and no overall score for studies can be calculated from this.

Papers were assessed with the SYRCLE tool overall, meaning that independent studies within each paper were not rated individually. Papers were coded as 'yes' if they explicitly addressed the criteria, and 'no' if they did not demonstrate that they had met the criteria.

Publication bias

In academic research, non-significant results are far less likely to be published than positive results, and publication bias is thus a factor that needs to be considered when assessing overall effect sizes. To account for this in the current study, a funnel plot was used, and asymmetry was assessed with the Egger test.

Analytical strategy

The effect size for overall placebo analgesia in rodents was analysed in CMA using a random effects model, as this is an appropriate for studies with high heterogeneity (Schroll, Moustgaard, & Gøtzsche, 2011). Analysis compared control groups (no intervention) to the placebo/intervention groups using the reported measure of pain as the outcome measure. Comparison groups included within (pre-conditioning vs post), unpaired control, saline control, and/or naïve controls. Studies were included in analysis if they had $n \geq 2$ per group and included a control. Meta-regression was used to analyse continuous and dichotomous categorical moderators. Non-dichotomous categorical moderators were analysed using Q test. Each moderator was analysed in independent simple regression models. Continuous moderators needed to have at least 4 independent studies to be analysed, and dichotomous at least 2.

Effect sizes and moderator analysis were computed in both the complete set of studies (all studies) and separately in studies that included opioids as the active substance in the conditioning paradigms (opioid studies).

Results

Search results

The search strategy yielded 7848 papers, and 6376 remained after duplicate removal (Figure 18). Titles and abstracts were reviewed by one author (RS) and 6301 records were removed because they did not meet the inclusion criteria. The remaining 75 were downloaded and their full text reviewed for inclusion. Those that were excluded (n=54) were done so because they were not explicitly looking at analgesic placebo (n=43), were reviews themselves (n=5), did not include laboratory rodents as their subjects (n=5), or because they had a single subject (n=1). 21 papers met the inclusion criteria for the meta-analysis.

Figure 18 – PRISMA flow diagram

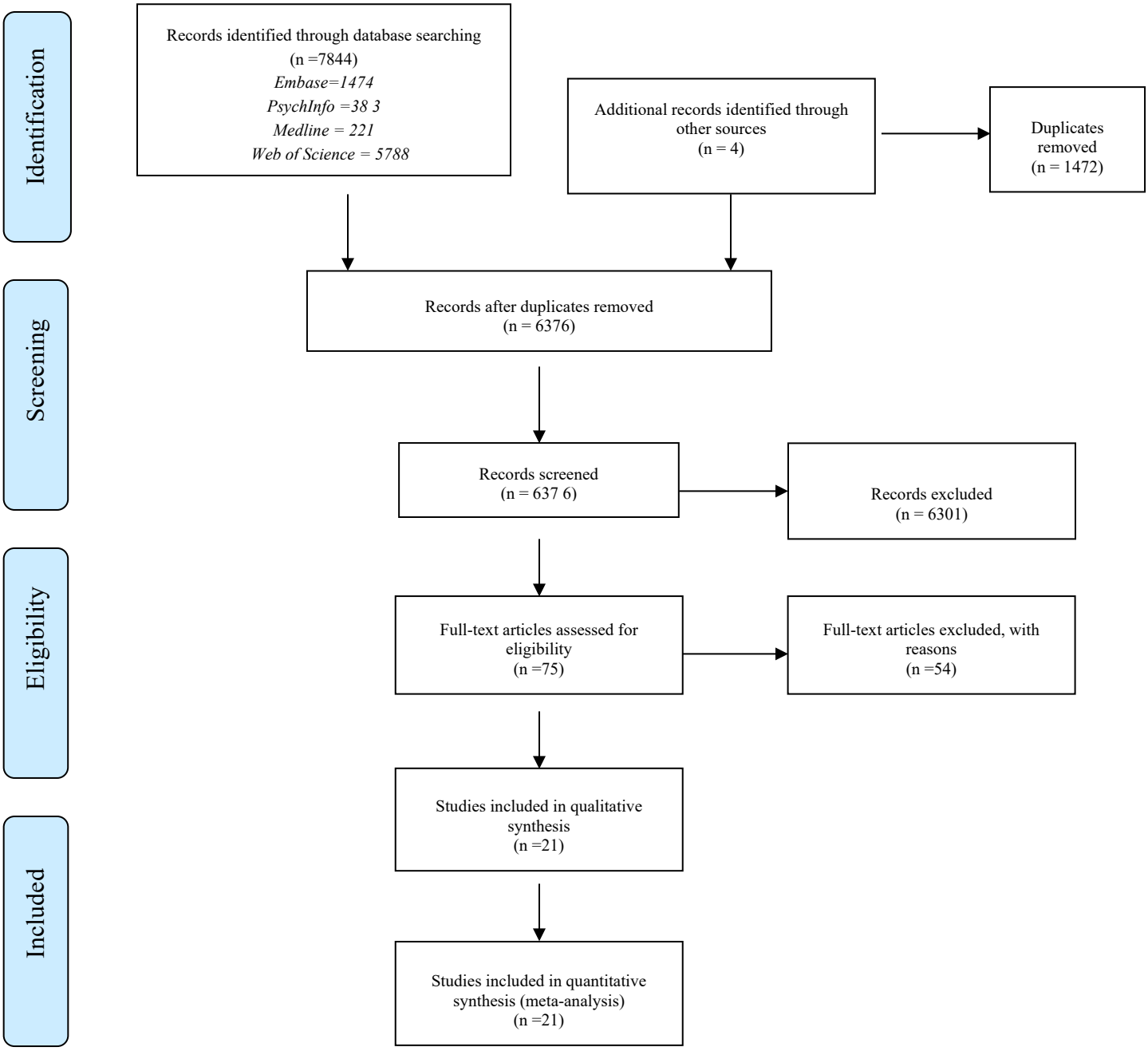


Table 7 *Study characteristics*

Paper #	Authors	study	Animal	Outcome measure	N	Control group	Placebo manipulation	Cue	Experience pain during conditioning	Dose	Trials	Main findings
1	Krank, Hinson, & Siegel (1981)	a (i)	Rat	HPWL	24# (8 per group)	Unpaired morphine	Drug - morphine	Injection + Visual, auditory. c	No	5 mg/kg	9	Conditioned hyperalgesia
		a (ii)	Rat	HPWL		Saline only	Drug - morphine	Injection + Visual, auditory. c	No	5 mg/kg	9	Conditioned hyperalgesia
		b (i)	Rat	HPWL	24# (8 per group)	Unpaired morphine	Drug - morphine	Injection + Visual, auditory. c	No	5 mg/kg	3	Conditioned hyperalgesia
		b (ii)	Rat	HPWL		Saline only	Drug - morphine	Injection + Visual, auditory. c	No	5 mg/kg	3	Conditioned hyperalgesia
2	Kehoe & Blass (1989)	a (i)	Rat (pups)	HPWL	30# (10 per group)	Saline only	Drug - morphine	Injection + olfactory	No	0.5 mg/kg	1	Placebo analgesia
		a (ii)	Rat (pups)	HPWL		Natural history	Drug - morphine	Injection + olfactory	No	0.5 mg/kg	1	Placebo analgesia
3	Miller, Kelly, Neisewander, McCoy, Bardo (1990)	a (i)	Rat	HPWL	33## (5/6 per group)	Saline only	Drug - morphine	Injection + taste.	No	15 mg/kg	2	Placebo analgesia
		a (ii)	Rat	HPWL		Saline only	Drug - morphine	Injection + taste.	No	15 mg/kg	2	Placebo analgesia
		a (iii)	Rat	HPWL		Saline only	Drug - morphine	Injection + taste.	No	15 mg/kg	2	Placebo analgesia
		b (i)	Rat	HPWL	48# (16 per group)	Unpaired morphine	Drug - morphine	Injection + taste.	No	15 mg/kg	3	Placebo analgesia
		b (ii)	Rat	HPWL		Natural history	Drug - morphine	Injection + taste.	No	15 mg/kg	3	Placebo analgesia
4	Randall, Kraemer, Valone,& Bardo (1993)	a	Rat	HPWL	21# (7 per group)	Unpaired morphine	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		b	Rat	HPWL		Natural history	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		c	Rat	HPWL	21# (7 per group)	Unpaired morphine	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		d	Rat	HPWL		Natural history	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
5	Bardo & Valone (1993)	a (i)	Rat	HPWL	50^ (10 per group)	Saline only	Drug - morphine	Injection + olfactory	No	1 mg/kg	3	No effect
		a (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	1 mg/kg	3	No effect
		a (iii)	Rat	HPWL		Saline only	Drug - morphine	Injection + olfactory	No	3 mg/kg	3	No effect
		a (iv)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	3 mg/kg	3	No effect
		a (v)	Rat	HPWL		Saline only	Drug - morphine	Injection + olfactory	No	10 mg/kg	3	No effect

Paper #	Authors	study	Animal	Outcome measure	N	Control group	Placebo manipulation	Cue	Experience pain during conditioning	Dose	Trials	Main findings
		a (vi)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	10 mg/kg	3	No effect
		a (vii)	Rat	HPWL		Saline only	Drug - morphine	Injection + olfactory	No	30 mg/kg	3	No effect
		a (viii)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	30 mg/kg	3	No effect
		b (i)	Rat	HPWL	44^ (11 per group)	Saline only	Drug - morphine	Injection + olfactory	No	15 mg/kg	3	No effect
		b (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	15 mg/kg	3	Placebo analgesia
		b (iii)	Rat	HPWL		Saline only	Drug - morphine	Injection + olfactory	No	15 mg/kg	3	No effect
		b (iv)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	15 mg/kg	3	Placebo analgesia
6	Bevins, Valone, Bradley, Bardo (1995)	a (i)	Rat	FPL	54 (9 per group)	Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	1	No effect
		a (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	3	No effect
		a (iii)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	6	Placebo analgesia
		b (i)	Rat	FPL	40^^ (10 per group)	Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	3	No effect
		b (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	3	No effect
		b (iii)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	6	Placebo analgesia
		b (iv)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	6	No effect
		c (i)	Rat	FPL	44^^ (11 per group)	Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	6	Placebo analgesia
		c (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + taste	No	15 mg/kg	6	Placebo analgesia
7	Valone, Randall, Kraemer, Bardo (1998)	a (i)	Rat	FPL	20 (10 per group)	Saline only	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		b	Rat	FPL	20 (10 per group)	Unpaired morphine	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		c (i)	Rat	FPL	40 ^ (10 per group)	Saline only	Drug - morphine	Injection + olfactory	No	3 mg/kg	4	No effect
		c (ii)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	10 mg/kg	4	Placebo analgesia
		c (iii)	Rat	FPL		Saline only	Drug - morphine	Injection + olfactory	No	30 mg/kg	4	No effect
8	Bryant, Robertsm Culbertson, Le,	a (i)	Mouse	HPWL	43#	Unpaired fentanyl	Drug - fentanyl	Injection	No	0.2 mg/kg	1	Placebo analgesia

Paper #	Authors	study	Animal	Outcome measure	N	Control group	Placebo manipulation	Cue	Experience pain during conditioning	Dose	Trials	Main findings
	Evans & Fanslow (2009)	a (ii)			(19 fentanyl; 14 unpaired;10 saline)	Saline only	Drug - fentanyl	Injection	No	0.2 mg/kg	1	Placebo analgesia
9	Guo, Wang, & Luo (2010)	a (i)	Mouse	HPWL	36^ (12 per group)	Saline only	Drug - morphine	Injection + context chamber	Yes	10 mg/kg	4	Placebo analgesia
		a (ii)	Mouse	HPWL		Saline only	Drug - Aspirin	Injection + context chamber	Yes	400 mg/kg	4	Placebo analgesia
10	Guo, Yuan, Sui, Zhang, Wang, Luo & Luo (2011)	a	Mouse	HPWL	24 (12 per group)	Pre conditioning (within group)	Drug – morphine	Injection + context chamber	Yes	10 mg/kg	4	Placebo analgesia
11	Nolan, Price, Caudle, Murphy, & Neubert (2012)	a	Hairless rat	Successful licks Orofacial pain	27 (8 in control, 19 in morphine)	Saline only	Drug – morphine	Injection + tactile.	Yes	1 mg/kg	2	No effect
12	Zhang, Zhang, Wang, & Guo (2012)	a	Rat	HPWL	28 (14 per group)	Saline only	Drug - morphine	Injection + context chamber	Yes	10 mg/kg	4	Placebo analgesia
13	Jeon (2013, grey literature)	a	Rat	HPWL	16 (8 per group)	Saline only	Drug – morphine	Injection + visual, olfactory	Yes	10 mg/kg	4	No effect
		b	Rat	HPWL	16 (8 per group)	Saline only	Drug – morphine	Injection + visual, olfactory	Yes	10 mg/kg	4	No effect
14	McNabb, White, Harris, Fuchs (2014)	a	Rat	MPWT	19 (9 in gabapentin, 10 in control)	Saline only	Drug – gabapentin	Injection + visual, olfactory, taste, tactile.	Yes	90 mg/kg	4	No effect
		b	Rat	MPWT	21 (11 in loperamide, 10 in control)	Saline only	Drug – loperamide	Injection + visual, olfactory, taste, tactile.	Yes	3 mg/kg	4	No effect
		c	Rat	MPWT	21 (10 in morphine, 11 in control)	Saline only	Drug – morphine	Injection + visual, olfactory, taste, tactile.	Yes	6 mg/kg	4	No effect
15	Lee, Lee, Park, Olausson, Enck, &Chae (2015)	a	Rat	HPWL	26 (16 in placebo group, 10 in control)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	6	Placebo analgesia
16	Liu, Wang, Tsai, Kuo, Hou, & Lu (2017)	a	Rat	Electrom-yography	14	Pre conditioning (within group)	Drug – morphine	Injection + visual	No	10 mg/kg	4	Placebo analgesia

Paper #	Authors	study	Animal	Outcome measure	N	Control group	Placebo manipulation	Cue	Experience pain during conditioning	Dose	Trials	Main findings
17	Zeng, Hu, Yang, Hayashinaka, Wada, Watanabe, Zeng, Cui (2018)	a	Rat	MPWT	35 (25 in gabapentin, 10 in control)	Saline only	Drug – gabapentin	Injection	No	100 mg/kg	4	No effect
18	Boorman (2018, grey literature)	a	Rat	HPWL (hotplate)	22 (control 8, intervention 14)	Saline only	Drug – morphine	Injection + context chamber	Yes	6 mg/kg	7	No effect
		b	Rat	HPWL (cold plate)	22 (control 8, intervention 14)	Saline only	Drug – morphine	Injection + context chamber	Yes	6 mg/kg	7	No effect
19	Xu, Wan, Ma, Zheng, Han, Lium, Yi & Wan (2018)	a	Rat	HPWL	16 (8 per group)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	7	Placebo analgesia
		b	Rat	HPWL	16 (8 per group)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	7	Placebo analgesia
20	Akintola, Tricou, Raver, Castro, & Colloca (2019)	a (i)	Rat	OFWT	31 (13 in fentanyl, 11 in saline, 7 in natural history)	Saline only	Drug – fentanyl	Injection + visual, audio, olfactory, taste	Yes	25 µg/kg	7	No effect
		a (ii)	Rat	OFWT		Natural history	Drug – fentanyl	Injection + visual, audio, olfactory, taste	Yes	25 µg/kg	7	No effect
21	Swanton (2019 grey Literature)	a	Rat	HPWL	31 (15 in control, 16 in placebo)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	6	No effect
		b	Rat	HPWL	32 (16 in each group)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	6	No effect
		c	Rat	HPWL	32 (16 in each group)	Unpaired	Conditioning – surreptitious reduction in pain	Context chamber	Yes	NA	6	No effect

Notes #these studies had the same intervention group with multiple controls. ^These studies had the same control group and different intervention groups ## individual groups differentiated by delay between cue and stimulus (not presented) ^^ individual groups differentiated by prior exposure to the testing environment (not presented) **Individual groups differentiated by exposure to different hot plate temperature (not presented)

Study characteristics

Overall, 21 papers with a total of 45 independent studies involving 1001 animal subjects were included in the meta-analysis. Of these, 17 were peer reviewed publications, 1 was a conference presentation, and 2 were grey literature dissertations. The results from experiments 1, 2 and 3 of the current experiment were also included in analysis. Table 7 outlines the characteristics of all papers.

All but four of the studies used adult rats as their subjects. One study used rat pups (10 days old) and 3 used mice. Most studies (16) utilised a Hot Plate Test (HPT) as their model of pain, and of those, all except two use the hind paw withdrawal latency (HPWL) as a measure of pain threshold. Front paw lick (FPL) was used as well as HPWL by one study, and two used FPL only. Three studies used nerve ligation as their pain model, two at the L5 spinal level, and one on the infra orbital facial nerve. Mechanical Paw Withdrawal Threshold (MPWT) on the Von Frey test, was used as the outcome measure for both L5 ligation studies, and a similar measure using Von Frey on the face was used for infraorbital ligation.

All studies except for three used active drugs in their placebo analgesia conditioning, and morphine was used as the drug in conditioning by 83% of experiments that included a pharmacological substance. Other drugs utilised included fentanyl (n=2), gabapentin (n=2), loperamide (n=1), aspirin (n=1). Three studies did not include an active substance in their conditioning, and instead used conditioning which involved pairing a cue with a high pain or a low pain stimulus.

Data used to compute effect size

All effect sizes were computed using the means and standard deviations for the intervention (placebo) group and the control group. Control groups included pre-conditioning within group comparison (n=2), unpaired control (n=14) and saline control (n=35).

Publication bias

Publication bias was assessed using the funnel plot and eggers test. The funnel plot is presented in Figure 19 and demonstrates a symmetrical distribution of studies, indicating there is no issue of publication bias amongst the literature. Importantly, publication bias is often not detected when study heterogeneity is high (Sterne et al., 2011).

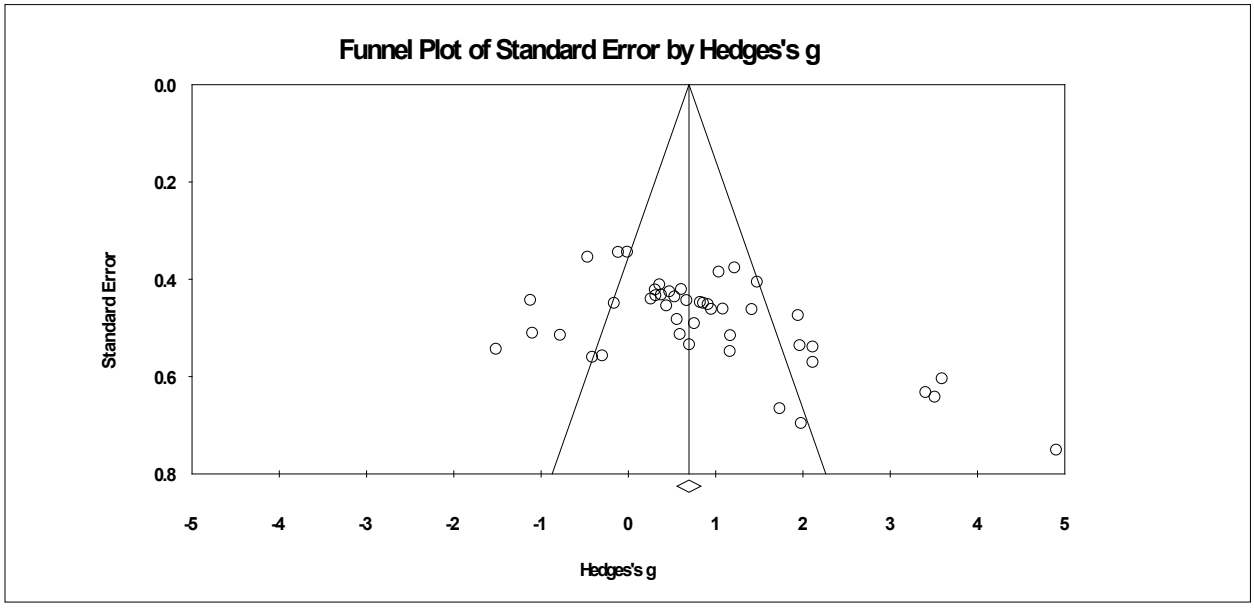


Figure 19 Publication bias of papers

Risk of bias

Table 8 – *Risk of Bias (RoB) outcomes*

Study	Was the allocation sequence adequately generated and applied☐	Were the groups similar at baseline or were they adjusted for confounders in the analysis☐	Was the allocation adequately concealed☐	Were the animals randomly housed during the experiment☐	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment☐	Were animals selected at random for outcome assessment☐	Was the outcome assessor blinded☐	Were incomplete outcome data adequately addressed☐	Are reports of the study free of selective outcome reporting☐	Was the study apparently free of other problems that could result in high risk of bias☐
Krank, Hinson, & Siegel (1981)	✘	✓	✘	✘	✘	✘	✘	✘	✓	✘
Kehoe & Blass (1989)	✘	✘	✓	✘	✘	✘	✓	✘	✘	✘
Miller, Kelly, Neisewander, McCoy, Bardo (1990)	✘	✓	✘	✘	✘	✘	✓	✘	✓	✘
Randall, Kraemer, Valone & Bardo (1993)	✘	✓	✘	✘	✓	✘	✓	✘	✘	✘
Bardo & Valone (1993)	✘	✓	✘	✘	✘	✘	✓	✘	✘	✘
Bevins, Valone, Bradley, Bardo (1995)	✘	✓	✘	✘	✘	✘	✘	✘	✘	✘
Valone, Randall, Kraemer, Bardo (1998)	✘	✘	✘	✘	✘	✘	✓	✘	✘	✘
Bryant, Robertsm Culbertson, Le, Evans & Fanslow (2009)	✘	✘	✘	✘	✘	✘	✘	✘	✘	✘
Guo, Wang, & Luo (2010)	✘	✘	✘	✘	✘	✘	✘	✘	✘	✘
Guo, Yuan, Sui, Zhang, Wang, Luo & Luo (2011)	✘	✘	✘	✘	✘	✘	✘	✘	✘	✘
Nolan, Price, Caudle, Murphy, & Neubert (2012)	✘	✘	✘	✘	✘	✘	✘	✘	✘	✘
Zhang, Zhang, Wang, & Guo (2012)	✘	✘	✘	✘	✘	✘	✘	✘	✘	✘

Study	Was the allocation sequence adequately generated and applied☐	Were the groups similar at baseline or were they adjusted for confounders in the analysis☐	Was the allocation adequately concealed☐	Were the animals randomly housed during the experiment☐	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment☐	Were animals selected at random for outcome assessment☐	Was the outcome assessor blinded☐	Were incomplete outcome data adequately addressed☐	Are reports of the study free of selective outcome reporting☐	Was the study apparently free of other problems that could result in high risk of bias☐
Jeon (2013, grey literature)	✖	✓	✖	✖	✖	✖	✖	✖	✓	✓
McNabb, White, Harris, Fuchs (2014)	✓	✓	✓	✖	✖	✖	✓	✓	✓	✓
Lee, Lee, Park, Olausson, Enck, &Chae (2015)	✖	✓	✖	✖	✖	✖	✖	✖	✖	✖
Liu, Wang, Tsai, Kuo, Hou, & Lu (2017)	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖
Zeng, Hu, Yang, Hayashinaka, Wada, Watanabe,Zeng, Cui (2018)	✖	✓	✖	✖	✖	✖	✖	✖	✖	✖
Xu, Wan, Ma, Zheng, Han, Lium, Yi & Wan (2018)	✖	✓	✖	✖	✓	✖	✓	✖	✖	✖
Boorman (2018 grey literature)	✓	✓	✖	✖	✖	✖	✖	✖	✖	✓
Akintola, Tricou, Raver, Castro, & Colloca (2019)	✖	✓	✓	✓	✓	✖	✓	✓	✓	✓
Swanton (2019 grey literature)	✓	✓	✖	✖	✖	✖	✖	✖	✓	✓

Results of the SYRCLE risk of bias tool are outlined in Table 8. No studies reported positive results for all factors included in the assessment. Only 1 published paper reported a distinct unbiased method of assigning randomisation of subjects to groups (McNabb et al., 2014), 1 unpublished dissertation also did this (Boorman 2018). In total, 10 papers reported that they assessed animals to be similar at baseline before allocating them to groups (Akintola et al., 2019; Bardo & Valone, 1994; Bevins et al., 1995; Krank et al., 1981; Lee et al., 2015; McNabb et al., 2014; Miller et al., 1990; Randall et al., 1993; Xu et al., 2018; Zeng et al., 2018), and 8 papers ensured that their outcome assessors were blinded to the grouping of animals at the time of test (Akintola et al., 2019; Bardo & Valone, 1994; Kehoe & Blass, 1989; McNabb et al., 2014; Miller et al., 1990; Randall et al., 1993; Valone et al., 1998; Xu et al., 2018).

Placebo Analgesia in Rodents

Table 9 *effect size of placebo analgesia in rodents*

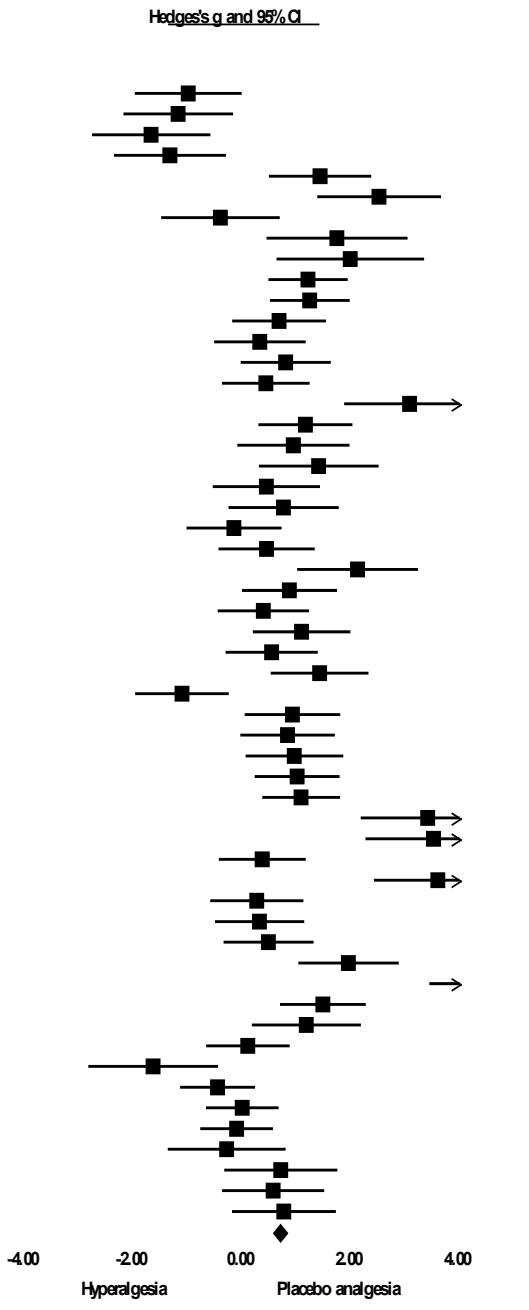
Group	**Sample size**		**Heterogeneity**			**Global effect size**		
	k	q	df	p	I^2	Hedges g	95%CI	p
All studies	45	224.921	44	**<0.001**	80.438	0.842	0.53-1.15	**<0.001**
Opioid only	35	183.156	34	**<0.001**	81.437	0.969	0.59-1.35	**<0.001**

The effect size of placebo analgesia in laboratory animals was assessed in 45 studies from 21 papers. A forest plot of all effect sizes is shown in Table 10. As a random effects model was utilised, results need to be interpreted as an estimate of the average effect across the studies, not as an estimate of the overall effect. The findings (k=45, g=0.842, p<0.001, 95% CI 0.529-1.154) indicate that the estimated average effect is large, and that this is significant. Tests of heterogeneity, however, were extremely significant (Q=224.921, I^2 =80.438%, p<0.001) indicating that there are factors contributing to the variability of the effect beyond chance, and

that interpreting the estimated average effect size needs to be done with caution. The results of placebo analgesia in rodents are presented in Table 9

Table 10 *Forest Plot of studies included in the meta-analysis*

Study name		Outcome	Hedges's g	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
Krank a (i)	vsNatHist	HPWL	-1.003	0.505	0.255	-1.992	-0.013	-1.986	0.047
Krank a (ii)	vsUnpaired	HPWL	-1.189	0.517	0.268	-2.203	-0.175	-2.299	0.022
Krank b (i)	vsNatHist	HPWL	-1.686	0.559	0.312	-2.781	-0.590	-3.016	0.003
Krank b (ii)	VsUnpaired	HPWL	-1.340	0.529	0.280	-2.376	-0.304	-2.534	0.011
Kehoe a (i)	vsNaive	HPWL	1.425	0.484	0.234	0.476	2.373	2.944	0.003
Kehoe a (ii)	vsNatHist	HPWL	2.513	0.584	0.341	1.368	3.658	4.301	0.000
Miller a (i)	vsNatHist	HPWL	-0.409	0.560	0.314	-1.508	0.689	-0.730	0.465
Miller a (ii)	vsNatHist	HPWL	1.738	0.666	0.444	0.432	3.044	2.609	0.009
Miller a (iii)	vsNatHist	HPWL	1.983	0.697	0.485	0.618	3.349	2.847	0.004
Miller b (i)	vsNatHist	HPWL	1.204	0.376	0.141	0.467	1.941	3.202	0.001
Miller b (ii)	vsUnpaired	HPWL	1.237	0.378	0.143	0.496	1.977	3.274	0.001
Barcb a (i)	Combined	FPLICK	0.672	0.444	0.197	-0.198	1.542	1.514	0.130
Barcb a (ii)	Combined	HPWL	0.317	0.434	0.188	-0.534	1.168	0.730	0.465
Barcb a (iii)	vsNatHist	FPLick	0.793	0.427	0.183	-0.045	1.630	1.855	0.064
Barcb a (iv)	vsNatHist	HPWL	0.427	0.415	0.172	-0.387	1.241	1.029	0.304
Barcb b (i)	vsNatHist	FPLick	3.077	0.619	0.384	1.864	4.291	4.969	0.000
Barcb b (ii)	vsNatHist	HPWL	1.156	0.446	0.199	0.282	2.030	2.594	0.009
Randall a	vsNatHist	HPWL	0.934	0.531	0.282	-0.106	1.974	1.761	0.078
Randall b	vsUnpaired	HPWL	1.401	0.566	0.321	0.292	2.511	2.475	0.013
Randall c	vsNatHist	HPWL	0.437	0.507	0.257	-0.557	1.431	0.862	0.389
Randall d	vsUnpaired	HPWL	0.752	0.520	0.271	-0.268	1.771	1.445	0.148
Bevins a (i)	vsNatHist	FPLick	-0.159	0.450	0.202	-1.041	0.722	-0.354	0.723
Bevins a (ii)	vsNatHist	FPLick	0.441	0.455	0.207	-0.451	1.332	0.969	0.333
Bevins a (iii)	vsNatHist	FPLick	2.117	0.571	0.326	0.998	3.236	3.707	0.000
Bevins b (i)	vsNatHist	FPLick	0.862	0.449	0.202	-0.019	1.743	1.918	0.055
Bevins b (ii)	vsNatHist	FPLick	0.381	0.433	0.187	-0.467	1.228	0.880	0.379
Bevins b (iii)	vsNatHist	FPLick	1.087	0.461	0.213	0.182	1.991	2.354	0.019
Bevins b (iv)	vsNatHist	FPLick	0.534	0.437	0.191	-0.322	1.389	1.222	0.222
Bevins c (i)	vsNatHist	FPLick	1.418	0.463	0.214	0.511	2.325	3.066	0.002
Bevins c (ii)	vsNatHist	FPLick	-1.118	0.443	0.197	-1.987	-0.249	-2.521	0.012
Valone a	vsNatHist	FPLick	0.916	0.452	0.204	0.030	1.802	2.026	0.043
Valone b	VsUnpaired	FPLick	0.826	0.448	0.201	-0.051	1.704	1.845	0.065
Valone c	Combined	FPLick	0.953	0.462	0.213	0.047	1.859	2.063	0.039
Bryant a (i)	VsNatHist	HPWL	1.003	0.402	0.162	0.216	1.791	2.497	0.013
Bryant a (ii)	VsUnpaired	HPWL	1.078	0.368	0.136	0.356	1.799	2.925	0.003
Guo10	Combined	HPWL	3.410	0.633	0.401	2.169	4.651	5.387	0.000
Guo11	VsPreWithin	HPWL	3.515	0.642	0.413	2.256	4.774	5.471	0.000
Nolan	VsNatHist	Lick%	0.361	0.412	0.169	-0.446	1.168	0.877	0.381
Zhang	VsNatHist	HPWL	3.597	0.605	0.366	2.412	4.782	5.948	0.000
McNabb a	VsNatHist	MPWT	0.261	0.441	0.194	-0.603	1.126	0.593	0.553
McNabb b	VsNatHist	MPWT	0.311	0.422	0.178	-0.517	1.138	0.736	0.462
McNabb c	VsNatHist	MPWT	0.476	0.426	0.181	-0.359	1.310	1.117	0.264
Lee	VsUnpaired	HPWL	1.948	0.475	0.225	1.018	2.879	4.104	0.000
Liu	VsPreWithin	ELECMYO	4.907	0.751	0.565	3.434	6.380	6.530	0.000
Zeng	VsNatHist	MPWT	1.479	0.406	0.165	0.683	2.275	3.643	0.000
Xu	Combined	FPlick	1.172	0.516	0.266	0.161	2.183	2.271	0.023
Akintola a (i)	VsNatHist	Facewithdraw	0.095	0.396	0.157	-0.680	0.871	0.241	0.810
Akintola a (ii)	VsUnpaired	Facewithdraw	-1.650	0.612	0.375	-2.849	-0.450	-2.695	0.007
Swanton a	VsUnpaired	HPWL	-0.463	0.355	0.126	-1.159	0.232	-1.305	0.192
Swanton b	VsUnpaired	HPWL	-0.008	0.345	0.119	-0.684	0.667	-0.024	0.981
Swanton c	VsUnpaired	HPWL	-0.111	0.345	0.119	-0.787	0.565	-0.323	0.747
Boorman a	VsNatHist	HPWL	-0.295	0.568	0.311	-1.388	0.798	-0.529	0.597
Boorman b	VsNatHist	HPWL	0.703	0.535	0.286	-0.345	1.751	1.314	0.189
Jeon a	VsNatHist	HPWL	0.561	0.483	0.233	-0.386	1.508	1.162	0.245
Jeon b	VsNatHist	HPWL	0.759	0.491	0.241	-0.204	1.722	1.545	0.122
			0.677	0.063	0.004	0.553	0.800	10.753	0.000



Moderator analysis

Table 11 *moderators for placebo analgesia in rodents*

Moderator	Group	k	b	95% CI	Q	df	p
Drug type: Other drug (ref) v opioids	All studies*	37	0.23	-1.44 – 0.98	0.14	1	0.710
Pain present during conditioning: no (ref) v yes	All studies	45	-0.02	-0.67 – 0.63	0	1	0.956
	Opioid only	37	0.36	0.48	1.20	1	0.399
Conditioning type: drug (ref) v behavioural	All studies	45	-0.43	-1.40 – 0.54	0.76	1	0.382
Cue type	All studies[#]	44	-	-	19.05	5	**0.002**
	Opioid only	37	-	-	29.94	5	**<0.001**
Pain test: HPT (ref) v other	All studies	45	0.04	-0.84 – 0.91	0.01	1	0.937
	Opioid only	37	0.29	-0.74 – 1.32	0.31	1	0.577
Morphine dose	Morphine only	43	0.02	-0.02 – 0.07	0.83	1	0.361
Number of conditioning trials	All studies	45	-0.16	-0.33 – 0.02	3.19	1	0.074
	Opioid only	35	-0.16	-0.35 – 0.03	2.59	1	0.108

Notes *Excluding non-drug behavioural studies [#] Excluded paper where n<2 for cue type. Ref = reference group for regression model.

Drug utilised

In order to determine if type of drug effected the outcome in rodent placebo analgesia studies, each independent study (n=37) was coded as either having an opioid as the active drug in the conditioning process (n=33) or having another drug type as a part of the conditioning process (n=4). Studies were excluded if they did not use an active drug in their conditioning model. Meta regression indicated that there was no significant effect of drug type on placebo analgesia in rodents (Table 11).

Pain present during conditioning

In order to determine if experiencing pain throughout the conditioning process affected the outcome in rodent placebo analgesia studies, each independent study (n=45) was coded as either including pain in the conditioning process (n=17), or not (n=28). Studies were excluded if they did not use an active drug in their conditioning model. Meta regression indicated that there was no significant effect of pain presence during conditioning on placebo analgesia in rodents.

In opioid only studies (n=37) pain during conditioning was also assessed and found to have no effect on placebo analgesia in rodents.

Conditioning type

To assess if the type of conditioning (drug conditioning or behavioural conditioning) influenced placebo analgesia in rodents, studies (n=45) were coded as utilizing a drug based conditioning procedure (n=40) or a behavioural based conditioning procedure (n=5). Meta regression indicated that there was no significant effect of conditioning type on placebo analgesia in rodents.

Cue type

Table 12 – *effect size by cue type*

Cue type	k	Hedges g	p
Injection	2	1.257	0.038
Injection + Context chamber	5	2.108	0.000
Injection + Olfactory	6	1.06	0.004
Injection + Taste	18	0.744	0.000
Injection + Combination of 2 or more	8	-0.097	0.759
Specific context chamber (no injection)	5	0.444	0.250

To assess if cue type could affect the development of placebo analgesia, studies (n=45) were coded by cue type alongside injection unless injection was not a part of the paradigm. Table 12 shows the effect sizes by cue type. Cue types coded were: Injection alone (n=2), Injection and a specific context chamber (n=5), injection and a visual cue (n=1, not included in analysis due to requirement of n≥2 studies for inclusion), injection and an olfactory cue (n=6), injection and a taste cue (n=18), injection and a tactile cue (n=0), injection and an auditory cue (n=0), injection and at least 2 other cues (n=8) and specific context chamber with no injection (n=5).

Cue type presented as a significant factor in development of placebo analgesia in rodents (p<0.001). The highest effect (g=2.12) was observed in studies that utilised injection and a specific context chamber. This was followed by injection alone (g=1.26), injection and an olfactory cue (g=1.06), and injection and a taste cue (g=0.74).

In opioid only studies, cue type also presented as a significant factor in the development of placebo analgesia (p<0.001).

Pain test type

To assess if the type of pain test used moderated the effect size of placebo analgesia in rodents, studies were coded as using a hot plate (n=38) or using a different pain test (n=7). Pain test did not have a significant effect on placebo analgesia in rodents in the main group, or in the opioid only group.

Dose of drug

Within the morphine only studies (n=43), dose of morphine delivered had no significant effect on the development of placebo analgesia in rodents.

Number of conditioning trials

Number of conditioning trials had no significant effect on the development of placebo analgesia in rodents in the whole group or within the opioid only studies.

Discussion

The current meta analysis examined placebo analgesia in rodents and measured the effect size when compared to a control group. Results indicate that there is a moderate to large significant effect size of placebo analgesia in rodents generally (g=0.842) and when conditioned with an opioid analgesic (g= 0.969). These results are consistent with a recent meta analysis in human placebo analgesic effects (Forsberg et al., 2017) that demonstrated a high effect size in people. The findings have important implications in the theoretical and clinical setting.

This is the first-time rodent models of placebo analgesia have been meta analysed and is thus the most compelling evidence we have to date that animals are capable of experiencing placebo analgesia. Ensuring all included studies had a control group means that we can be confident these findings are not attributable to other non-placebo factors such as regression to the mean. Theoretically, this is an important finding as it justifies the continued effort to use models of placebo analgesia in rodents as a method of understanding the underlying mechanisms of placebo analgesia. There still exists some existential debate about whether animals can experience placebo at all (McNabb et al., 2014). Together with the considerable research into immune response placebo effects in rodents (Lückemann, Stangl, Straub, Schedlowski, & Hadamitzky, 2019), the current findings suggest strongly that placebo effects are not exclusive to humans.

The high Q and I^2 scores in the main meta-analysis indicates that there are significant differences in the study designs of the included papers, which makes comparing them to each other difficult. While some of the variance in the effect across the included studies is attributable to actual observable differences in effect sizes, a large proportion of the variability in the

estimated average effect size of placebo analgesia in rodents is caused by differences in the experimental design. That is, the differences noted are potentially not actual differences in effect sizes across samples, but rather differences caused by variation in sample type, equipment and procedural design, and outcome measurement or statistical assessment. In future, as models of placebo analgesia in rodents become more refined and the experimental design becomes more cohesive, heterogeneity should reduce, and observable differences should be more related to real differences between effect sizes rather than study design.

Moderator analysis was conducted to try and explain some of the heterogeneity, and to help determine which experimental factors were more closely linked to the outcome of placebo analgesia. There is considerable research implicating the endogenous opioid system in the development of placebo analgesia (Levine, Gordon, & Fields, 1978; Schafer et al., 2018; ter Riet, de Craen, de Boer, & Kessels, 1998) including in rodents (J. Guo et al., 2010; Lee et al., 2015). The current results, however, indicate that while there is a large effect of placebo analgesia in opioid conditioned studies, non-opioid paradigms are not statistically different. The use of different drug types in conditioning does not account for the differences in reported outcomes. This is important because it adds to the building evidence that there are non-opioid mechanisms underlying placebo analgesia (J. Guo et al., 2010; Schafer et al., 2018) that warrant further systematic investigation.

The presence of pain during conditioning was investigated as a potential moderator of development of hyperalgesia versus placebo analgesia resulting from similar conditioning models. A key difference between recent placebo paradigms and past research on drug conditioning is the involvement of pain and the experience of pain reduction in the conditioning process. The current meta-analysis did not include drug tolerance papers in the

inclusion criteria for analysis, because this was beyond the scope of a placebo effect size analysis. This could, however, explain why we did not see any moderator effect of pain presence. Further systematic investigation that includes the full scope of conditioned analgesia (beyond just placebo specific models) could help delineate the factors that lead to hyperalgesia versus placebo analgesia in similar conditioning models.

A small number of studies utilized behavioural conditioning models with no active drug. Moderator analysis revealed that conditioning type (behavioural versus drug) was not a factor in development of placebo analgesia. This is consistent with human literature, where significant placebo effects have been reported in both active drug studies and behaviourally induced analgesia studies (Yeung et al., 2014). To date, all behavioural conditioning papers have used similar models with varying outcomes. More research using behavioural models would allow for meta assessment of the factors that contribute to the development of behaviourally induced placebo analgesia.

There is some discussion in the literature about the validity of some pain assays in studying placebo analgesia. In particular, the use of chronic neuropathic models (Akintola et al., 2019; Boorman, 2018; McNabb et al., 2014; Zeng et al., 2018) has been questioned due to their intense affect and non-responsiveness to clinical treatment (Akintola et al., 2019). The current moderator analysis did not show any significant impact of pain test type on placebo analgesia. Further research using models other than the hot plate test would help tease out the impact of pain test type on the placebo analgesic effect.

Moderator analysis of the continuous variables of dose and number of trials were not significant. Prior research in humans (Luana Colloca et al., 2010) indicates that number of trials has a direct effect on the magnitude of placebo but this was not supported by the current

findings. In their 2010 experiments, Colloca and colleagues tested placebo responding after 1 and 4 conditioning trials. Both groups demonstrated placebo analgesia at test, but in the single trial group the effect extinguished rapidly (Luana Colloca et al., 2010). All studies in the current review tested for placebo analgesia at a single time point so the impact of trial numbers was possibly not captured in the data. Repeated tests for placebo analgesia in rodents would help us understand factors that contribute to a robust effect.

Meta regression revealed dose was not a factor in the development of placebo analgesia in morphine conditioning models in rodents. Papers included in this study themselves reported differences in placebo analgesia related to morphine dose (Bardo & Valone, 1994), but this was largely related to giving a dose high enough to induce analgesia (> 3 mg/kg). Almost all papers in the current review reported doses higher than this, suggesting that as long as the dose used in conditioning is sufficient to induce analgesia, dose does not have an effect on development of placebo analgesia in rodents.

Finally, Q test revealed that cue type was a significant moderator of placebo analgesia in rodents. When hedges g was examined across the different cue types, results ranged from -0.097 for injection and a combination of 2 or more cues, to 2.108 for injection and specific context chamber. Cue chambers were defined as small boxes designed to be sensory stimulus for the subjects and included 2 or more cues within them. This is distinct from the 'injection and two or more cues' option because the animals were contained within a chamber that they never otherwise entered. This is important for cue saliency, and for ensuring latent inhibition caused by the general experimental environment is avoided (Gershman, Norman, & Niv, 2015). Akintola et al (2019) raise the issue of presenting more than one cue potentially complicating learning for rodents, and the current results provide evidence for this being a possible moderator of placebo

analgesia when the cues are delivered within the general experimental environment, but not when they are delivered within a specific cue chamber.

Overall, this meta-analysis provides evidence for a strong placebo analgesic effect in rodents and suggests that an animal model of placebo analgesia is indeed possible. This is significant as it shows placebo analgesia is not exclusive to 'higher order' animals like humans and gives validity to the continued quest to develop a reliable animal model of placebo analgesia.

The finding that context cue chambers have a strong effect on development of placebo analgesia is an important development, as inclusion of this cue type could reduce the variability and rate of null results in animal placebo analgesia research in the future. Lack of significant results in other domains does not necessarily mean that they are irrelevant to the development of placebo analgesia. More detailed reporting of methods and analytical strategy, as well as capturing multiple time points when testing placebo analgesia would improve our ability to assess the contribution of different moderators to the effect size.

Chapter 4 - General conclusions and discussion

Summary of aims and main findings

This project examined animal models of placebo analgesia and had two main aims. The first was to replicate a recent model of placebo analgesia. The study chosen for replication (Lee et al., 2015) was selected because the approach mirrored recent models of placebo analgesia in humans (Yeung et al., 2014) and, if successful, would be a valid animal model that could be utilized to further our understanding of the analgesic placebo effect and it's potential for therapeutic benefit. The second aim of the project was to conduct a meta-analysis of the existing published literature and determine the overall effect size of placebo analgesia in rodent models. If an effect was observed, the analysis would then assess factors that mediated the size of the placebo analgesic effect in rodents.

Experiment 1 sought to replicate the findings presented by Lee et al (2015) by following their protocol in the local environment with female rats. The protocol paired a context cue chamber (cCX) with a high pain stimulus, and another context cue chamber (pCX) with a low pain stimulus over an 11 day conditioning schedule in a paired and unpaired design. At test, pCX was presented before the high pain stimulus and it was predicted that rats in the paired group would demonstrate placebo analgesia induced by the pCX cue. Results from experiment 1 did not support the hypothesis and showed no evidence for placebo analgesia in rodents.

Experiment 2 was a second attempt to replicate the findings of Lee et al (2015). The second attempt adjusted the potential limitations from experiment 1 such as cue chamber intensity and re-ran the protocol using rodents of the same sex and strain as the original paper.

Results from experiment 2 were similar to experiment 1 and did not support the hypotheses or provide any evidence for placebo analgesia in rodents.

Experiment 3 used a simplified version of the behavioural conditioning methodology from experiments 1 and 2, but was modified to reduce handling stress, to increase the salience of the to-be-conditioned placebo cue, and to reduce stress-induced analgesia. In experiment 3, animals were also exposed to the 50°C hotplate more frequently in order to increase discrimination learning between the cue that signalled high pain, and the cue that signalled no pain. As in experiment 1 and 2, it was hypothesized that rats in the paired group would experience placebo analgesia when presented with the low pain cue followed by high pain hotplate at test. Results from experiment 3 did not support this hypothesis and no evidence for placebo analgesia in rodents was observed.

Given the failure to replicate previous empirical research, a meta-analysis of the literature was conducted to explore if there were important boundary conditions for the placebo effect in laboratory animals. The meta-analysis of 45 studies across 21 papers revealed a moderate to large effect of placebo analgesia in rodents ($g = 0.842$). Importantly, studies included in the meta-analysis were highly heterogeneous. High heterogeneity indicates that there are significant differences in the study designs that make comparing them to each other difficult. Moderator analysis is one way to try and account for this heterogeneity and make it more valid to compare different studies. The moderator assessment conducted included the categorical moderators of conditioning type (drug or behavioural), drug type (opioid or other), cue type (general or specific) and outcome measure used to assess pain (HPT or other). The included continuous moderators were dose of morphine and number of conditioning trials. Moderator analysis revealed that cue type was the only factor that had a significant effect on the development of

placebo analgesia in animals. Of all the cue types, injection coupled with a cue-context chamber had the highest effect size (g = 2.108).

Overall, the experiments undertaken in this project did not provide evidence for placebo analgesia in rodents and demonstrated that there is an issue with replicating findings from previously successful models of the effect. Results from the meta-analysis however, contradicted the empirical findings and demonstrated a moderate to large effect of placebo analgesia in rodents in the available literature. Factor analysis revealed that cue type moderates the size of the placebo analgesic effect in rodents and showed that context cue chambers paired with an injection were significantly associated with the development of placebo analgesia more than any other cue type. There are several considerations to be made about these findings.

Replicability in animal studies

Replicability of results is a significant issue in pre-clinical research (Pedro-Roig & Emmerich, 2017), and animal models of human experiences are not often replicated or validated by further research (M. Baker, 2016). A recent survey (M. Baker, 2016) of researchers found that 70% of respondents had tried and failed to replicate previously published results. Recommendations to improve replicability include detailed documentation of methods including statistical analysis, and public availability of raw data (Peng, 2015). Prior research in the area of behaviourally conditioned placebo analgesia was perhaps not comprehensive enough when reporting methodologies, which could have contributed to the current null results. Details such as explanation of statistical approach, descriptions of the procedural rooms and apparatus, as well as information regarding blinding of experimenters and housing arrangements for animals are all factors that impact experimental results and were not clearly described in previous research. Specifically, Lee et al (2015) state that they use visual and tactile cues in their CPP chambers,

but do not describe these cues in a way that means they can be re-created by another researcher, thus meaning the cues used were not identical between their work and the current experiments. With vision being a significant issue for particular strains of rats (Green et al., 1991), this could have been a critical difference between the two procedures. The current paper has tried to include replicability recommendations as best as possible and has thus provided more detail than would usually be found in a scientific report. It is recommended that future studies into animal models of placebo analgesia consider this and try to include as much detail as possible, particularly regarding cue type. At the very least making detailed procedural documents available online or by request would be beneficial. The finding from the meta-analysis that cue type significantly effects the development of placebo analgesia in rodents makes the need for detailed reporting of cue types even more pertinent.

Cue types in placebo analgesia

Experiment 3 adapted the model presented by Lee et al (2015) and resulted in no observable signs of placebo analgesic effect in rodents. The study utilised a context cue chamber like those identified as being related to placebo analgesia by the meta-analysis and included olfactory and taste as well as audio and visual cues. The null results are similar to those published in recent literature (Akintola et al., 2019; McNabb et al., 2014). Both of these papers attempted to establish placebo analgesia in rodents using an opioid drug conditioning paradigm in a well-documented model of chronic pain. Both studies used multi-faceted cues that were delivered in the broad experimental space, and neither were able to observe placebo analgesia at the end of their conditioning period.

Both Akintola (2019) and McNabb (2014) suggested that having too many cues could have confused the rats and limited their learning. In experiment 3 of the current project there

were also numerous cues presented, but all within a context chamber. There is a possibility that although a context chamber was used, the same problem of too many cues occurred in the current experiment. Results from the meta-analysis support this idea; studies that used 2 or more cues were found to have a much smaller effect size than those using a single cue or using a complex cue chamber. After the context cue chamber, olfactory and taste cues as individual cues were significantly associated with the development of placebo analgesia.

Early ideas on the difference between placebo analgesia and compensatory tolerance thought that the type of cues were important (Bardo & Valone, 1994), such that gustatory cues produced placebo analgesia and exteroceptive cues produced compensatory tolerance or hyperalgesia (Miller et al., 1990). However, the evidence to support this distinction is mixed as some studies show evidence for morphine tolerance using flavour cues (McNally & Westbrook, 1998), and others use environmental cues to demonstrate placebo analgesia (Bryant et al., 2009).

Differences in cue preparedness have not been studied in non-drug models of placebo analgesia like the one utilized in the current experiments, but if differential preparedness extends to non-drug analgesia conditioning then it is possible that protocols applying competing cue types (e.g. exteroceptive AND gustatory), as was done in experiment 3, result in placebo analgesia paired with 1 cue being inhibited by compensatory tolerance paired with the other cue, resulting in no effect.

Clearly, cue type plays a significant role in the development of placebo analgesia in rodents and needs to be examined further. Future studies should carefully consider the type of cue in regard to the outcome being measured. For placebo analgesia, using a simplified context chamber that includes only relevant cues identified by the meta-analysis - olfactory and taste – is most appropriate.

Defining the other parameters of placebo analgesia in rodents

The current meta-analysis revealed that context cue chambers are the most effective cue to use in conditioning models of placebo analgesia, but the other parameters within which placebo analgesia can occur are still uncertain. While prior research in humans has demonstrated that dose of drug and number of trials are both important factors in placebo analgesia studies, there was no evidence that these factors played a role in mediating the effect size in rodents. Importantly, these two factors were not often the only differentiator between studies, so there could be an issue with confounding variables masking effects of dose and trials. In future, reducing the number of elements in study design that change from paper to paper would lead to a better understanding of which factors, and to what degree, influence the onset of placebo analgesia in rodents. Methodical research that systematically assesses the factors contributing to placebo analgesia in rodents and establishes the boundaries of each of these conditions needs to be done if a reliable model of placebo analgesia is to be developed. This is not a small task that can be undertaken by one group or entity. A collaborative approach across institutions would enable more in-depth research into specific elements on the effect in animals.

Humans versus animals

The main findings from the meta-analysis suggests that, according to the published literature, rodents *can* experience placebo analgesia. This indicates concerns that the placebo effect is a cognitive construct too complex for non-human animals are incorrect, and that the effect can be studied in animals. Obviously, there are many notable differences between people and rodents, and these differences are not irrelevant to development of placebo analgesia.

Even though the meta-analysis revealed a moderate to large effect size of placebo analgesia, the empirical studies in the current thesis failed to show any effect of placebo

analgesia in rodents. Previous work in the same university has also failed to find any evidence of the effect (Jeon, 2013; Boorman, 2018), and while it is possible there is a local problem inhibiting the response, published literature reporting null results (Akintola et al., 2019; McNabb et al., 2014) suggests that there is still an element to animal placebo analgesia we are not accounting for. The issue of the 'bottom drawer effect' i.e. publications favouring positive results could be one explanation for this. While the meta-analysis did not reveal any evidence for publication bias, this could have been masked by the significant heterogeneity between studies that was observed. Reporting null results in the literature is essential to garnering an accurate understanding of the magnitude of placebo analgesia in rodents and is also an integral part in building a reliable model of placebo analgesia. If we do not know what doesn't work, how can we know to exclude it from future models?

One consideration that has been developed in human placebo literature is the idea of 'states' being integral to the development of expectation, and thus placebo (Luana Colloca & Miller, 2011). This conceptualisation of expectancy proposes that in order for expectancy to develop, a state or representation of the world must first be formed (Gershman et al., 2015). This state is developed by the subject connecting both observable and unobservable stimuli to construct a 'causal' environment in which an outcome can occur (Gershman et al., 2015). Colloca and Miller (2011) propose a theory that placebo analgesia develops in humans when a series of 'signs', both implicit and explicit, that have been established and understood build an expectation of pain relief in a person so that when they are presented all together, pain relief occurs (Luana Colloca & Miller, 2011). While the experience of rats is not as intricate as the human experience, for a concept as complex and poorly understood as the placebo response a

more layered approach that better reflects the human placebo experience may result in a stronger response in animals.

It is well recorded that there are significant homologies in descending pain inhibitory systems between rats and humans (Mogil et al., 2010), and that these systems appear to be involved in the modulation of placebo analgesia (Geuter et al., 2017). In humans, these systems can be triggered by complex environmental cues that involve higher order executive functioning, and also via simple conditioning paradigms (Eippert et al., 2009). Future research into animal models of placebo analgesia should therefore utilize procedures that produce conditioned placebo effects and have been shown to activate these descending pathways in people. This consistency across rodent and human studies would also help to standardize animal research into the effect.

The problem of conditioned hyperalgesia

One significant limitation of the current research is that the conflicting construct of the nocebo effect was not examined comprehensively in the meta-analysis. Here, meta-analysis search terms were designed to capture the placebo analgesia literature. However, in animal literature, it is well documented that paradigms similar to those used to induce placebo analgesia can result in tolerance or hyperalgesia – a nocebo response – occurring instead. Examples include Sherman et al (1979) who paired morphine with a novel cue in one group, and saline with the same cue in another. At test, rats who had previously experienced the cue with morphine exhibited hyperalgesia compared to those who had never been exposed to the association between the drug and cue. The significant body of work contributed by Siegel and colleagues (Siegel, 1976; Siegel, 1979; Siegel et al., 1978) further demonstrates that rats with prior exposure to a cue paired with morphine exhibit hyperalgesia when presented with the cue alone. Including

the expansive body of work around drug tolerance was out of scope for the current project, which was specifically trying to measure placebo analgesia in paradigms proclaiming to examine the effect in rodents.

Theoretically, Eikelboom and Stewart's (1982) argument suggests that further investigating the presence of pain during conditioning could help tease apart the conflicting outcomes of hyper or hypoalgesia. Their model suggests that conditioning occurs only if the unconditioned stimulus stimulates afferent nerves and generates a response by the efferent nervous system. Because morphine directly mimics the terminal efferent response (i.e., release of opioids on targets), then there is no stimulation of afferent nerves by morphine. So conditioning occurs when the homeostatic disturbance caused by morphine is detected by the nervous system and the efferent homeostatic opponent process is triggered. In this way, you get conditioned tolerance and/or hyperalgesia to reliable morphine injections.

However, this model can theoretically also account for conditioned analgesia under a specific condition. That is, pain presence during conditioning is essential for the development of placebo analgesia. When an opioid is given during a painful experience, it will affect pain sensory nerves (the afferent input into the CNS) and the efferent response of analgesia via descending pain modulation pathway follows. In a conditioning paradigm, the conditioned response of placebo analgesia would result. However, while presence of pain was included in the meta-analysis as a factor, it was not statistically significant. As many drug tolerance studies do *not* include pain as part of their conditioning paradigm (unlike placebo analgesia specific studies) including opioid conditioning in rodents more broadly could change this result.

The current meta-analysis attempted to draw out some of the potential contributing factors to placebo analgesia such as cue type, pain test used, and whether pain being present

during conditioning affected the observed outcome. Without the inclusion of the full body of nocebo literature, it is not possible to fully measure the impact of these factors in the development of either conditioned hyperalgesia or placebo analgesia. Future research to delineate between these two constructs and attempt to understand the factors that lead to placebo analgesia versus conditioned hyperalgesia would be beneficial in helping establish a reliable animal model of placebo analgesia.

Final conclusions

To conclude, the current project attempted to replicate an animal of placebo analgesia but was not successful. Subsequent alterations to this model also resulted in no effect. A meta-analysis that assessed available literature on placebo analgesia in rodents found that there is a moderate overall effect of placebo analgesia in rodents, and that cue type was significantly related to the size of the effect. Using a context cue box as the cue type was found to be the most effective approach to establishing placebo analgesia in rodents.

The findings of a moderate to large effect size of placebo analgesia in rodents from the meta-analysis is contrasted with the null-results from the 3 experiments conducted. The current research has highlighted the replicability problem in pre-clinical research and as such it is recommended that animal studies looking at placebo analgesia aim to include more methodological detail in their reporting, particularly in regard to cue type. Additionally, a concerted effort to methodically assess the factors that may contribute to placebo analgesia in rodents, rather than developing new models in each new study, would help to further the development of a reliable model. Ensuring laboratory models of animal placebo analgesia align closely with human laboratory models would also allow for better comparison between the two. Finally, a limitation of the current project was the exclusion of drug tolerance literature that

results in nocebo responses. Future research should compare and contrast this body of work more closely with research into placebo analgesia in rodents.

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Appendix 1 – search strategy for meta-analysis

Question

1. What is the overall effect size for placebo analgesia in rodents?
2. Which factors moderate the size of placebo analgesia in rodents?

What we already know

Many papers have reported findings that indicate the observation of placebo analgesia in rodents. Very few studies have been reliably replicated, and there is not a model that has been repeatedly tested and found to be stable and valid. It is not clear why some laboratories report successes, while others report no findings, or contradictory findings.

Objectives

The goal of this paper is to review published data on rodent placebo analgesia and assess if there is a significant effect across the literature. Additionally, we aim to understand the moderators/factors in those with successful results, those with no results, and those with opposing results (e.g. tolerance or conditioned hyperalgesia).

Selection Criteria

	Inclusion	**Exclusion**
Studies	RCT Quasi experimental Grey or unpublished literature (e.g. dissertations, conference papers)`	Qualitative Non-experimental

Animals	Small lab animals - Mice - Rats - Hamster - Guinea pig	All other lab animals Humans
DV	Placebo Analgesia HPWL Thermofacial Tail flick	Non analgesic placebo Nocebo
Moderators	Pain assay (reflexive etc) Pain included in conditioning Animal (species, sex, age) Number of conditioning trials Drug (and dose)	NA

Search Strategy

The search strategy will include a search of EmBase, Pubmed (MEDLINE), Web of Science, and PsycINFO, and will include the following terms:

EmBase

1. placebo effect/ or placebo/
2. placebo analgesia.mp.
3. conditioned analgesia.mp.
4. pain/
5. exp nociception/

6. pain threshold/

7. antinoceception.mp.

8. endogenous opi*.mp.

9. opiate/

10. animal model/

11. rat/

12. rodent/

13. rodent model/

14. mouse/ or mouse model/

15. analgesia/

16. conditioning/

17. 4 or 5 or 6 or 7 or 8 or 9 or 15

18. 1 or 2 or 3 or 16

19. 10 or 11 or 12 or 13 or 14

20. 17 and 18 and 19

MEDLINE bold indicates MeSH term

1. Placebo Effect

2. Placebo

3. Placebo analgesia

4. Conditioned analgesia

5. OR 1-4

6. Pain
7. Nociception
8. Nocicept*
9. Pain threshold
10. Pain tolerance
11. Analgesia
12. antinociceptive
13. OR 7-12
14. Rats
15. Rodentia
16. Mice
17. Animal model
18. OR 14-16
19. AND 6, 11, 16

WEB OF SCIENCE

1. Conditioned analgesia
2. Placebo
3. Placebo effect
4. OR 1-3
5. Analgesia
6. Pain relief

7. Reduced pain
8. Pain threshold
9. Pain perception
10. Antinociceptive
11. OR 6-11
12. Animal model
13. Rat
14. Rodent
15. Mice
16. OR 13-16
17. AND 5, 12, 17.

PsychINFO

1. conditioned analgesia.mp.
2. exp PLACEBO/
3. placebo effect.mp.
4. exp CONDITIONING/
5. exp ANALGESIA/
6. exp Pain/ or exp Pain Management/
7. exp Pain Thresholds/
8. exp Pain Perception/
9. exp Pain Measurement/

10. antinociceptive.mp.
11. exp Animal Models/
12. exp RATS/
13. exp RODENTS/
14. exp MICE/
15. 1 or 2 or 3 or 4
16. 5 or 6 or 7 or 8 or 9 or 10
17. 11 or 12 or 13 or 14
18. 15 and 16 and 17

Methods of the review

Article selection

The search strategy will identify all relevant articles and titles will be reviewed by two reviewers (RS and IJ). Abstracts will then be read by RS and included according to the inclusion criteria. These will be reviewed by IJ. Discrepancies in exclusion/exclusion will be discussed and agreed upon.

Data extraction

Will be extracted and entered into a table by RS and reviewed by IJ. Differences in data extraction will be resolved by referring back to Characteristics in the original paper and discussing reasons for different views.

Data for extraction:

- Year
- Authors
- Title
- Animals used
- CS
- CS Presentation
- US
- Drug
- Dose
- Pain present during conditioning
- Conditioning type
- Pain test type
- Pain apparatus novel at test

Data will be coded as follows:

Characteristics

- Animals
- SD rat F 1
- SD rat M 2
- Wistar rat F 3
- Wistar rat M 4
- Mice F 5
- Mice M 6

- Other 7

CS

- Environment (general) 1
- Room (explicit cue) 2
- Light 3
- Sound 4
- Scent 5
- Temporal 6
- Taste 7
- Combination of 3 or more 8

CS Presentation

- Pre 1
- Post (immediate) 2
- Both (continuous) 3
- Delayed 4

US

- Drug 1
- Temperature 2
- Other 3

Drug

- Morphine 1
- Asprin 2

- Fentanyl 3
- Gabapentin 4
- Loperamide 5

Dose

- 1 mg/kg 1
- 3 mg/kg 2
- 5 mg/kg 3
- 10 mg/kg 4
- 15 mg/kg 5
- 30 mg/kg 6
- Other 7

Pain present during conditioning

- Yes 1
- No 2

Conditioning type

- Classical 1
- Operant 2

Pain test type

- HPT 1
- MPWT 2
- Tail flick 3
- Orofacial 4

Pain apparatus novel at test

- No 1
- Yes 2

Quality Assessment

Quality of studies and potential for bias will be assessed using the SYRCLE RoB tool. RS will review papers and IJ/BC will cross check the initial assessment. Disagreements will be resolved by referring to the original paper and finding a consensus.

Synthesis

If studies that meet the selection criteria a meta analysis will be applied. If not, a narrative systemic review will be completed. If appropriate, some sub-group analysis (such as animal type and pain assay) may be completed.