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# Assessing the impact of non-steroidal antiinflammatory drugs in the hot plate test: An alternative model

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Assessing the impact of non-steroidal antiinflammatory drugs in the hot plate test:

An alternative model

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Abstract

The current paradigm for the hot plate pain test is problematic in several ways. It uses very limited behavioral criteria to define pain; traditionally, the hot plate pain test measures rats' latencies to performing a specified behavior (hind paw mouthing or jumping) when placed on a warm surface. Also, the hot plate test yields significant results for only certain analgesics. Non-steroidal antiinflammatory drugs (NSAIDs) have an analgesic effect in humans. They do not, however, affect hot plate latencies in rats, unlike opioid analgesics, such as morphine. This study was intended to develop a new paradigm for using the hot plate to determine the effectiveness of different analgesics.

This study had two main components; first, an inventory of morphine and saline treated rats' behavior on the hot plate was compiled. Videotaped sessions of rats being placed on the hot plate were used to operationally define several behaviors not commonly employed in hot plate analysis. Then the frequencies of these behaviors were determined from the tapes and used to develop a paradigm intended to yield significant results for rats treated with NSAIDs.

In the second component of this study, rats treated with ibuprofen, an NSAID, were subjected to the new paradigm. These rats displayed certain behaviors at a significantly different frequency than control rats suggesting that there are in fact behavioral changes on the hot plate in response to NSAIDs, and they are detectable with the new paradigm.

## Chapter 1

## Introduction

The information provided by pain research is already extensive; however, the field is still developing. One major issue faced by neuroscience today is pain management, especially for chronic pain. Both doctors and patients continue to search for more effective ways of reducing or eliminating pain. In this process, many analgesic drugs have been developed and refined for short term or acute pain, but such drugs typically have negative side effects if used for an extended period of time. Many non-steroidal antiinflammatory drugs, for instance, cause gastrointestinal or renal problems if used repeatedly (Gilman, 1993). Morphine, while an excellent analgesic, has well known psychological effects that make it undesirable as a long term pain solution, not to mention its effects on respiration and gastrointestinal disturbances (for a review, see Gilman, 1993). Present pain research is being conducted not only to expand knowledge of the pain system's function and structure, but also to develop drugs that will more adequately help people manage pain.

One of the primary ways to both explore the nature of an organism's pain systems and develop new treatments is via the application of pain assessments. These tests often employ animal models, especially in the early stages of research and development of a treatment. While this is more complicated than testing on humans (because animals cannot describe the amount or quality of pain they are experiencing), it is a necessary precursor to human testing to meet ethical standards. As a result, objective measures of behavior must be used in order to determine the *nociception*, or pain related to stimulation of neurons in the pain systems, that animal subjects experience. In addition,

for treatments that will be adapted for humans, the animal model must provide the closest possible parallel to the human pain system. For these reasons, and in an attempt to expedite the development of effective treatments, it is crucial that pain tests are accurate and efficient. Each type of pain test has different aspects and mechanisms designed to ensure its accuracy and reliability. Unfortunately, some tests, including the hot plate test, commonly used to measure acute pain in rats and mice, have guidelines and traditional procedures that may actually detract from the accuracy of the test (Mikhail, 2000, p. 433).

Although numerous methodologies for measuring acute and chronic pain in animal models have been well developed, such measures still lack in the consistency needed in development of new pain treatments to be used in humans. The inconsistency is partially a result of the historical development of pain tests. Early pain research strongly focused on acute pain; such tests used for this purpose include the hot plate and tail flick tests (Mogil, 2004). The hot plate test (Adams, 1969) involves placing a rat on a uniformly hot (49-54°C), enclosed surface for a limited number of seconds or until the rat displays a certain specified behavior. The latency to displaying the behavior is used as a measure of pain. The tail flick test (D'Amour & Smith, 1941) involves placing a restrained rat's tail over a focused heat source and measuring the time that the tail remains on the heat source before it is reflexively jerked, or *flicked* away. Again, latency, measured repeatedly over a time course, is used as the measure of pain. The information provided by these paradigms is far from the complete picture of pain; such tests fail to provide information about chronic pain or the range of behaviors related to pain. They focus only on latency to a pain response, not magnitude or duration of pain. Further tests, including the formalin test, were developed to assess chronic pain. In this test,

researchers inject a rat's paw with an irritant (such as a 2.5% Formalin solution) and record the frequency of flinching or favoring behavior during a time course (Manning, 1995). Although there are now many tests for both acute and chronic pain, drug studies provide ample evidence that collectively, these tests still do not provide researchers with sufficient information (Lavich et al, 2005). For instance, non-steroidal anti-inflammatory drugs (NSAIDs) have *no effect* on the hot plate that can be consistently demonstrated (Lavich et al, 2005; See also: Le Bars, 2001; Vogel, 1997; Taber, 1974). These findings contrast with the pain relief associated with such drugs reported by humans. The inability of the hot plate test to assess the analgesic properties of NSAIDs is a serious issue, especially in developing new treatments for relieving pain. Pain tests should reliably reflect the analgesic properties of a treatment in humans, so that new drugs can be effectively tested in animals before humans with a minimal risk of discarding a new and effective treatment. Although there is no human equivalent to the hot plate test, in other comparative tests of analgesics, participants in multiple studies have reported an unspecified, but greater pain relief (on a validated scale of pain) from NSAIDs than that obtained from opioids (Holdgate, 2004). Based on current hot plate models, it remains unclear why there is a discrepancy between these drugs in the hot plate test. NSAIDs may be ineffective because they unreliably affect rats and mice, or perhaps, because they do not reduce thermal nociception. These reasons are unlikely affecting the hot plate test, however, because NSAIDs do have a significant effect on other thermal nociception rodent tests, such as the Hargreaves test, which assesses pain by the latency of lifting a paw exposed to a focal radiant heat source (Hargreaves, 1988) or the "modified hot plate"

(MHP) examined by Lavich et al, (2005) which also assesses pain by the latency of lifting a paw, but uses a uniform heat source.

These tests, when used to determine the analgesic ability of NSAIDs, however, also involve use of an irritant such as carrageenan to cause pain in the paw being subjected to the heat source. In such usage, this models chronic pain and tests an antihyperalgesic response, which is a very different paradigm than the traditional hot plate. The traditional hot plate test measures (or is intended to measure) *acute* pain. During the test, the rat experiences pain only when placed on the hot plate, instead of receiving an injection of carrageenan or formalin prior to the thermal pain stimulus. If NSAIDs affect longer term pain management differently than sudden, acute pain, the hot plate test logically may well be unaffected by them. This is the second major difference between the traditional hot plate and the MHP test. Although both measure pain reduction, the hot plate methodology is meant to assess a drug's ability to reduce an acute, thermal nociceptive stimulus. In contrast, the MHP measures a drug's capacity to reduce a response to an acute, thermal nociceptive stimulus from hyperalgesic, or sensitized, increased pain behaviors, to normal. NSAIDs have been shown to consistently reduce hyperalgesia (MHP), but not induce analgesia (hot plate) in thermal pain tests. The ineffectiveness of the traditional hot plate test may, therefore, result from an attempt to measure a response unaffected by NSAIDs.

Opioids, including morphine (a drug that *has* a significant and consistent analgesic effect on hot plate behavior) have different pharmacological properties than NSAIDs, such as ibuprofen. Morphine is an opioid agonist that, in addition to analgesia, causes drowsiness, mood changes, nausea and vomiting, and decreases in respiration and

gastrointestinal motility (Martin, 1977). Clearly, the analgesic mechanisms of morphine are the effects most relevant to the hot plate test; however, it is important to recognize that drugs have a multitude of effects in an organism, any of which could be influencing, confounding, or even controlling a behavioral assay. Opioid analgesia is relatively selective to different types of receptors in different parts of the body (Gilman, 1993). The nonspecific effect of morphine in humans raises additional questions about using the simple, traditional hot plate test to measure analgesia. Anecdotal evidence suggests that humans on morphine still experience pain, but that it causes less discomfort; this draws into question the basis of the hot plate test. When animals display significantly different behavior on the hot plate after receiving morphine, does this actually signify that they are experiencing pain relief or simply behavioral change? Rats display less hind paw licking and less jumping, but this may not necessarily be due to analgesia; it could, for example, be attributed to a reduction in motor activity.

The three subtypes of opioids,  $\mu$ ,  $\delta$ , and  $\kappa$  agonists cause analgesia by modulating the effects of neurotransmitters (like substance P) involved in pain mediation (Gilman, 1993).  $\kappa$  agonists, however, seem to be only slightly involved in the suppression of thermal nociception (Lewis *et al.*, 1987). When injected intrathecally, morphine seems to limit nociceptive processing descending in the spinal cord (Gilman, 1993); when administered both spinally and supraspinally, morphine seems to have a synergistic effect, resulting in more pain relief (*see* Advokat, 1988). Morphine has significant euphoric effects in addition to its analgesic properties. The euphoric effects of morphine in humans are less well understood; rats will work to administer morphine to the nucleus accumbens (Gilman, 1993) implying a reinforcing effect may exist in animal models.

Euphoric effects could also be related to reduced anxiety and panic caused by agonist binding to the numerous opioid receptors in the locus ceruleus (Gilman, 1993). This effect seems particularly related to the traditional hot plate test; panic or fear is a logical response to a novel situation in a dim room during which the rat is subjected to an unexpected, noxious stimulus. Lastly, consider that opioids affect the hypothalamus causing a reduction in body temperature (Martin, 1983). This could possibly make the hot plate an even more negative situation in which the magnitude of the noxious pain stimulus may be affected, strengthening the significance of the perceived analgesic effect.

Non-steroidal antiinflammatory drugs have different mechanisms of action from that of opiates. Primarily, these drugs function by inhibiting cyclooxygenase; this enzyme is necessary for the synthesis of prostaglandins, which are in turn important in inflammation and fever (Vane and Botting, 1987). Postoperatively, NSAIDs can be more effective analgesics than opioids, especially reducing pain related to inflammation or sensitization (Holdgate, 2004). Their function is very different from opioids in that NSAIDs inhibit the synthesis of a pain inducing enzyme, instead of directly binding to neurons. Because of genetic variation, each animal is distinct and may display a slightly different response to a dose of a drug, affecting hot plate results even when phenotypically identical rats are used (Gilman, 1993).

Although many types of drugs (including both opioids and NSAIDs) are grouped together as pain reducing, analgesic drugs, they actually serve a variety of functions that reflect the many types of pain; for instance, they are taken to reduce headaches, reduce the hyperalgesia related to fever, and to alleviate injury-related pain, to name a few. Pain can be fundamentally divided into pain *as a sensation* and pain *as suffering*. Pain as a

sensation is characterized by a sharp feeling that may result in activation of a spinal reflex, instead of a more cognitive escape reaction. Pain as suffering is characterized by a long term source of pain that results in learned, escape behaviors. Nociceptive pain is defined as pain that results from stimulation of neurons in the pain systems, whereas neuropathic pain describes pain that exists without an external stimulus, due to damage to nerves. This type of pain is relatively constant because the nerve-damage results in continuous stimulation of neurons in the pain systems. Neuropathic, or chronic pain, is a more negative stimulus ranging from dull to severe persistent pain (Gilman, 1993). This type of pain often causes a supraspinal reaction that the animal can control. This chronic pain resulting in a supraspinal reaction is the type of pain the hot plate is intended to assess (Sora, 1997). Additionally, types of pain can be divided into categories based on the stimulus; for example, the hot plate test is clearly intended to measure thermally-induced pain. Other types of pain that are studied in animal models include visceral, mechanical and chemical or irritant-induced pain (Mogil, 2004; Lizarraga, 2006; Dubuisson, 1977).

Despite their different methods of action, both opioids and NSAIDs can have an analgesic effect on a variety of pain “types”. Opioids are traditionally used for dull, persistent pain (Gilman, 1993). NSAIDs are often used to treat more severe pain caused by inflammation (Gilman, 1993). Their effectiveness in acute, sharp pain is less well documented because it is impractical to administer drugs as a preventative measure against sudden, unexpected pain.

In addition to the effect of NSAIDs on certain types of pain remaining unstudied, there are other reasons that such drugs may not yield significant hot plate results. For

instance, the traditional hot plate test lacks an appropriate standardized methodology to yield results. Although there is some variation seen, typically the hot plate is set to about 50°C, and rats are placed on the plate and monitored by a researcher until they perform a target behavior, or, until a certain amount of time (usually 30 or 40 seconds) has passed (Adams, 1969). This time limit is intended to ensure that the rats will have no tissue damage if the target behavior does not occur. The rats subjected to the hot plate test are usually experimentally naïve or, at very least, have never previously experienced the hot plate. The rationale for using inexperienced rats is that repeated exposure to the painful stimulus provided by the hot plate will result in learned coping behaviors that the rat will display in subsequent hot plate tests regardless of the pain they are actually feeling (Espejo, 1992, p. 1161).

This central methodology of the hot plate test is based on assumptions and measures that limit the tests' ability to yield useful results in a variety of ways. First, the assumption that a repeated measures paradigm would result in confounding learned behaviors may not be valid. In one study, weekly hot plate exposures were shown to elicit *more* pain response behaviors in subsequent tests. (Espejo, 1992, p. 1157) This increase in pain or hyperalgesic effect demonstrates an effect contrary to the theory behind standard hot plate methodologies. Instead of appearing to habituate to the noxious painful stimulus, rats in the study conducted by Espejo seemed to be sensitized to the heat or prepared to react to it, responding more than they had initially. Although repeated measures hot plate paradigms have been avoided in an effort to prevent habituation to the stimulus, this study actually suggests that a more pronounced effect would be visible on the hot plate with repeated exposures. It could then be argued by critics of a repeated

measures paradigm that the increased responding would not reflect the actual nociceptive effect, but a learned response to the hot plate setting. However, since the specific pain related behaviors of rats may not, themselves, provide information about the analgesic affect of the drug, the increased responding would simply make smaller differences in drug effects visible and comparative studies between drugs more useful.

Many acute pain tests are based on a stimulus threshold that results in a response. The tailflick test measures the time before a rat moves its tail off of a heat source, the Hargreave's test measures latency of a paw lift, and the hot plate test measures the latency of either licking a hind paw or jumping. There are several practical reasons for the use of latency and threshold measurements; the data is easily gathered and quantified, and in some cases reflects behaviors exhibited by humans (Mogil, 2004) However, these measurements do not account for spontaneously exhibited behaviors resulting from chronic pain, and only a small fraction of pain studies have looked at spontaneous behavior (Mogil, 2004). The more recently-designed tests meant to assess chronic pain, like the formalin test, however, measure behavior quantity. Since a variety of spontaneous behaviors are more relevant to chronic pain research, it follows that an adaptation of the hot plate test to assess such behaviors may provide valuable new data about drugs' analgesic effects.

Despite the fact that research has identified many behaviors that rats and mice perform on the hot plate, the widely accepted methodology remains limited to determining hind paw lick or jump latencies. This is problematic because hind paw lick latencies are affected by many *non*-analgesic drugs. In one study (Carter, 1991), two hot plate methodologies were compared using a variety of analgesic and non-analgesic drugs

such as clozapine and haloperidol, among others. One method used only hind paw lick latencies and the other used hind paw lick *or* jump latencies. The former method showed significantly different results between control rats and experimental rats treated with non-analgesic drugs. Drugs have varied physiological and behavioral effects that can affect behaviors in ways that may masquerade an analgesic effect if the definition of an analgesic response is too imprecise. Since the hot plate does not take into account any motor limitations caused by a drug, it seems logical that tracking more behaviors would improve the accuracy of the hot plate (Carter, 1991). The blind acceptance of the traditional hot plate paradigm is the only limitation on the behaviors chosen in the collection of hot plate data. Consider that Espejo et al., for example, tracked 14 distinct behaviors in their daily and weekly exposure of rats to the hot plate test (1992, p. 1158). Since the hot plate test has been unable to yield significant results with certain types of analgesic drugs, it is possible that the rats' behavior is not being analyzed in an appropriate way. If other behaviors, in addition to the traditional hind paw mouthing and jumping, have significantly different frequencies after drug treatments, they might prove to be useful to track and yield more sensitive results to a treatment assessed using the hot plate test.

The hot plate is rather unique in its use of completely naïve rats. Research on the effects of training history suggests that any previous experience with behavioral paradigms, even if it is unrelated to nociception, can affect analgesic response on the hot plate (McIlwain, 2001). There is clear evidence for the confounding effects of previous experience; however, it is nearly impossible for any two rats to have the exact same experience in every aspect of their life before the test. Since McIlwain found that even

unrelated experience can be confounding, it may be the assumption that naïve rats will behave identically is itself naïve. Many other pain tests have taken this possibility into account; instead of assuming reliable behavior in a new setting, a training or baselining procedure creates a reliable comparison for each rat in the test. Although other pain tests may start with naïve rats, the tests often take place over a period of time, or a baseline measure is found for each rat. The hot plate test traditionally uses neither as a measure in order to prevent the development of a learned response unrelated to pain. In finding animals' baselines in other tests, the initial reading is often very different than the baseline, possibly because the new stressful situation causes the rat to be anxious and display more atypical behaviors. If this is the case, the use of naïve rats for the traditional hot plate could make it prone to such errors. Even the modified hot plate (MHP), a measure of antihyperalgesia, starts with a measurement of baseline latencies before injections of carrageenan and the analgesic drug (Menendez, 2002).

In addition to the logical pitfalls of the accepted hot plate methodology, the results it produces are unreliable. Opiates show significant analgesic results in hot plate paradigms; however, a consistent response among other drugs known to have an analgesic effect in humans has not been found (Lavich et al., 2005). The reason for this discrepancy is uncertain; perhaps other types of analgesics affect different behaviors, so effects are missed when researchers are looking for too narrow of a behavioral change. Another possibility is that other factors *unaccounted for* during the hot plate test affect the rat's behavior. This might include anxiety in the test situation, development of learned helplessness in repeated measures, being raised in isolation, engaging in aggression towards another rat before being placed on the hot plate. Isolation at various

ages has resulted in decreased threshold for foot shock in rats (Arakawa, 2002). If variations in the environment in which the rat was raised (in this case isolation) can cause hyperalgesia in some pain tests, it is possible that it could affect the hot plate test also. This suggests that knowing the history of the subjects may be critical for accuracy. In another study, King et al. (2003) demonstrated that stress can increase latencies for hind paw licking behavior, and shorten the actual time spent engaging in the behavior. If there is variation in the amount of stress rats experience such as differences in handling by the experimenter, then results could be confounded by this fact and not simply reflect analgesia. Further, a study on the effects of defeat concluded that mice have an analgesic response to the hot plate test immediately after defeat (Siegfried et al., 1984). This result, however, only occurred in one of two strains of mice. Variations in nociceptive responses exist among rats of different sex and strain (Vendruscolo, 2004). Presumably these factors are kept consistent within an experiment; however, in reviewing the literature, and comparing results between studies, such factors could cause confounds.

Although some of the problems with the traditional hot plate test have been alleviated by modifying the hot plate and by using radiant heat tests, a suitable hot plate model has still not been developed. Most of the tests of thermal pain that yield significant results measure antihyperalgesia or use hind paw latencies, which have been demonstrated to lengthen in response to drugs with no analgesic property.

Because of the numerous problems with the current hot plate model and the growing evidence that more behaviors should be considered in measuring nociception, the current study sought to develop a better methodology for the hot plate that would be responsive to NSAIDs. First, a behavioral inventory for rat behavior on the hot plate was

compiled. This served to document the variety and frequency of behaviors emitted by both control rats and rats that received morphine, an analgesic drug known to yield significant results in the traditional hot plate paradigm. The behaviors exhibited at significantly different frequencies between groups of rats in the inventory group were subsequently employed in a second study to analyze the behavior of rats treated with NSAIDs. These behaviors were used because the varying frequency between groups suggest that they reflect the analgesic effect of a drug. This study sought to find possible differences in behavioral responding on the hot plate between NSAID-treated rats and controls that are overlooked when the traditional measure of hind paw lick and jump latencies is used.

Chapter 2

Experiment 1

Methods

*Subjects*

Thirty experimentally naïve adult male Sprague-Dawley rats were used for this study. The rats were divided non-systematically into three groups: moderate morphine dose, low morphine dose, and saline. The rats had access to food and water ad libitum and were housed two to a cage on a 12 hour light/dark schedule. Because rats were run at two different times of day to accommodate the researcher's schedule, some of the rats were housed with light from 7am to 7pm and other received light from 10am until 10pm daily. All procedures were conducted in accord with protocols approved by the Macalester College IACUC.

*Materials*

The rats were placed on an IITC Inc. Model 39D Hot Plate Analgesia Meter set at  $47.9 \pm 1^\circ\text{C}$ ; this temperature was used because the noxious heat threshold for rats has been established at  $45.3 \pm 0.3^\circ\text{C}$  (Almási, 2003), and a lower temperature was preferred in an effort to prevent tissue damage in the repeated measures paradigm. The trials were conducted in a small room illuminated with a red light and videotaped for later behavioral analysis. This illumination level was intended to ensure that the rats could not see the apparatus, and to prevent possible confounds of the rat's anticipation being placed in a visible chamber. A timer was employed to ensure the intervals between trials were the correct length of time. The rats received subcutaneous morphine (6.0mg/ml; medium-strength dose or 0.6mg/ml; low dose) or equivolume saline at 1ml/kg 30 minutes before

the initial hot plate trial. Morphine was used as a drug challenge because it has well-established effects on hot plate behavior.

### *Procedure*

Two rats were run at once (alternating trials) in order to expedite the data collection process; the animals received an assigned injection of either morphine or saline 2.5 minutes apart, and were then placed in the hot plate experimental room in their home cage to acclimate for 30 minutes. In order to account for the possible effects of circadian rhythms, injections were given near either 1:30pm, for the rats on the earlier light schedule, or 4:30pm, for the rats on the later light schedule. Before each trial, the video camera was turned on, but was not left on between trials. Each trial lasted 30 seconds, and after the 30 minute period following the injection, there were trials at zero, five, ten, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes. After this initial time-course, five more follow-up trials were conducted, at 90 minutes, 120 minutes, 150 minutes, 240 minutes, and 420 minutes. These trials were to track the behavioral changes the rat displayed after repeated experience on the hot plate and as the drug wore off. Because two rats were run at the same time, one rat's trials were always 2.5 minutes after the first rat's trials. (e.g., minute 2.5, 7.5, 12.5, etc.) Between each of the follow-up trials, rats had access ad libitum to food and water. After the minute 420 trial, rats were returned to their home room. Any unusual behavior or problems were recorded on a datasheet for each rat.

After all 30 rats had been run, six of the session tapes were watched in order to code behaviors. After behaviors were coded, *all* of the tapes were viewed and frequency of the behaviors and latency to displaying a traditional hot plate behavior (jump or hind

paw mouthing) were recorded. In addition, the last behavior performed at the end of 30 seconds was recorded for each trial.

## Results

### *Behavioral Inventory*

Based on the observation of six rats' hot plate behavior, 16 specific behaviors were defined. These behaviors were used to create a behavioral inventory. Although most of the behaviors were analyzed based on frequency, some were simply noted based on their occurrence (or not) during the trial. These include *pausing* and *frantic* behaviors. Since the rats generally walked around while on the hot plate, this was not considered a behavior; therefore pausing was defined as more than two seconds without movement. This behavior often occurred immediately after the rat was placed on the hot plate. Frantic was defined as when one or both of the rat's hind paws moved up and down rapidly, in a seemingly uncontrollable manner, three or more times. Although this was common during early trials, and occasionally present during the trial at minute 420, it generally seemed to be replaced by controlled lifting of the hind paws.

There were 14 frequency-based behaviors. Corner digging was when the rat rapidly alternated putting each front paw in a corner at the base of the chamber. Corner sniffing was defined as the rat putting its nose directly in one of the corners at the base of the walls of the hot plate chamber. The rat could easily have been looking at or feeling the corners, too; corner sniffing is simply a name given to the behavior based on its appearance. Side sniffing was the rat putting its nose anywhere along the base of a single side of the enclosure. Side standing was when the rat supported itself by placing one or both of their front paws on a side of the hot plate chamber. This behavior often seemed

to be used in order to smell the air higher up in the chamber. Corner standing was defined as the rat supporting itself with one or both front paws directly in the corner of the walls or one on each side of a corner. Center standing was defined as the rat standing on its hind paws with no support from its front paws. This was distinguished from the sitting position the rat assumed in order to engage in mouthing behaviors (described later) by requiring that the rat lift its body weight off the hot plate.

Jumping was defined as both hind paws coming off of the hot plate while the front paws were already off. Hind paw lifting, which was differentiated between left and right paws, was when the rat lifted a hind paw completely off of the hot plate in a way that was in no way related to mouthing, jumping, or taking a step. Although front paw lifting was initially tracked, this measure was not included because it was not distinguishable from walking in many cases.

Mouthing was defined as anytime that the rat's mouth made contact with its paws. This behavior was separated for each paw. Front mouthing was an additional behavior because much of the time the rat simultaneously mouthed both front paws.

### *Behavioral Frequencies*

The tapes of the remaining 24 rats in addition to the first six were analyzed. The frequencies of behaviors and the latencies to hind paw mouthing or jumping were used to distinguish differences among the dosage groups' responses to the hot plate test. The differences in frequency of each behavior based on dosage were analyzed using ANOVA. The frequencies for each trial of both rats in a single condition were combined. (e.g., the ten saline rats' frequencies for each of their 18 trials were combined resulting in 180 data

points for each behavior for the saline condition.) Post hoc analysis revealed several statistically significant comparisons.

All of the 14 frequency behaviors with the exception of sidestand yielded significant results. The majority of behaviors were exhibited most by the rats treated with saline and least by the rats treated with the medium dose of morphine. Cornersniff (Figure 1) was displayed significantly less by the medium dose rats,  $F(2,531)=29.70$ ,  $p<.01$ ; but the low dose rats and control rats were not significantly different. The frequency of cornersniff behaviors (Figure 2) was significantly different between all three groups,  $F(2,531)=34.36$ ,  $p<.01$ . The medium dose rats displayed the behavior significantly less than the low dose rats, and the low dose rats displayed the behavior significantly less than the saline rats. Sidestand (Figure 3) and centerstand (Figure 4) frequencies were similar to cornersniff; the medium dose rats displayed this behavior much less than the other two groups,  $F(2,531)=82.90$ ,  $p<.01$  and  $F(2,531)=44.42$ ,  $p<.01$ , respectively. Jumping behavior was significantly different between all the groups with the control rats displaying it the most and medium dose rats displaying no jumping behavior,  $F(2,531)=48.68$ ,  $p<.01$  (Figure 5). The saline rats' right hind paw lifting frequencies were significantly higher than the two experimental groups,  $F(2,531)=4.94$ ,  $p<.0$ . The low dose frequency was significantly higher than the medium dose frequency at the .05 level. Figure 6 shows that these frequencies, although significantly different, follow a very similar trend. The frequency of right hind paw lifting was significantly higher,  $F(2,531)=5.79$ ,  $p<.01$ , for the saline group than for the two morphine groups which were not significantly different from each other (Figure 7). Cornerdigging was

also displayed significantly more by the saline group than by the other groups,  $F(2,531)=8.96, p<.01$  (Figure 8).

The mouthing behaviors all followed different trends among the doses. Mouthing of the left front paw (Figure 9) and of the right front paw (Figure 10) showed the reverse trend; the medium dose frequencies were the highest while the saline frequencies were the lowest. The medium dose groups were significantly different from the other groups,  $F(2,531)=60.23, p<.01, F(2,531)=37.46, p<.01$ , respectively; the low dose and saline groups were only significantly different at the .05 level. Simultaneous mouthing of the front paws was displayed significantly more by the medium dose rats than by the other two groups,  $F(2,531)=16.39, p<.01$ . Although the difference was not significant, saline rats displayed more front paw mouthing than low dose rats (Figure 11). Mouthing of the left hind paw (Figure 12) as well as the right hind paw (Figure 13) follow a similar trend in which saline frequencies fall in between the two morphine groups. For mouthing of the left hind paw, all of the frequencies were significantly different,  $F(2,531)=27.47, p<.01$ , and, as in the behaviors described earlier, the medium dose group displayed the lowest frequencies. The frequencies for mouthing of the right hind paw were significantly higher for the low morphine dose  $F(2,531)=9.17, p<.01$ , and the other two groups were not significantly different.

In addition to the frequency behaviors, the presence of frantic behavior was significantly less common among rats in the medium dose group and the other two groups,  $F(2,531)=15.51, p<.01$  (Figure 14). Also traditional latencies, the latency to either hind paw mouthing or jumping, was significantly slower for the medium dose group than for the other two groups,  $F(2,531)=54.91, p<.01$  (Figure 15).

## Discussion

Eight of the 14 frequency behaviors tracked in this study yielded significant results with a trend that corresponds with the expected morphine effects; that is, the rats treated with saline performed the most behaviors and the rats that received a medium dose of morphine displayed the fewest. Assuming that the behaviors emitted on the hot plate are in response to pain, more behavioral responding would suggest more pain. Therefore, it would be expected that rats treated with morphine would display fewer behaviors. For several of the mouthing behaviors, morphine show the reverse effect; the morphine-treated rats displayed significantly more mouthing behaviors than the rats administered saline. This is important to note because the traditional hot plate assumes that behavior displayed on the hot plate is a response to pain, and also that lower latencies to mouthing of the hind paws as well as jumps provides information about the pain. If these two behaviors show an opposite trend with morphine, it may suggest that they should not be grouped together and analyzed in the same way on the hot plate. Also, three of the mouthing behaviors showed an interesting trend in which saline frequencies fell in between the two morphine groups' frequencies. This could suggest that a low dose of morphine has an analgesic affect with respect to saline and a medium dose has a hyperalgesic effect. Although this is possible, previous research has shown morphine to reduce pain more at higher doses than at lower doses.

The traditional latencies agreed with previous research demonstrating that morphine is related to a longer latency before displaying either mouthing of the hind paw or jumping.

Several confounding factors could have influenced the results. For instance, because two different light schedules were used for housing the rats, several rats that had to be moved experienced inconsistent lighting in the week preceding data collection. It is possible that their sleep was affected; sleep and circadian rhythms may influence analgesia. Experimenter error is also a possible confounding variable because the hot plate test requires a significant amount of handling the rats.

Many of the behavior frequencies are significantly different based on dose condition; therefore, despite the possible errors in this study, it is possible to draw some conclusions about behaviors that might be useful to consider when running the hot plate test. In the traditional methodology only the mouthing of one hind paw and jumping are used to determine analgesia. The results from this study suggest that sniffing the corner or standing in the hot plate chamber, jumping, cornerdigging or hind paw lifting might be even more useful behaviors to analyze during the hot plate test.

Some of the behaviors that were more frequent for rats in the saline condition like jumps may not, however, always reflect a reaction to pain. For instance, jumps and corner digging were not consistently displayed by all of the rats in a certain condition. They therefore provide insufficient information for judging the effect of a drug treatment on hotplate behavior. Jumps, in particular, may be misleading because often the rats jumped at a time corresponding to the 30 second trial ended, so they were allowed to escape. In subsequent trials jumping was much more frequent; it seems possible that jumping was associated with getting off of the hot plate, and was therefore elicited more often regardless of the severity of nociception. In the development of a new

methodology, it may be important to note the impact on behavior the negative reinforcement of escape has when a repeated measures paradigm is used.

The graphs reveal a possible pattern that might be useful in developing a repeated measures hot plate methodology (Figures 1-13). The increased behavioral responding as morphine wears off (trial 14, or minute 90 trial) seems more consistent than the initial behavioral response differences between groups. If the behavior frequency decline during the time-course and increase during follow-ups is compared to the overall pattern of behavior of the morphine groups, the effects of a drug may be more evident.

There are more variables that affect hot plate performance than those mentioned here, and many more variables need to be addressed in changing the hot plate methodology. For instance, the panic factor in the traditional method can be alleviated in a repeated measures scenario, so the rat is familiar with the surroundings. Perhaps the learned coping behaviors of repeated measures and the panic factor could both be minimized in a training trial where the rat is placed on a room temperature “hot” plate several time before given a real injection and being subjected to heat. Although the hot plate test is still in need of changes, this initial study provides a basis with which to begin research towards developing a better methodology.

Figure 1.

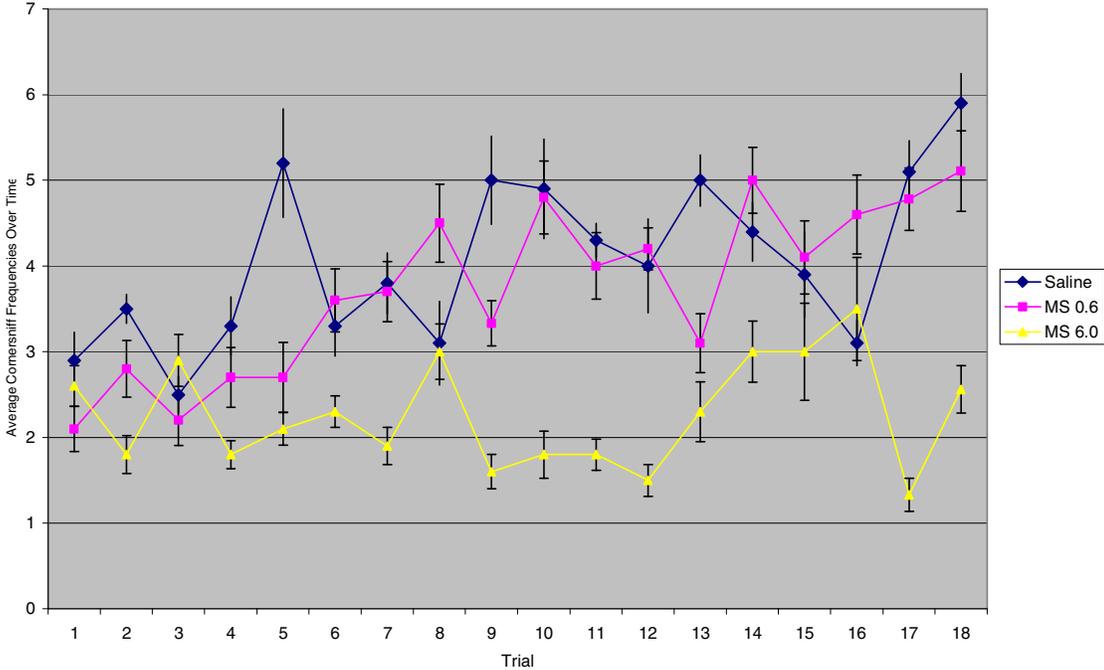


Figure 2.

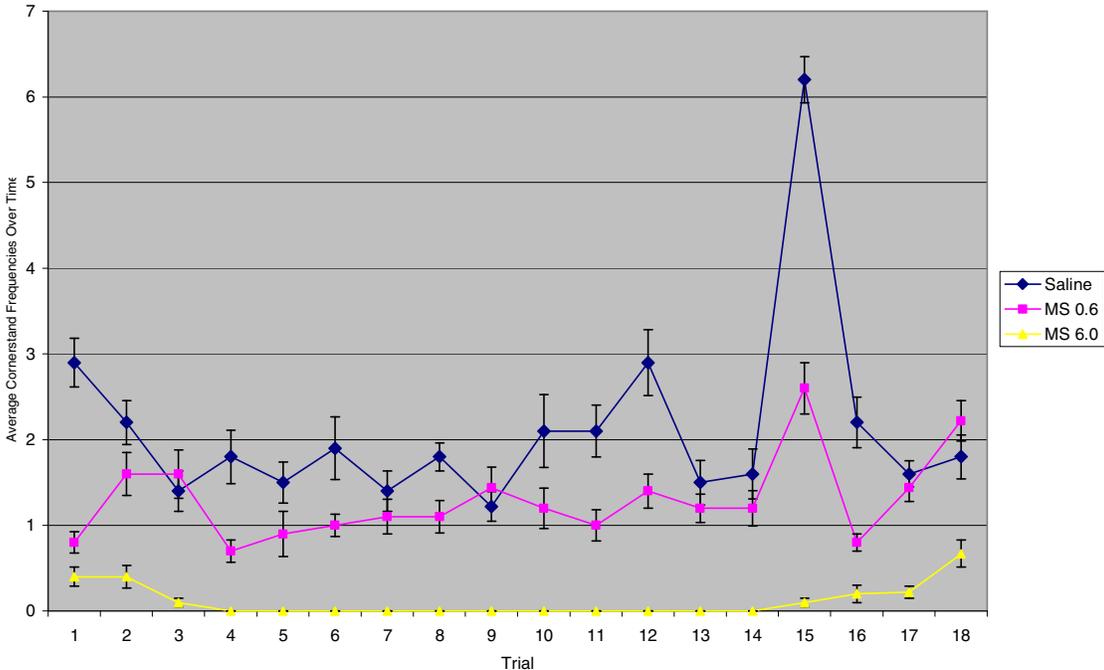


Figure 3.

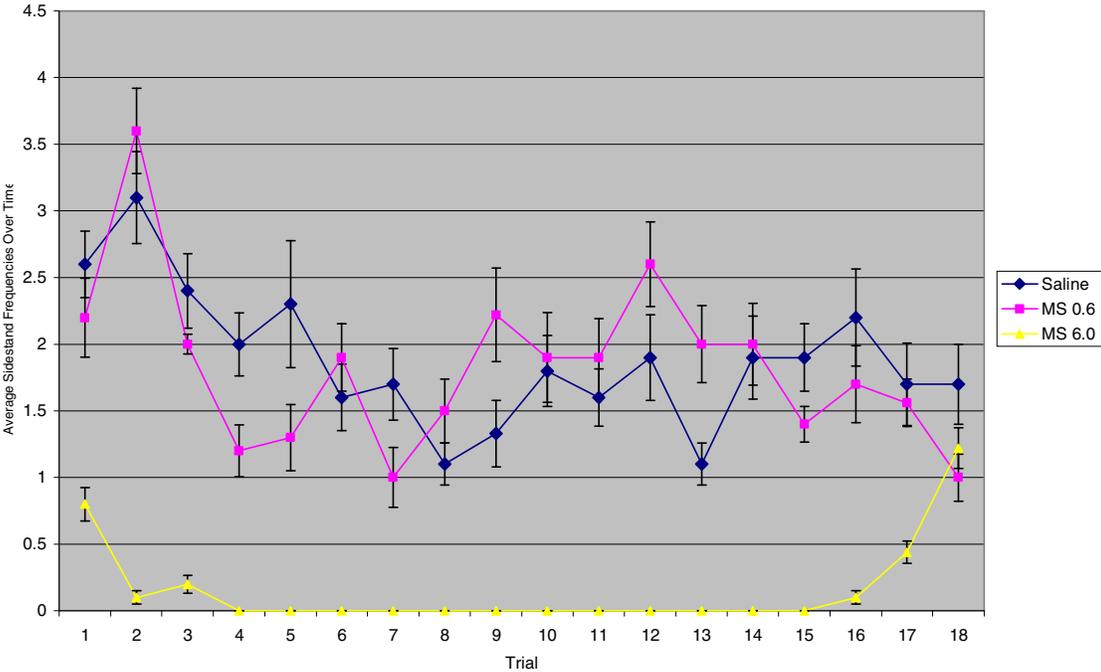


Figure 4.

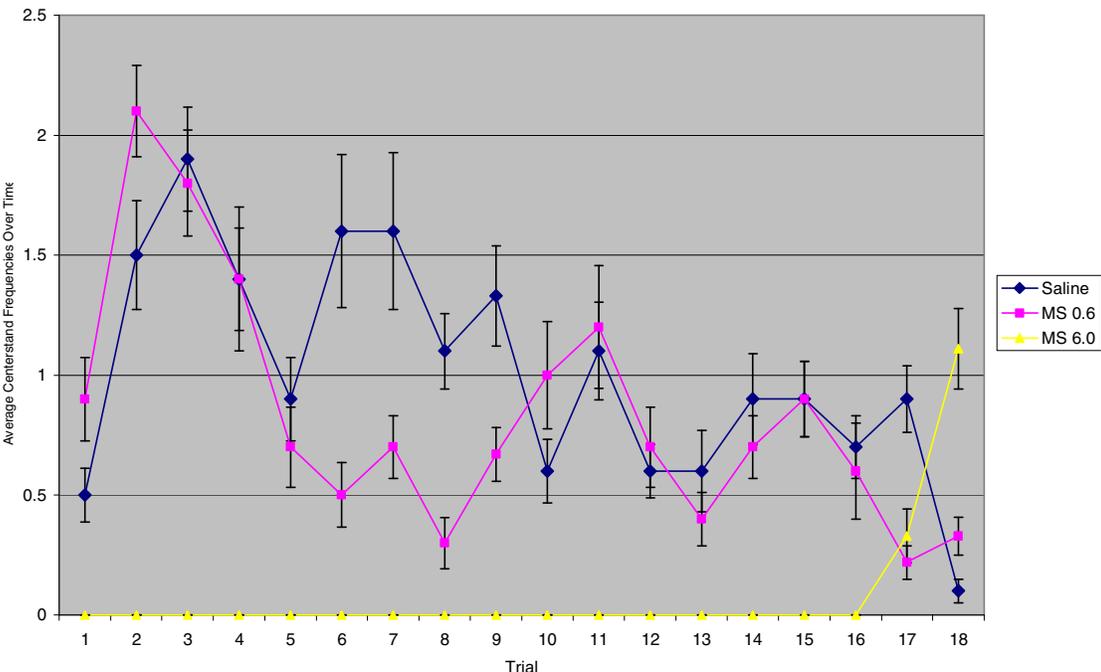


Figure 5.

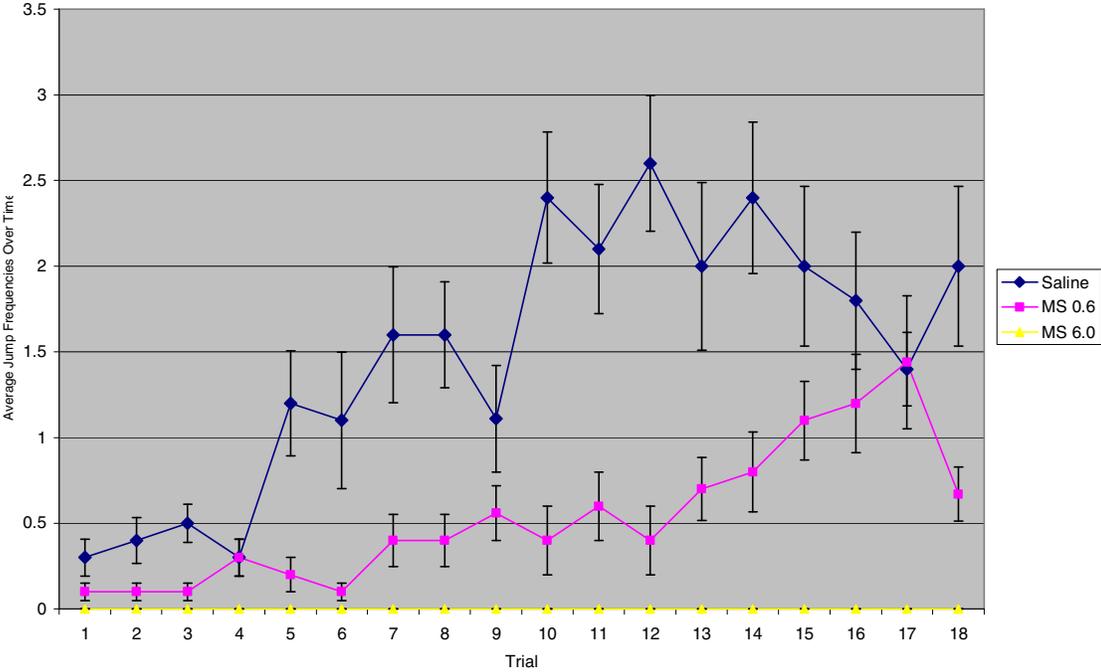


Figure 6.

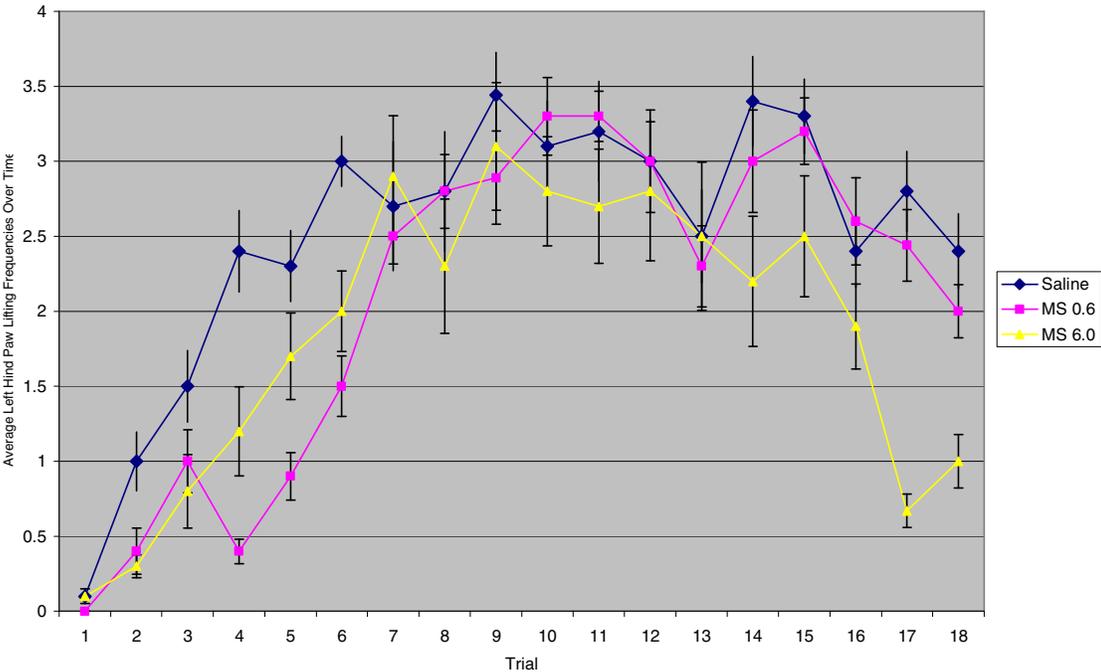


Figure 7.

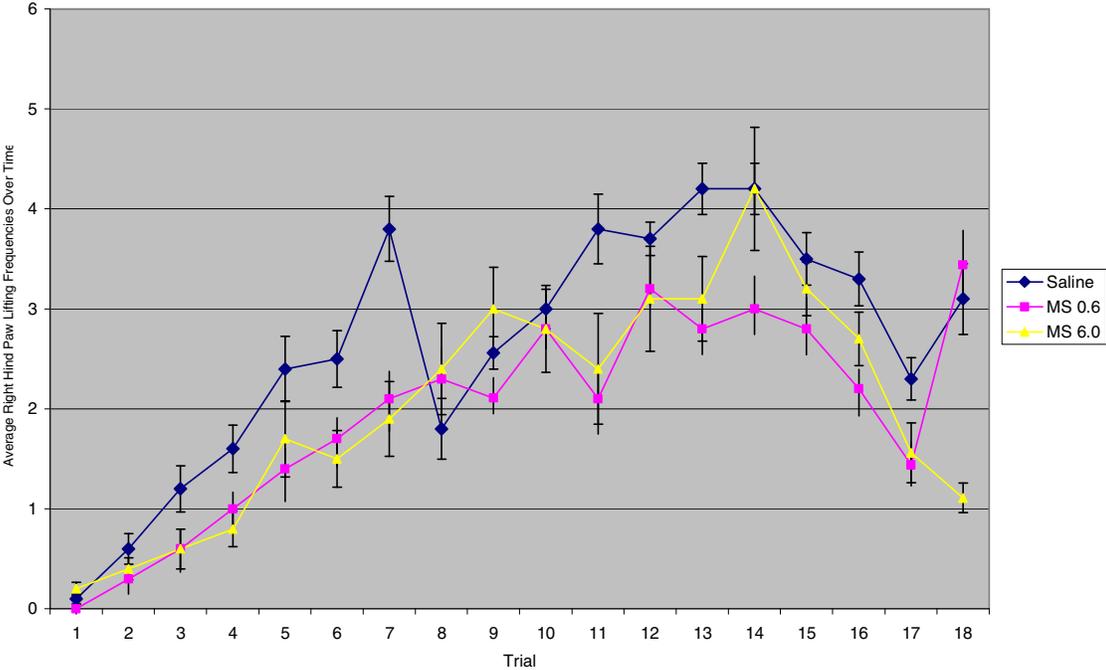


Figure 8.

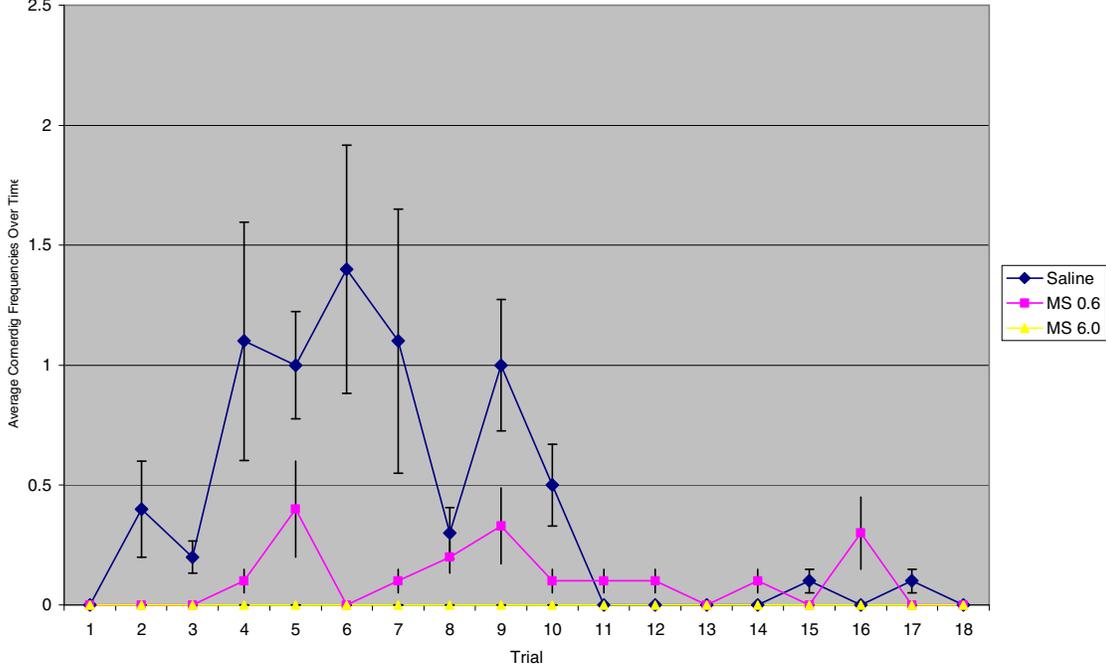


Figure 9.

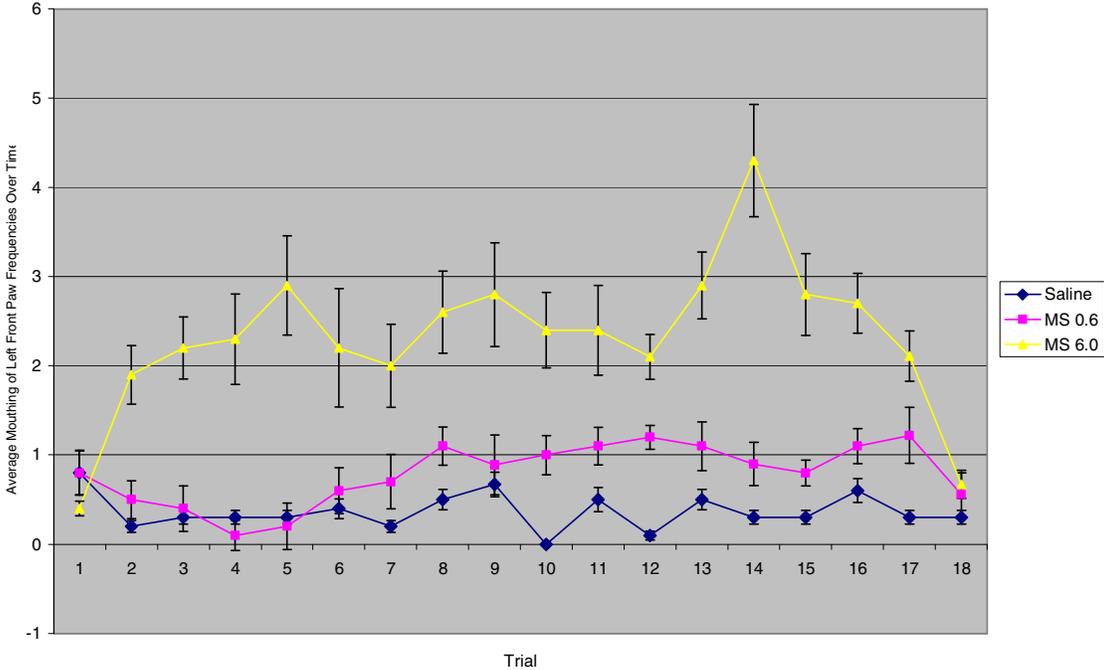


Figure 10.

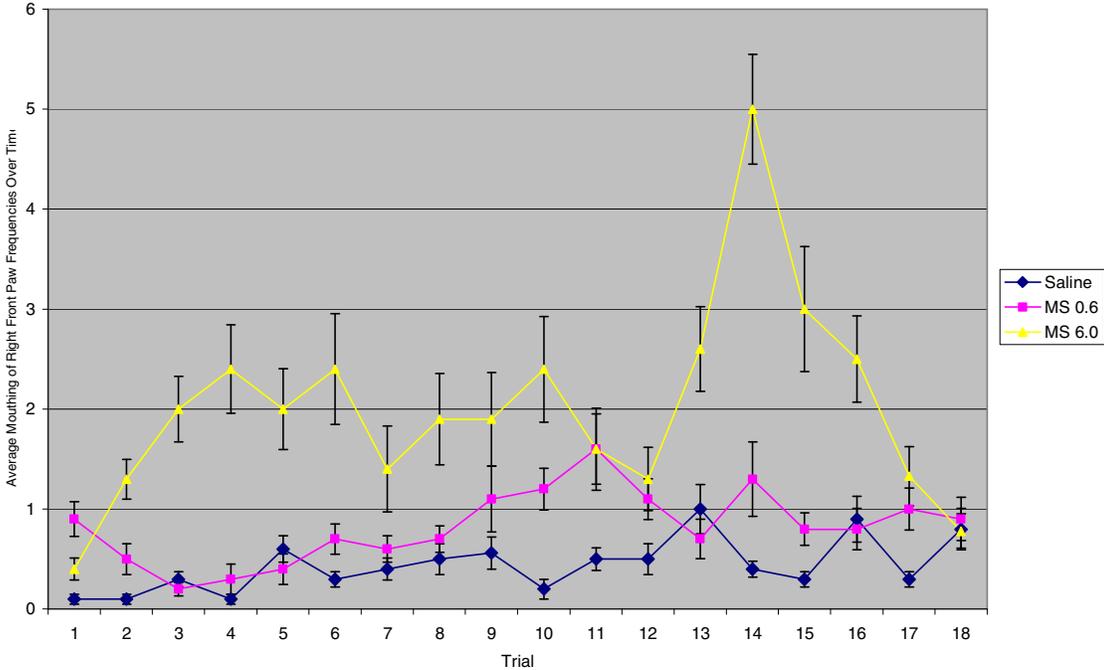


Figure 11.

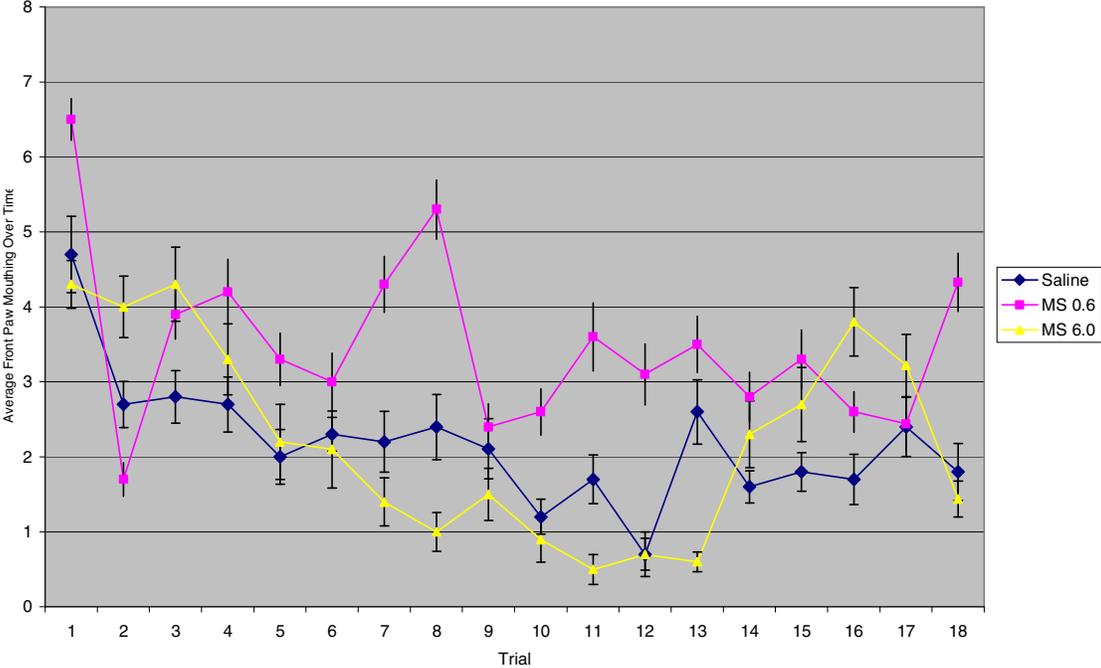


Figure 12.

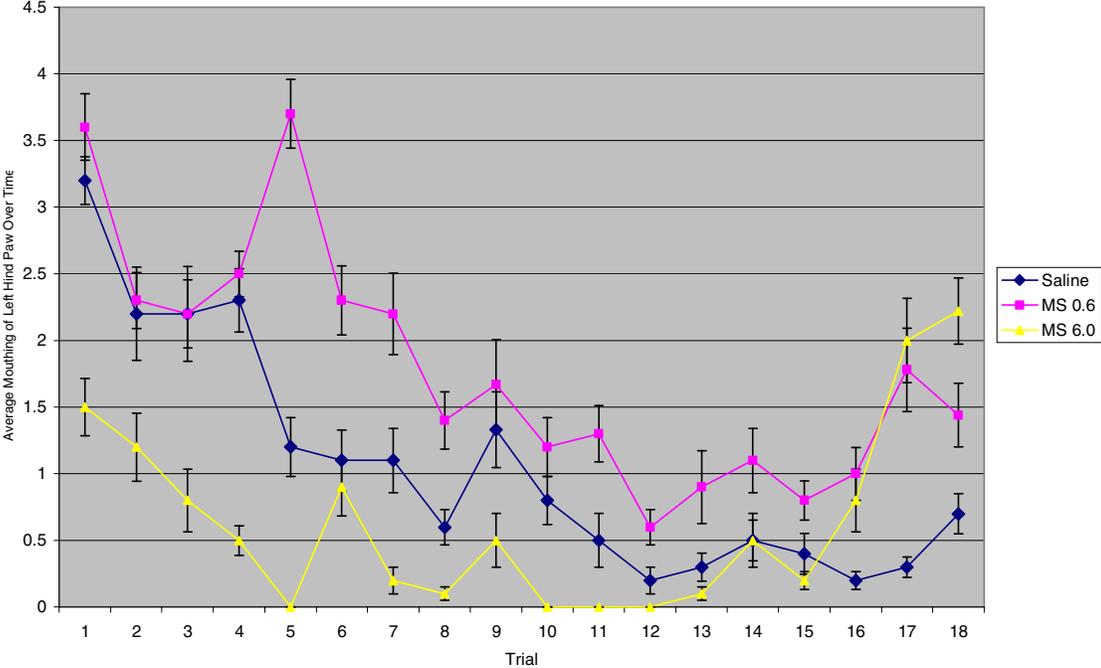


Figure 13.

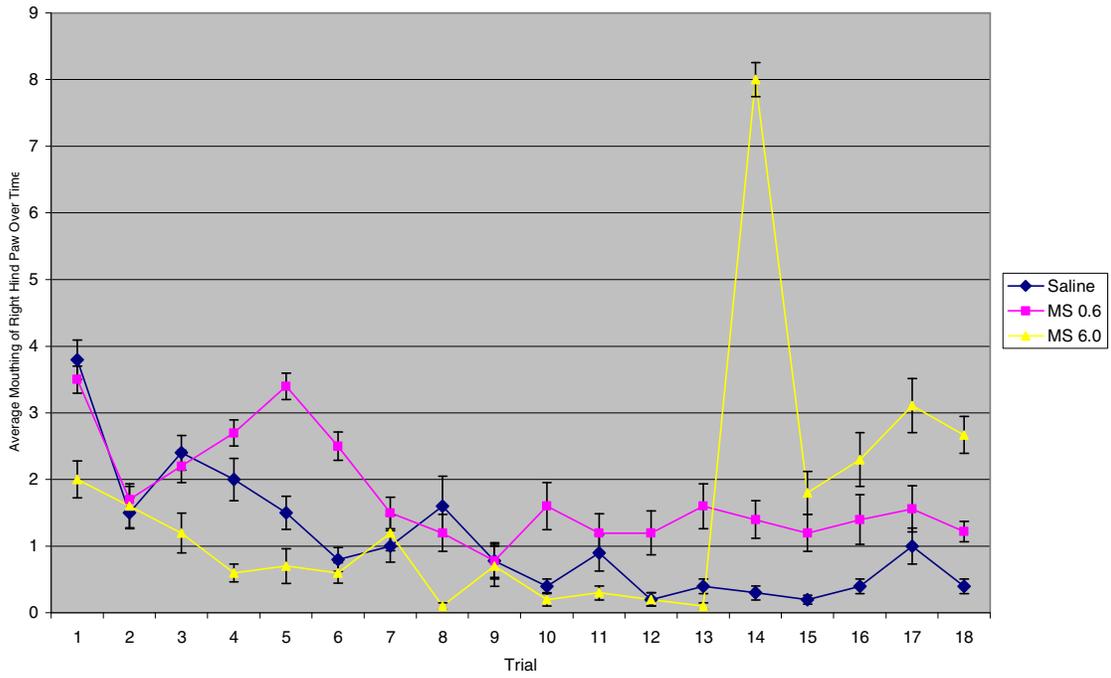


Figure 14.

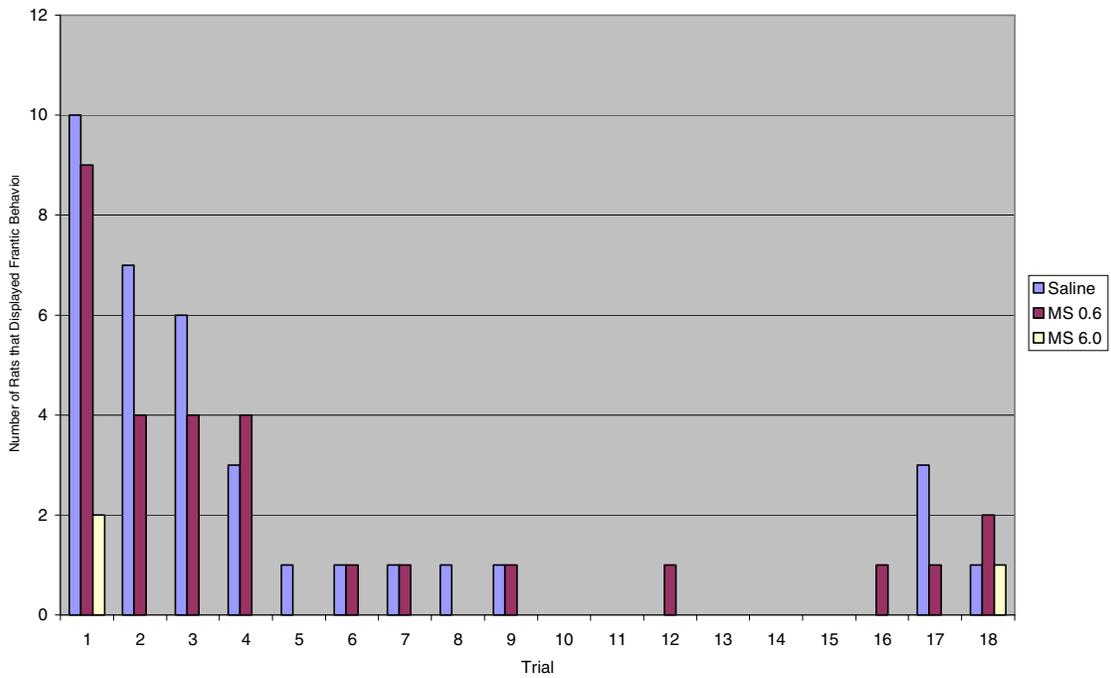


Figure 15.

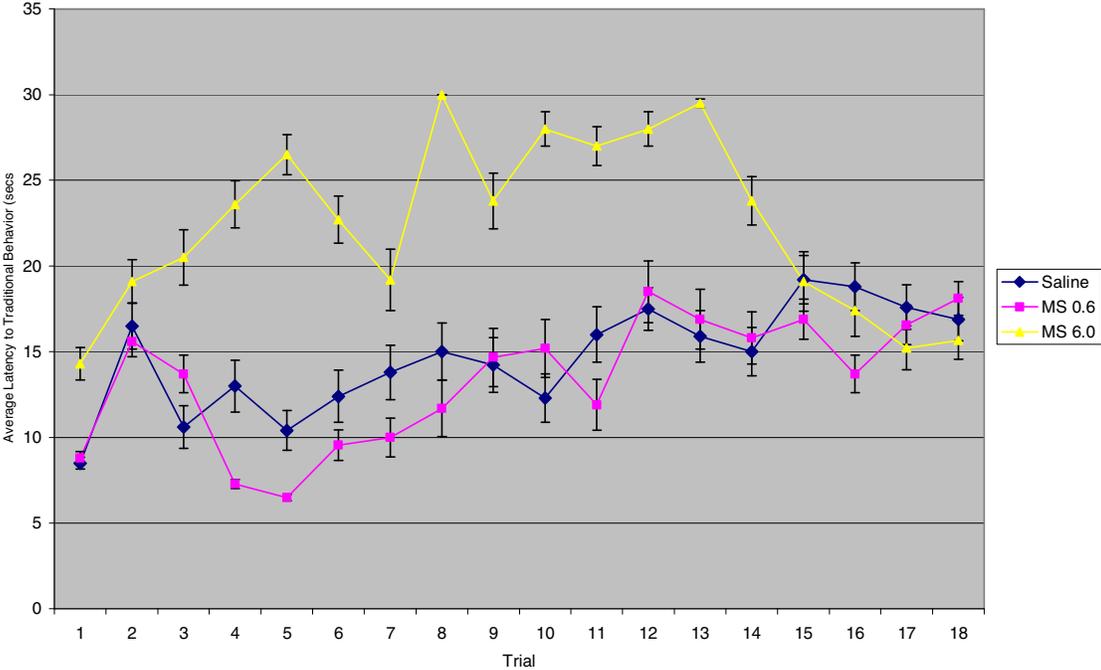


Figure 16.

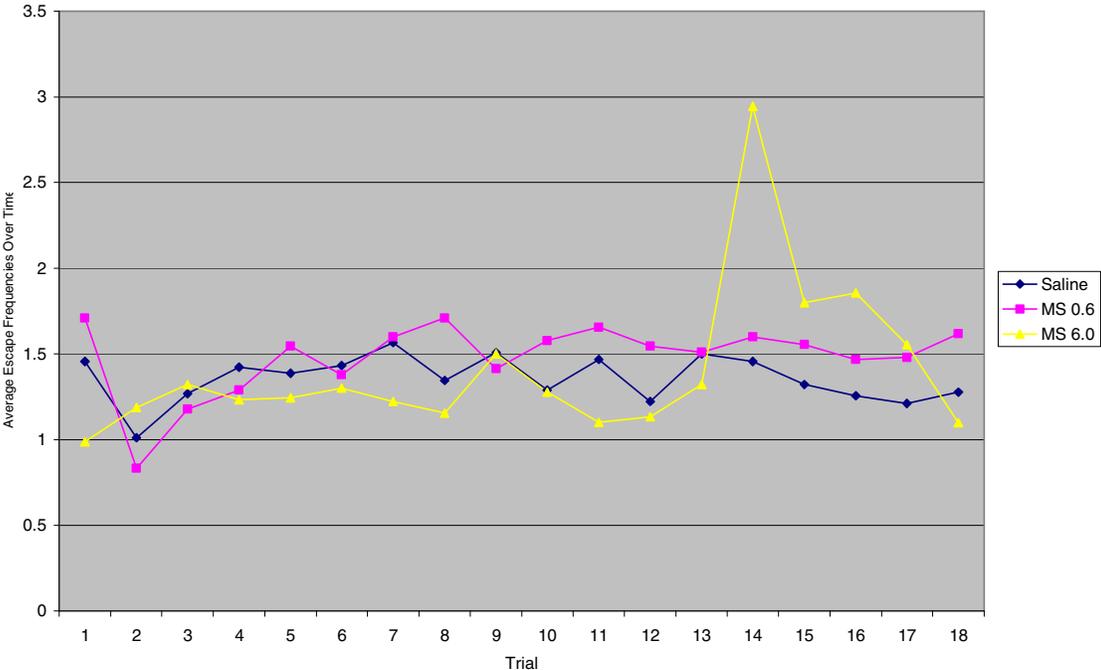
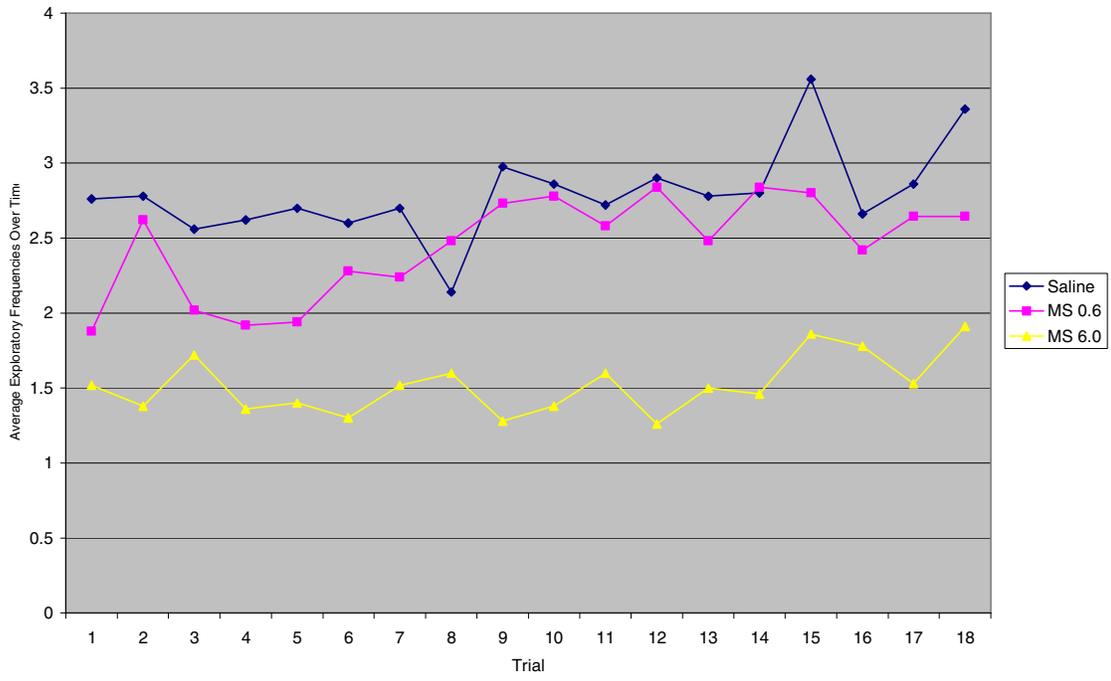


Figure 17.



## Chapter 3

## Experiment 2

Non-steroidal antiinflammatory drugs (NSAIDs) are a group of analgesics that, unlike opioids, do not demonstrate a consistent analgesic effect on the hot plate. Although this could be due to a variety of factors, the results of the previous experiment suggest that the lack of effect demonstrated by NSAIDs may be due to limitations of the traditional hot plate methodology. Traditionally, only hind paw mouthing and jumping behaviors are tracked on the hot plate, but (as demonstrated in the previous experiment) when other behaviors (eg. cornerstanding, hind paw lifting) are tracked, their frequencies are significantly different among rats receiving morphine or saline. The next study was intended to explore the effects of ibuprofen (an NSAID) on hot plate behavior. The results of the previous study suggest that frequency, as well as latency, of a variety of hot plate behaviors is affected by *morphine*. Using the behavioral inventory and the behavioral frequency results gathered in that study, a new methodology focusing on frequencies of certain behaviors was used for analyzing hot plate results gathered by rats treated with ibuprofen.

## Methods

*Subjects*

Sixteen naïve adult male Sprague-Dawley rats were used for this study. The rats were non-systematically divided into two groups, one experimental group, n=8; (that received ibuprofen) and one control group, n=8; (that received saline). The rats had access to food and water ad libitum and were housed two to a cage and maintained on a 12 hour light/dark schedule. Rats were run at two different times of day in order to

accommodate the researcher's schedule, so some of them were housed with light from 7am to 7pm and other received light from 1pm until 1am daily. All procedures were conducted in accord with protocols approved by the Macalester College IACUC.

### *Materials*

The rats were placed on an IITC Inc. Model 39D Hot Plate Analgesia Meter set at  $51.0 \pm 1^\circ\text{C}$ . Since NSAIDs do not traditionally affect hot plate behavior, a higher temperature (than in experiment 1) was used in order to elicit more behavioral responses to noxious heat. All trials took place in a small room illuminated with a red light to control for confounds that could result from the rat seeing the apparatus and anticipating being placed in it, and a timer was used to make sure the intervals between trials were the correct length of time. The trials were videotaped so that the emitted behaviors could be carefully analyzed. The rats received saline or ibuprofen via gavage in a volume of 1ml/kg 30 minutes before the initial hot plate trial. Ibuprofen (20mg/ml) was employed as the NSAID challenge because suggested animal doses were readily available (Ducommun, 2001).

### *Procedure*

Two rats were run at once (alternating trials) in order to expedite the data collection process. They received an assigned dose of either ibuprofen or saline 2.5 minutes apart, and were then placed in the hot plate room in their home cage to acclimate for 30 minutes. In order to account for the possible effects of circadian rhythms, rats on the earlier light schedule were given the drug or saline near 1:00pm, and the rats on the later light schedule were given the drug or saline around 6:00pm. Before each trial, the

video camera was turned on, but was not left on between trials. Each trial lasted 30 seconds, and following the 30 minute period after the injection, there were trials conducted at zero, five, ten, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes. These trials were intended to assess changes in hot plate behavior due to learning and to the effects of the drug over time. After this initial time-course, four more trials were conducted, at 90 minutes, 120 minutes, 150 minutes, and 240 minutes; these trials were used to assess the longer term effects of learning and the effects of the drug wearing off. Between each of the trials, the hot plate was cleaned to control for effects of scents left in the hotplate chamber. Between each of the follow-up trials, rats had access ad libitum to food and water. After the minute 240 trial, rats were returned to their home room. Any unusual behavior or problems were recorded on a datasheet for each rat.

#### *Data Analysis*

After all 16 rats had been run, the videotaped sessions were viewed and frequency of the behaviors was recorded for each trial. The frequencies were analyzed using ANOVA.

#### Results

After collecting all of the data from the tapes, the behavioral frequencies for each of the behaviors described in the behavioral inventory were analyzed. Operational definitions for all of the behaviors analyzed can be found in the previous section (chapter 2). The graphs (18-26) show trends in behavioral frequencies during the timecourse; each data point represents the average of the eight rats in that condition. Frequencies for each trial of the rats in a single condition were combined. ANOVA revealed that several behaviors had significantly different frequencies for rats who received ibuprofen than for

saline rats. Four of these behaviors were displayed significantly fewer times by rats who received ibuprofen than by control rats. The graph in Figure 18 shows the average number of times that the eight rats in each condition displayed the cornerstand behavior. The saline rats displayed consistently more cornerstand behaviors during the timecourse,  $F(1,270)=14.28$ ,  $p<.001$ . Figure 19 shows similar data for sidestand behaviors. The saline rats again displayed more sidestand behaviors than the ibuprofen-treated rats in most of the trials,  $F(1,270)=9.52$ ,  $p<.001$ . The graph of average jumping behavior during the timecourse (Figure 20) shows drastically greater frequencies for saline rats than for the ibuprofen-treated rats,  $F(1,270)=52.92$ ,  $p<.001$ . Both groups of rats displayed very few jumps during the first and second trials, and the ibuprofen treated rats maintained this low frequency while the control rats displayed much more jumping behavior over the time. Lifting of the left hind paw was also displayed more in the control rats,  $F(1,270)=8.58$ ,  $p<.005$ ; however, frequencies of lifting of the right hind paw were not different between the groups (Figures 21, 22).

Although several behaviors were exhibited more often by the saline-treated rats, other behaviors, specifically mouthing behaviors, were displayed more frequently by the rats that were administered ibuprofen. The average frequency of left hind paw mouthing was greater in experimental rats than in control rats throughout the whole time course,  $F(1,270)=27.70$ ,  $p<.001$  (Figure 23), and as shown in Figure 24, the average frequency for right hind paw mouthing was greater in experimental rats in all but one trial,  $F(1,270)=28.06$ ,  $p<.001$ . Although mouthing of each front paw individually was not significantly different between groups (data not shown), the average frequency of

simultaneously mouthing both front paws was greater in ibuprofen-treated rats in all but two trials,  $F(1,270)=13.75$ ,  $p<.001$  (Figure 25).

The latencies to displaying a traditional hot plate behavior (jump or hind paw mouthing) were also determined for each trial; if the behavior was not displayed, a cut off value of 30 seconds was used. During the first few trials this latency was similar; however, during the majority of the timecourse the latency for saline treated rats was longer than for ibuprofen rats,  $F(1,270)=7.06$ ,  $p<.01$  (Figure 26). During the minute 90 trial, trial 14, the average latency for the ibuprofen-treated rats was longer than for saline rats.

Figure 18.

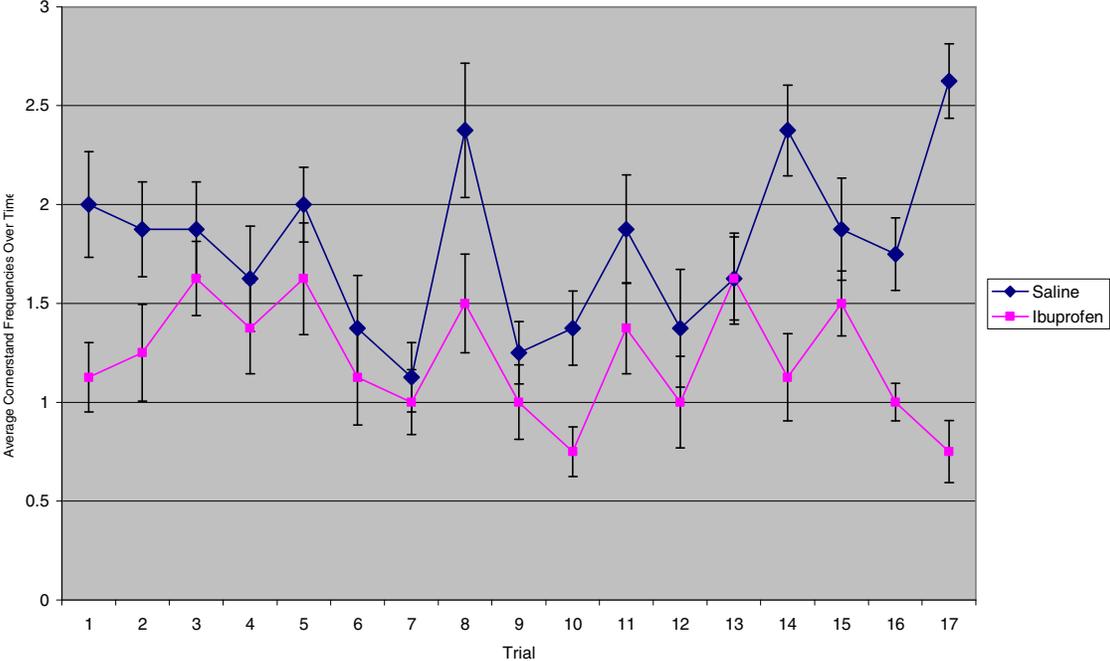


Figure 19.

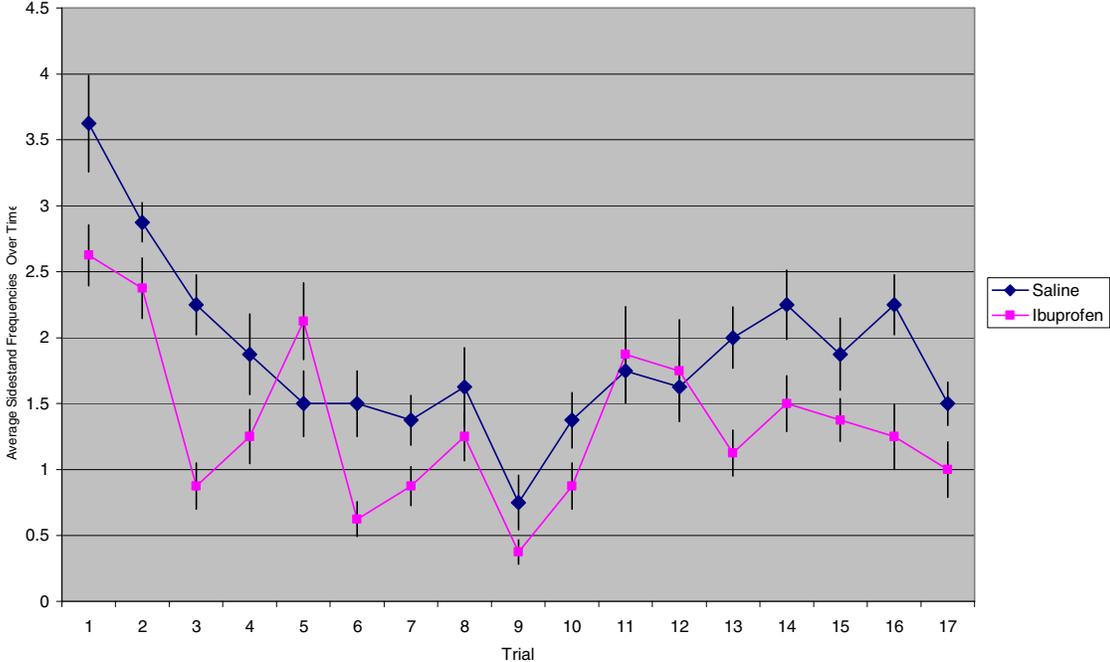


Figure 20.

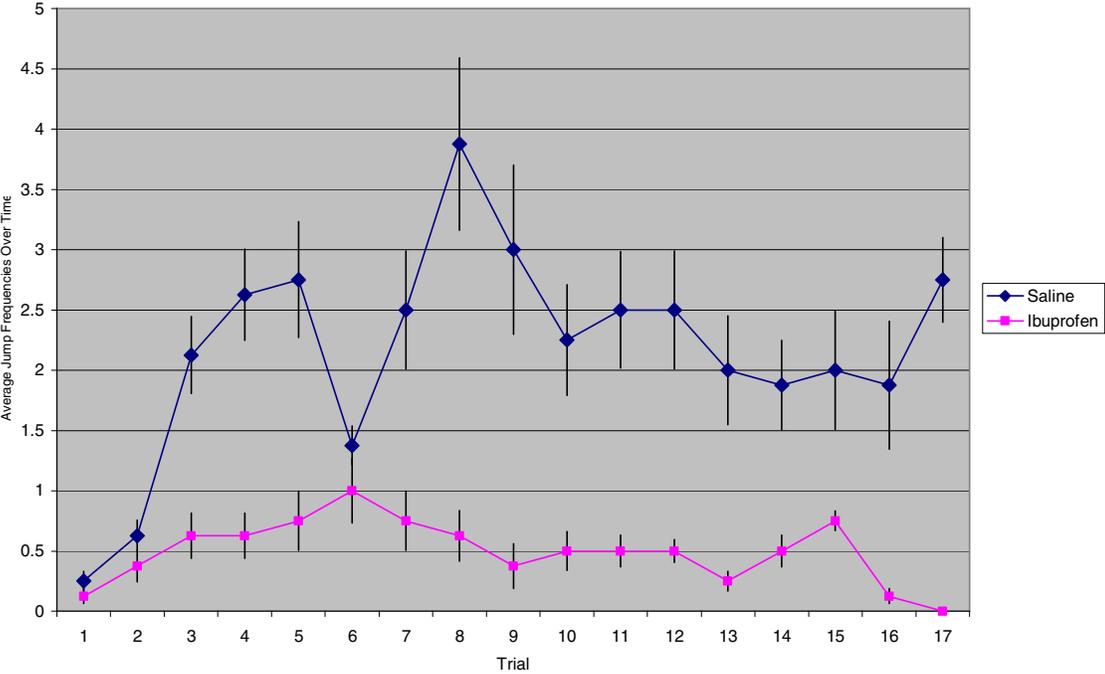


Figure 21.

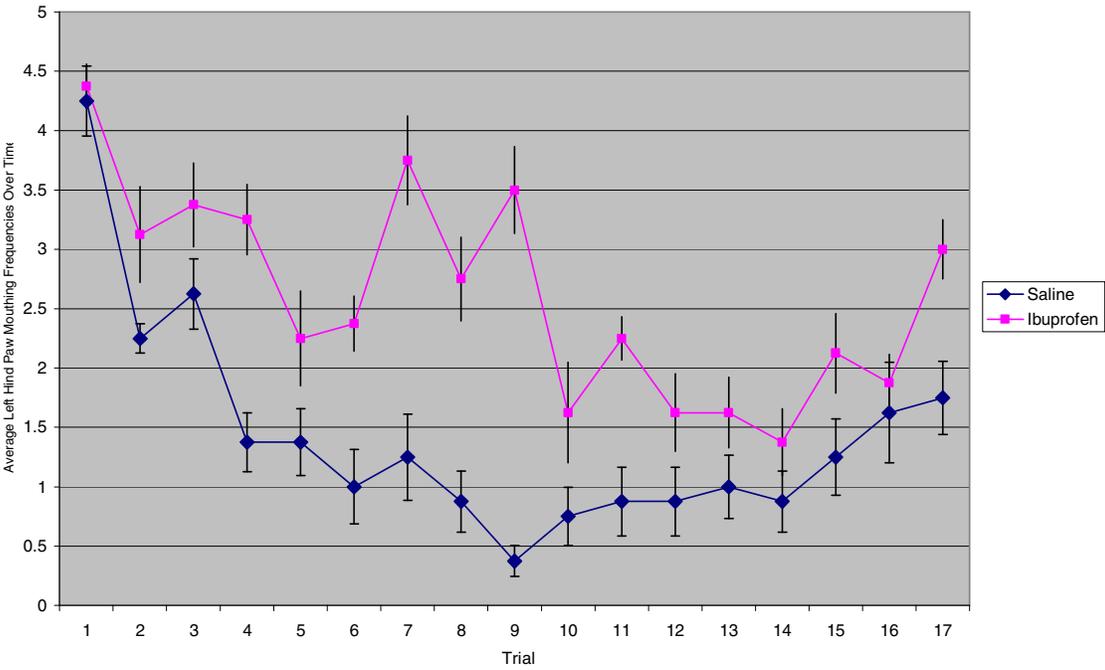


Figure 22.

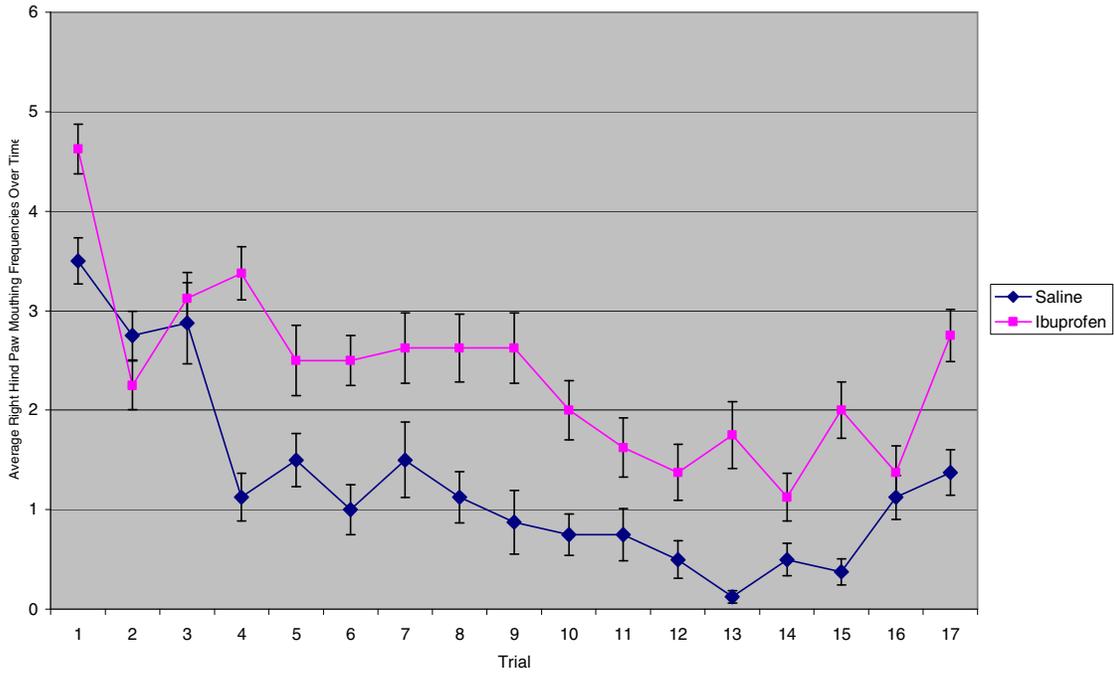


Figure 23.

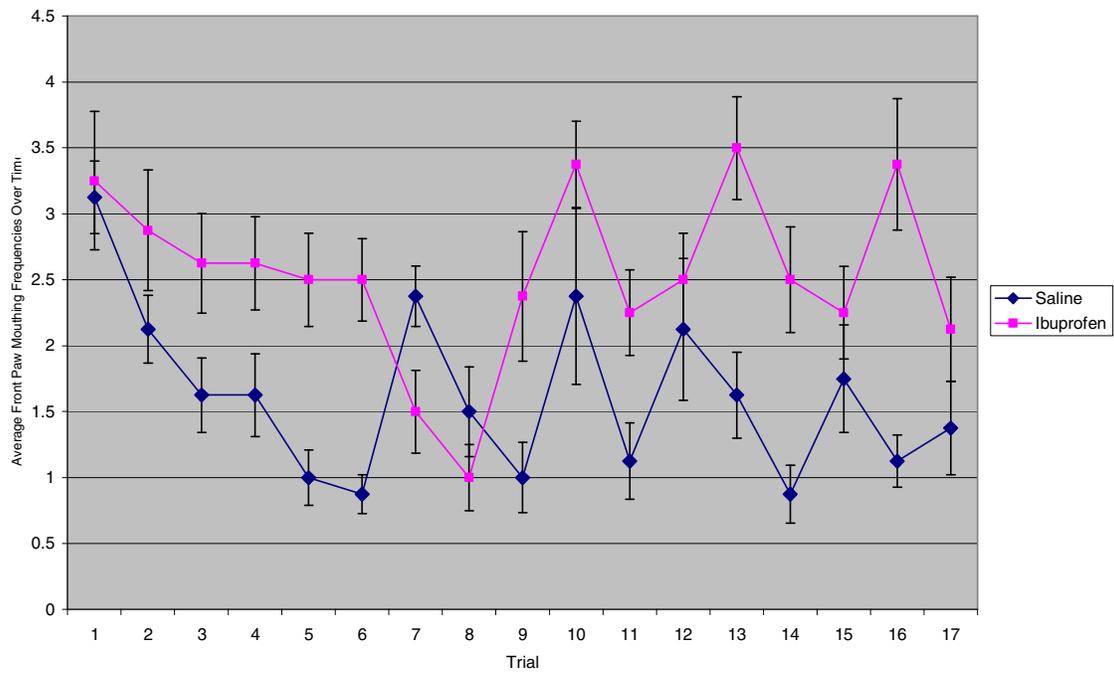


Figure 24.

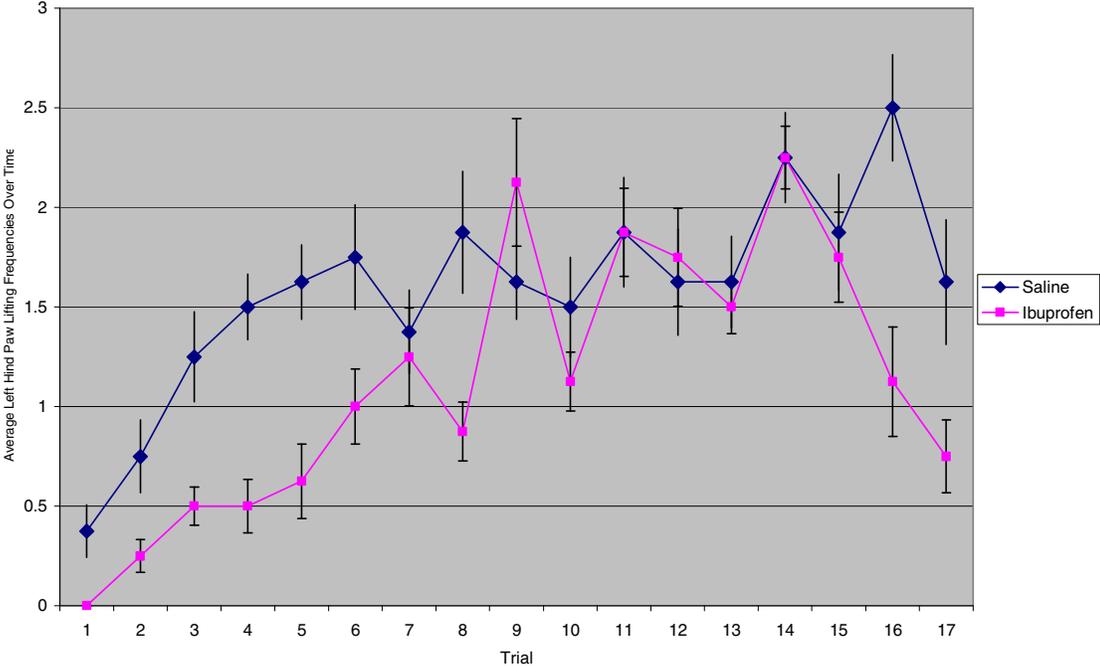


Figure 25.

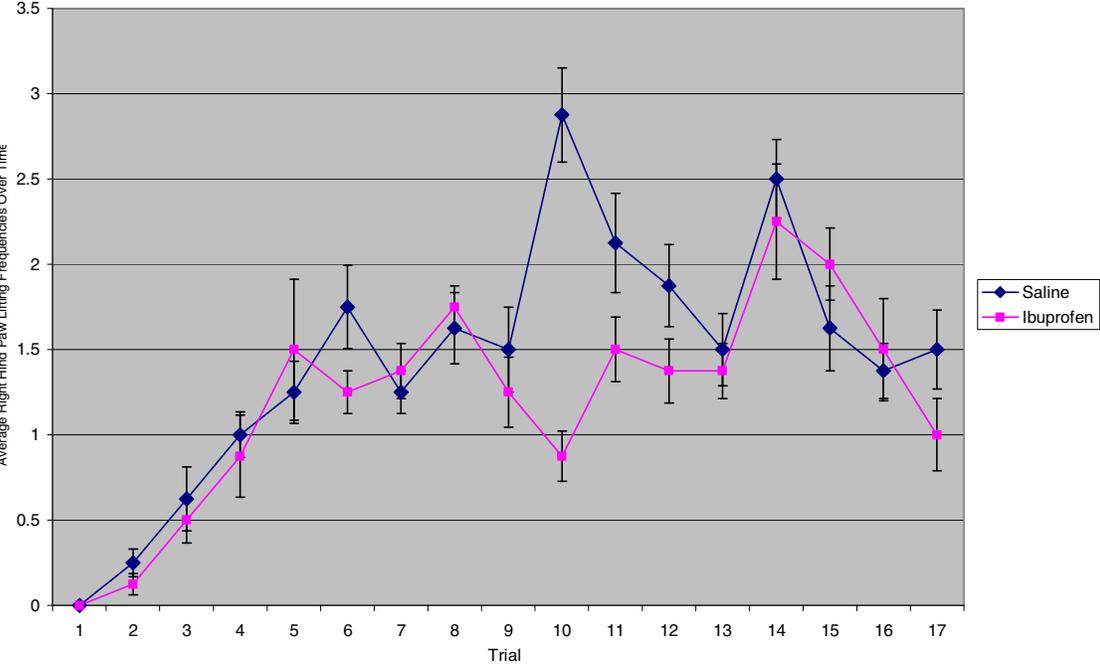
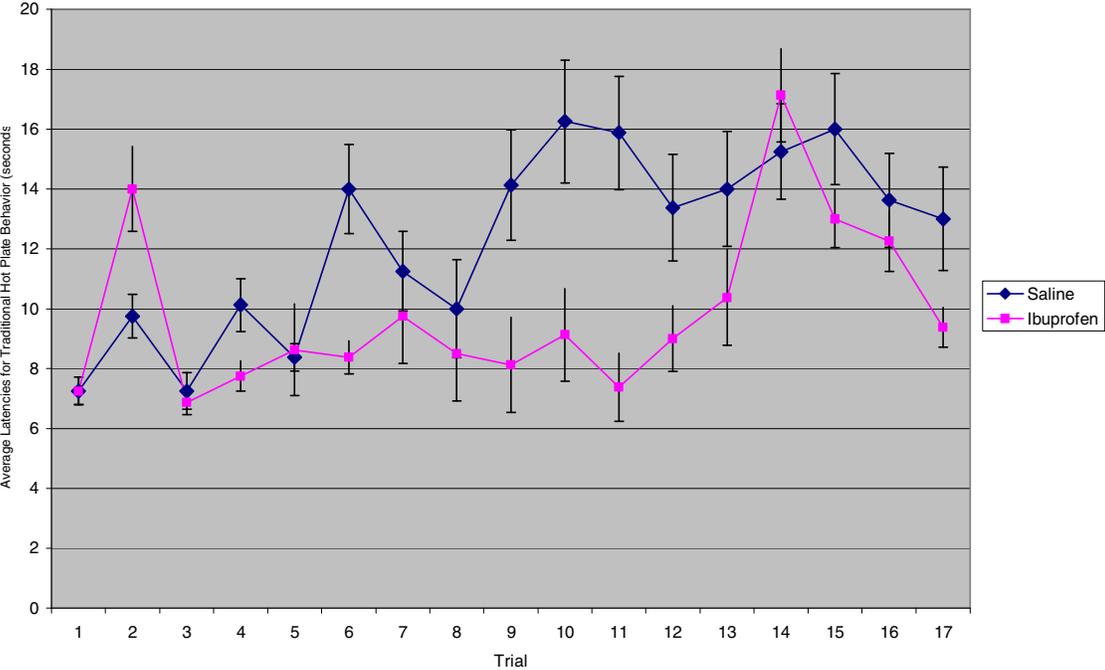


Figure 26.



## Chapter 4

## General Discussion

The traditional hot plate, one of the central tests used to assess pain treatments, fails to yield significant results for the analgesia caused by non-steroidal antiinflammatory drugs, despite the robust demonstration of the effect of opioids it yields. There are numerous possible causes for this inconsistency; these fall into two major categories. First is the possibility that NSAIDs do not actually provide any analgesic effect for acute thermal pain in rats or mice. However, this is unlikely based on the significant results found on Hargreaves and modified hot plate paradigms (Lavich, et al., 2005); these results show a reduced hyperalgesic response to acute thermal stimuli. Alternatively, methodological problems with the current hot plate paradigm might be affecting the measurement of analgesia such that the effects of NSAIDs are lost. Because of the extensive experimental evidence of NSAIDs' analgesic properties in humans, in addition to the research on NSAIDs' ability to suppress hyperalgesia in animals, this study focused on the methodological issues related to the traditional hot plate test.

The hot plate test, unlike the tail flick test (another acute thermal pain test), does not traditionally include baseline measurements of pain. The justification for this is that the rats might learn a certain behavioral response to the hot plate setting, and thus, subsequent trials on the hot plate might be affected by this learning. For this reason, the hot plate traditionally uses naïve rats, and for a single trial. Although this ensures that no previous learning will influence behavioral responses, it also allows for additional confounds such as anxiety about the novel situation that could interfere with the actual nociception experienced by the rats.

The quantitative measure of nociception employed by the hot plate is the latency to either mouthing of a hind paw or jumping. As discussed previously, latency to a certain behavior as a measure of pain is characteristic of pain tests developed earlier in pain research. Later, when chronic pain became a focus of pain research, frequencies of behavior (eg. flinching during the formalin test) became a more common measure of pain. Unlike latency, frequency may provide some clue to the magnitude and persistence of pain. Although the latency measure works well for morphine on the hot plate, it does not reveal significant differences between rats treated with saline and rats treated with NSAIDs.

The purpose of the current study was to utilize a different, more recently established quantitative measure of pain, behavioral frequency, to analyze hot plate behavior. The measure of frequency was intended to allow the examination of previously unrecognized, differences in behavior exhibited on the hot plate between rats treated with saline and rats treated with an NSAID.

In order to establish some basis for frequencies of behaviors displayed by rats on the hot plate, a behavioral inventory was developed, examining the pain-related behaviors of rats given morphine or a saline vehicle. However, in addition to measuring traditional hot plate behavior latencies, frequencies of several other behaviors were tracked in a repeated-measures paradigm. Most of these behaviors, with the exception of mouthing behaviors, yielded significant results with the expected trend that rats administered the medium dose displayed the fewest behaviors and saline-treated rats displayed the most. This trend was expected, as presumably the behaviors displayed on the hot plate are seen in response to the noxious thermal stimulation and provide information about the

nociceptive experience of the rat; rats treated with an analgesic should therefore display fewer behaviors or different behaviors unrelated to pain. The results obtained for the mouthing behaviors were less expected, and suggested that higher doses of morphine actually result in more mouthing responses.

The results of the behavioral inventory study provided evidence for several possible modifications to the hot plate paradigm. First, it suggested that the effects of analgesics are different for mouthing and jumping behaviors. If their frequencies change in the opposite directions with the same doses of morphine, their presence in hot plate behavior may reflect differential responses to nociception or heat. This follows well from previous research that has shown very different “analgesia” measures for rats treated with the same drugs but analyzed with latencies for mouthing or mouthing and jumping (Carter, 1991). Although grouping these two behaviors yields the expected latency results for morphine, it is possible that the test was developed to work with certain analgesics and may not have been based on behaviors that actually reflect analgesia.

Another interesting trend in the behavioral inventory that could be considered in modifying the traditional hot plate methodology is the “rebound” effect seen during the follow up trials. During the last five trials, the medium dose rats often displayed an increase in behavioral frequencies compared to during the first hour of the time course (Figures 3, 4, 7, 9, 10, 11, 12, 13, 16). This suggests that as the morphine wears off, the rats with the medium dose experienced a hyperalgesic effect because they had not been experiencing as much nociceptive stimulation in the subjective sense during the first hour, due to the analgesic effect of morphine. This pattern of responding may be useful in analyzing the effects of other treatments for pain. Even if latencies and preliminary

frequencies are similar between groups, a subsequent increase in behavior after a drug begins to wear off could suggest that it had analgesic properties. In the absence of the analgesia, a perceived hyperalgesia could cause increased responding.

Another possible conclusion from the results of the behavioral inventory is that morphine generally suppresses behavior, with the exception of mouthing, so the “analgesic” effect is actually a result of all behaviors being suppressed. Clearly if there is motor inhibition as a result of opioid analgesics, the latency to specific behaviors are likely to be longer. This is unlikely, however, due to the extensive research on opioids and their accepted analgesic effect in the hot plate.

The behavioral inventory results also suggest that instead of jumping and mouthing, other behaviors might be more useful to detect analgesia. Specifically, cornersniffing and standing should be considered. Interestingly, when the behaviors were divided into exploratory (sniffing and standing) and escape (jumping, mouthing, lifting and cornerdigging) behaviors the exploratory behaviors were the ones noticeably suppressed by a medium dose of morphine (Figure 17). Perhaps these behaviors should be used instead of, or in addition to, mouthing and jumping when analyzing hot plate results.

This last possibility was explored in the second study presented here. The new behaviors defined in the behavioral inventory that had demonstrated significantly different frequencies among the different doses and saline in the first study were used to analyze the hot plate behavior of rats treated with ibuprofen. Several of the exploratory behaviors followed the same frequency pattern expected of morphine; the rats who received the analgesic drug, in this case ibuprofen, displayed the behaviors significantly

less than the saline-treated rats. These behaviors included jumping, cornerstanding, and sidestanding. Both morphine and ibuprofen, two very different drugs, suppressed jumping and exploratory behaviors; the fact that the drugs have different methods of action and different side effect profiles, suggests that this behavioral suppression may be due to analgesia. Although this effect must be studied in more detail, it seems like a hot plate methodology that analyzes these behaviors may provide more information about analgesia caused by such different treatments.

Interestingly, as in the first study, mouthing behaviors followed a different pattern. In addition to again suggesting that mouthing and jumping behaviors should not be grouped together for behavioral analysis, this presents another similarity between morphine and ibuprofen's effects on the hot plate. One possible explanation for the unexpected frequencies of mouthing behavior is that mouthing is a response to heat instead of pain. If this were the case, it would make sense that rats who received the analgesic drugs displayed more mouthing behavior than saline rats. The saline rats presumably experienced more intense nociception, and if there was any behavioral competition between responses to pain versus responses to heat, the latter might be suppressed.

Despite the similarities between the behavioral frequency effects of the two drugs, the latency to traditional hot plate behaviors was inconsistent between drugs. Morphine was related to longer latencies than saline, and ibuprofen was related to shorter latencies than saline. This result follows from previous research that has claimed that NSAIDs do not demonstrate an analgesic effect, like morphine does, on the hot plate. However, the similarities in behavioral frequencies between the two drugs suggest that latency

measures, not NSAIDs, themselves are the reason for the lack of significant effects from NSAIDs on the traditional hot plate test.

The traditional latency measure for the second study suggests a possible problem with the results of this study. During trial 14, a much longer latency was observed for the ibuprofen group than during the other trials. Trial 14 corresponds to the minute 90 trial; after the rats had been on the hot plate every five minutes for an hour, ibuprofen had a noticeable effect on the latency. Perhaps this was due to inflammation caused by the exposure to the hot plate; ibuprofen may have had a greater impact on this type of pain than on the acute, nociceptive thermal pain caused in the previous 13 trials. As mentioned above, this study addressed the possibility that the routine lack of significant effects associated with the use of NSAIDs on the hot plate in the literature is due to a methodological problem. The data for trial 14 could be interpreted to suggest that, in fact, affect acute, nociceptive pain, and this could be the reason behind insignificant NSAID effects on the hot plate.

The results of this study may also have been affected by experimental error. This includes the effects of being handled, as well as irregular lighting schedules. The rats that were maintained on the later light/dark schedule, from 1pm to 1am, were occasionally exposed to light during their dark hours so that the animal facility staff could care for them; this was minimized as much as possible.

The results of the first study reported here suggested several different ways of analyzing hot plate behavior that might allow significant effects of NSAIDs to be detected. The second study only addressed two of these: that more behaviors should be analyzed, and that frequency, not latency should be the focus of analysis. In addition to

further exploring other behaviors on the hot plate, further research on the rebound effect shown by the medium-morphine-dose rats, and possible overall behavioral suppression of morphine is needed to modify the traditional hot plate methodology so that it is a more reliable pain assessment. The rebound effect displayed by many of the rats treated with the medium dose of morphine during the follow up trials may have significant implications for determining the magnitude of the analgesia experienced during the initial timecourse.

This study provided evidence for several non-traditional hot plate behaviors that were performed at significantly different frequencies by rats treated with either saline or an analgesic drug. However, future examination of these behaviors and other behaviors performed on the hot plate is necessary before a new methodology can be definitely established.

Figure Caption

\*All trial numbers correspond to minutes when rats were run (ie. Trial 1 corresponds to Minute 0, Trial 2 corresponds to Minute 5, Trial 17 corresponds to minute 240).

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