2014

Modeling Nitroglycerin-Induced Migraine in Rats

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MODELING NITROGLYCERIN-INDUCED MIGRAINE IN RATS

by
Stephanie Marie Staszko

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2014

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DEDICATION

I dedicate this thesis to my family and friends, without whose support I could not have completed this work. Thank you for encouraging me through the difficult times – all of my victories are greater when being shared with you.
ACKNOWLEDGEMENTS

I would like to acknowledge the Sally McDonnell Barksdale Honors College and Dean Michael Allen for their financial support of this project. Additionally, I would like to acknowledge Dr. Kevin Lewellyn for assistance with drug preparations, graduate student Rachel Davis for assistance with data analyses, and the graduate students and research assistants of the psychopharmacology laboratory for their generous assistance throughout the experimental process.
The present research sought to determine whether nitroglycerin (NTG) produced changes in clinically relevant endophenotypes of migraine. Rats were given a single injection of NTG or vehicle with the following dependent measures recorded: Rat Grimace Scale, hot and cold tail flick latency, Rotor-Rod performance, and photophobia and movement in traditional and modified light/dark boxes. NTG increased rat grimace scores but did not produce thermal alldynia nor photophobia. Further, NTG produced paradoxical increases in Rotor-Rod performance and movement. These results demonstrate that a single injection of NTG does not produce behaviors that parallel clinical symptoms of migraine.
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1. Introduction

Migraine is a debilitating headache disorder caused by the inflammation of the nerves and blood vessels of the brain (World Health Organization, 2012). Migraine is recognized as one of the most common neurovascular disorders, with epidemiological studies reporting an overall prevalence of 12% in adults of Western countries (Lipton et. al, 2007). Additionally, gender influences the development of migraine, with approximately 18% of women and 6% of men suffering from migraine (Lipton et. al, 2007). Migraine is one of the most costly forms of headache: the World Health Organization reports 25 million working days are lost per year in the United Kingdom due to migraine (World Health Organization, 2012).

Migraine is a complex disorder that can be broadly categorized as migraine with aura and migraine without aura and may present as a variety of subtypes (Ferrari, 1998). Migraine without aura is more common, forming 75% of the migraine population (Ferrari, 1998). Aura consists of visual, sensory, or aphasic symptoms that typically onset prior to the migraine attack (Ferrari, 1998). Migraine may further be classified as episodic, with patients experiencing less than 15 headache days a month, or chronic, with patients experiencing 15 or more headache days a month for at least 3 months (Katsarava, Buse, Manack, & Lipton, 2012).

Migraine symptoms include moderate to severe head pain that is typically unilateral in nature and characterized by a pulsing sensation, lasting from hours to days
Migraine presents with a variety of associated symptoms, which include nausea and/or vomiting as well as sensitivity to light (photophobia), sound (phonophobia), and exacerbation of symptoms with movement (Goadsby, Lipton, & Ferrari, 2002). Although not a diagnostic criterion, many migraine patients also experience cutaneous allodynia, or the perception of pain in response to non-noxious stimuli (Silberstein, 2004). Additionally, to meet the criteria for migraine with aura, patients must experience at least one neurological aura symptom lasting from 4-60 minutes, with a migraine attack following or accompanying the aura (Ferrari, 1998).

The current understanding of the pathophysiology of migraine is limited, but significant advances have been made in recent years. Originally, the vascular theory of migraine dominated the field. The primary mechanism of migraine was believed to be vasodilation of cerebral and extracranial blood vessels (Bigal, Ferrari, Silberstein, Lipton, & Goadsby, 2009). However, migraine is now identified as a multifaceted neurological disorder, characterized by vasodilation, central sensitization, and cortical spreading depression (Dodick & Silberstein, 2006; Bigal et al, 2009).

It is hypothesized that migraine pain is caused by the sensitization of nociceptive pathways (Dodick & Silberstein, 2006). Sensitization occurs when, over time, a neuron requires a less intense stimulus to reach threshold potential (Dodick & Silberstein, 2006). Peripheral sensitization of the dural and meningeal nociceptors leads to increased firing on target neurons in the trigeminal nucleus caudalis, causing the hyperexcitability and sensitization of those neurons (Dodick & Silberstein, 2006). Cortical spreading depression is a wave of depolarization that moves across the cortex at a rate of 6 mm/minute, which results in neural suppression (Bigal et al, 2009). Cortical spreading
depression is believed to be responsible for the presence of aura in some migraine patients (Bigal et al., 2009). Activation of the trigeminal nucleus caudalis initiates the release of inflammatory neuropeptides, causing the meningeal vasodilation thought to be responsible for the throbbing pain characteristic of migraine (Bigal et al., 2009).

A variety of treatment options exist for migraine attacks, but the most common acute treatment is pharmacological intervention with triptans. Triptans are widely used due to their selective activity as well as their well-established safety and efficacy (Goadsby et al., 2002). Triptans are 5-HT₁B/₁D receptor agonists and were originally hypothesized to cause vasoconstriction via 5-HT₁D receptors on intracranial vessels, in accordance with the vascular theory of migraine (Silberstein, 2004). More recent evidence suggests, however, that triptans activate inhibitory 5-HT₁D receptors, inhibiting neuronal transmission by sensitized peripheral nociceptors (Dodick & Silberstein, 2006). Although sumatriptan is one of the most widely prescribed acute treatments for migraine, a meta-analysis of 53 clinical trials revealed that sumatriptan has a response rate of only 59% (Ferrari, Roon, Lipton, & Goadsby, 2001). The lack of efficacious treatments for migraine may be due to a lack of comprehensive animal models.

A valid animal model can accomplish a variety of goals, including serving as a screening assay, which can correctly identify efficacious and non-eficacious compounds; a biobehavioral assay, which focuses on uncovering the underlying physiology of a disorder; or a simulation, which mimics behavioral responses that accompany a disorder (Willner, 1991). A valid model of migraine could be used to identify novel mechanistic targets for treatment as well as to determine the efficacy of novel therapeutic compounds. Current migraine models are based on the findings that in humans nitroglycerin (NTG)
induces headache in non-migraine participants and migraine in migraine patients (Iverson, 2001). NTG is proposed to serve as a nitric oxide (NO) donor, causing vasodilation and activating pathways involved in nociception (Iverson, 2001). Existing literature of animal models of NTG migraine examines NTG-induced allodynia.

A study in mice by Bates et. al investigated the effects of varying NTG doses on thermal and mechanical allodynia, as well as reversal of allodynia by sumatriptan (Bates et. al, 2010). This study was designed based on the knowledge that NTG triggers headache in humans and that many migraine patients experience thermal and mechanical allodynia (Bates et. al, 2010). Mice were injected IP with NTG at doses of 0.5, 1, 5, or 10 mg/kg then administered sumatriptan or saline either IP (300 µg/kg or 600 µg/kg) or intrathecally (0.06 µg in 5 µL) (Bates et. al, 2010). Thermal allodynia was evaluated using the Hargreaves assay 30, 60, 90, 120, and 240 minutes post-NTG injection (Bates et. al, 2010). The 5 and 10 mg/kg doses produced a significant effect for thermal allodynia at 90 and 120 minutes that was reversed by sumatriptan (Bates et. al, 2010). Collectively, this study identified doses of NTG that produce mechanical and thermal allodynia and identified these measures as behavioral endpoints for a model of migraine (Bates et. al, 2010).

Valid animal models of clinical syndromes are based on disease-specific endophenotypes, which are a variety of observable behavioral, biochemical, endocrinological, and neuroanatomical characteristics that are related to a syndrome or disorder (van der Staay, 2006). In humans, migraine is typified by head pain, photophobia, and the exacerbation of symptoms by movement. Additionally, although not a diagnostic criterion, migraine may occur with peripheral allodynia. If the NTG
model is a valid model of migraine, these features should be observable in the model. The present research attempts to validate the NTG model of migraine by administering a single injection of NTG and observing responses on assays corresponding to behavioral endophenotypes of the human clinical syndrome. This includes the quantification of: (i) pain, using the Rat Grimace Scale; (ii) thermal allodynia, using a thermal tail flick test; (iii) photophobia, using traditional and modified light/dark boxes; and (iv) movement differences, using a Rotor-Rod as well as traditional and modified light/dark boxes.
2. Materials and methods

2.1. Subjects and housing characteristics

Adult male Sprague Dawley rats were obtained from Harlan Laboratories (Indianapolis, Indiana, USA). Animals were housed in 13.34 cm x 20.96 cm x 22.38 cm cages with two animals per cage. Food (Teklad 7001, Teklad Diets, Madison, WI, USA) and water were made available ad libitum via a wire cage top throughout the study. Room temperature was maintained at 22 +/- 4°C and overhead florescent illumination was maintained on a 12/12-h light/dark cycle (lights on at 7:00 am). All procedures were approved by the University of Mississippi IACUC (protocol number 13-023).

2.2 Drugs

Nitroglycerin (NTG) was obtained from Copperhead Chemical Company Inc. (SDM®27, Tamaqua, PA, US). Saline was chosen as the vehicle based on reports that injections comprised of saline, alcohol, and propylene glycol did not display different effects than injections of saline alone (Pradhan, Smith, McGuire, Tarash, Evans, & Charles, 2014). The concentration and dosing of the NTG solution were selected based on previous studies by Costa et. al (2005) and Pradhan et. al (2014). NTG was
diluted to a concentration of 5 mg/mL with propylene glycol. NTG or saline was injected IP at a volume of 2 mL/kg leading to a final dose of NTG of 10 mg/kg.

2.3. Behavioral collection assays

2.3.1. Rat Grimace Scale

Rats were placed in 31.12 cm x 21.59 cm x 26.04 cm Plexiglas chambers with wire bottoms for the Rat Grimace Scale (Sotocinal et. al, 2011). Rats were individually photographed in these chambers using a digital camera at 60, 90, and 120 minutes post-injection. A dry-erase board containing the date, subject number, and photograph time point was placed behind the apparatus for photo identification. The apparatus was wiped down with a Clorox wipe between animals. Rats were returned to their home cage between photographs. Photographs were scored by two trained research assistants blind to the treatment condition on four facial “action units”: (i) orbital tightening, or the apparent “squeezing” shut of the eye; (ii) nose/cheek flattening, or the disappearance of a characteristic crease between the cheek and whiskers; (iii) ear changes, or the outward folding and curling of the ears; and (iv) whisker changes, or the bunching, forward movement of whiskers (Sotocinal et. al, 2011). Rats were scored individually on each of these four units, with a score of 0 denoting the absence of the feature, a score of 1 denoting a “moderate” appearance or uncertainty of presence of the feature, or a score of 2 denoting obvious appearance of the feature (Sotocinal et. al, 2011).
2.3.2. Tail flick test

Both hot and cold tail flick tests were performed. For hot tail flick test, water was added to a 250 mL Erlenmeyer flask and water temperature was maintained at a temperature of 46°C +/- 0.1°C using a hot plate (Harvard apparatus). For cold tail flick test, water was added to a 8.5 cm x 18 cm glass cylinder to a depth of 14 cm. Crushed ice was added to maintain a temperature of 15°C +/- 0.1°C. One day prior to testing, all rats’ tails were marked with a black felt tip maker 5 cm from the tip of the tail. Additionally, rats were acclimated to a terry cloth towel used to restrain the rat during testing by gently wrapping the rat in the towel and holding it above the apparatus for 40 seconds. On test day, the rat was wrapped gently in a terry cloth towel, its tail submerged to the 5 cm mark, and a timer started. Latency to tail flick was recorded in seconds, with a test cut-off of 40 seconds. A “tail flick” was defined as a prominent curling or flicking in an obvious attempt to remove the tail from the water. Rats were then removed from the towel and placed in the traditional light/dark box.

2.3.3. Traditional light/dark box

The light/dark box measures 40 cm x 40 cm x 20 cm in total area, and is divided into two compartments: light and dark. Each compartment measured 20 cm x 20 cm x 20 cm. The light chamber was composed of Plexiglas and the dark chamber was composed of black polycarbonate. A 10 cm x 10 cm opening allowed movement between the two chambers. Sliding lids over each compartment allowed experimenters access into the
apparatus. The apparatus was placed on a solid metal sheet covered with pine chip bedding (Alfapet, St. Louis, MO, USA). Rats were placed in the dark compartment facing away from the entrance to the light compartment, initiating the 5-minute test session. The time spent in the light compartment and number of entries into the light compartment were recorded.

2.3.4. Rotor-Rod

A Rotor-Rod (San Diego Instruments, San Diego, CA, USA) was used with a rat drum (diameter = 6.99 cm). The door of each lane was covered with black poster board to darken the apparatus. Rats were given three 60-second training sessions one day prior to testing. For both training sessions as well as test day, the ramp speed increased by 15 rotations/min every 15 seconds for 45 seconds, to reach a maximum speed of 60 rotations/min. Animals were tested for 5 minutes and latency to fall was recorded.

2.3.5. Modified light/dark box with movement

A conditioned place preference apparatus (Model# MED-CPP-013, Med Associates Inc., St. Albans, VT, USA) was modified to create a light/dark box with automated movement measures. The clear Plexiglas lids of the gray and black chambers were covered with black poster board to create a dark environment. Two days prior to testing, rats were acclimated to the modified light/dark box and data was not collected. For acclimation, rats were placed in the gray chamber and allowed to acclimate for 30
seconds, the guillotine doors were manually lifted, and rats were allowed to explore the apparatus for 5 minutes. One day prior to testing, a baseline for total movement and time spent in the light box was collected for each rat using the procedure listed above. On test day, the same procedure was used. Total time spent in the “light box” (white chamber), total time spent in the “dark box” (gray and black chambers), and total movement throughout the apparatus were recorded with vendor supplied software.

2.4 Procedure

Two experimental groups (N=20) included rats (248 – 281 g) that received either a control saline injection or an NTG injection. Rats were injected then transported to a procedure room for the Rat Grimace Scale. Photographs were taken 60, 90, and 120 minutes post-injection. After taking the last photograph, the rat was transported to a second testing room and tested on the behavioral assays in the following order: hot or cold tail flick test (counter-balanced for order effects), traditional light/dark box, remaining tail flick test, and the Rotor-Rod. Following the Rotor-Rod, rats were transported to the original test room and were tested using the modified light/dark box. Following the modified light/dark box, rats were returned to their home cage.

2.5 Statistical analyses

All data was analyzed using SPSS software. Data for the Rat Grimace Scale was analyzed for percent agreement between raters prior to conducting any additional
statistical analyses. Data collected by two observers produced a percent agreement of 86.1%. Rat Grimace Scale data were then analyzed using a two-way repeated measures analysis of variance (ANOVA) and simple effects ANOVAs were conducted. All other data was analyzed using independent t-tests. Significance for all tests was set at p < .05.
3. Results

The effects of NTG on Rat Grimace Scale score were examined at three time points and are summarized in Figure 1. Vehicle rats minimally displayed grimace behavior throughout the test session, while NTG rats show a mild increase in grimace behavior. The main effect for treatment approached statistical significance $F(1, 18) = 2.78$, $p = .086$. The main effect for time was not significant $F(2, 36) = 2.78$, $p = \text{n.s.}$ Further, the treatment by time interaction was not significant $F(2, 36) = 0.035$, $p = \text{n.s.}$ Post-hoc analyses failed to reveal significant differences between treatment groups at any time point.

The effects of NTG on tail flick latency in the cold and hot thermal tests are summarized in Figure 2A and Figure 2B, respectively. NTG produced no differences in tail flick latency for either temperature manipulation. T-tests of these data failed to reveal significant group differences $t(18) = 0.450$, $p = \text{n.s.}$, $t(18) = 1.261$, $p = \text{n.s.}$, respectively.

The effects of NTG on time spent in the light box of the traditional light/dark box are summarized in Figure 3. NTG produced no differences on time spent in the light box. A t-test of these data failed to reveal significant group differences $t(18) = 0.812$, $p = \text{n.s.}$

The effects of NTG on number of entries into the light box of the traditional light dark box are summarized in Figure 4. NTG produced no differences in number of entries. A t-test of these data failed to reveal significant group differences $t(18) = 0.367$, $p = \text{n.s.}$
The effects of NTG on latency to fall in the Rotor-Rod test are summarized in Figure 5. Compared to vehicle rats, NTG rats displayed a longer mean latency to fall. A t-test of these data revealed group differences that approached significance $t(18) = 2.493$, $p = .059$.

The effects of NTG on time spent in the light box of the modified light dark box are summarized in Figure 6. NTG produced no differences in times spent in the light box. A t-test of these data failed to reveal significant group differences $t(15) = 1.671$, $p= n.s.$

The effects of NTG on movement in the modified light dark box are summarized in Figure 7. Compared to vehicle rats, NTG rats moved more throughout the apparatus. A t-test of these data revealed a significant treatment effect $t(15) = 3.957$, $p = .022$. 
4. Discussion

The purpose of this study was to determine if a single NTG injection in rats produced the expression of behavioral endophenotypes that accurately model the symptom expression of clinical migraine. Rats were tested 60, 90, and 120-minutes post-NTG or saline injection on the Rat Grimace Scale and 2-hours post-injection on hot and cold tail flick, traditional light/dark box, Rotor-Rod, and modified light/dark box.

NTG produced a modest increase in rat grimace score that remained stable across the test session. To our knowledge, this is the first reported use of the rat grimace scale on NTG-invoked pain. Previous research shows inducing pain with Complete Freund’s Adjuvant, Kaolin/Carrageenan, or laparotomy surgery produces grimace scores ranging from 0.6 to 1.1 (Sotocinal et. al, 2011). In the present research, NTG injection produced a grimace score of 0.207. Collectively, these findings suggest NTG migraine is a less severe pain state than these traditional pain models as measured by the rat grimace scale.

NTG did not produce thermal allodynia on the hot or cold tail flick tests. This contradicts previous findings that NTG induces thermal allodynia in mice (Bates et. al, 2010). These differences may be due to differences in the assays used to assess the presence of thermal alldynia. Previous research utilized the Hargreaves assay, a test involving supraspinal nociceptive processing, while the current research utilized water bath tail flick tests, which assess spinal nociceptive reflexes (Dirig, Salami, Rathbun,
Ozaki, & Yaksh, 1997). Our results suggest NTG may have a greater effect on supraspinal nociceptive processing than spinal nociceptive reflexes.

Increased activity exacerbates migraine symptom severity in humans (Goadsby et. al, 2002). To our knowledge, this is the first reported use of Rotor-Rod to measure changes in movement induced by NTG. In contrast to our predictions, NTG increased the amount of time on the Rotor-Rod. We hypothesize that this outcome may be due to a confound of the paradigm itself. The Rotor-Rod apparatus is designed to use the fear of falling into a dark space as a motivational factor to keep rats moving on a rotating drum. The present findings suggest that Rotor-Rod may be more sensitive to measuring NTG-induced anxiety rather than changes in movement. Interestingly, anxiety is highly comorbid with migraine (Wang, Chen, & Fuh, 2010). Future research may examine the relationship between NTG-induced migraine and anxiety.

Both a traditional light/dark box and modified light/dark box were used to examine the effects of NTG on movement and photophobia. NTG did not affect photophobic behavior or movement in the traditional light/dark box. Contrary to our hypothesis, NTG increased movement in the modified light/dark box. To our knowledge, this is the first attempt to quantify these clinically relevant symptoms of migraine.

We hypothesize our results reveal limitations of the single NTG injection as a valid model of migraine. In humans, migraine is a recurring condition in which the individual learns what triggers and exacerbates migraine by experiencing multiple episodes. A single NTG-induced migraine episode does not provide opportunity for this
learning component. Thus, animals have no motivation to avoid light or move less during NTG migraine episodes. A single NTG migraine may increase motor activity because animals are attempting to escape the pain state. Based on the human clinical population, one would predict multiple episodes of migraine should generate responses on these clinically relevant endpoints.

One limitation of the NTG model may be the agent used to induce migraine. Migraine is physiologically complex, yet NTG only induces cortical vasodilation, one of the many physiological conditions responsible for migraine. Therefore, if the NTG model were to be validated, it would only be valid for a subset of migraineurs.

Another limitation, but a major finding of this study, is that a single migraine episode does not appear to affect relevant behavioral indices that would be expected in a valid model of migraine. This is not surprising, considering clinical diagnosis requires 5 migraine episodes. We suggest that multiple injections of NTG may yield alterations in these relevant clinical endpoints. A recent study has explored the recurring episode paradigm.

Despite these limitations, this study has identified clinically relevant behavioral endpoints that may be useful in the development of a migraine model. The thermal tail flick test did not appear to be effective in measuring thermal alodynia, and Rotor-Rod was not an appropriate measure of movement due to the anxiety confound. The automated scoring within the modified light/dark box proved to be more sensitive than the traditional light box in measuring activity, and could be a useful measure in future studies.
The results of this study generate several lines of inquiry that can be used to explore the NTG model of migraine further. Future studies could investigate if multiple injections of NTG will induce behavioral responses mirroring the clinical picture. As mentioned previously, a recent study attempts to simulate the chronic nature of migraine by administering multiple NTG injections and measuring the development of hyperalgesia (Pradhan et al, 2014). Mice were injected with NTG IP at a dose of 10 mg/kg, followed by either saline or sumatriptan 1 hour and 15 minutes after the NTG injection (Pradhan et al, 2014). Mice were tested on mechanical or thermal sensitivity measures 2 hours post-NTG injection (Pradhan et al, 2014). For chronic experiments, mice were tested every other day for a total of 5 days. Mechanical sensitivity was tested using a von Frey, and NTG produced a significant decrease in the mechanical threshold required to produce a pain response. Observed mechanical hyperalgesia was reversed by sumatriptan administration and prevented by topiramate (Pradhan et al, 2014).

Although work by Pradhan et al more closely mirrors the clinical experience by addressing the issue of a single NTG administration and shows sensitivity to drugs used for migraine treatment and prevention, it fails to utilize behavioral endpoints required for migraine diagnosis. Hyperalgesia, while a valid measure of alterations in nociceptive processing, is not a diagnostic criterion of migraine in humans. Photophobia and sensitivity to movement are two clinically relevant behavioral endophenotypes that are diagnostic criteria in humans and easily quantifiable in rodent models. Research examining these behavioral endpoints under recurring NTG administration is ongoing in this laboratory.
LIST OF REFERENCES


Compared to vehicle animals, NTG animals displayed an increase in rat grimace score that remained stable 60, 90, and 120 minutes post-injection (P = 0.086).

*Figure 1.* Quantification of Rat Grimace Score. Data points represent means +/- SEM. Compared to vehicle animals, NTG animals displayed an increase in rat grimace score that remained stable 60, 90, and 120 minutes post-injection (P = 0.086).
Figure 2A. Quantification of thermal alldynia in the cold tail flick test. Bars represent means +/- SEM. No group differences were observed for tail flick latency.
Figure 2B. Quantification of thermal allodynia in the hot tail flick test. Bars represent group means +/- SEM. No group differences were observed for tail flick latency.
Figure 3. Quantification of photophobia using traditional light/dark box. Bars represent group means +/- SEM. No group differences were observed on photophobia in the traditional light dark box.
Figure 4. Quantification of movement differences using traditional light/dark box. Bars represent group means +/- SEM. No group differences were observed for movement in the traditional light/dark box.
Figure 5. Latency to fall from Rotor-Rod. This figure displays group means +/- SEM. Compared to controls, nitroglycerin animals exhibited an increased latency to fall (P = 0.059).
Figure 6. Quantification of photophobia using modified light/dark box. Bars represent group means +/- SEM. No group differences were observed for photophobia in the modified light/dark box.
Figure 7. Quantification of movement using modified light/dark box. Bars represent group means +/- SEM. Compared to controls, Nitroglycerin animals displayed significantly more movement throughout the test session (P < 0.05).