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The Influence of Dopamine on the Magnitude and Duration of the Placebo Effect

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Abstract

A placebo effect is a real and *beneficial* psychobiological phenomenon following the administration of a substance or procedure that has no inherent power to produce an effect. Nocebo effects, on the other hand are genuine and *detrimental* psychobiological phenomenon following the administration of and inert substance or procedure. These effects have been extensively studied but are not well understood. Central to the development of a placebo effect is the anticipation of benefit or the anticipation of harm. Indeed, expectancy and conditioning are thought to be the two primary mechanisms involved in the acquisition of the placebo effect. The neurotransmitter Dopamine (DA) is integral to expectancy and reward and as such has recently been considered a key player in the mechanisms of the placebo effect. Based on this line of inquiry this study sought to investigate the role DA might have in the development of the placebo effect as observed in pain using an animal (mouse) model. It was proposed that DA is involved in the acquisition and maintenance of the placebo effect. Specifically it was proposed that the DA agonist cocaine would enhance the magnitude and duration of the placebo analgesia and that the DA antagonists SCH23390 and eticlopride would together or separately block the acquisition of the placebo analgesia. These proposals were assessed by utilizing supra-spinal (hotplate) and spinal (tail flick latency) protocols. Results indicated that cocaine enhanced placebo analgesia in spinal but not supra-spinal measures and that the DA antagonists SCH23390 and eticlopride each contributed to the acquisition, rather than the blockade, of placebo analgesia in both spinal and supra-spinal models. In fact, the most profound effect was observed when both antagonists were administered together rather than separately on supra-spinal measures but not spinal measures resulting in an enduring *nocebo* effect contradicting all predictions. The novel results presented in this study raises more questions than they answer, warranting more detailed exploration of the mechanisms of DA and its relationship with placebo effects.

Keywords: placebo, nocebo, analgesia, supra-spinal, spinal, dopamine

Introduction

The placebo effect is a phenomenon that has been extensively studied but is not well understood (Benedett, Lanotte, Lopiano, & Colloca, 2007). The term placebo is practically ubiquitous in contemporary language and has a lengthy history. The use of the word placebo dates back several centuries in medical literature with the first reported controlled placebo study conducted in 1799 (Price, Finniss, & Benedetti, 2008). Geers, Helfer, Kosbab, Weiland and Landry (2005) pointed out that "placebos have been described as one of the most powerful agents of symptom relief in medicine" and argue that prior to the beginning of the 20th century most treatments for illness and disease were placebo.

It has been argued that even if there was not a specific term for it, the history of medicine prior to the 20th century is a history of the placebo effect (Benedetti, 2009; Geers, Helfer, Kosbab, Weiland and Landry, 2005; Shapiro and Shapiro 1997). With a few noted exceptions (e.g. opium, white willow bark, foxglove) medicines and treatments were as likely to harm (e.g. bleeding, blistering, purging) as to help. It is believed, for instance, that the bleeding of George Washington for tonsillar abscess (2.5 to 2.8 quarts in twelve hours) resulted in his death at age 57 (Shapiro and Shapiro, 1999). Even with treatments that were as likely to harm as to help, people did get better prior to the 20th century. Galen commented "He cures most in whom most are confident" (Shapiro and Shapiro, 1997), a strong indication that even if there was no specific word for it at the time healers in antiquity understood that the mind could improve the body.

As the 20th century approached and medical professionals began using the word placebo to describe a treatment primarily given to please a patient, it is not taken

seriously until the Henry Beecher's (1955) article *The Powerful Placebo* (Evans, 2004; Finiss, Kaptchuk, Miller and Benedetti 2010; Moerman, 2002; Shapiro and Shapiro 1997, 1999). Using an early meta-analytical technique Beecher evaluated patient responses across 15 different clinical trials. His conclusion, "Thus in 15 studies (7 of our own, 8 of others) involving 1,082 patients, placebos are found to have an average significant effectiveness of 35.2 ± 2.2%, a degree not widely recognized" (Beecher, 1955).

Beecher argued in this article that placebo effects were not just subjective responses; they could be observed and measured objectively. Further, he argued, the placebo effect was constant across a variety of conditions (e.g. pain, nausea, and mood) as demonstrated by the small standard error. Finally, he recommended that due to the large consistent response to placebos, it was important to use the "double unknowns technique" (Beecher, 1955) to eliminate the biasness of clinical impressions when evaluating a drug. This technique, he argued, would allow a drug's effect to be separated out from a placebo effect. Finally, he suggests that the placebo effect be studied seriously to determine its mechanism and the extent of its therapeutic benefit. Beecher, though, was wrong about placebo response size and how to measure it (Kienle and Kiene, 1997). As a result, researchers have been misquoting this important paper for decades. Despite the influence Beecher's paper may have had on the medical community there remains much confusion about how to operationalize the phenomenon and under what circumstances can it be observed.

Definitions

Placebo

Researchers have been trying to explicate what a placebo is, placebo effects and placebo response since the classic Beecher (1955) article and on the whole have not reached a consensus (de Craen, Kaptchuk, Tijssen, Kleijnen, 1999). It appears that much of the discord comes from the theoretical position the individual researcher is trying to advance. Shapiro and Shapiro (1997) provide the most cited definition of placebo:

> A placebo is any therapy (or that component of any therapy) that is intentionally or knowingly used for its nonspecific, psychological, or psychophysiological, therapeutic effect or that is used for a presumed specific therapeutic effect on a patient, symptom, or illness but is without specific activity for the condition being treated.

They go on to say "A placebo, when used as a control in experimental studies, is a substance or procedure that is without specific activity for the condition being treated." Stewart-Williams and Podd (2004) posited the following, "A placebo is a substance or procedure that has no inherent power to produce an effect that is sought or expected." Olchansky (2007) states a "placebo is a sham, often a pill, but any intervention purported to be therapeutic. Without direct physiologic or pharmacologic activity, a placebo somehow provides benefit or apparent benefit."

It should be clear that the definitional essence of placebo is that of an inert substance or procedure. This, however, leads to a paradox. If something is inert or a

sham, then by definition it is not capable of producing an effect (Finiss et al., 2010; Moerman and Jonas, 2002).

Nocebo

Not all effects elicited by an inert substance are beneficial. According to Benedetti and Amanzio (1996) the term *nocebo* was introduced by Kissel and Barrucand in 1974 to distinguish “the pleasing and salubrious effects of placebo from the noxious effects.” This distinction, though important, does little to eliminate confusion between the two terms.

Paradoxes notwithstanding, the definitions for placebo are fairly consistent varying primarily in scope. This changes, however, when definitions for placebo effect and placebo response are examined. Shapiro and Shapiro (1997) stated “The placebo effect is the nonspecific psychological or psychophysiological therapeutic effect produced by a placebo.” Price, Finiss and Benedetti (2008) suggested the placebo effect is “the responses of a population to placebo administration, such as in a clinical trial” and represent a group effect and a placebo response is that of an individual. Stewart-Williams (2004) sees the placebo response as “any change that occurs after the administration of a placebo” and the placebo effect as “the portion of the placebo response, if any, that is attributable to the placebo; that is, it would not have occurred if the placebo had not been administered.” Spiro (1999) said the placebo response is the “behavioral change in the person receiving the pill” and the placebo effect as “that part of the change attributable to the symbolic effect of the medication.” Some researchers think that the placebo effect/response should be renamed altogether. Moerman (2002),

for example, thinks we should change placebo effect to “meaning response” and Evans (2004) suggested we should use “the belief effect”.

In regard to the nocebo effect, Colloca and Benedetti (2007) state for the following, “If positive verbal suggestions, which are typical of the placebo effect, are reversed in the opposite direction, a nocebo effect can be obtained. Therefore, the study of the nocebo effect is the study of the negative psychosocial context around the patient and the treatment, and its neurobiological investigation is the analysis of the effects of this negative context on the patient’s brain and body.” In short, nocebo is the opposite of placebo either with pill or procedure and its observed effect or effects.

In addition to the general lack of consensus among placebo researchers clinical drug studies view placebo effects/responses as statistical noise to be controlled. In response Benedetti (2009) recommended that we abandon the terms “placebo effect and placebo response” when referring to the outcome of clinical trials and replace it with something like “improvement in the group that received the placebo”. Benedetti (2009) further suggested that placebo researchers use “placebo effect and response interchangeably to mean a psychobiological phenomenon occurring in an individual or in a group of individuals.”

Placebo effect: What it is not.

A number of things are often mistakenly called a placebo effect when in fact it may be something else. Beecher’s 1955 paper makes this error as do most clinical studies. This section will review what should not be considered the placebo effect.

Spontaneous remission

The majority of chronic conditions will exhibit spontaneous fluctuation in symptom intensity and/or length which are commonly known as natural history (Fields and Levine, 1984). For example, Beecher's (1955) paper included in its analysis a study on the common cold. Beecher, however, did not take into consideration that many patients will spontaneously improve after about 6 days (Benedetti, 2009). The result was Beecher reporting that 35% of people with the common cold receiving "satisfactory relief" after taking a placebo (lactose). Satisfactory relief of the common cold was determined by the original study's author (Diehl, 1933) as improvement of symptoms noted by the patients on a written response card about any changes observed over a two day period.

Pain provides another useful example. If, for example, one takes a placebo for headache pain and the discomfort associated with the headache lessens it may or may not be due to the placebo. The headache might have gotten better without the placebo, as the reader is likely to have experienced spontaneous improvement of headache pain without intervention. On the other hand, the placebo may have triggered a physiological response resulting in relief from the pain.

Regression to the mean

Regression to the mean can be considered a special case of spontaneous improvement. This phenomenon is a statistical tendency for extreme values to move toward the mean following repeated measurement. Regression to the mean is common in clinical trials because the persons enrolled in clinical trials typically have an extreme score for the condition being evaluated and these values will tend to be lower at the second measurement (Davis, 2002). Ruck and Sylven (2006) demonstrated that it is

sufficient to randomly generate numbers from 0.00 to 1.00 and place them in two even columns for a model of regression to the mean. This is accomplished by selecting predetermined high values (e.g. .70) in the first column and comparing them to the corresponding values in the 2nd column.

The author replicated these findings by generating 10 columns of 25 fractional numbers between 0.00 and 1.00 on RANDOM.org. There was no difference observed between any pair of columns examined. However, if any number greater than or equal to 0.70 is selected from the first column, the corresponding number from the second column was typically smaller. Selecting the data in this manner produced a significant difference each of the five times the exercise was performed.

Report bias

Biases are another source of error that can be mistaken for a placebo effect. Biases can occur for patients, doctors and investigators. Evidence suggests that patients many times want to please their doctors and as a result will exaggerate reports of clinical improvement (Kienle and Kiene, 1997; Roberts, 1995) or will exaggerate symptoms for inclusion in clinical trials (Kleinman, Guess and Wilentz, 2002). Additionally, investigators and/or patients may become unblinded during a clinical trial which will then alter expectations about the effectiveness of the trial (Benedetti, 2009).

Co-interventional improvement

The last source of potential symptom improvement that is often overlooked by investigators is that of additional interventions. In a study of angina pectoris (a painful heart condition) evaluated by Beecher (1955) the placebo group was allowed to take nitrates (Benedetti, 2009). In the Diehl (1933) study of patients with the common cold

patients were allowed to take hot baths, gargles and alter diets. In addition, patients assigned to a wait-list, which is common as a control in many types of studies, will seek interventions and fail to tell investigators (Benedetti, 2009; Evans, 2004; Shapiro and Shapiro, 1997).

Taken together, spontaneous remission, regression to the mean, report bias and co-interventional improvement represent the most common elements of apparent symptom improvement that can be mistaken for the placebo effect. In clinical or experimental studies that do not include a natural history or no treatment group as control it is not possible to determine whether improvement is due to a placebo effect/response or other factors (Benedetti, 2009; Evans, 2004; Price et al., 2010; Shapiro and Shapiro,1997, 1999; Stewart-Williams, 2004).

What is the placebo effect?

Benedetti (2008) argues that there is no placebo effect instead there are placebo effects. His argument is that placebo effects are induced under a wide variety of circumstances for a number of conditions. “The brain may anticipate a clinical benefit through different mechanisms, such as expectation of a reward or expectation that reduces anxiety, as well as classical conditioning, and this may occur in different systems and apparatuses of the body” (Benedetti, 2009). Thus, he argues, if the main mechanism of a given placebo effect is primarily reward, then the investigator is actually studying reward mechanisms. Likewise, for classical conditioning or any other theoretical mechanism, many of which will be discussed in other responses.

So what is the placebo effect? I would argue the placebo effect is the outcome difference between a placebo group and a natural-history group. Subtracting the latter

from the former will give you the placebo effect size or placebo response (Ernst and Resch, 1995). In agreement with Benedetti (2009) I will use placebo effect and placebo response interchangeably both of which will mean “a psychobiological phenomenon occurring in an individual or in a group of individuals” following the administration of an inert substance or procedure. Additionally, nocebo will be considered to fall under the umbrella heading of placebo, but only in the sense that an inert substance influences a biological change. If a specific direction, positive or negative, is to be discussed in the context of a biological change and this change is negative then nocebo will be used specifically.

Is there really a placebo effect?

Given the discord among researchers in defining placebo and placebo effects and the things that are often misconstrued as the placebo effect (e.g. spontaneous remission, regression to the mean) skeptics have emerged questioning the existence of the placebo effect. In a highly controversial article in the New England Journal of Medicine, Hróbjartsson and Gøtzsche (2001) conducted a meta-analysis to evaluate this question. They located 114 placebo-controlled clinical trials that included no treatment groups and concluded that they “found little evidence in general that placebo had powerful clinical effects” (Hróbjartsson and Gøtzsche, 2001) and recommended that placebo’s cease to be used clinically.

This meta-analysis was interpreted by many to mean that the placebo effect was in fact a myth (Stewart-Williams, 2004). Even though the analysis was generally accepted for statistical accuracy it received tremendous criticism on several points. First, all of the studies were clinical, no experimental studies were included (Stewart-

Williams, 2004). Second, the authors defined placebo “practically as an intervention labeled as such in the report of a clinical trial” (Hróbjartsson and Gøtzsche, 2001). Some of the things listed as placebos in their study would not typically be considered a placebo (e.g. relaxation, which was a placebo in some and a treatment in others, reading and favorite foods) (Kirsh, 2002). Lastly, while their loose definition may have been troubling to some critics, it was the range of disorders that deserves special attention. The trials included consisted of the common cold, alcohol abuse, smoking, poor oral hygiene, herpes simplex infection, infertility, mental retardation, marital discord, fecal soiling, pain, obesity, asthma, hypertension, anxiety, insomnia, Alzheimer’s disease, carpal tunnel syndrome and “undiagnosed ailments” (Hróbjartsson and Gøtzsche, 2001).

Hróbjartsson and Gøtzsche’s (2001) meta-analysis was conducted specifically to evaluate Beecher’s 1955 claim that the placebo effect was a powerful therapeutic agent to be employed more often in clinical settings. It is ironic that they make similar mistakes. Beecher did not consider the possibility that other factors may be included in the placebo effect and as a result overestimated its impact. Hróbjartsson and Gøtzsche assumed that there was a single mechanism underlying all of the conditions included in the analysis. Some of the disorders listed above have strong placebo effects, others have weaker effects and some may not have any placebo responsiveness (Kirsch, 2002). Pooling the disparate trials essentially washed out any useful information the analysis may have been able to provide. In 2004 Hróbjartsson and Gøtzsche conducted another analysis of 52 additional trials to evaluate whether newer studies had revealed a larger placebo effect in comparison to their original analysis. They did not change their

criteria or definition and the results proved to be similar. That said, I find that both analyses provide support for Benedetti's argument that there is not one placebo effect but many. Thus, illustrating how difficult it may be to determine placebo effects in general.

The Placebo Responder

Following Beecher's 1955 article more attention was paid to evaluating effective medicine more critically. The randomized control trial called the "double unknowns technique" by Beecher became more common and in the 70's was a mandated requirement by the FDA to evaluate the efficacy of new medications (Shapiro and Shapiro, 1997). Strenuous effort to identify responders was expended by drug manufacturers who wanted to eliminate responders from studies and by clinicians who wanted to utilize placebo effects (Wasan, Kaptchuk, Davar and Jamison, 2006).

During the 60's research investigating personality types and characteristics for placebo responders found anxiety, suggestibility, dependence on others and church going to be likely responders (Vallance, 2006). These results were not replicated and a hodgepodge of inconsistencies subsequently emerged. Responders were found to be extroverted and introverted, outgoing and not socially confident, of low intelligence and verbally skilled, well-adjusted and submissive (Shapiro and Shapiro, 1999). By the 1970's the general consensus by researchers was that no consistent placebo responder existed (Harrington, 1999).

If no consistent responder exists how does one proceed? Drug companies that considered the placebo effect as statistical noise to be controlled began implementing a run-in period (Vallance, 2006). A run-in period is a phase at the beginning of a drug trial

that gives all participants placebos for a period of time in order to identify responders. Once identified, the responders are eliminated and the next phase of the trial can begin. This procedure does not eliminate placebo responsiveness nor increase the drug-to-placebo effect size (Lee, Walker, Jakul and Sexton, 2004; Quitkin, McGrath, Stewart, Ocepek-Welikson, Taylor, et al., 1998; Vallance, 2006). Despite the lack of statistical improvement, drug companies continue to use the run-in period.

Armed with the knowledge of what a placebo effect is not, researchers have recently revisited the proposal that some personality traits may be associated with placebo response. De Pascalis et al. (2002) found differences in suggestibility contributing significantly to the magnitude of placebo analgesia. Geers et al. (2005, 2007) found personality and situation variables interact to produce placebo response. Their findings indicated that optimists were likely to be placebo responders and pessimists were likely to be nocebo responders. There is, however, a dearth of research that includes a natural history group currently pursuing whether additional personality or coping mechanisms may be involved in placebo responsiveness. Benedetti (2009) argues that it is too difficult to find individual responders and the effort should be spent evaluating group effects in an effort to uncover the underlying mechanisms. Hoffman, Harrington and Fields (2005) argue that it is critical to identify the individual responders because it is from them that researchers will gather insight into placebo responsiveness.

Conditions influenced by the placebo effect.

Pain is the most studied placebo condition and has provided the most insight into placebo and nocebo mechanisms (Benedetti, 2009). Pain provides an easy platform from which to manipulate variables. This ability has enabled researchers to articulate

the neurological mechanisms involved with pain and placebo responses. It has been demonstrated placebos activate endogenous opioids (analgesia) that decrease pain response and nocebos activates a pro-nociceptive hyperalgesic non-opioid system (cholecystokinin, CCK) that increase pain responsiveness (Amanzio and Benedetti, 1999; Benedetti, 2008; Benedetti & Amanzio, 1997; Benedetti et al., 2007; Colloca and Benedetti, 2007; Colloca, Siguado & Benedett, 2008; Enck, Benedetti & Schedlowski, 2008; Klosterhalfen and Enck, 2008; Kong et al., 2008).

Though pain has been one of the most intensively studied areas of placebo effects a number of other conditions have been studied using a placebo paradigm. Next to pain, Parkinson's disease has been well described and studied in placebo settings. It is generally thought to generate an expectation induced release of dopamine (DA) in the striatum and recorded changes of firing patterns of sub-thalamic nucleus neurons as a result have been observed (Benedetti et al., 2004). According to Benedetti's (2008) review of placebo and placebo effects across diseases and treatments, depression has differential metabolic responses in a variety of brain regions, thought to be related to inhibition of serotonin reuptake. Furthermore, the review indicated addiction had demonstrable changes in metabolic activity in various brain regions and the cardiovascular system has demonstrated reductions of β-adrenergic activity, all in response to placebo. Additionally, it has been demonstrated that conditioning of opioid receptors in respiratory centers have been seen as a result of pharmacological preconditioning and the immune system has been documented to respond to pharmacological preconditioning as well, especially to immunosuppressive drugs.

Finally, it has been reported that conditioning of some hormones has been observed for the endocrine system as a result of pharmacological preconditioning with 5-HT receptor agonists.

Neurotransmitters and the placebo/nocebo effect.

Opioids

Opioids are powerful analgesics that can be classified generally into two categories, weak (e.g. codeine) and strong (e.g. morphine), which describes their relative efficacy and receptor site affinity (Twycross, 1994). It has long been known that opium and its derivatives like morphine relieve pain but it wasn't until the discovery of stereospecific binding sites for opioids by Pert and Snyder (1973) that researchers were able to articulate how they may function. Once receptor sites for opiates were located, the hunt began for their natural ligands. This was accomplished by Hughes in 1975, demonstrating an endogenous opioid system in the central nervous system.

Opioid receptors are found throughout the brain, brainstem and the spinal cord (Benedetti, 2009). Receptors are found to be particularly dense in the cingulate cortex, prefrontal cortex (Pfeiffer, Pasi, Mehraein and Herz, 1982), periaqueductal grey (PAG) and the rostral ventromedial medulla RVM (Fields, 2004). The latter two areas are particularly important for the blockade of ascending pain signals. The question is how this information is relevant to the placebo response.

Levine, Gordon and Fields (1978) began exploring the mechanisms of placebo analgesia. They conducted a study in a clinical setting asking whether patients that had undergone a 3rd molar tooth extraction would respond to a placebo administration (saline) and if so whether it could be disrupted. To determine whether a placebo

analgesic effect involved endogenous opioids naloxone (an opioid antagonist) was given to placebo responders and non-responders. It was observed that naloxone would reverse analgesia experienced by placebo responders and had no effect on non-responders, indicating that placebo analgesia involved the endogenous opioid system. Even though this study did not include a natural history group it was the first study to give scientific credibility to the placebo effect by suggesting the underpinning of a biological mechanism (Benedetti, 2009) and later better controlled studies supported the conclusion that placebo analgesia can be mediated by endogenous opioids (Benedetti, 1996; Levine and Gordon, 1984).

It has been demonstrated that not only can a placebo induce analgesia it can do so in a very specific fashion. Montgomery and Kirsh (1996) demonstrated that placebo analgesia could be achieved in only one finger despite a stimulus being applied to all fingers. This was achieved by conditioning subjects to believe that an analgesic cream applied to one of their fingers would effectively reduce pain. Benedetti (1999) similarly demonstrated that if a noxious stimulus was simultaneously applied to both feet and both hands and a placebo cream was applied to one hand, analgesia could be induced in only that hand. This highly specific effect could also be blocked by naloxone suggesting that a placebo activated endogenous opioid system can be precise and is perhaps somatotopically organized. In addition, imaging studies have indicated that placebo analgesia can activate the same brain regions known to contain opioid receptor sites (Petrovic, Kalso, Petersson and Ingvar, 2002; Wager, Scott and Zubieta, 2007; Zubieta, Bueller, Jackson, Scott, Xu et al., 2005). Lastly, it should be mentioned that expectancy, conditioning and/or experience are necessary to induce placebo analgesia.

When placebos are given in hidden injection paradigms analgesia is not produced (Benedetti, 2009). In other words a placebo response requires some level of situational awareness.

Cholecystokinin (CCK)

To maintain homeostasis, most body systems contain opposing systems. This is true as well for endogenous opioids in the form of the endogenous peptide cholecystokinin (CCK). Where endogenous opioids can mediate placebo analgesia, CCK mediates nocebo hyperalgesia. In other words, CCK increases pain perception and exacerbates symptoms. CCK, then, is considered to be a pro-nociceptive system (Enck, Benedetti and Schedlowski, 2008). Because a nocebo effect involves symptom worsening, its induction, presumably, would be stressful and anxiogenic. For this reason, it is ethically problematic to study nocebo effects in many conditions and as a consequence, less is known about its mechanisms (Benedetti, Lanotte, Lopiano and Colloca, 2007). That said, a 1997 study with post thoracotomy patients given suggestions of pain worsening did indeed produce hyperalgesia. When subjects were given the non-specific CCK receptor antagonist proglumide, hyperalgesia was prevented in a dose dependent manner (Benedetti, Amanzio, Casadio, Oliaro and Maggi, 1997) even though proglumide is not specifically considered an analgesic.

CCK is known to be involved in anxiety mechanisms (Benedetti, 2009) and in efforts to better understanding its role in hyperalgesia, a number of experiments were conducted. One study found that an oral administration of placebo and verbal suggestion of hyperalgesia would produce hyperalgesia and hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis. This was assessed by measuring

adrenocorticotropic hormone (ACTH) and cortisol plasma levels (Benedetti, Manzio, Vighetti and Asteggiano, 2006). Subsequently, diazepam, a benzodiazepine, and proglumide were given to different groups of subjects. Results indicated that diazepam prevented nocebo-induced hyperalgesia and HPA activation providing evidence of anxiety mechanisms in nocebo hyperalgesia. Subjects receiving proglumide, on the other hand, experienced a blockade of hyperalgesia but had unaffected levels of ACTH and cortisol. This finding suggests a very specific involvement of CCK in hyperalgesia but not in the anxiety provoking aspect of the nocebo response (Benedetti et al., 2006; Benedetti, 2008; 2009).

CCK has been shown to reverse opioid analgesia by acting on the RVM (Mitchell, Lowe and Fields, 1998; Heinricher, McGaraughty and Tortorici, 2001) and to activate pain facilitating neurons within the RVM (Heinricher and Neubert, 2004). It is worth noting that there is a discrepancy between the hyperalgesia induced by a CCK/anxiety interaction and the analgesia that can be produced in certain situations (Benedetti, 2009). It is suggested that hyperalgesia may be induced when the anxiety experienced is about the impending pain (Sawamoto, Honda, Okada, Hanakawa, Kanda et al., 2000; Koyama, Tanaka and Mikami, 1998; Benedetti et al., 2006) and that analgesia can occur when the stressor shifts attention from the pain being experienced (e.g. battlefield situations, specific goal focused activities) (Flor and Grusser, 1999). However, much more research needs to be conducted to elucidate the discrepancies between anxiety provoked hyperalgesia and stress induced analgesia and the role CCK may play in these situations.

Dopamine

There are two primary DA cell groups that will be discussed, the substantia nigra pars compacta and the ventral tegmental area, each of which has neuronal projections to different areas of the brain (Alexander, Delong and Strick, 1986; de la Fuente-Fernández and Stoessl, 2002). The substantia nigra projects primarily to the dorsal striatum, is known as the nigrostriatal pathway and is primarily involved in motor function (de la Fuente-Fernández and Stoessl, 2002). The ventral tegmental area (VTA) projects to subcortical limbic structures (ventral striatum, amygdala, hippocampus, olfactory tubercle and septal region) known as the mesolimbic pathway. This pathway is primarily involved in emotional responses (Alexander et al., 1986). In addition, there is a projection from lateral regions of the VTA to frontal cortical regions and is known as the mesocortical pathway (de la Fuente-Fernández and Stoessl, 2002).

The effects of DA are mediated by two classes of receptors termed D1 and D2 receptors. The D1 class is made up of the molecularly distinct D_1 and D_5 subtype receptors, and the D2 class is made up of the molecularly distinct D_2, D_3 and D_4 subtype receptors. The D_1 and D_2 receptors *per se* vastly outnumber the D_3, D_4 and D_5 receptors and are densely present in the dorsal and ventral striata. Remarkably, under normal conditions, the behavioral and functional effects of DA require concomitant stimulation of both D_1 and D_2 receptors, a phenomenon called requisite synergism (LaHoste & Marshall, 1992).

It is important to note, however, that basal DAergic tone at D_1 receptors is sufficient to synergize with D_2 receptor stimulation. Thus, in the absence of exogenous drug administration, it is necessary to block one receptor with an antagonist in order to probe the function of the heterotypic receptor (LaHoste, Henry & Marshall, 2000).

The nucleus accumbens (NAc) is the major structure of the ventral striatum and its association with reward mechanisms has been studied intensely (Holt, Graybiel, and Saper, 1997). The NAc is particularly known for its association with the rewarding nature of substances of abuse. Dopamine release in the NAc appears to be related to expectation of reward rather than to the reward itself (de la Fuente-Fernández and Stoessl, 2002). The salient point at this juncture is the association with DA and expectation. Expectation and conditioning (discussed later) are the two primary theoretical positions of placebo responding.

The limbic and prefrontal cortex via DA input can influence opioid release directly in the periaqueductal gray (PAG) (Christie, James, and Beart, 1986). The VTA has projections directly to the PAG (Beitz, 1982) as does the NAc through the hypothalamus (Yu and Han, 1989). In addition, the PAG has projections to limbic structures to include the VTA, NAc, amygdala and limbic frontal areas (Cameron, Khan, Westlund, Cliffer and Willis, 1995). These reciprocal connections indicate a potential influential relationship between DA release and the perception of pain (de la Fuente-Fernández and Stoessl, 2002). This line of research is under investigation. Scott, Stohler, Egnatuk, Wang, Koeppe et al. (2008) in an fMRI study examined this relationship and found that placebo responders (10) had right NAC activation in association with the μ-opioid system (particularly the PAG) explaining up to 30% of the variance in regional μ-opioid

system response to placebo administration. Conversely, nocebo responders (5) indicated an opposite response, experiencing a deactivation of opioid neurotransmission and dopamine transmission. While more research needs to be done to replicate these findings, it does support the hypothesis that dopamine plays a major role in placebo analgesia and its suppression in nocebo hyperalgesia.

Parkinson's disease is primarily a disorder of movement even though cognitive, mood, sensory and sleep disturbances may be observed as well (Benedetti, 2009). It is characterized by three important characteristics, tremor at rest, bradykinesia (slowness of movement)/akinesia (lack of movement initiation) and rigidity (Benedetti, 2009; de la Fuente-Fernández and Stoessl, 2002). These characteristics are produced by loss of dopamine producing cells in the substantia nigra pars compacta that project to the striatum, specifically the caudate and putamen. It has been estimated that it takes approximately an 80% loss of striatal dopamine before symptoms begin to appear (de la Fuente-Fernández and Stoessl). In addition to pain, Parkinson's disease has been associated with a strong placebo effect. Shetty, Friedman, Kieburtz, Marshall and Oakes (1999) reviewed 36 drug studies and found 12 of them reporting a 9 to 59% improvement of motor symptoms after placebo administration. Watts, Freeman, Hauser, Bakay, Ellias et al. (2001) demonstrated a substantial 18 month motor performance improvement after intrastriatal implantation of fetal porcine ventral mesencephalic tissue in both the real *and* sham surgery groups.

Controlled experimental studies have also demonstrated strong placebo responding in Parkinson's patients. Patients with implanted subthalamic nuclei electrodes for stimulation have demonstrated improvement in movement velocity after

the electrodes are switched off but the patients *believe* the electrodes are still on (Benedetti, Pollo, Lopiano, Lanotte, Vigheti et al., 2003; Pollo, Torre, Lopiano, Rizzone, Lanotte et al., 2002). Benedetti et al. (2003) were also able to induce motor worsening by telling patients that the electrodes had been switched off when in fact they had been left on. Providing demonstrable evidence that motor performance can be modified in opposing directions as has been shown in pain. Imaging studies have demonstrated placebo induced dopamine release in both the dorsal (de la Fuente-Fernández, Ruth, Soss, Schulzer, Calne et al., 2001) and ventral (de la Fuente-Fernández, Phillips, Zamburlini, Sossi and Calne, 2002) striatum. De la Fuente-Fernándes (2002) reported that there was no difference in the amount of dopamine release in the ventral striatum in patients that perceived or did not perceive a clinical benefit. They argue that it is the expectation of reward and not the experience of a reward that triggers dopamine release and is the underlying mechanism in placebo responsiveness.

Finally, imaging studies examining placebo effects in depression observed unique ventral striatal and orbital frontal changes in both drug and placebo responders after one week of treatment, which is well before clinical benefit should be seen (Benedetti, 2009). The suggestion is in keeping with the hypothesis of reward expectation (expectation of clinical improvement) leading to dopamine release through activation of the ventral striatum (nucleus accumbens). The evidence is compelling that dopamine release is significantly involved in placebo responsiveness in a number of conditions (pain, Parkinson's disease, depression) and lack of dopamine release is involved in nocebo responses, at least for pain and Parkinson's disease.

Serotonin

Serotonin re-uptake inhibitors (SSRIs) are one of the leading classes of drugs involved in the treatment of depression (Benedetti, 2009). Kirsch and Sapirstein (1998) conducted a large meta-analysis of 19 double-blind clinical trials (2,318) and found that 75% of response to active drug is attributable to placebo effects when compared to natural history groups. It should be noted that the natural history groups were obtained from waitlisted depression patients in psychotherapy studies and not in the drug studies. Kirsch and Sapirstein's conclusion was that natural history accounted for 23.87%, drug effects for 25.16% and placebo effect for the remaining 50.97%. Considering the large response in clinical trials, it is tempting to speculate that serotonin is involved. However, it appears that due to the ethical limitations involved with depression studies, no one has expressly studied the role of serotonin in depression placebo response. Although as noted above, it may be that dopamine is playing the major role here as well.

Furmark et al. (2008) examined genetic variants related to serotonin and its role in placebo responding to social anxiety. It found that only subjects homozygous for the long allele of the 5-HTTLPR (serotonin transporter-linked polymorphic region) or the G variant of the TPH2 (tryptophan hydroxylase-2) gene promoter G-703T exhibited reduced stress related activity in the amygdala during placebo response. Additionally, the TPH2 polymorphism was found to be a significant predictor of clinical placebo response. Clearly, much more research is needed to determine whether serotonin may be involved in placebo responses and to what extent.

Non-Physiological mechanisms of the placebo effect

Expectancy

"Expectancy is the experienced likelihood of an outcome or an expected effect" (Price, Finiss and Benedetti, 2008). Expectancy is believed to be one of the principal components in eliciting a placebo effect (Benedetti, 2008; 2009; Evans, 2004; Finiss, Kaptchuk, Miller and Benedetti, 2010; Hoffman, Harrington & Fields, 2005; Moerman, 2002; Price, Finiss and Benedetti, 2008; Stewart-Williams, 2004; Stewart-Williams and Podd, 2004). It is the expectancy that if one takes a certain drug or receives a certain treatment that one will have a resultant experience (e.g. pain relief, symptom improvement).

Expectancy is thought to be acquired in a number of ways, direct personal experience, verbal instructions (suggestion), observational learning and context factors (Stewart-Williams, 2004). Through these acquisitions we come to "know" lots of things and through this acquisition we derive meaning about the situation (Moerman, 2002).

Some of the things we "know" are that two pills are stronger than one, an injection is more powerful than a pill and surgery is more potent still (Moerman, 2002). Does the research bear this out? In a study of medical students that were given two different colored placebo pills and told one was a sedative and one a stimulant, some of the students received two pills and reported stronger perceived effects than students that took one pill (Blackwell, Bloomfield and Buncher, 1972). One explanation for this observation is that the students taking two pills expected a stronger effect (Moerman, 2002). In an evaluation of 117 studies for ulcer treatment, Moerman (2000) reported similar findings. Additionally, in the treatment of migraines it has been reported that

placebo injections are more effective than oral placebo (de Craen, Tijssen, de Gans and Kleijnen, 2000). The point so far is that among placebo manipulations the difference in response magnitude has presumably been a difference in what participants expected. (Moerman, 2002). It is important to point out that without natural history groups included in a study other placebo like effects could be responsible as well.

Because of the dangers in administering anesthesia, surgery is rarely scrutinized with placebo comparisons (Wall, 1994). Even so, there have been a few cases. In the 1950's it was a common practice to ligate the internal mammary arteries to relieve the painful condition known as angina pectoris (Wall, 1994). Angina is a painful condition that is thought to occur due to inadequate blood supply to muscle in the heart. It was thought that ligation of the internal mammary arteries would force blood to find alternative routes (presumably through routes that were not as clogged) to the heart (Moerman, 1997; 2002; Wall, 1994). The first fifty patients in the United States to undergo the procedure reported improvement rates anywhere from slight to complete (68%) in two and six month follow ups (Moerman, 2002) and the procedure gained popularity. However, pathologists were not finding any of the "new" blood routes, which called the entire procedure into question (Wall, 1994). Two independent teams of surgeons and cardiologists explored the question by conducting double blind trials (Cobb, Thomas, Dillard and Marendo, 1959; Dimond, Kittle and Crockett, 1960). The surgeons performing the procedure were not informed until the moment of surgery whether the patient would receive the real or sham procedure, which involved everything except ligating the internal mammary arteries. Results indicated that after a six month follow up by cardiologists (who were also unaware of which patients received

which procedure) 67% of patients receiving the full surgery reported substantial improvement and 82% of patients receiving the sham surgery reported substantial improvement with the remaining patients, of both groups, reporting slight improvement. In both conditions patients were, on average, able to exercise longer, took fewer nitroglycerine tablets, reported less pain, and a few had improved ECG readings (Wall, 1994).

There are other instances of surgery leading to symptom improvement even though no change had been made. Spangfort (1972) noted 346 patients reporting complete relief of sciatic pain associated with a slipped disc (burning pain down the leg and lower back pain) after a surgery to correct the problem. In all of these cases, however, the surgery was exploratory and no tissue was excised from the disc. Finally, Moseley, Wray, and Kuykendall (1994) and Moseley, O'Malley, Peterson, Menke, Brody et al. (2002) conducted two randomized controlled placebo trials using patients with osteoarthritis of the knee. Placebo patients were put to sleep, draped, injected with a local anesthetic and given three stab wounds to the skin, as would have been done in an arthroscopic debridement. The arthroscopic instruments were inserted and a debridement was simulated in case the patient was aware during the surgical procedure. Results measured at several time points for two years indicated placebo treatment was significantly better then debridement for up to a year and at two years there was no significant difference, although placebo still outperformed debridement. The 2002 study was unique among the surgical studies in that each group had a number of psychological measures evaluated, including anxiety, depression, expectancy, optimism, health satisfaction, somatization, stress and vitality. There were

no differences between groups on any of these measures. In keeping with typical clinical trial procedures, all patients were informed about the possibility of receiving a placebo surgery. One could argue that perhaps the results of the surgical interventions are not generalizable, or something was special about the subject pool, or that no natural history groups were included in the comparison. All valid points. Nevertheless, there is no need to generalize these specific surgeries for our purposes and though no natural history groups were included, each of these conditions is not known for spontaneous improvement (Moerman, 2002).

Taken together, these studies provide persuasive evidence that surgical interventions can have powerful long lasting placebo effects. It is important to point out the long lasting duration of the placebo effect in these studies. Placebo effects, more often than not, are believed to be transient occurrences (Wall, 1994).

Manipulation of expectancy

The studies reported so far have presumed that expectancy has led to clinical improvement but is that actually the case? It was mentioned earlier that informed consent stated a placebo surgery may take place. This wording, it turns out, matters. Pollo, Amanzio, Arsianian, Casadio, Maggi et al. (2001) investigated whether there was any difference in the double-blind procedure and a deceptive paradigm. They followed post-operative thoracotomy patients treated with buprenorphine (an opioid analgesic). For three consecutive days, three groups of patients received buprenorphine on request via basal infusions of saline solution that had been started shortly after surgery. In group one, patients were told nothing about the saline infusion, representing the natural history group. In group two, standard double-blind clinical procedures were followed and

they were told the basal infusion may be a painkiller or a placebo Group three was told specifically that the basal infusion was a pain killer. The placebo effect was measured by how many times the patients asked for buprenorphine over the three days. Compared to the natural history group a 20.8% decrease was seen in the double-blind group and a significantly different 33.8% decrease was seen in the deceptive administration group.

Awareness of treatment has also been demonstrated to be an important component in analgesia. Analysis of five widely administered postoperative analgesics (morphine, buprenorphine, tramadol, ketorolac, metamizole) using an open vs. hidden paradigm have been conducted (Amanzio, Pollo, Maggi and Benedetti, 2001; Benedetti, Maggi, Lopiano, Lanotte and Rainero, 2003; Colloca, Lopiano, Lanotte and Benedetti, 2004). Doctors would carry out an open administration (bedside) for each of these drugs telling them the injection was a powerful analgesic and the pain would subside after a few minutes. Contrasting this, an automatic infusion pump administering the same dose of each of the medications was carried out when no doctor or nurse was in the room. The analyses found the dose required to achieve 50% pain reduction (AD_{50}) was significantly increased when the administration was hidden for each of the five drugs. In short, it requires more analgesic to receive the same benefit if you do not know about it. This result was replicated by Amanzio et al. (2001) experimentally using ischemic arm pain and the non-opioid ketorolac.

If expectancy assists in relieving pain, can it increase pain? Yes. An experimental study by Dworkin, Chen, LeResche and Clark (1983) changed the direction of nitrous oxide from that of an analgesic to a hyperalgesic using verbal suggestion alone.

Benedetti, Amanzio, Casadio, Oliaro and Maggi (1997) and Benedetti, Amanzio, Vighetti and Aseggiano (2006) demonstrated hyperalgesia in both clinical and experimental settings. In the clinical setting a straight forward increase of pain was observed when a placebo was administered after suggestion of hyperalgesia. In the experimental setting, hyperalgesia was observed using verbal suggestion of pain and ischemic arm pain. Finally, open/hidden paradigms have been explored in clinical settings as well. In postoperative patients that had been receiving morphine for 48 hours some patients were told that their morphine had been stopped (open condition) and some patients were told nothing about their morphine being discontinued (hidden condition). At 10 hours after morphine interruption, a significantly larger number of patients in the open condition requested more morphine than the hidden condition (Benedetti et al. 2003; Colloca et al. 2004). Thus, expectations in clinical and experimental settings can lead to both pain relief and hyperalgesia. Next, the role of conditioning in pain will be examined.

Conditioning

Classical conditioning comprises the second major theoretical approach to the placebo effect. In general, applying conditioning to the placebo effect requires the drug or active ingredient to be the unconditioned stimulus (US) and the unlearned response to the active ingredient to be the unconditioned response (UR). In the course of any number of paradigms, the US would be paired with a neutral stimulus such as pill casings, syringes or even to objects, places, people and the procedures themselves. Through repeated associations with the US the neutral stimuli become conditioned stimuli (CS) capable of producing an effect similar to that of the active ingredient, which

would be considered a conditioned response (CR). Thus, in a conditioning framework the placebo would be considered the CS and the placebo effect the CR (Stewart-Williams and Podd, 2004). Much of the support for the classical conditioning paradigm comes from research on nonhuman animals and has been demonstrated with a variety of drugs and systems. Herrnstien (1962) demonstrated that rats conditioned with injections of amphetamines when injected with saline exhibited behavior similar to that seen by amphetamine injection. Ader and Cohen (1975) paired novel saccharine flavored liquid with cyclophosphamide, an immunosuppressant. After several pairings, the saccharine solution (CS, placebo) would elicit immunosuppression (CR, placebo effect) (Stewart-Williams, 2004).

What about human conditioning? Voudouris, Peck and Coleman (1989;1990) conditioned subjects over a period of three days. On day 1, they were exposed to iontophoretic stimulation, an adjustable electric current generator, at a tolerance level. Meaning the current was increased until the subjects said they could no longer tolerate the discomfort. On day 2, participants received a placebo cream and were told that it was an analgesic. In addition, they were told that the iontophoretic stimulation intensity was the same as the day before; when in reality the voltage had been surreptitiously turned down or turned up. On the third day, voltage was returned to day one levels. Subjects that had been exposed to lessened voltage reported much less pain, while subjects exposed to more voltage experienced heightened pain.

Benedetti, Pollo, Lopiano, Lanotte, Vighetti et al. (2003) conditioned two groups of subjects pharmacologically with ketorolac (a non-opioid analgesic) for two days. Two other groups received no conditioning and a natural history group was included. On day

3 the conditioned groups were given a saline injection and were told it was a powerful analgesic or were told it was hyperalgesic. The unconditioned groups were simply given a saline injection and told that it was a powerful analgesic or hyperalgesic. Results indicated that for the conditioned analgesia group, they experienced a dramatic reduction in pain. The unconditioned analgesia group experienced a reduction in pain compared to the natural history group, but was nowhere near the magnitude of the conditioned group. The second conditioned group when administered saline and the suggestion of pain worsening experienced a complete reversal of all conditioned analgesia. A number of other studies have observed similar conditioning/abolishment results (Montgomery and Kirsch, 1997; Price, Milling, Kirsch, Duff, Montgomery et al. 1999). In short, analgesia and hyperalgesia can be conditioned in humans. When additional verbal expectancy is combined to the conditioning process, analgesia can be greatly increased or conditioning can be completely abolished. Thus, indicating that the placebo effect is not only observed in conditioning paradigms but that its effect can be mediated or moderated by expectancy as well.

Expectancy and Dopamine as a Unifying Construct

The placebo effect, as mentioned earlier, has been observed in conditions ranging from pain and Parkinson's disease, to depression and immune system modulation. Each of these conditions is served by disparate systems of the body and brain yet all have been implicated in placebo reactivity. Benedetti (2009) argues that there is not one placebo effect but many placebo effects, a position the author agrees with, nonetheless, a question remains of whether there is a unifying construct that could explain these disparate phenomena without invoking separate mechanisms. Is there a

mechanism that could be responsible for engaging the far flung systems mentioned thus far? De la Fuente-Fernández and Stoessl (2002) argued that such a mechanism could be found in dopamine release within the ventral striatum, specifically the NAC. This structure receives major DAergic input from the VTA.

Subsequent to the proposal by de la Fuente-Fernández a number of studies have investigated this question. Scott, Stohler, Egnatuk, Wang, Koeppe et al. (2008), as mentioned above, observed a right NAC response with placebo responders and a deactivation of DA observed with nocebo responders. This study, however, constrained observation to areas of the brain consistent with μ opioid receptors and $D_{2,}$ D_3 receptors. The results, however, support a relationship with DA ($D_{2,}$ D_3) release and placebo/nocebo response. Lidstone, Schulzer, Dinelle, Mak, Sossi, Ruth et al. (2010) explored whether the degree of expectation would modulate DA release. In a group of 35 Parkinson's patients experimenters explained that there was a 25%, 50%, 75% or 100% chance of receiving an active medication or placebo (in fact all participants received placebo) and compared that to [^{11}C]raclopride (a $D_{2,}$ D_3 radio ligand) uptake in a PET scan. Results indicated the larger the probability of receiving an active drug the stronger DA output. However, this ceased to be true when there was a 100% probability of receiving an "active" drug. This is consistent with conditioned studies in which DA activation is associated with anticipation of a likely event vs. a certain event (O'Doherty, Dayan, Schultz, Deichmann, Friston et al., 2004). In addition, the Lidstone et al. study was unable to disentangle whether there was an effect of experience with levodopa as all of the subjects were Parkinson's patients and had experience with that drug and therefore could form an anticipation of likely outcome. Lastly, Scott, Stohler, Egnatuk,

Wang, Koeppe et al. (2007) evaluated whether anticipation of monetary reward would be related to placebo outcomes in an fMRI study. It was observed that subjects with greater right NAC activation in anticipation of the largest monetary reward had the largest placebo responses as well as those that had anticipated the greatest benefit from the placebo analgesic.

Taken together, these studies provide strong support for the hypothesis that DA is involved in the placebo effect, the individual variation that is often observed and to a certain extent the magnitude of the response in relationship to anticipation of benefit. However, these studies focused on DA D_2 and D_3 receptor subtypes as these two DA receptor subtypes are strongly involved in the acquisition of expectancy and its relationship to nociception (Magnusson and Fisher, 2000). These studies did not look at the role of DA and conditioning in the formation of the placebo response. Nor, did they look at the duration of the placebo response and to this author's knowledge no study to date has looked at these unique variables. The D_1 and D_2 DA receptor subtypes in the NAC have been implicated in the acquisition of conditioned reward (Koch, Schmid & Schnitzler, 2000) and it is known that DA neurons are "able to use contextual information in addition to information from explicitly conditioned stimuli" (Tobler, Fiorillo & Schultz, 2005), thus linking the role DA has to the two major theoretical constructs, expectancy and conditioning, and potentially providing another piece for the placebo puzzle.

Purpose and hypotheses

The studies investigating the role of DA and the placebo effect thus far have restricted their observations to DA receptor subtypes D_2 & D_3 and have used primarily

imaging studies (radio ligand binding) and probabilities or monetary gain to manipulate expectancies and presumably, by extension, DA levels in the NAC and other areas. These studies provide support for the hypothesis that DA is involved in the acquisition and perhaps the magnitude of the placebo effect. However, all of these studies have been conducted exclusively on humans and as a result none of them have manipulated DA levels directly. In addition, none of these studies examined DA and conditioning or whether DA receptor subtype D_1 may have a role in the acquisition and magnitude of the placebo effect.

It is proposed by this author that a basic animal model utilizing a conditioning paradigm and neuropharmacological manipulation of DA levels will provide information about the placebo effect, its acquisition, magnitude and duration that human studies have so far been able to address. Animal models allow for the direct manipulation of DA levels in addition to demonstrating robust placebo effects (Bryant, Roberts, Culbertson, Le, Evans et al., 2009; Guo, Lang & Luo, 2010; Nolan, Price, Caudle, Murphy & Keubert, 2012). Based on the literature reviewed here the author posits that an increase of DA will have a subsequent increase in the magnitude and by extension the duration of the placebo effect as compared to the placebo effect observed in non-DA manipulated animals and natural history controls. In addition, it is asserted that the blockade of DA, specifically from D1 and D2 receptor subtypes will circumvent the acquisition and/or magnitude of the placebo effect (respectively) in comparison to controls.

Methods

Animals

Male CD-1 outbred mice weighing 35g at the start of training were used as subjects in all experiments. A total of 80 mice were used. All animals were housed in groups of 4 with free access to food and water. Animals were on a 12 hour light cycle with all experiments conducted in the first 6 hours of the light cycle.

Materials and Apparatus

Drugs

The following drugs were administered either singly or in combination:

1) morphine sulfate(Paddock Laboratories; Minneapolis, MN) 5 mg/kg body weight; this drug served as the primary vehicle for conditioning a placebo response

2) cocaine HCL (Sigma; St. Louis, MO),10 mg/kg

3) *SCH23390 HCL* (Sigma; St. Louis, MO), 0.1 mg/kg; DA D1 antagonist

4) *Eticlopride HCL* (Sigma; St. Louis, MO), 0.1 mg/kg; DA D2 antagonist

All drugs were dissolved in a saline solution of 0.9% and prepared just prior to administration. In addition, drugs were dissolved so that an injection of 10 ml/kg intraperitoneal (i.p.) delivered the appropriate dosage.

Apparatus

Two Plexiglas® containers with four chambers (eight total) measuring 10 cm^2 x 10 cm in height with a single clear Plexiglas® lid for every two chambers were utilized as cue environments. These chambers also included opaque interior Plexiglas® walls on three walls and white plastic flooring to further distinguish the cue environment from the home cage environment. Two measures of analgesia were employed in this study.

The hotplate apparatus was a commercially available (IITC model 35D) analgesic hotplate with an electronically controlled metal plate and a Plexiglas® enclosure (11cm^2 x 17cm) that restricted the animals to the surface of the hotplate. The tail flick latency apparatus is a commercially available (IITC, Series 8, model 336T) instrument that focuses an adjustable radiant heat from a halogen light source to the dorsal surface of the tail. Tail flick latency is recorded by an automatic sensor situated underneath the light source.

Hotplate test

Animals were placed on the hotplate which maintained a set temperature of 55 ± 0.5 °C, (Guo, Wang, & Luo, 2009) to measure supra-spinal nociception. Time from placement on the hotplate floor to the occurrence of a nociceptive behavior was recorded to the hundredth of a second with a digital stopwatch. A nociceptive behavior was defined as licking of either hind paw or a vertical leap from the surface of the hotplate. A cutoff time of 60 seconds was established to prevent tissue damage (Guo, Wang, & Luo, 2009).

Tail Flick test

In order to measure spinally mediated nociceptive responses, animals were loosely restrained in a towel with the tail placed under a halogen light emitting a 1 cm^2 source focused approximately two to three centimeters from the distal end. Time from placement of the tail to a spinally mediated nocifensive response was automatically recorded. A pre-set maximum withdrawal latency of 12 seconds was instituted to prevent tissue damage (Lee et al., 2011). Every effort was made in the experimental design to limit exposure of nociceptive stimuli, so that the duration of the stimulus is

limited by the animal. One could argue that this assures that the stimulus is minimally painful.

Procedure

Eighty mice were randomly assigned to ten groups of eight animals, each group was distinguished by a drug or drug combination (see table 1). In addition, due to scheduling difficulties, only two groups could be tested in a single day, one group at 10 a.m. and one group at 2 p.m. In order to compensate for any potential effect that time of day might have on testing, each group was split into a counterbalanced design of two groups of four so that one half of the group would be tested in the morning and the other half in the afternoon.

The experiment took place over five days for each group. The first two days were drug conditioning days. Conditioning, in this context, refers to the timeframe that an animal is exposed to an active drug and either acquires or fails to acquire a placebo response when an inert substance is subsequently administered. The last three days (days three, four, and five) were placebo test days. On these days saline, which is normally ineffectual in relieving pain, was injected in lieu of the active drug. Because all drugs used are soluble in saline, combinations of drugs were dissolved in the same injectate during active drug days and a single injection was given.

Every day, each animal was removed from its home cage and placed on the hotplate two times, separated by five minutes, for baseline supra-spinal assessment. Following the second hotplate baseline test the animal was loosely wrapped in a towel and a baseline tail flick test was conducted. Subsequent to the tail flick baseline each animal received an IP injection of drug, drug combination, or saline, depending on group

membership and test day. Following IP administration, each animal was placed in an individual cue chamber for 15 minutes to maximize expectation and drug effect. Tests for drug effect or placebo effect were given on the hot plate followed by the tail flick apparatus at 15, 30, 45, and 60 minutes post injection. Following each assessment period the subject was placed back in its cue chamber until the next assessment.

Finally, because day three was the first saline (placebo) administration for all drug groups supra-spinal hotplate assessments were video recorded. The video was recorded with a digital camcorder (Canon VIXIA HF R40 HD) from a distance of 2 feet from the hotplate Plexiglas container. A mirror was placed behind the container so that the subject could be viewed from multiple angles. Prior to each animal being placed in the hotplate container an index card with a number indicating one through eight, for later coding identification purposes, was presented in front of the camera. A group of eight animals constituted one video session and each session's recording was labeled with the date, time and group number and was given to an independent rater who was blind to all group conditions and trained in what constituted a nociceptive response.

Groups 1 and 2 provided a standard for the development of the placebo effect. Group 1 received saline only for all five days and is considered the natural history control group against which all other groups were compared. Group 2 received morphine only and was considered the baseline placebo group against which the magnitude of any remaining group's placebo effect could be assessed. Testing on days 4-5 was used to determine the duration of the placebo effect. The true test of the placebo effect, however, is the comparison of behavior of Group 1 with that of any drug group on Day 3.

Groups 3-10 were designed to test for the effects of dopamine (DA) manipulations on the development of the placebo effect. In Groups 4, 6, 8, and 10, each mouse received morphine concomitantly with a drug that alters the interaction of DA with its D1 class and D2 class receptors: In addition to morphine, Group 4 received a DA agonist, cocaine, which releases DA from nerve terminals allowing postsynaptic stimulation of both D1 class and D2 class receptors, thereby increasing the basal tone of DA activity; Group 6 received morphine plus the selective D1 class DA antagonist SCH23390 (thereby blocking the D1 pathway of basal DA activity); Group 8 received morphine plus selective D2 class antagonist eticlopride, thus blocking the D2 pathway of basal activity. Group 10 received a combination of SCH23390, eticlopride and morphine, thereby blocking D1class and D2 class basal activity.

The development of the expectation of a beneficial effect was predicated on the delivery of morphine to provide that benefit. To control for the possibility the DA modulations may alter pain sensitivity by means other than the placebo effect, four additional groups (Groups 3, 5, 7 and 9) were treated identically as Groups 4, 6, 8 and 10 respectively, except that no morphine was given.

Table 1, group membership by active drug administration

	Group Membership									
	1	2	3	4	5	6	7	8	9	10
Saline	X									
Morphine		X		X		X		X		X
Cocaine			X	X						
SCH23390					X	X			X	X
Eticlopride							X	X	X	X

Statistical Analysis

Data consisting of latency to exhibit a nociceptive behavior (hind-paw lick or jump, and tail flick) during testing sessions on all days were analyzed for statistically significant differences by a group by time two-way repeated measures analysis of Variance (ANOVA). Drug Treatment (see Groups in Table 1) was a 10-level between groups factor where time was a 4-level repeated measures factor representing different time-points relative to the injection on a given test day (see above); ANOVAs for each day and for each measure (hotplate and tail flick) were calculated. Results were considered significant at $p < 0.05$ and planned follow up comparisons were made subject to Tukey's post hoc tests. Additionally, day 3 recorded responses were assessed for inter rater reliability using intra-class correlation coefficient (ICC) on all six measurements (both baselines and all four drug effect times).

It should be noted that the above statistical analyses differs from the original planned analysis in the following way. Original planned analysis included averaging both baseline scores to compute percent maximum possible effect (%MPE), [(latency – baseline) / (cutoff – baseline)] x 100. Percent MPE scores were then to be used in the same fashion as above. However, unreliable baseline data and highly positively skewed time points (that would necessitate log10 transformations) resulted in inflated %MPE scores. In order to conservatively avoid the risk of committing a type I error %MPE and baseline scores were consequently dropped from the analysis.

Results

To assess reliability of measurement an Intra-class Correlation Coefficient (ICC) was conducted on six, day three, measurements between the experimenter and a blind rater. A median ICC value of .970 was observed among the six variables. Table 2 reports each variable's ICC, mean and standard Deviation.

Table 2, Intra-Class Correlation

Day 3 Variable	ICC	Rater	Mean	SD
Base Line 1	.959	Experimenter	18.80	10.16
		Blind Rater	18.20	10.46
Base Line 2	.995	Experimenter	14.41	8.69
		Blind Rater	14.29	8.81
Time 1	.988	Experimenter	16.77	12.16
		Blind Rater	16.02	12.23
Time 2	.981	Experimenter	16.17	9.59
		Blind Rater	15.53	9.75
Time 3	.933	Experimenter	15.20	8.28
		Blind Rater	14.11	8.11
Time 4	.931	Experimenter	17.21	12.33
		Blind Rater	16.32	12.93

During the course of the experiment three subjects expired. Two subjects (34 & 36) from group 5 on days 4 and 5, respectively and one subject from group 6 (46) on day 4. In order to deal with the subsequent missing data it was decided to substitute the mean of the subjects assigned group for each variable on days 4 & 5 rather than removing the cases altogether.

An exploration of the data revealed that hotplate (HP) baselines one and two were highly variable from and among each other. It was observed that for days 3 and 5 base line one was significantly higher than baseline two, $t(49) = 4.51$, $p < .001$ and $t(49) = 4.77$, $p < .001$, respectively. Additionally, the data indicated a significant difference between Day 1 and all other baseline days, $F(9, 70) = 13.26$, $p < .001$. Due to the variability observed in the HP baseline data and several baseline scores in both HP and tail flick tests reaching cut-off, it was determined that %MPE calculations would be unreliable and was subsequently not conducted. In addition, all baseline data was excluded from examination in subsequent repeated measures ANOVA analyses.

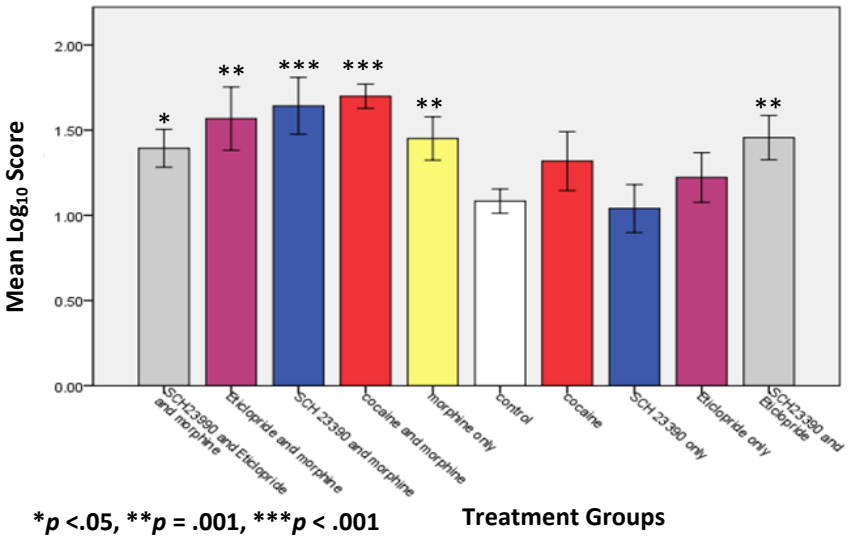
Further exploration of the data revealed that several variables were substantially positively skewed (skewness value greater than 2). A LOG10 transformation as recommended by Tabachnick and Fidell (2006) was performed on each variable to be included in further analysis.

Drug conditioning (supra-spinal), days 1 & 2

Examining the effect of Day 1 drug administration on supra-spinal latency response, repeated measures ANOVA indicated a between subjects effect, F (9, 70) = 14.79; p = 0.000, η_p^2 .66, no within subjects effect of time F (3, 210) = 1.43; p = .237, η_p^2 .02, and an interaction of drug and time F (27, 210) = 1.67, p = .024, η_p^2 .18. Tukey post hoc comparisons indicated significantly greater latency in the morphine group (p = .001), cocaine & morphine group (p < .001), SCH23390 and morphine group (p <.001), eticlopride & morphine group (p < .001) SCH23390, eticlopride, & morphine group (p = .011), and the SCH23390 & eticlopride group (p = .001) contrasted to the saline only control group. There were no significantly different latency times for the cocaine group (p = .136), SCH23390 group (p = 1.00), or eticlopride group (p = .799), contrasted to the saline only control group, figure 1. Additionally, Tukey post hoc comparisons identified significant latency differences from the SCH23390, eticlopride and morphine group to the cocaine & morphine group (p = .014), and to the SCH23390 only group (p = .002), between the morphine only group and the SCH23390 only group (p < .001), between the cocaine only group and the cocaine & morphine group (p = .001), the SCH23390 only group (p = .036), and the SCH23390 & morphine group (p = .007), between the cocaine & morphine group and the Eticlopride only group (p < .001), and the SCH23390 only group (p < .001), and finally between the SCH23390 only group and SCH23390 &

eticlopride only group (p < .001). Due to complex higher order interactions means and 95% confidence intervals are represented in Figure 1 as averaged time point data for clarity of presentation.

Figure 1, Day 1 Hotplate mean and 95% Confidence Intervals

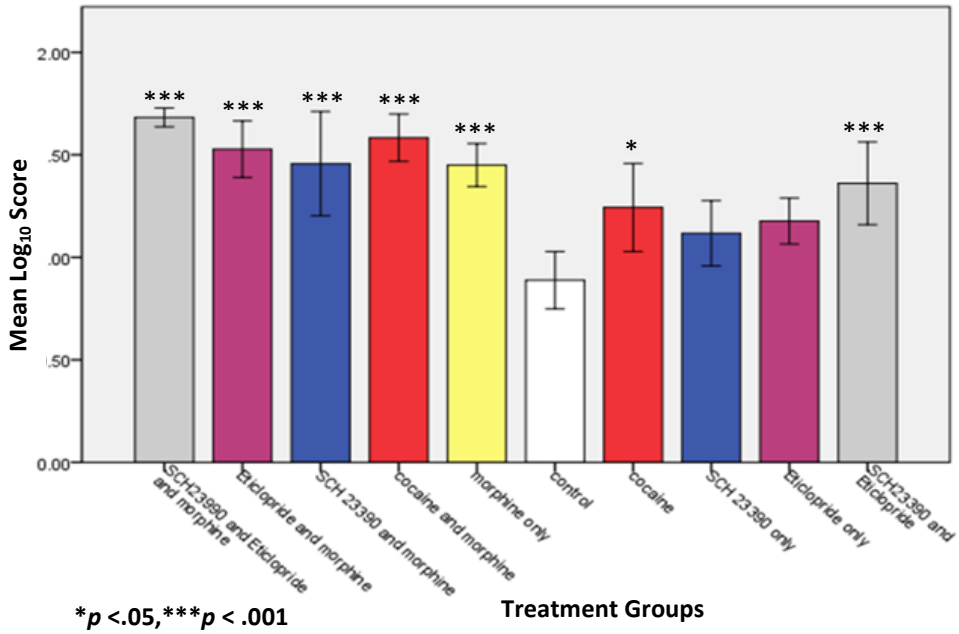


***p* <.05, ****p* = .001, *****p* < .001

Day 2 supra-spinal latency response, repeated measures ANOVA indicated a between subjects effect, $F(9, 70) = 12.85$, $p < .001$, η_p^2 .62, no within subjects effect of time $F(3, 210) = 1.40$, $p = .247$, η_p^2 .02, and no interaction of drug and time $F(27, 210) = 1.09$, $p = .024$, η_p^2 .12. Tukey post hoc comparisons indicated significantly greater latency in the morphine group ($p < .001$), cocaine only group ($p = .014$), cocaine & morphine group ($p < .001$), SCH23390 and morphine group ($p < .001$), eticlopride & morphine group ($p < .001$) SCH23390, eticlopride, & morphine group ($p = .011$), and the SCH23390 & eticlopride group ($p < .001$) contrasted to the saline only control group. There were no significantly different latency times for the SCH23390 only group ($p = .344$), or eticlopride only group ($p = .094$), contrasted to the saline only control group. Additionally, Tukey post hoc comparisons identified significant latency differences from the SCH23390, eticlopride & morphine group to the cocaine only group ($p = .001$), to the

SCH23390 only group (p < .001), the eticlopride only group (p < .001) and the SCH23390 & eticlopride only group (p = .037); between the morphine only group and the SCH23390 only group (p = .027), between the cocaine only group and the cocaine & morphine group (p = .022); between the cocaine & morphine group and the eticlopride only group (p = .002), and the SCH23390 only group (p < .001), and finally between the SCH23390 only group and SCH23390 & morphine group (p = .022). Figure 2 represents the means and confidence intervals for day two drug administration.

Figure 2, Day 2 Hotplate mean and 95% Confidence Intervals



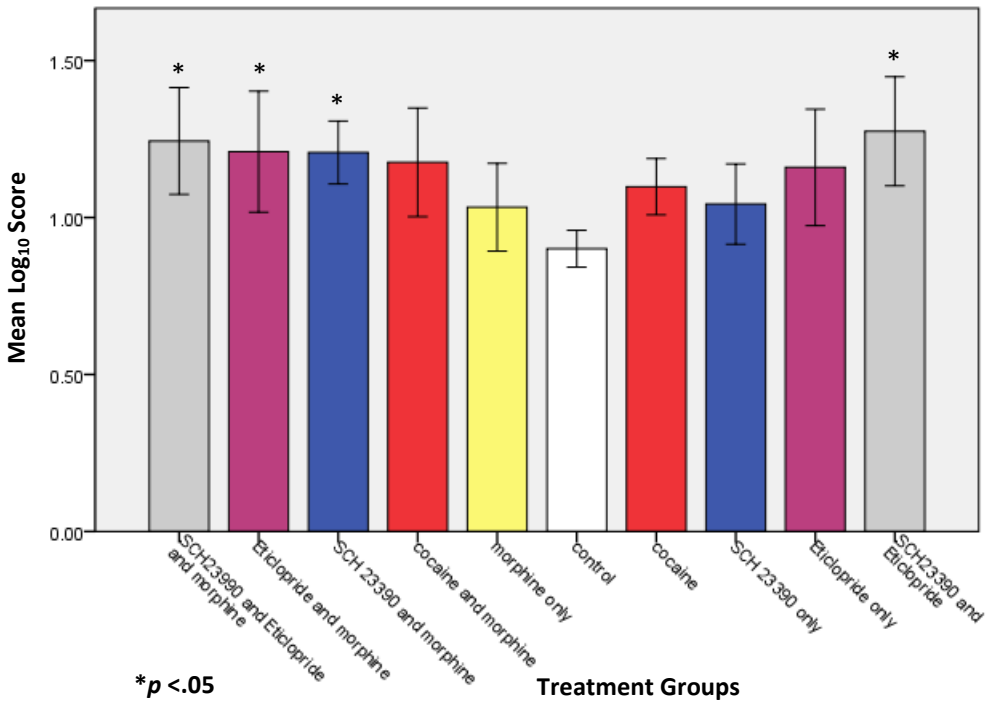
p* <.05,*p* < .001**

Saline placebo (supra-spinal), days 3, 4, & 5

Day 3 Evaluation of the hypothesis that increased dopamine during conditioning would enhance the magnitude of the placebo effect and that removal of dopamine during conditioning with D1 class antagonist (SCH23390), D2 Class antagonist (eticlopride), or combination would abolish the development of the placebo effect was conducted using repeated measure ANOVA. Repeated measures ANOVA indicated a

between subjects effect, $F(9, 70) = 3.40$, $p = .002$, η_p^2 .30, no within subjects time effect $F(3, 210) = .20$, $p = .899$, $\eta_p^2 = .003$, and no interaction of time and group, $F(27, 210) = 1.22$, $p = .135$, η_p^2.15. Tukey post hoc comparisons indicated significantly greater latency between SCH23390 & eticlopride & morphine ($p = .008$), SCH23390 & morphine ($p = .029$), eticlopride and morphine ($p = .027$), and SCH23390 & eticlopride ($p = .003$) groups compared to the saline only control group. No significant latency was observed between the remaining groups, though the cocaine & morphine group trended toward significance ($p = .075$), and the saline only group. Figure 5 illustrates time averaged data by group.

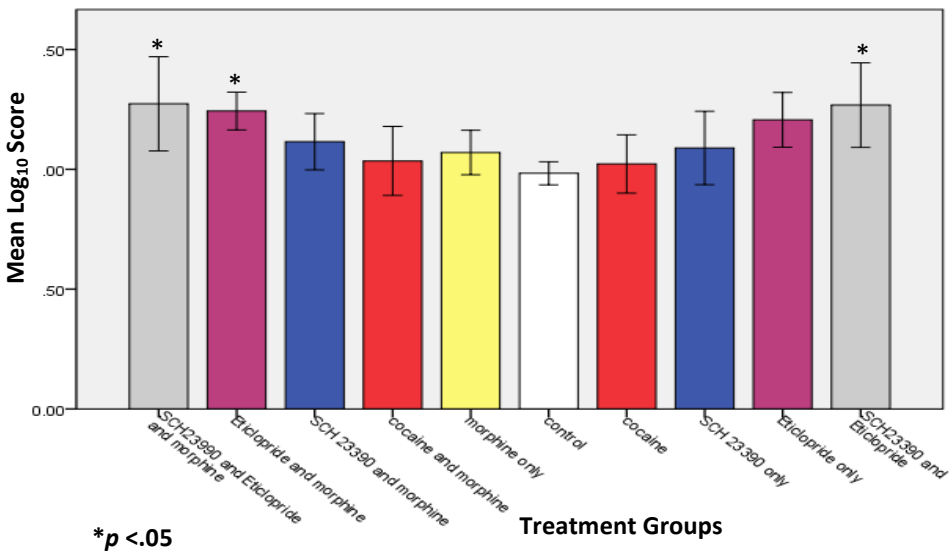
Figure 3, Day 3 Hotplate placebo mean and 95% confidence intervals



Day 4 continued evaluation of the hypothesis that increased dopamine (cocaine) during conditioning would extend the duration of the placebo effect and that removal of dopamine during conditioning with D1 class antagonist (SCH23390), D2 Class

antagonist (eticlopride), or combination would abolish the development of the placebo effect was conducted using repeated measure ANOVA. A between subjects effect $F(9, 70) = 3.81$, $p = .001$, η_p^2 .33, no within subjects time effect $F(3, 210) = 1.014$, $p = .39$, $\eta_p^2 = .01$, and an interaction of time and group, $F(27, 210) = 1.80$, $p = .012$, η_p^2.19 was observed. Tukey post hoc comparisons indicated significantly greater latency between SCH23390, eticlopride & morphine ($p = .015$), eticlopride & morphine ($p = .045$) and SCH23390 & eticlopride only ($p = .018$) compared to the saline only control group. No significant latency was observed among or between the remaining groups. Figure 4 illustrates time averaged data by group.

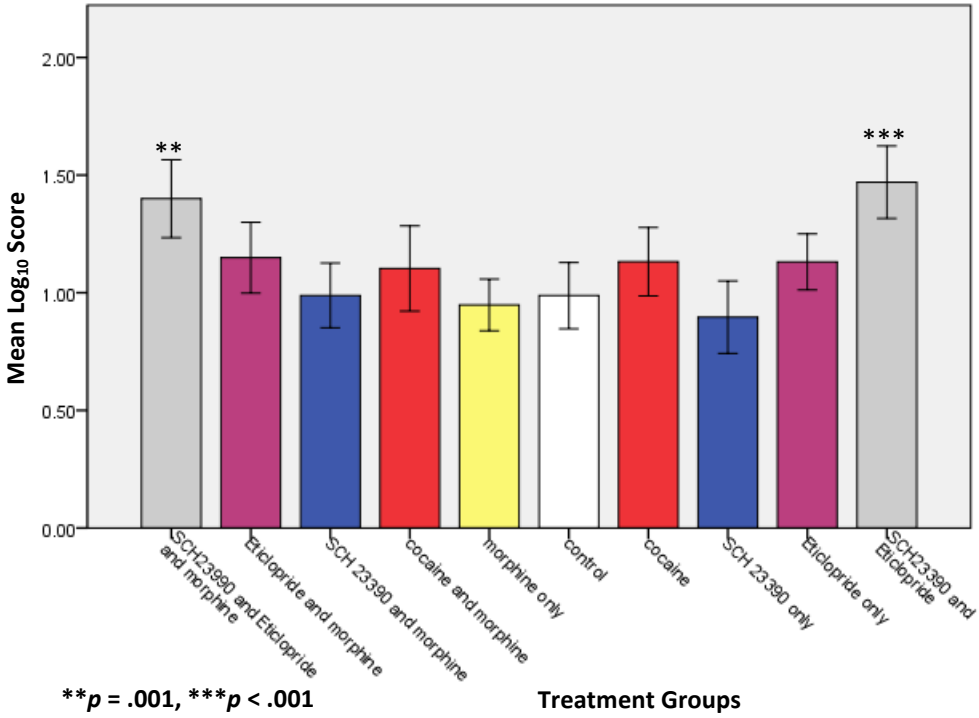
Figure 4, Day 4, Hotplate placebo mean and 95% confidence intervals



Day 5 evaluation of the extinction rate of the placebo was conducted. Repeated measures ANOVA indicated a significant between subjects effect $F(9, 70) = 9.09$, $p <$ .001, η_p^2 .539, no within subjects time effect $F(3, 210) = 2.57$, $p = .056$, $\eta_p^2 = .035$, and an interaction of time and group, $F(27, 210) = 1.88$, $p = .008$, η_p^2.195. Tukey post hoc comparisons indicated significantly greater latency between SCH23390, eticlopride &

morphine (p = .001), SCH23390 & eticlopride (p < .001) and the saline only control group. Additionally, Tukey post hoc comparisons identified significant latency differences between the SCH23390, eticlopride & morphine group and the morphine only group (p < .001), the cocaine & morphine group (p = .038), the SCH23390 only group (p < .001), and to the SCH23390 & morphine group (p = .001).

Figure 5, Day 5 Hotplate placebo mean and 95% confidence intervals

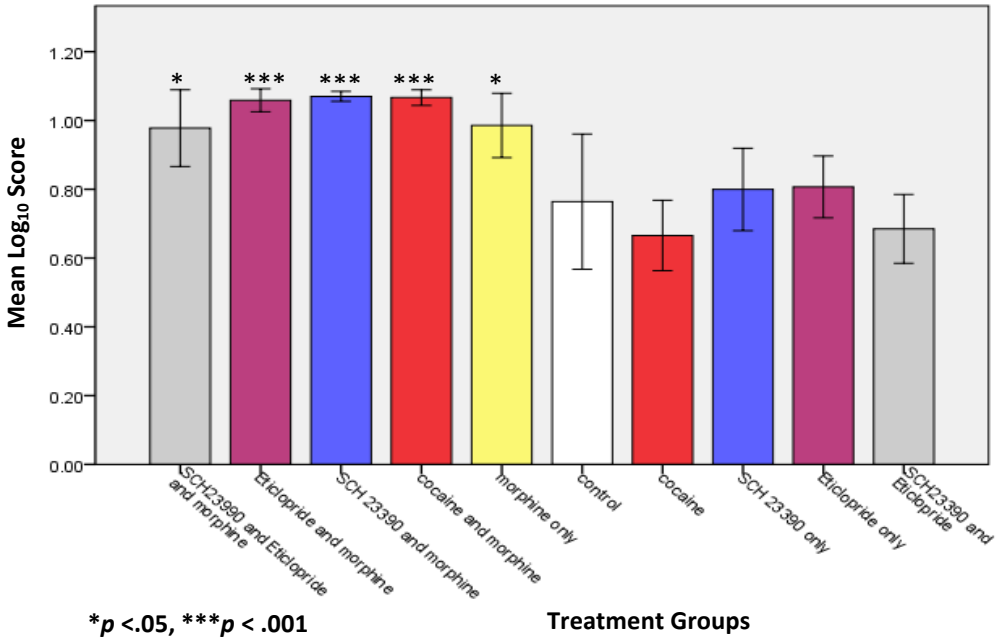


Finally, group differences were observed between the SCH23390 & eticlopride only group and the morphine only group (p < .001), the cocaine only group (p = .009), the cocaine & morphine group (p = .003) the SCH23390 only group (p < .001), the eticlopride only group (p = .009) and lastly the eticlopride & morphine group (p = .017). Due to complex higher order interactions means and 95% confidence intervals are presented in Figure 5 as averaged time point data for clarity of presentation.

Drug conditioning (spinal), days 1 & 2

Examining the effect of Day 1 drug administration on spinally mediated tail flick latency (TFL), repeated measures ANOVA indicated a between subjects drug effect, $F(9, 70) = 13.80$, $p < .001$, η_p^2 .640, no within subjects time effect $F(3, 210) = .95$, $p =$ .418, $\eta_p^2 = .013$, and no interaction of time and group, $F(27, 210) = 1.37$, $p = .115$, η_p^2 .150. Tukey post hoc comparisons indicated significantly greater latency between morphine containing groups (morphine only ($p = .018$), cocaine & morphine ($p < .001$), SCH23390 & morphine ($p < .001$), eticlopride & morphine ($p < .001$), SCH23390,

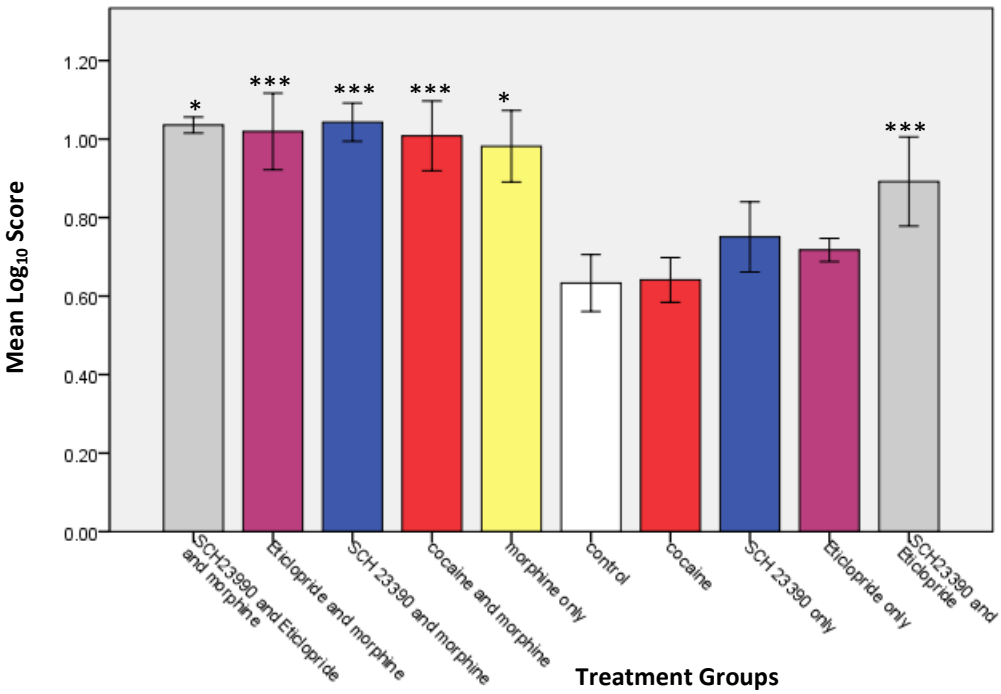
Figure 6, Day 1 Tail Flick Latency mean and 95% confidence intervals



eticlopride & morphine ($p = .02$) and saline only control. No difference was observed between active drug control groups (cocaine only, $p = .84$; SCH23390 only, $p = 1.0$; eticlopride only, $p = .99$; and SCH23390 & eticlopride, $p = .95$) and the saline only control group. Figure 6 illustrates averaged group means.

Day 2 drug administration on spinally mediated TFL, repeated measures ANOVA indicated a between subjects drug effect, $F(9, 70) = 27.17$, $p < .001$, η_p^2 .777, no within subjects time effect $F(3, 210) = .99$, $p = .398$, $\eta_p^2 = .01$, and significant interaction of time and group, $F(27, 210) = 1.94$, $p = .115$, $\eta_p^2 = .200$. Tukey post hoc comparisons indicated significantly greater latency between morphine only ($p = .018$), cocaine & morphine ($p < .001$), SCH23390 & morphine ($p < .001$), eticlopride & morphine ($p < .001$), SCH23390, eticlopride & morphine ($p = .027$), SCH23390 & Eticlopride ($p < .001$) and saline only control. No difference was observed between the remaining active drug control groups (cocaine only, $p = 1.00$; SCH23390 only, $p = .256$; and eticlopride only, $p = .710$) and the saline only control group. Additionally, Tukey post hoc comparisons identified significant latency differences from the SCH23390, eticlopride & morphine group to the cocaine only group ($p < .001$), to the SCH23390 only group ($p < .001$), and the Eticlopride only group ($p < .001$); between the morphine only group and the cocaine only group ($p < .001$), SCH23390 only group ($p < .001$), and the eticlopride only group ($p < .001$); between the cocaine only group and the cocaine & morphine group ($p < .001$) the eticlopride only group ($p < .001$) and the SCH23390 & eticlopride group ($p < .001$); between the cocaine & morphine group and the SCH23390 only group ($p < .001$), and the eticlopride only group ($p = .002$); and finally between the SCH23390 only group and SCH23390 & morphine group ($p < .001$). Due to complex higher order interactions means and 95% confidence intervals are presented in Figure 7 as averaged time point data for clarity of presentation.

Figure 7, Day 2 Tail Flick Latency mean and 95% confidence intervals



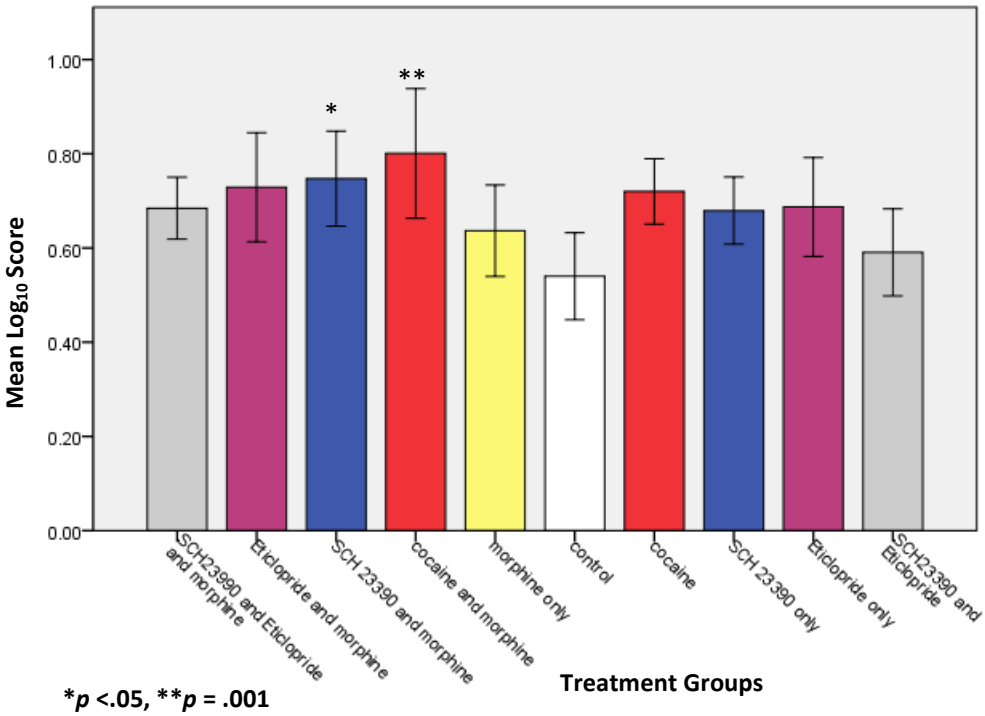
***p* <.05, ****p* = .001, *****p* < .001

Saline placebo (spinal) days 3, 4, & 5

Evaluation of the hypothesis that increased dopamine during conditioning would enhance the magnitude of the placebo effect and that removal of dopamine during conditioning with D1 class antagonist (SCH23390), D2 Class antagonist (eticlopride), or combination would abolish the development of the placebo effect in a spinally mediated reflex was conducted using repeated measure ANOVA. Results indicated a between subjects effect F (9, 70) = 3.47, p = .001, η_p^2 .309, no within subjects time effect F (3, 210) = .20, p = .894, η_p^2 = .003, and no interaction of time and group, F (27, 210) = 1.02, p = .438, η_p^2.116. Tukey post hoc comparisons indicated significantly greater latency between cocaine & morphine (p = .001) and SCH23390 & morphine (p = .021), compared to the saline only control group. No significant latency was observed between the remaining groups, though the eticlopride & morphine group trended toward

significance (p = .052), and the saline only group. Figure 8 illustrates time averaged data by group.

Figure 8, Day 3 placebo, Tail Flick Latency mean and 95% confidence intervals

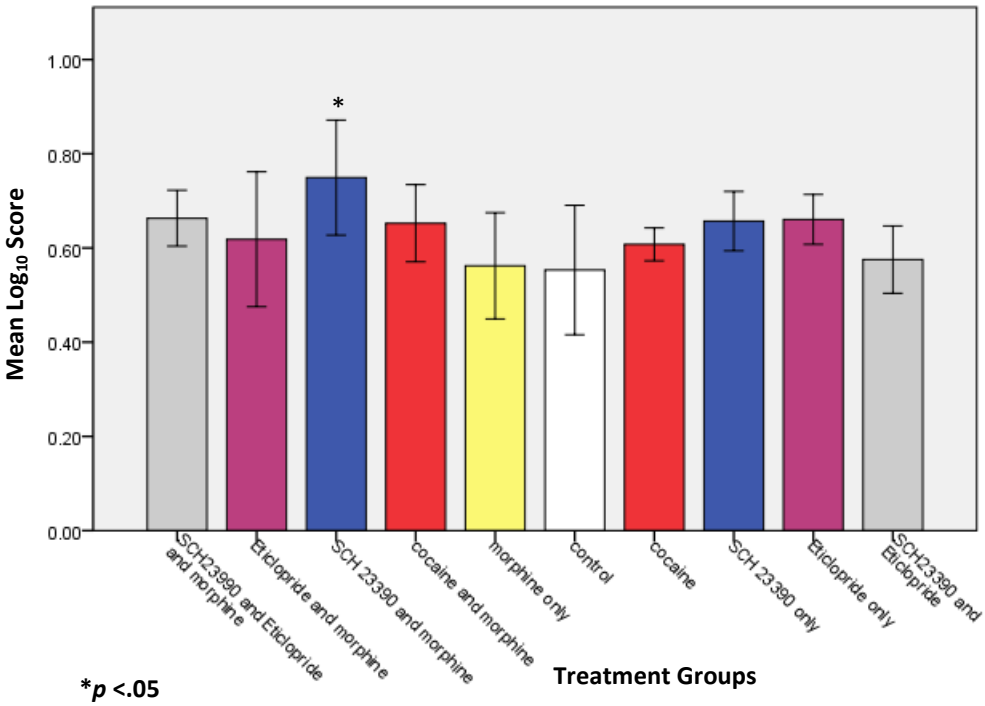


*p <.05, **p = .001

Day 4 evaluation of the hypothesis that increased dopamine (cocaine) during conditioning would extend the duration of the placebo effect and that removal of dopamine during conditioning with D1 class antagonist (SCH23390), D2 Class antagonist (eticlopride), or combination would abolish the development or extension of the placebo effect in a spinally mediated measure was conducted. Repeated measures ANOVA indicated a between subjects effect $F(9, 70) = 2.174$, p = .34, η_p^2 .218, no within subjects time effect $F(3, 210) = 1.13$, p = .335, η_p^2 = .016, and no interaction of time and group, $F(27, 210) = .83$, p = .693, η_p^2.096.

Tukey post hoc comparisons indicated significantly greater latency between SCH23390 & morphine (p = .030) and the saline only control group. No significant latency was observed among or between the remaining groups.

Figure 9, Day 4 placebo, Tail Flick Latency mean and 95% confidence intervals



*p <.05

Day 5 final evaluation of the hypothesis that increased dopamine (cocaine) during conditioning would extend the duration of the placebo effect and that removal of dopamine during conditioning with D1 class antagonist (SCH23390), D2 Class antagonist (eticlopride), or combination would abolish the development or extension of the placebo effect in a spinally mediated measure was conducted. Repeated measures ANOVA indicated no between subjects effect $F(9, 70) = .293$, $p = .975$, η_p^2 .04, a within subjects time effect $F(3, 210) = 2.73$, $p = .045$, $\eta_p^2 = .04$, and no interaction of time and group, $F(27, 210) = 1.49$, $p = .064$, η_p^2.16. Pairwise time-point comparison indicated a

significant difference from time-point 1 to time-point 4 (p = .012) and from time-point 2 to time-point 4 (p =.004). No other time-points were significant.

Discussion

The placebo effect is based on the expectation of reward, a phenomenon that is known to involve the actions of DA. As such, this study sought to examine the role of dopamine in the acquisition and maintenance of the placebo effect. Specifically it was posited that an increase of DA bioavailability would have a subsequent increase in the magnitude and, by extension, the duration of the placebo effect as compared to the placebo effect observed in non-DA manipulated animals and natural history controls. Additionally, it was asserted that the blockade of DA, specifically at D1 and D2 class receptor subtypes would prevent the acquisition and/or alter the magnitude of the placebo effect (respectively) in comparison to controls. These hypotheses were tested using morphine, cocaine (an indirect DA agonist), SCH23390 (a specific D1 class antagonist), and eticlopride (a specific D2 class antagonist) via supra-spinal (hotplate) and spinal (tail flick) measures.

These hypotheses were in part supported and in part refuted, depending on which measure was used to assess the development of the placebo effect and which class of receptor was being investigated. The DA agonist cocaine elicited a strong placebo effect, but this effect was only observed in the spinally mediated tail flick test and was confined to only one day of observation. Cocaine had no effect on the supra-spinal measure. This partly supports the assertion that DA would enhance the acquisition and duration of the placebo effect. The assertion that D1 and D2 class antagonists would prevent or attenuate the development and magnitude of the placebo effect was refuted. In fact, quite the opposite was observed in both supra-spinal and spinal measurements.

Supra-spinal Analgesia and Placebo

Supra-spinal mechanisms of analgesia and placebo were assessed by means of the hot plate test (Jóhannesson & Woods, 1964; Kayan, Woods, & Mitchell, 1969). The expectation from the experimental design was that a placebo effect would be observed on Day 3, during which no drug treatments were given. Placebo effects were not observed in the morphine and the cocaine/morphine group, although the cocaine/morphine group was trending toward significance. By contrast, placebo effects were observed in the D1 class antagonist/morphine group, the D2 class antagonist/morphine group, and in both combined antagonist groups, with and without morphine. On Day 4, the second day without drug treatments, placebo effects had already been extinguished in the D1 class antagonist/morphine group, whereas it was retained in the D2 class antagonist/morphine group and in the combined antagonist groups, with or without morphine. On Day 5, the third day of placebo administrations, the placebo effect had extinguished in the D2 antagonist/morphine group but was still observed in the combined antagonist groups, with and without morphine. To summarize, morphine and morphine + cocaine, yielded no placebo effect with two days of drug administration/conditioning. However, both D1 class and D2 class antagonists, when combined with morphine, did produce a placebo effect. Further, the combination of DA class antagonists, with or without morphine, in a single injectate produced the most enduring effect.

These observations are exactly opposite to predicted outcomes and more than a little puzzling. One possible explanation is that the antagonists in combination produced an enduring effect that worked through *motor* pathways rather than pain pathways.

Spinal Analgesia and Placebo

Spinal mechanisms of analgesia and placebo were assessed by means of the tail flick test. A different pattern of results was obtained using this measure than in the hot-plate test. Contrary to supra-spinal observations, Day 3 administrations produced placebo responses in the cocaine/morphine group and the D1 antagonist/morphine group yet was not observed in all other groups, though the D2 antagonist/morphine group was nearly significant. On Day 4 only the D1 antagonist/morphine group showed a placebo response and by Day 5 placebo administrations resulted in no observed placebo effects. In line with predicted outcomes the administration of a DA agonist produced a placebo effect that contrasted with morphine administration alone. Contrary to predicted outcomes, DA administration did not produce a lasting placebo effect, yet stimulation of D2 receptors alone by endogenous DA (achieved by administration of a selective D1 antagonist) in the presence of morphine produced a spinally mediated placebo effect lasting two days. This would indicate that D2 class receptors are more involved in the observed spinally mediated placebo effect than D1 class receptors.

In order to tease apart the discrepancies observed between supra-spinal and spinal observations it is necessary to revisit the anatomy of the systems involved as they relate to analgesia and the placebo effect.

Dopamine revisited

Two primary DA cell groups have been discussed previously, the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), each with neuronal projections to a number of areas in the brain (Alexander, Delong and Strick, 1986; de la Fuente-Fernández and Stoessl, 2002).

The (SNc) projects primarily to the dorsal striatum, (the nigrostriatal pathway) and is primarily involved in motor function (de la Fuente-Fernández and Stoessl, 2002). In addition to the projections made to the dorsal striatum, SNc DA neurons project to the medio-ventral striatum (caudate, putamen), (Jarcho, Mayer, Jiang, Feier, & London, 2012) and to the spinal cord via the PAG.

The VTA projects to several subcortical limbic structures, specifically the ventral striatum (nucleus accumbens, (NAc), amygdala, hippocampus, olfactory tubercle and septal region and is known as the mesolimbic pathway. This pathway is primarily involved in emotional responses (Alexander, DeLong, & Strick, 1986). In addition, there are projections from lateral regions of the VTA to frontal cortical regions, specifically the medial prefrontal cortex (mPFC), known as the mesocortical pathway (de la Fuente-Fernández and Stoessl, 2002) and projections to the medial thalamus (Altier and Stewart, 1999), rostral agranular insular cortex (RAIC) and the anterior cingulate cortex (ACC) (Potvin, Grignon, & Marchand, 2009). The latter two structures are strongly involved in the affective qualities of pain (Peyron, Laurent, & Garcia-Larrea, 2000; Rainville, Duncan, Price, Carrier & Bushnell, 1997), and interestingly previous research has indicated that micro-injections of DA agonists in both of these areas produces analgesia (Coffeen et al., 2008; Lopez-Avila et al., 2004).

Reciprocal connections among the structures described above also indicate a potential influential relationship between DA release and the perception of pain (de la Fuente-Fernández and Stoessl, 2002). DA input to the limbic and prefrontal cortex can influence opioid release directly in the periaqueductal gray (PAG) via DA input (Christie, James, and Beart, 1986). The VTA has projections indirectly to the PAG (Beitz, 1982),

as does the NAc, through the hypothalamus (Yu and Han, 1989). In addition, the PAG has projections to limbic structures including the VTA, NAc, amygdala and limbic frontal areas (Cameron, Khan, Westlund, Cliffer and Willis, 1995).

The SNc and VTA have both been directly and indirectly implicated in the perception and processing of pain (Magnusson & Martin, 2002).Unfortunately the pathways associated with ascending and descending pain control is not well described or understood, especially as it relates to the NAc or reward (Potvin, Grignon, & Marchand, 2009). Imaging studies, however, have demonstrated placebo induced dopamine release in both the dorsal (de la Fuente-Fernández, Ruth, Soss, Schulzer, Calne et al., 2001) and ventral striatum (de la Fuente-Fernández, Phillips, Zamburlini, Sossi and Calne, 2002).

While the SN and VTA have a large body of research associating many of their projection structures and complex reciprocal connections with pain, they are not the only DA producing cell groups. Dopamine neurons also exist in small groups caudal to the hypothalamus and are known as the diencephalon dopamine neurons (Qu et al., 2006). Specifically, there are four small, distinct groups identified as A11, A12, A13 and A14 (Qu et al., 2006). Notably, these groups show remarkable species variation in number, size and exact location (Qu et al., 2006). Relevant to this discussion is group A11 and possibly group A13. Group A11 projects from the caudal region of the diencephalon ipsilaterally to the entire length of the spinal cord, primarily to segmental dorsal horns, and while this projection has been known for some time, knowledge regarding DA function in the spinal cord is still limited (Qu et al., 2006). Finally, group A13 neurons project to the lateral PAG (Messanvi, Eggens-Meijer, Roozendaal, & van

der Want, 2013) which is known to be involved in fear responses, while the central PAG is involved in pain modulation (Da Costa Gomez & Behbehani, 1995). Since fear is known to induce analgesia (Ford, Kieran, Dolan, Harhen & Finn, 2011), the DA projections of A13 to the lateral PAG could be a factor in certain types of analgesic states.

As mentioned previously the effects of DA are mediated by two classes of receptors termed D1 and D2 receptors, initially distinguished by pharmacological and second messenger differences (Stoof & Kebbian, 1984). Based on more recent genomic studies, the D1 class has been shown to be made up of two molecularly distinct receptor subtypes termed, D_1 and D_5; the D2 class is made up of three molecularly distinct receptor subtypes termed D_2, D_3 and D_4. In the present study we have distinguished between the two major classes—D1 and D2—by using drugs that are highly specific for each receptor class but do not distinguish among subtypes within a class. The D_1 and D_2 receptors *per se* vastly outnumber the D_3, D_4 and D_5 receptors and are densely present in the dorsal and ventral striata. Remarkably, under normal conditions, the behavioral and functional effects of DA require concomitant stimulation of both D_1 and D_2 receptors, a phenomenon called requisite synergism (LaHoste & Marshall, 1992). It is important to note, however, that basal DAergic tone at D_1 receptors is sufficient to synergize with D_2 receptor stimulation. Thus, in the absence of exogenous drug administration, it is necessary to block one receptor with an antagonist in order to probe the function of the heterotypic receptor (LaHoste, Henry & Marshall, 2000).

Placebo or Nocebo?

When considering the placebo effects observed in the supra-spinal hot plate measurement it is important to keep in mind that the relevant literature is inconsistent. It has been observed that DA antagonists attenuate or abolish analgesia and that DA antagonists can be analgesic (Kiritsy-Roy, Standish & Cass, 1984; Ozdemir, Bagcivan & Gursoy, 2013); the circumstances under which DA antagonists will be analgesic, or not, are unclear and likely to be a function of anatomical location, receptor type and dosage. Nonetheless, it is clear from the present results that when tested in the absence of drugs on Day 3, an analgesic placebo effect was observed in mice treated previously (Days 1-2) with morphine in combination with either the D1 class antagonist SCH23390, or the D2 class antagonist eticlopride. By contrast, neither SCH23390 nor eticlopride given alone on Days 1-2 resulted in a placebo response on Day 3. This observation implies analgesia rather than motor impairment. Additionally, when pre-treated with eticlopride/morphine, mice developed a more enduring effect, as evidenced by the presence of a placebo effect on Day Four, which had already extinguished by that time in mice pre-treated with SCH23390/morphine.

When one turns to the effects of combined D1 and D2 antagonists, with and without morphine, the implication becomes less clear. Although these two experimental groups displayed a strong effect across all three placebo days, it is not clear whether this effect is analgesia or motor inhibition misconstrued as an analgesic response. It should be remembered, however that no drug was given on the placebo days. When one considers the literature, and data observed elsewhere in this experiment, it is possible that combining SCH23390 with eticlopride produced an analgesic response on

the placebo days. However, given the observation that SCH23390 & eticlopride only group was significantly different from the saline only control group during drug conditioning days one and two, the author asserts that it is *more likely* the observed effect is a result of motor inhibition and not analgesia, therefore a *nocebo* effect. In other words, the effect observed in both combined antagonist groups was a slow-to-extinguish deleterious motor effect.

When one looks at the spinally mediated tail flick response observations the picture is a little clearer. There is an evident and predicted analgesic placebo effect for the cocaine + morphine group on Day 3 placebo administration, however this effect does not extend past Day 3. On the other hand, as mentioned previously, there was an observed effect for the D1 class antagonist + morphine for Days 3 and 4, extinguishing on Day 5. This would indicate that D2 receptors in the spinal cord responded more strongly to morphine than D1 receptors.

Within the context of this study, why then is there a discrepancy between the observed spinally mediated tail flick responses and the supra-spinal hotplate responses? The author asserts that these differences are largely due to functional and anatomical separation. Monoamine neurotransmitters cannot be said to have uniform effects across the central nervous system. DAergic axons making synapses on neurons that differ in either their location or chemical phenotype modulate different functions and in different ways. Moreover DA exerts different effects *within the same synapse* depending on which specific receptor subtype is activated. It is possible that differences in the role of DA on supra-spinal vs. spinal pain and placebo mechanisms can be explained by these anatomical and molecular distinctions. A supra-spinal

hotplate response, for example, requires an effortful and coordinated cortical response in order for the mouse to either lick its hind-paw or to jump. This response would necessarily recruit a number of brain regions with much more elaborate reciprocal connections between and among the SNc and VTA. The tail flick response, on the other hand, requires little to no cognitive effort as it is a reflex response with only one small, if poorly understood and articulated, DA cell group projecting from the caudal hypothalamus directly to the spinal cord (Qu et al., 2006). It is known, however, that spinal responses can be influenced by supra-spinal connections (Goffaux, Redmond, Rainville & Marchand, 2007; Kiritsy-Roy et al., 1994), indicating potentially complex relationships with cortical areas, although this is more often the case for tonic pain as opposed to phasic pain (Altier & Stewart, 1999; Wood, 2006).

Study weaknesses

The analgesic placebo response observed following prior exposure to morphine alone has been robustly observed in animal studies (Guo, Wang, & Luo, 2010; Nolan, Price, Caudle, Murphy & Neubert, 2012), but the literature is not consistent as to dosage or number of conditioning days required. To aid us in designing our experiments, we conducted several small pilot studies (four groups of three mice).Based on the results of these pilot studies a decision was made to reduce the originally planned dose of morphine from 10mg/kg to 5 mg/kg to avoid tolerance confounds observed in one group. Additionally, the placebo effect was observed at this dose for animals conditioned two or three consecutive days with no significant difference observed between them. Thus, it was also decided to reduce the number of conditioning days to two days in consideration of possible animal discomfort and

experimenter logistical concerns. Because we did not observe a morphine analgesic placebo response in the main study, the decision to reduce the number of conditioning days from four to two based on sample sizes of *n = 3* should be considered a potential study weakness. Additionally, pilot studies were conducted on C57/Bl mice, raising the possibility that we would have obtained different pilot results if we had used the same strain (CD1/outbred) as used in the main study. Further, the inconsistent baseline data observed in this study suggests that a different baseline collection procedure is warranted. Finally, the addition of the opioid antagonist naloxone to the study would have helped to identify whether some response where analgesic or motor in nature.

Future directions

This study yielded some very interesting but conflicting results. Based on the results observed here the author concludes that the results have more heuristic than definitive value. The study is rich in the number of hypothesis yielded to be tested in future research. Specifically, future research should continue to use combinations of DA antagonists (this is the only study the author is aware of that combined DA antagonists) as well as single administrations. These efforts should be combined with precise anatomical administration of drugs into the central nervous system (via intracerebral or intraspinal cannulation) and/or precise destruction of anatomically or neurochemically distinct neuronal clusters while sparing axons of passage, as can be achieved by intracerebral or intraspinal injection of soma-specific neurotoxins or DA-specific 6-hydroxydopamine. Additionally, exploration of serotonergic and noradrenergic involvement in the placebo effect is suggested. It is with these methods and additional scope that a more nuanced view of pain and the placebo effect can be observed.

Conclusion

The role DA pays in the placebo effect and pain, as highlighted by this study, is complex. It is apparent that much more work elucidating the circumstances under which a placebo or nocebo effect will develop is necessary. A portion of that exploration will come with a better understanding of the cellular, anatomical, and functional properties of DA. This study contributed to that understanding by producing previously unobserved effects with the novel use of D1 + D2 antagonists in combination. The novel results presented here raise even more questions regarding the relationship between DA and the placebo effect.

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Appendix

Institutional Animal Care and Use Committee

UNIVERSITY OF NEW ORLEANS

DATE: **January 22, 2014**
TO: **Gerald LaHoste, Ph.D.**
FROM: Bernard B. Rees, Chair
RE: *IACUC Protocol* # 13-004
Entitled: The influence of dopamine on the magnitude of the placebo effect

After reviewing the pilot data you generated, the IACUC has approved your use of the full number of animals (96) in the above referenced protocol. As reminder, this is a one year approval, beginning August 8, 2013 and expiring August 7, 2014.

The University of New Orleans has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), National Institutes of Health. The assurance number is A3299-01.

Vita

The author was born in Sallisaw, Oklahoma. He obtained his bachelor's degree in psychology from Rogers State University in 2008. He joined the University of New Orleans graduate psychology program to pursue a PhD in applied biopsychology in 2009. The author specifically began studying pain and placebo effects under Dr. Denis Soignier. He completed his MS in applied biopsychology with a study examining the factors that might predict a person's susceptibility to placebo and nocebo effects with Professor Emeritus Kevin Greve. Upon completion of his masters in 2011, he began studying the neurobiological underpinnings of dopamine and the placebo effect with Dr. Gerald (Jerry) LaHoste.