Using The Rat Grimace Scale to Detect Orofacial Pain in Mechanically-induced Temporomandibular Joint Pain in Rats

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Abstract

Introduction: Orofacial pain in animal models of TMJ disorders is typically evaluated by measuring evoked reflexive responses. Since the rat grimace scale (RGS) was adopted recently to assess spontaneous pain in other pathologies, this study evaluated its effectiveness for TMJ pain in the rat. RGS was evaluated using a well-defined pain model of TMJ loading.

Material and Methods: Female Holtzman rats were assigned to separate groups: loading (n=10); sham (n=4); loading with naproxen (n=4) or vehicle (n=3) on days 4 and 5 after pain developed. Jaw loading was imposed for 7 consecutive days under anesthesia by repeated mouth-opening for 1hr. Sham had no mouth-opening. Naproxen or vehicle (1mg/kg) was given intravenously. Rats were videotaped for 30mins daily after loading, and for 7 days after loading was stopped. Images were randomized and quantitatively scored using 4 action units: orbital tightening, nose/cheek flattening, ear change, whisker change. The RGS score was compared between groups using a repeated-measures ANOVA and Tukey’s post-hoc test.

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Conclusion: Orofacial pain can be detected by the RGS, which may provide a useful new method to evaluate TMJ pain.

Degree Type
Thesis

Degree Name
MSOB (Master of Science in Oral Biology)

Primary Advisor
Beth A. Winkelstein

Keywords
Rat grimace scale, temporomandibular joint, orofacial pain, spontaneous pain, non-evoked pain, behavioral testing

Subject Categories
Dentistry | Medicine and Health Sciences

Comments

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Using The Rat Grimace Scale to Detect Orofacial Pain in Mechanically-induced Temporomandibular Joint Pain in Rats

THESIS

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July 25th, 2017

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ACKNOWLEDGEMENTS

Many thanks to my principal investigator, Dr. Beth Winkelstein, and Dr. Eric Granquist for giving me the opportunity to join their research team and conduct this study. Without their patience, guidance and encouragement, my masters program would have not been like this. I sincerely thank them for all the support and guidance. I would like to thank Dr. Kelly L. Jordan-Sciutto, for connecting all wonderful researchers and helping me meet them to pursue my research. Lastly, but not least, I would also like to thank Dr. Bekir Karabucak for being my committee member, and putting insight to enrich this thesis.

I would also like to thank all the members of SPRL for their assistance and support in learning laboratory techniques and protocols. Sonia Kartha, one of the Phd students in the lab, devoted much of her time to teaching me the techniques for setting up the TMJ pain model, doing behavioral testing, performing tail injection in rats, and dissecting. Your contributions to this study were essential and I owe much of my learning to your instruction. It was a pleasure working with you. One member of the lab, Megan Sperry, has been incredibly helpful with this study. I appreciate your insights into the experimental design, your generosity, and your hospitality. Your profound knowledge of brain in rats and TMJ knowledge were essential to this study and allowed me to deepen my understanding of the topic. Also, I learned how to do frozen section and IHC from you. Thank you for the effort you devoted to my learning basic and complex lab techniques; it is much appreciated.

I would like to thank the Department of Endodontics for allowing me this opportunity to develop research skills through the Masters of Science in Oral Biology program. It has been a worthwhile experience I hope to benefit from throughout my career. I look forward to bringing my newfound skills to help projects in our department. In addition to the Endodontic residency program, this masters program enriched my study not only in Endodontic perspective but in science as well.

Finally, I would like to thank my family for their continuous love, help and support. I am grateful to my parents, they selflessly encouraged me to explore new directions in life. Also, many thanks to other significant family member, this journey would not have been possible if not for them.
ABSTRACT

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LITERATURE REVIEW

Anatomy of the temporomandibular joint

The bilateral temporomandibular joints (TMJs) play an important role in facilitating and limiting the articulation between the cranium, mandible and upper cervical spine. Each TMJ is a synovial joint, composed of a fibrocartilaginous disc and articular cartilage covered condyle. The bony part of the TMJ is formed by the condyle of the mandible, which inserts into the glenoid fossa of the temporal bone (Figure 1). The articular disc lies between the two bony components. The disc is biconcave and is made up of dense fibrous connective tissue attached to bilaminar zone which is richly vascularized and innervated. Collateral ligaments are attached to both the medial and the distal sides of the mandibular condyle, which keep the disc in place relative to the head of the condyle during movements without displacement (Meyenberg et al. 1986).

The movement of the upper joint space is mainly associated with translations of the condyle and the lower joint space is responsible for condyle rotation. Several muscles permit those frictionless translation and rotation movements, typically allowing for painless and efficient functional movements, including chewing, swallowing, and speaking. Such anatomical characteristics not only allow the joint to move back and forth in one plane, but also permit gliding movements between the temporal and mandibular articular bone (Alomar et al. 2007). The primary muscles that provide mandibular movements are the masticatory muscles, which include the masseter muscle, the medial pterygoid muscle, the lateral pterygoid muscle, and the temporal muscle (Figure 2). There are additional muscles associated with neck and head, supporting movements (Scrivani et al. 2008). Parafunctional behaviors, such as bruxism — which is the abnormal wear on the dentition that results from continuous grinding and clenching pressures — can involve the muscles of the TMJ (Glaros et al. 1977). Other parafunctional habits include grinding of the teeth,
clenching, empty-mouth movements and other non-functional, involuntary mandibular compensatory movements (Glaros et al. 1977, Scrivani et al. 2008). Because the joint capsule and surrounding muscles are innervated by nociceptive fibers, they may be sources of pain in TMJ disorders (Buescher 2007).

Figure 1. The anatomy of TMJ associated tissue
Figure 2. The anatomy of TMJ and its associated tissues
Overview of pain

Pain is defined as, “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey et al. 1994).

Approximately 9% of the adult population in the United States suffers from moderate to severe non-cancer related chronic pain (Jeffery et al. 2011). Even more people (10% to 20% of the population) report having persistent pain lasting over three months (Gureje et al. 1998, Verhaak et al.1998, Blyth et al. 2001, Gatchel et al. 2006), which is defined as “chronic” pain. People older than 50-years-old are twice as likely to be diagnosed with chronic pain as compared to the younger population (Gatchel et al. 2006). Management of chronic pain is an important clinical issue, particularly for the future health care of aging population (Campbell et al. 2006, Gatchel et al. 2006). Accurate and reliable clinical questionnaires and scales are used to evaluate pain using human self-ratings (Price et al. 1983). However, studies are challenging not only because they are fundamentally subjective but also ethically self-limiting (Mogil 2009). New techniques, such as functional-imaging scans or genetic biomarkers, are being developed that may provide both reliable and more objective measurements to study pain in the future. For now, non-human animals are widely used in pain studies (LeBars et al. 2001, Mogil 2009).

Pain is multi-factorial and dynamic, made more complex since inhibition and nociceptive amplification can take place at many places in the central nervous system (CNS) with synaptic communication. Pain can be classified into three major categories from a neurobiological perspective: (1) nociceptive pain; (2) inflammatory pain; and (3) pathological pain. Nociceptive pain and inflammatory pain are both adaptive and protective, while pathological pain is maladaptive, resulting from abnormal functioning of the nervous system (Woolf 2010).
(1) **Nociceptive pain**

The most common type of pain is nociceptive pain, brought on by sensing noxious stimuli. A nociceptor is a high-threshold pain receptor only activated by intense stimuli. Nociceptive pain produces an autonomic response and withdrawal reflex. If something extremely cold or hot or something sharp is touched, the terminals of the nociceptive nerve will induce a protective response (Woolf 2010). First-order nociceptive afferents (primary sensory neurons) collect information about non-noxious and noxious stimuli, and are classified into four major groups. Large diameter, myelinated fibers (A-α and A-β fibers) conduct the fastest. A-δ fibers are lightly myelinated; and unmyelinated C fibers are the slowest responders (Milligan et al. 2009).

Normally, A-β fibers only respond to low-frequency and non-noxious stimulation (Torebjork et al. 1992), which are interpreted as light touch. However, A-β fibers can begin to transmit pain signals after injury (Woolf 2010). Nociceptive neurons contain a bifurcating axon, with A-δ fibers and C fibers projecting to nociceptive interneurons and second-order pain-projection neurons in the dorsal horn of the spinal cord (Figure 3) (Mannion et al. 2000). A-δ fibers and C fibers both respond to high-frequency and painful mechanical stimuli (Merrill et al. 2007). A-δ fibers transmit impulses faster than C fibers due to their myelination. C fibers stimulate second-order neurons to release many different kinds of neurotransmitters, which can maintain persistent pain (Mannion et al. 2000, Bolay et al. 2002, Merrill et al. 2007, Milligan et al. 2009). If there is no stimulus that is potentially noxious, the nociceptors are normally silent. However, nociceptors become hypersensitive and develop pathological spontaneous activity after peripheral nerve injury (Woolf 2010). Spontaneous pain in the absence of external stimuli can occur and is believed to be due to increased messenger RNA for voltage-gated sodium channels in primary afferent neurons, lowering the threshold for generating an action-potential leading to revival in hyperactivity (Lai et al, 2003, Merrill et al. 2007, Milligan et al. 2009, Woolf 2011).
Figure 3. Activation of nociceptive nerve fibers and spinal transmission

First-order nociceptive afferents in the periphery (primary sensory neurons) collect information about non-noxious and noxious stimuli: A-\(\beta\) fibers (yellow), A-\(\delta\) fibers (blue), and C fibers (red). A-\(\delta\) fibers and C fibers project to nociceptive interneurons and second-order pain-projection neurons (green) in the dorsal horn of the spinal cord.

(2) Inflammatory pain

Inflammatory pain can be caused by tissue injury or infection which activates the immune system response (Woolf 2011). Inflammation of peripheral tissue is believed to be associated with the release of chemical mediators from cells or from the nociceptive afferent endings themselves. Spreading the mediators through the tissue, the adjacent tissue will also have the same reaction inflammation reaction (Sessle 2011). Inflammatory reactions can be protective and help in healing by creating a protected environment through discouraging physical contact, such as in rheumatoid arthritis or cases of extensive injury. The inflammatory pain comes with tenderness, spontaneous pain and/or pain hypersensitivity to reduce the risk of damage and promote
recovery (Woolf 2010). Nociceptive pain and inflammatory pain are usually short-term, while the pathological pain often last months to years (Mogil 2009).

(3) Pathological pain

Pathological pain is associated with inflammation and/or trauma of peripheral tissues or nerves after injury. There are different mechanisms to cause pathological pain. When injury or inflammation is prolonged and causes tissue damage, noxious stimuli are no longer required to induce the pain, Woolf defined this as “dysfunctional pain” (Woolf 2011). If the inflammation or injury damaged neural tissue, it is called “neuropathic pain” (Milligan et al. 2009).

Normally, acute pain processing begins with stimuli that activate specialized receptive endings on peripheral sensory nerve fibers. Acute pain is self-limited, inducing an adaptive and protective response which helps to prevent further tissue damage (Jeffery et al. 2011, Woolf 2011). Acute pain results from a transient, high-intensity activation of specialized receptive endings on nociceptive sensory fibers which often leads to tissue damage (Milligan et al. 2009). When injury or inflammation is prolonged, primary nociceptive neurons can become hyper-excitable, causing chronic pain. The CNS can also sensitise neurons in the spinal cord, leading to chronic pain (Milligan et al. 2009). Different from acute pain, chronic pain is considered to be a disease condition (Mogil 2009). Chronic pain has negative effects on many aspects of quality of life. Patient with long-lasting pain have a higher chance to experience negative emotions and to distrust people around him/her (Mienna et al. 2012). In one European survey, 19% of the adult population suffered from moderate to severe chronic pain, and their daily activities, social and working lives were seriously affected (Breivik et al. 2006).
Temporomandibular joint disorders

Temporomandibular joint disorders (TMD) is a group of musculoskeletal, neuromuscular, and combined conditions associated with the tissues in and surrounding the TMJ (Buescher et al. 2007, Alomar et al. 2007, Scrivani et al. 2008). The earliest study of TMD is reported by an otolaryngologist — James Costen. He described a syndrome of ear and sinus symptoms related to loss of lower posterior teeth and mandible over-closure (Costen et al. 1934). In 1982, the American Dental Association (ADA) defined TMJ disorders as a craniomandibular disorder (CMD) (Griffiths et al. 1983). TMD is a major cause of non-odontogenic pain in the orofacial region, with the primary presenting symptom of pain, localized to the masticatory muscles and/or pre-auricular area. In addition to pain, TMD patients also frequently present with limited jaw movement and TMJ sounds (De Leeuw and Klasser 2013).

The overall prevalence of TMJ disorder is over 5% (Liu et al. 2013). In the United States (US), the prevalence of TMJ disorder is 8.4%, and 6% of the US population has a symptom involving joint pain (Lipton et al. 1993). Another study reports the prevalence of TMJ pain in women in the US as estimated to be 10.5% (Janal et al. 2008). One of the most prevalent TMJ pathologies is osteoarthritis (OA) (Scrivani et al. 2008). TMJ-OA leads to low-grade inflammation and joint degeneration, and it is often associated with persistent pain (Stegenga et al. 1989, Scrivani et al. 2008). Previous result has shown that 19% of the dental students at Umeå University in Sweden have persistent signs and/or symptoms from the TMJ in a 2-year follow up period (Marklund et al. 2010). Females have twice the likelihood of persistent TMJ signs or symptoms compared to males (LeResche et al. 1997, Marklund et al. 2010, Anastassaki Kohler et al. 2012). Initial treatment for treating TMD patients is noninvasive therapy, including rest, occlusal appliances, heat, muscle relaxants, nonsteroidal antiinflammatory drugs (NSAIDs), and physical therapy (Scrivani et al. 2008). However, persistent TMJ pain is a challenge for clinical management.
because it cannot always be resolved through traditional courses of treatment. Surgical interventions, such as intra-articular injection with steroids, arthrocentesis, or arthroscopy, is needed in patients with persistence of high levels of pain and mandibular dysfunction (Scrivani et al. 2008, Israel et al. 2010).

Functional brain imaging, including positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), are used in more recent studies to understand the functional and structural changes in the CNS. fMRI demonstrates changes in cortical brain circuitry in TMD patients, supporting the hypothesis that TMJ disorder is similar to other chronic pain disorders (de Leeuw et al. 2005). TMD patients may also have different pain processing than normal in the trigeminal system, a phenomenon known as “central sensitization” (Merrill et al. 2007, Scrivani et al. 2008, Woolf 2011). Central sensitization is a condition in which the somatosensory pathways exhibit enhanced synaptic efficacy and decreases in inhibition (Woolf 1983). As a result, patients develop a lower central threshold to noxious stimuli (hyperalgesia) or/and have pain to non-noxious stimuli (allodynia) (Iwata et al. 1999, de Leeuw et al. 2005, Woolf et al. 2011). In addition, different studies suggest that there is a connection between chronic TMJ disorders and coexisting psychopathology conditions such as anxiety, depression, and physical abuse histories (Campbell et al. 2000, Ferrando et al. 2004). Early detection of psychological symptoms could enable adequate treatment earlier to slow down the development of psychological symptoms (Ferrando et al. 2004). Overall, the etiology of TMJ disorder remains unclear. However, it is considered to be multifactorial disorder, with physiological and psychological components (Scrivani et al. 2008).
Animal models of TMJ pain

Different animal models have been developed to study TMJ disorder. TMD studies use different species including rodents, rabbits, pigs, sheep, goats, and cattle. In recently studies, rats are widely used due to the research involving nociceptive and nervous system in rats increase rapidly (Herring 2003). There are only minor differences in anatomy between rats and human: the angles between the condyle and the mandible corpus, condyle axis, and there are no anterior eminence in rats' temporal fossa. Overall, rats are easy to handle and the TMJ structure is also comparable to human (Orset et al. 2014).

There are two main ways used to induce TMD: chemical approaches and surgical or mechanical approaches (Almarza et al. 2011). For chemical approaches, chemical agents are injected into the TMJ region (Roveroni et al. 2001, Krzyzanowska et al. 2012). There are different chemical agents in the chemical approaches: Complete Freund's Adjuvant (CFA), mustard oil, formalin, and carrageenan. CFA is heat-killed Mycobacterium tuberculosis in an oil and saline emulsion, and can produce acute inflammation. CFA-induced allodynia can last up to 18-days (Ren 1999, Iwata et al. 1999, Hutchins et al. 2000). Contacting to formaldehyde results in the DNA-protein cross-links formed, primary genotoxic effect, which cause tissue inflammation (Lu et al. 2010). However, the effect of formalin only lasts for about 45minutes (Roveroni et al. 2001, Clemente et al. 2004, Gamerio et al. 2005, Almarza et al. 2011). Both mustard oil and carrageenan are used to irritate the TMJ to create inflammation. Mustard oil is only used to alter muscle activity via electromyography (EMG) in a short-time (30minutes). There are no studies discussing the effect to the articulating tissue of the TMJ of mustard oil (Hathaway et al. 1995, Yu et al. 1996, Almarza et al. 2011). The effect of carrageenan is usually seem within 3 hours of its application. And carrageenan can also prime the TMJ afferents to develop hyperalgesia (Swift et al. 1998, Oliveira et al. 2005, Rodrigues et al. 2006).
TMD is also induced by surgical or mechanical approaches. Most of the surgical procedures are used on larger animals, such as rabbits, monkeys, and sheep. The surgical techniques are including disc displacement, condylectomy, disc perforation, and discectomy (Almarza et al. 2011). All the surgical procedures mimic more severe injury of the TMJ tissue. Various methods of mechanical perturbation have been used to create TMD (Almarza et al. 2011). These procedures are more close to mimicking how TMD is induced in reality, such as bite changes, altered dietary consistency, tooth extraction, and orthodontic appliance (Fujita and Hoshino 1989, Cicochon et al. 1997, Mao et al. 1998, Liao et al. 2014). All of the above studies indicate that mechanical perturbation does have an effect in inducing TMD.

However, all of the above approaches artificially damage the TMJ but are not clinically relevant. OA is the primary pathology of the TMD. Mechanical overloading of the TMJ is the major factor inducing OA onset in TMJ tissue (Stegenga et al. 1989, Israel et al. 1991). Several studies found that forced jaw-opening models in rabbits and rats can induce OA by histological evaluation (Fujisawa et al. 2003, Tanaka et al. 2005, Kawai et al. 2008). However, none of those studies mentioned the loading protocol details, the onset time of pain, and how long the pain was maintained. Our lab has developed a model of mechanically-induced TMJ pain in the rat to mimic the joint loading that occurs in clinical TMD to enable study of the orofacial pain and joint tissue responses (Nicoll et al. 2010, Kartha et al. 2016). The TMJ loading protocol uses mouth-opening for 1 hour repeated for seven continuous days, using a 2-N or 3.5-N load to the TMJ. The behavioral hypersensitivity increases immediately and persists for about 14 days. Then by 2 weeks after the loadings stopped, the threshold of head withdrawal gradually went back to the baseline level. Histologic findings showed thinning of both condylar cartilage and articular disc in the TMJ tissue after loading for 1-week. However, there was no evidence of tissue repair in the 2-N group on day 14 despite behavioral hypersensitivity resolving. Ongoing work from our lab altered the jaw-opening
force from 2-N to 3.5-N, which is the maximum load below the biomechanical threshold for jaw dislodging in rats (Kartha et al. 2016). That work showed the 3.5-N loading induces a constant and non-resolving pain at 14-days after loading, and the head withdrawal threshold in the 3.5-N loading group is significant lower than the 2-N loading group on days 13 and 14. Also, the upregulation of the inflammatory marker, matrix metalloproteinase-13 (MMP-13), hypoxia-inducible factor-1a (HIF-1a), and tumor necrosis factor-α (TNF-α), only in the 3.5-N loading group. From the results of the two studies, conditions of the forced mouth-opening that can create either acute (2-N loading) or persistent (3.5-N) orofacial pain, depending on the forced jaw-opening loading.
Pain assessment methods

Mechanical reflex testing

The majority of pre-clinical pain studies assess mechanical sensitivity by measuring head withdrawal thresholds to von Frey filament stimulation (Ren et al. 1999, Mogil et al. 2004). That type of testing measures reflexes at the level of the spinal cord after a thermal, chemical, electrical or mechanical stimulation. Most TMJ studies measure pain using mechanical stimulation of the bilateral TMJ regions by von Frey filaments to measure head-withdrawal threshold (Ren et al. 1999, Krzyzanowska et al. 2012, Nicoll et al. 2010, Kartha et al. 2016).

However, hypersensitivity is only one component of pain (Mogil 2009) and is only a measure of evoked pain. It requires trained handling of the rat during behavioral testing, and is best suited to acute stimuli, which assesses nociceptive pathways only. However, the evoked pain is an indirect way to measure the pain. It is also possible that the observed withdrawal reflexes reflect only an avoidance of the stimulus (Bove 2006, DeRantere et al. 2015). Although evoked behavioral testing is a useful measurement for pre-clinical pain studies, it is challenging to translate these outcomes into clinical practice. There is a survey showing patients with neuropathic pain present with spontaneous pain in 96% of that population, while the evoked testings — mechanical and thermal hypersensitivities — which were widely used in laboratory are only observed in 38-64% of the patients with chronic pain (Backonja et al. 2004). The prevalence of evoked pain in patients with chronic pain is much lower than the prevalence of non-evoked or spontaneous pain. That work also shined that spontaneous pain is a much better predictor of pain than the evoked testings (Backonja et al. 2004, Mogil 2009).
**Spontaneous behavioral testing**

Spontaneous pain is a more common and reliable symptom reported clinically (Backonja et al. 2004). Indeed, nerve injury produces spontaneous pain in animal models as well (Kupers et al. 1992, Baron et al. 2000). However, it is still unknown whether it is possible to measure spontaneous pain in the TMJ in rodents. This is challenging because animals do not self-report their pain levels (Kupers et al. 1992, Choi et al. 1994). Different measurements have been used in different studies, such as bite force, grooming behavior, guarding, and weight loss (Whittaker et al. 2014). However, such spontaneous behaviors are difficult to quantify, and it is also difficult to differentiate whether they indicate stress, pain, paresthesia, or avoidance behavior (Krzyzanowska et al. 2012).

**Human grimace scale**

Pain assessment in humans usually relies on self-reporting (Chambers et al. 2015). Many doctors use pain scales to gather more detailed information of patients’ pain (Williamson et al. 2005, Ferreira-Valente et al. 2011). The most common pain scales are the visual analog scale (VAS), the numerical rating scale (NRS), and the verbal rating scale (VRS). The VAS is presented as a 100-mm line; the patient is asked to mark on the line to indicate pain intensity. The VAS, 0-mm as “no pain” and 100-mm as “worst imaginable pain”, provides 101 levels of pain intensity. One of the disadvantages of the VAS is the scale must be presented on papery. The NRS has different point scale (11/21/101 levels), the most widely used is the 0-10 NRS where the end points are extremes of “no pain” and “worst imaginable pain”. The NRS can be delivered either graphically or orally. The commonly used VRS is more simplified with only four categories: no pain, mild pain, moderate pain, and severe pain. All three scales are reliable and easy for clinical use, and the
NRS has good sensibility and can be statistically analysis (Chanques et al. 2010). However, all of the pain scales rely on self-report. For those who cannot express themselves in words — such as infants, young children, and verbal or cognitive impairments — facial expression can be used as a tool to quantify pain intensity. Ekman and Friesen developed the Facial Action Coding System, which transferred human facial expression movement into action units (Ekman and Friesen, 1977). The Neonatal Facial Coding System is now widely used in infant populations (Grunau et al. 1987, Chambers et al. 2015). With these approaches as models, there are now several objective methods to evaluate expression of pain in humans. But, experiments on human are practically challenging and ethically self-limiting, thus, laboratory animal models are widely used in pain models (Mogil 2009).

Animal grimace scale

In recent years, the animal grimace scale has been developed (Chambers et al. 2015). The facial coding system was brought from the human to mice via the mouse grimace scale (MGS), which consisted of five facial features as indicators to evaluate pain (Langford et al. 2010). In that study, both chemical injection and surgical approaches were used to produce the pain in the mice, and an analgesic agent was given to reduce the pain as well. Compliantly the MGS, von Frey filaments were used to do mechanical reflex behavior testing. Langford’s group found that pain scores on the MGS were related to the intensity of stimulus. And also that there is a positive and linear relationship between dosage of analgesia and MGS scores. The MGS is highly accurate and reliable method to evaluate pain across different pain models. Moreover, both MGS and mechanical reflexing testing have been shown to exhibit a high positive correlation to each other (Leach et al. 2012).
Grimace scales were then developed for different species, including the rat, horse and cat, with high accuracy and reliability by quantifying pain through facial expression (Mogil 2009, Chambers et al. 2015). The rat grimace scale (RGS) was developed to evaluate spontaneous pain responses (Sotocinal et al. 2011). The RGS has been used in different pain models: intraplantar CFA injection, intra-articular kaolin/carrageenan injection, plantar incision, laparotomy, experimental tooth movement, and acute chemotherapy-induced mucositis (Sotocinal et al. 2011, Liao et al. 2014, De Rantere et al. 2015, Whittaker et al. 2016). Our lab has recently used the RGS to evaluate pain in a rat model of spinal nerve root compression (Philips et al. 2017). The RGS scores were significantly higher than the sham group, and scores remained higher than baseline for as long as 48 hours. The RGS in that study had a very good interobserver reliability and excellent internal consistency. All of the findings suggest that RGS is a useful pain assessment tool to identifying and monitoring acute neuropathic pain in rats. There is only one study using RGS to evaluate orofacial pain: orthodontic tooth movement pain (Liao et al. 2014). As per our knowledge, there is no evaluation TMJ pain using RGS as an assessment approach.
Objective

In this study, TMJ OA is induced by mechanical loading of the TMJ. TMJ pain following the onset of TMJ OA is evaluated using both the RGS and the mechanical reflex test (Nicoll et al. 2010, Kartha et al. 2016, Sperry et al. 2017). Additionally, analgesic interventions are also used to evaluate the sensitivity of RGS measurements to TMJ disorders treatment. Pain associated with TMJ disorders is multifactorial, but is strongly associated with tissue inflammation. Naproxen, a NSAIDs, is used as a first-line pharmacologic intervention for TMJ disorders (Dym et al. 2016). This study evaluated if anti-inflammatory treatment has an effect on facial expression after TMJ pain is induced. To quantify the consistency and reproducibility of RGS scoring, the intraclass correlation coefficient is calculated across multiple scorers.

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83. Woolf CJ. What is this thing called pain?. The Journal of clinical investigation. 2010 Nov 1;120(11):3742.
Introduction

Temporomandibular disorders (TMD) is one of the most common sources of orofacial pain (De Leeuw and Klasser 2013). It is defined as a subgroup of orofacial pain disorders involving the temporomandibular joint (TMJ), masticatory muscles, and associated head and neck musculoskeletal structures (Magnusson et al. 2000; Aggarwal et al. 2007). TMD can negatively impact on individual’s quality of life due to the symptoms of TMD, including joint pain and impaired jaw function (Dahlstrom et al. 2010, Mienna et al. 2012). The overall prevalence of TMD in the United States (US) is 8.4%, and 6% of US population has a symptom involving joint pain (Lipton et al. 1993). Osteoarthritis (OA) is the primary pathology of the TMJ, resulting in low-grade inflammation and joint degeneration (Stegenga et al. 1989; Israel et al. 1991). Although OA is a peripheral pathology, central sensitization also contributes to OA pain (Arendt-Nielsen et al. 2010). The etiology of TMD is multifactorial, with physiological and psychological symptoms (Scrivani et al. 2008).

Current preclinical pain research relies heavily on rat models. Mogil and his group developed the rat grimace scale (RGS) to evaluate and quantify the pain of chemical injection and surgical assays in rats using their facial expression (Sotocinal et al. 2011). The RGS has been used in several different pain models, including intraplantar CFA injection, intra-articular kaolin/carrageenan injection, plantar incision, laparotomy, experimental tooth movement, and acute chemotherapy-induced mucositis (Sotocinal et al. 2011, Liao et al. 2014, De Rantere et al. 2015, Whittaker et al. 2016). Our lab has recently used the RGS to evaluate neuropathic pain in a rat model of spinal nerve root compression (Philips et al. 2017). RGS scores of the painful loading group were significantly higher than the sham group at 6 hours after loading, and scores remained higher than baseline for as long as 48 hours. The RGS in that study had a very good interobserver
reliability and excellent internal consistency. All of the findings suggest that RGS is a useful pain assessment tool to identify and monitor acute neuropathic pain in rats. However, there are only one studies using RGS to evaluate orofacial pain: orthodontic tooth movement pain (Liao et al. 2014). To our knowledge, there is no study evaluating TMJ pain using RGS as an assessment approach. We hypothesize that the RGS can be the used to evaluate mechanically-induced TMJ pain in the rat.

In this study, pain was evaluated with the RGS using a repeated mouth-opening model that induces sustained hyperalgesia in the TMJ region (Nicoll et al. 2010, Kartha et al. 2016). Naproxen, a non-steroidal anti-inflammatory drug (NSAID), is used to test if treatment has effect on facial expression after TMJ pain is induced. To quantify the consistency and reproducibility of RGS scoring, intraclass correlation was performed to evaluate the consistency of quantitative measurements made by different observers scoring the RGS values.
Material and Methods

Animals

All studies used adult female Holtzman rats (Hsd: Holtman Sprague Dawley; 250-300g at acquisition) obtained from Harlan Laboratories (Indianapolis, IN). Rats were housed in groups of two or three in standard polycarbonate caging (AnCare, Bellmore, NY), with 0.25-inch corncob bedding (Bed-o’Cobs; The Andersons Lab Bedding Products, Maumee, OH) and ad libitum access to food (LabDiet 5001; LabDiet, St Louis, MO) and water (acidified to pH=3). Rats were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited vivarium under a 12:12 hour light:dark cycle in a temperature-controlled environment in accordance with recommendations set forth in *The Guide for Care and Use of Laboratory Animals, 8th edition* (Institute for Laboratory Animal Research 2011). All procedures were approved by the IACUC at the University of Pennsylvania and adhered to the guidelines for research and ethical issues of the International Association for the Study of Pain (IASP) (Zimmermann 1983).

Rats (n=23) were randomly assigned to four groups: (1) loading only (n=10); (2) loading with naproxen-injection (n=6); (3) loading with vehicle-injection (n=3); and (4) sham (anesthesia only, n=4). The sham group did not receive loaded, but all other conditions of the experiment were identical to the loading groups. Mechanical reflex testing for mechanical hyperalgesia took place at approximately 8:00 AM daily, and was before any TMJ loading (Figure 1). Three rounds of reflex testing were performed bilaterally, with 10 minutes of rest between each round. The rats were loaded with a 3.5-N weight for 1hour daily for 7 continuous days (Nicoll et al. 2010, Kartha et al. 2016). Digital video recordings of all four groups were collected at three hours after loading and the isoflurane exposure were complete (Figure 2).
Figure 1. The overall timeline of the entire study.

The three loading groups (loading-only, loading with naproxen-injected, loading with vehicle-injected) undergo three stages; baseline, the loading phase, and the unloading phase. In the loading phase, the rats were loaded with 3.5-N weight for 1 hour/day for 7 continuous days (D0-D6). For the naproxen-injected and vehicle-injected groups, the intravenous injection (inj.) was given immediately after loading via the lateral tail vein under isoflurane anesthesia on day 4 (D4) and day 5 (D5) after pain developed. Rats in the sham group were under isoflurane exposure 1-hr/day for 7 continuous days. Both mechanical reflex testing and digital video recording were taken in the all three phases (baseline, loading phase, and unloading phase).

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Loading phase (3.5-N, 1-hr/day for 7 days)</th>
<th>Unloading phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2</td>
<td>D-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D0</td>
<td>D1</td>
</tr>
</tbody>
</table>

**Figure 2. The daily timeline for all rats.**

Mechanical reflex testing was performed in the morning at 8:00AM before any procedure. After evoked reflex behavioral testing, the rats were loaded with 3.5-N weight 1 hour/day for 7 continuous days. The sham group was exposed to isoflurane anesthesia but not loaded. All rats were digitally videotaped for 30 minutes, at least three hours after the loading/anesthesia.
Mechanically-induced TMJ pain

A mechanically-induced TMJ pain model in the rat was used in all experiments, as previously described (Nicoll et al, 2010; Kartha et al, 2016). All rats were exposed to isoflurane inhalation anesthesia (4-5% for induction and 2.5-3% for maintenance) during the loading procedure mixed with oxygen. Rats in the loading groups were placed into an acrylic chamber in the prone position. The mandible was fixed with an acrylic loop and the maxilla was held open with a sling attached to a 3.5-N weight for 60 minutes per day (Figure 3). The procedure was applied daily for 7 continuous days (D0-D6) (Figure 1). The loading groups received the same conditions, while the sham group was unloaded and received isoflurane anesthesia for 60 minutes each day for 7 continuous days (D0-D6) (Figure 1).

Figure 3. Methods of mechanically-inducing TMJ pain.
The mandible of the rat was fixed with an acrylic loop and the maxilla was held open with a sling attached to a 3.5-N weight for 60 minutes each day for 7 continuous days.
**Treatment Injections**

After loading, in the groups receiving treatment, an intravenous injection was performed via the lateral tail vein under isoflurane anesthesia on day 4 (D4) and day 5 (D5). The volume of administration Naproxen was given according to the weight of the rats (1mg/kg) (Jakubowski et al. 2007). The dose is about one-eighth of the human oral dose of 500mg recommended for treating patient (8mg/kg for a 60-kg person) (Jakubowski et al. 2007). The vehicle-injected group received only saline (control group) in the same volume. All injection procedures were performed by a single operator (MMS) after loading on D4 and D5, but before digital video taping for RGS scoring.

**Mechanical reflex behavioral testing of orofacial area**

Mechanical reflex testing took place at approximately 8:00 AM, before the application of loading. Head-withdrawal thresholds were measured by a series of von Frey filaments of increasing strengths from 0.6g to 60g (Nicoll et al. 2010, Kartha et al. 2016). Three rounds of reflex testing were performed bilaterally, with 10 minutes of rest between each round, and each strength of von Frey filament stimulation was applied five times to each site. The lowest-strength filament evoking a response was recorded as the sensitivity threshold if the next higher filament also elicited a response. Responses were taken as a head withdrawal or immediate pawing of the stimulated area. All procedures were performed by a single operator (MMS).
Rats were placed in a 23x10x10 cm³ high transparent Plexiglas chamber with a removable stainless steel top (Figure 4). A digital video camera (Sony HDR-CX380/B High Definition Handycam) was placed in front of the wider side of the box (Figure 4). Rats were videotaped for 30 minutes, 3 hours after exposure to the loading/isoflurane anesthesia. All procedures were performed in a quiet environment and personnel remained out of visual contact with the rats for the duration of the recording session. The loading-only group had two subgroups for which digital video was taken at different time points. The L1 group underwent a short-term observation period. The data collected in that group were from baseline (D-2 and D-1), the 7-days of continuous loading (D0—D6), and the first day after loading (D7). The L2 group had a longer observation period; baseline (D-2 and D-1), only on four days of the 7-days continuously loading (D0, D1, D3, and D5), and at five times doing the rest (unloading) (D7, D8, D10, D12, and D13). The days picked from the loading phase is based on the data from the L1 group.

Digital video of the two injection groups were taken at the same times as L1. The sham group was recorded at baseline (D-1), during the loading phase (D0, D1, D5) and after unloading phase (D8, D13). All procedures were performed by single operator (YHY) and videos were acquired as mp4 files (.mp4).
Figure 4. Set-up digital video for RGS.
Rats were placed into a 23x10x10 cm³ high transparent Plexiglas chamber with a removable stainless steel top. The camera was placed at the wider side of the box. Digital video taping (30 minutes duration) was performed three hours after loading/isoflurane exposure on those days.

Image selection and RGS scoring

Elmedia Player (Eltima Software), free software for the Mac, was used as the media player in this study to play the mp4 files. A total of 10-images was captured from each 30-minute video session at 3-minute intervals with the built-in application Image Capture (Apple), as portable network graphics files (png files). Images were required to have a clear view of the four action units (eyes, nose/cheek, ears, and whiskers) (Figure 5) and were not taken during grooming, sleeping, or active sniffing activity. When the images could not be extracted at the 3-minute interval, the video was advanced to the closest time point until the image could be used. The image-capture operator (YHY) was completely blinded to the groups. All of the images were inserted into Microsoft PowerPoint for Mac with one image per slide and a black background (Figure 6). A PowerPoint macro, Random Slides (http://www.tusharmehta.com/powerpoint/randomslideshow/index.htm), was used to randomize the slide order before assessment.
Figure 5. The four action units of the RGS.
In each individual component, a score of “0” indicated the action unit (AU) was absent. A score of “2” indicated there is an obvious appearance of the AU. A score “1” indicated a moderate appearance of the AU.
Before scoring any of the images from this study, RGS training was given to the scorer using a standard method, previously used to train RGS scorers from our lab team (Philips et al. 2017). For the training, after carefully reading the RGS scoring instruction, twenty-eight practice slides were used to establish scoring consensus. Each image had four scores of the RGS action units (AUs), including orbital tightening, nose/cheek flattening, ear change, and whisker change (Figure 5). The scorer assigned a value of intensity number from 0 (absent) to 2 (obviously present) for each of the four action units for each image. If the action units of the image could not be scored by the rater, the value would be assigned as “not scored”. The RGS score for each image is the average of the action unit scores. The mean RGS score at an individual time point is the average of the RGS scores across the 10-images acquired (Figure 10). All of the images included in this study were scored by a single observer who was blinded to treatments (YHY) (Figure 7).
Figure 7. RGS scoring methods.

The four action units (AUs) were scored along a scale from 0 to 2. The RGS score of single image is the average of the scores from the four AUs (left). The average of 10 RGS score is the rat's RGS score (right).

Comparison across RGS raters

The agreement between four trained RGS raters was calculated using the intraclass correlation coefficient (ICC). The ICC for the overall average RGS scores and by individual AUs were compared across all treatment groups from previous nerve root compression (NRC) rats model from our lab was used to assess variability across RGS raters. Slides (n = 230) of 6hour post-surgery were evaluated by four scorers (BAW, BHP, CLW, and YHY). Inter-rater reliability was calculated with one-way random average measures. The strength of agreement was categorized in five groups: (1) very good (0.81-1.00); (2) good (0.61-0.80); (3) moderate (0.41-0.60); (4) fair (0.21-0.40); and (5) poor (<0.20) (Landis et al. 1977, Altman et al. 1990).
Statistical analyses

All statistical analyses were performed with RStudio: Integrated Development for R (RStudio, Inc., Boston, MA, URL http://www.rstudio.com/). For the time-course study, the RGS score was compared between two groups by repeated-measures ANOVA and Tukey's post-hoc testing, with significance defined at a P-value of less than 0.05. RGS values are reported as mean± 1 standard deviation. The ICC was calculated with a one-way random average measures (ICC1k).
Results

A total of 2030 images was collected in this study and scored by a single blinded scorer (YHY). Four action units were scored in all images, and all action units were scored (no “not scored” action units). The baseline of RGS average value in the sham group (0.45±0.04) was not significantly different than the three other groups (Table 1). The RGS value of the sham group increased to 0.67±0.21 on day 0 after exposure to isoflurane for 1 hour, but it was not significantly different from the baseline value \((p = 0.86)\). The peak in RGS scores for the sham group was on D1 (0.79±0.13). The value decreased slightly after D1, remaining consistent with the D0 value (D5=0.71±0.12, D8= 0.69±0.13, and D13= 0.6±0.28). There were no significant differences from baseline after exposure to inhalation anesthesia at any time points (Figure 8) (Table 1).
The RGS score for the three loading groups increased immediately after loading (D1). The RGS scores of the L1 group (red) increase until D6 with continuous 7-days loading, and once unloaded, the score begins to decrease. The two injected-groups with vehicle (blue) or naproxen (yellow) have their tail injection on D4 and D5 (red arrows). The sham group (black) has slightly elevated scores after exposure to isoflurane 1hour/day. However, the RGS scores stabilize after 2 days of exposure (D1).

#: Significant difference between the loading group and the sham group ($p<0.05$)

◎: Significant difference between the loading group and the naproxen-injected group ($p<0.05$)
<table>
<thead>
<tr>
<th>Day</th>
<th>Mean RGS score (0-2)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1 (n=6)</td>
<td>Vehicle (n=3)</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-2</td>
<td>0.475</td>
<td>0.400</td>
</tr>
<tr>
<td>D-1</td>
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<td>0.542</td>
</tr>
<tr>
<td>Loading phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0</td>
<td>1.071</td>
<td>1.000</td>
</tr>
<tr>
<td>D1</td>
<td>1.129</td>
<td>1.017</td>
</tr>
<tr>
<td>D2</td>
<td>1.275</td>
<td>1.250</td>
</tr>
<tr>
<td>D3</td>
<td>1.304</td>
<td>1.267</td>
</tr>
<tr>
<td>D4</td>
<td>1.379</td>
<td>1.367</td>
</tr>
<tr>
<td>D5</td>
<td>1.375</td>
<td>1.175</td>
</tr>
<tr>
<td>D6</td>
<td>1.388</td>
<td>1.300</td>
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<tr>
<td>Unloading phase</td>
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</tr>
<tr>
<td>D7</td>
<td>1.250</td>
<td>1.067</td>
</tr>
<tr>
<td>D8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. RGS scores for all groups: the L1 group, the naproxen-injected group, the vehicle-injected group, and the sham group.
In this study, both the RGS scale scoring and the mechanical reflex testing were performed for pain assessment. The mechanical reflex testing was done before the start of loading on each day; however, the RGS videotapes were recorded daily, 3 hours after loading is complete (Figure 2). So, when looking at those data, comparing the data from the first day of RGS with the data from the second day of mechanical reflexing testing is more appropriate than comparing days. For example, the D0 of mechanical reflex testing is baseline (applied before loading). Conversely, the data at D0 to RGS scoring is in the first day of loading, and measured three hours after loading. In addition to different time points, the scoring scale is different between the two methods. If pain levels increase, the mechanical sensitivity threshold is decreased but the RGS score is increased (Figure 9). However, in both methods, data trends are the same, with withdrawal threshold and RGS score reaching extreme values, which indicate pain. Yet, there are still some differences between the two assessments. Although withdrawal threshold for the sham group is not significantly different before and after isoflurane exposure, the RGS scores are slightly elevated over the baseline score. After mechanically-induced TMJ pain, the L2 group has a steadily decreasing threshold from D1 to D14 (Figure 9b). On the other hand, the RGS score increases with time and peaks on D5 (Figure 9b). The RGS scores on the first two unloading days (D7 and D8) returns to lower levels, similar in magnitude to D0 through D3. The scores return to sham levels from D10 to D13. From these data, both methods seem to detect pain in rats, and the RGS detects minor changes.
Rats in the L1 and L2 groups were randomly assigned and testing conditions for the two groups were the same. The only difference was the digital video recording time points. The RGS scores in the L1 group are elevated after the first day after loading (D0 = 1.07±0.17) compared to baseline (0.49±0.13), and the RGS scores stay steady throughout the loading phase after loading for 3 continuous days (D2 = 1.28±0.10). To test the effect of mechanical TMJ loading, we compared the combined L1/L2 group and the sham group at matching time points. The two loading groups have significantly higher RGS scores compared with the sham group within the loading phase on D5 (Figure 10) (Table 2) (Figure 11) (Table 3). In the loading phase, there are no differences until D5. One reason for this may be the limited data from the sham group and that
all the time points were not matched in the loading groups. After loading was stopped, the RGS score of the L2 group returns to the sham levels on D13 (RGS score: L2 = 0.62±0.21; sham = 0.60±0.28).

Figure 10. The L1 and sham groups.
#: Significant difference between the loading group and the sham group (p < 0.05)

<table>
<thead>
<tr>
<th>L1 &amp; Sham</th>
<th>D-1</th>
<th>D0</th>
<th>D1</th>
<th>D5</th>
<th>p-value</th>
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<tbody>
<tr>
<td>D-1</td>
<td>1.000</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D0</td>
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<td>D1</td>
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<td></td>
</tr>
<tr>
<td>D5</td>
<td></td>
<td></td>
<td></td>
<td>0.0000010</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. P-values for the L1 group and the sham group
#: Significant difference between the loading group and the sham group (p<0.01)
Figure 11. The time course of RGS scores in the L2 group and the sham group. 
#: Significant difference between the loading group and the sham group ($p < 0.01$)

<table>
<thead>
<tr>
<th></th>
<th>Mean RGS (0-2)</th>
<th>Standard deviation</th>
<th>$p$-value</th>
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<tbody>
<tr>
<td></td>
<td>Loading (n=4)</td>
<td>Sham (n=4)</td>
<td></td>
</tr>
<tr>
<td>D-2</td>
<td>0.519</td>
<td>0.072</td>
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</tr>
<tr>
<td>D-1</td>
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<td>0.450</td>
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<tr>
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<td></td>
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<tr>
<td>D1</td>
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<td>D3</td>
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<tr>
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<td>0.360</td>
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<tr>
<td>D7</td>
<td>0.950</td>
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<td>D8</td>
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<tr>
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<tr>
<td>D13</td>
<td>0.619</td>
<td>0.600</td>
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</tr>
<tr>
<td></td>
<td>0.213</td>
<td>0.280</td>
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</tr>
</tbody>
</table>

Table 3. RGS scores of the L2 group and the sham group.
Lateral tail vein injections of vehicle or naproxen were delivered after loading on D4 and D5. In this study, naproxen was hypothesized to reduce TMJ pain. Overall, the RGS scores of the vehicle group were not significantly different from the L1 group at any time points (Figure 12) (Table 4). However, on D5, the RGS score rebounds back to original levels. This may occur because one of the rats in the group had a substantially lower score (0.83) at the time point compared to the other two rats (1.13 and 1.58), lowering the average RGS score (1.18±0.38) on D5.

After injection of naproxen, the value of RGS decreased immediately (D4=1.09±0.17, D5=1.03±0.18), but was not significantly different from loading or vehicle injection with correction for multiple comparisons (Figures 13 and 14) (Tables 5 and 6). Continued loading without naproxen injection was performed on D6, at which point the RGS score increased to 1.2±0.17. The naproxen-injected group had no significant differences compared to sham at matched time points (D-1, D0, D1, D5). Comparison of the vehicle-injected group and the naproxen-injected group, also showed no statistical differences (Figure 13) (Table 5).
Table 4. P-values for the comparison of the L1 group and the vehicle-injected group.

<table>
<thead>
<tr>
<th>L1&amp;V</th>
<th>D-2</th>
<th>D-1</th>
<th>D0</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-value</td>
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<td>1.000</td>
<td>1.000</td>
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<td>0.970</td>
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<td>0.968</td>
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</table>

Table 5. P-values for the comparison of the vehicle-injected group and the naproxen-injected group.

<table>
<thead>
<tr>
<th>V&amp;N</th>
<th>D-2</th>
<th>D-1</th>
<th>D0</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
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<th>D6</th>
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</thead>
<tbody>
<tr>
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<td>0.972</td>
<td>1.000</td>
<td>0.746</td>
<td>1.000</td>
<td>0.995</td>
<td>0.897</td>
</tr>
</tbody>
</table>
The RGS scores in the naproxen-injected group continuously decreased on D4 and D5. However, there is no statistical differences when compared to the L1 group. The score rebounds to higher levels on D6, during which the rats were in the loading phase without injection. Interestingly, the RGS score is dramatically lowered, almost to sham levels, on D7 (unloading phase, $0.77\pm0.26$) and is significantly different from the L1 group (Figure 14) (Table 6). The large variation in the RGS score may contribute to the lack of significant difference between the two injection groups on D7. From the results here, naproxen appears to be reducing pain somewhat but higher doses may be required to completely resolve pain. The reason why the RGS scores decrease significantly after unloading in the naproxen group is still unclear.

![Figure 14. The L1 group and the naproxen-injected group.](image)

©: Significant difference between the loading group and the naproxen-injected group ($p<0.01$)

<table>
<thead>
<tr>
<th>L1&amp;N</th>
<th>D-2</th>
<th>D-1</th>
<th>D0</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-value</td>
<td>0.985</td>
<td>1.000</td>
<td>0.732</td>
<td>1.000</td>
<td>0.674</td>
<td>0.985</td>
<td>0.268</td>
<td>0.080</td>
<td>0.926</td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>

Table 6. P-values for the comparison of the L1 group and the naproxen-injected group.
The agreement between four trained RGS raters was calculated using the intraclass correlation coefficient (ICC). According to prior investigations (Landis et al. 1977, Altman et al. 1990), there are five categories of ICC levels indicating agreement: (1) very good (0.81-1.00); (2) good (0.61-0.80); (3) moderate (0.41-0.60); (4) fair (0.21-0.40); and (5) poor (<0.20). For the individual AUs, orbital tightening is the only highly reliable indicator component when compared to the other three AUs (ICC=0.86, very good). The second is whisker change, with moderate level of reliability (ICC=0.64). And the nose/cheek flattening and ear change are the two least reliable AUs with fair level of reliability (ICC=0.44 and 0.45 respectively). The ICC of the average RGS scores between four raters is 0.85, which is represent a “very good” level of reliability (Figure 15) (Table 7) (Landis et al. 1977, Altman et al. 1990).
Figure 15. RGS scores across four raters.

Table 7. Intraclass correlation coefficients (ICC) of the four raters.

The average RGS scores between four raters is 0.85, which is representative of a “very good” level of reliability. For the individual AUs, orbital tightening is the only highly reliable indicator component (ICC=0.86, very good). The second is whisker change, with moderate level of reliability (ICC=0.64). And the nose/cheek flattening and ear change are the two least reliable AUs with fair level of reliability (ICC=0.44 and 0.45 respectively).
Discussion

This is the first study applying RGS to mechanically-induced TMJ pain over an extended observation period. From the results, both reflex testing and RGS scoring appear to be alter to detect pain in rats subjected to TMJ loading. The withdrawal reflex is a measurement of hypersensitivity. However, it might also indicate that the animals want to avoid the stimulus. LaBuda and Fuchs found that rats avoid behavior during the first 10-15 minutes of mechanical stimulation (LaBuda and Fuchs, 2000). The withdrawal reflex testing is technically sensitive, requiring experienced animal handler to apply the test. And the nociceptive reaction acquired from the test is not always present as the “actual pain” (Le Bars et al. 2001, Mogil and Crager, 2004). The RGS scoring is a non-evoked test. It has been used on different pain models but not with long-term observation. Most studies using RGS scoring have only a single exposure to chemical injection or surgical intervention, with 3 to 48 hours of observation (Sotocinal et al. 2011, Oliver et al. 2014, Whittaker et al. 2014). De Rantere's group uses data on day 7, 48 hours after a one time exposure to the stimuli (De Rantere et al. 2015). That study, however, recorded data for up to 14 days with “per day” as an observation unit instead of “hour”. To our knowledge, there is only one study similar to ours with continuous mechanical force applied to the teeth with orthodontic device, and RGS scoring on setting days (Liao et al. 2014). That study did not show the original data of RGS. Each rat had two RGS scores in a day; a baseline RGS score of the day, and another RGS score after activating closed-coil spring. The statistic analyses is based on the RGS difference score of the day which is different from the other studies. Previously, our lab applied the RGS scoring system to spinal nerve root compression rat model. According to the study, the peak of RGS scores appeared at three to six hours after nerve root injury (Philips et al. 2017). Our choice of videotaping three hours after TMJ loading is based on this evidence.
This study used female rats. According to clinical studies, the female population has a two-fold higher risk of persistent TMJ signs or symptoms compared to males (LeResche et al. 1997, Marklund et al. 2010, Anastassaki Kohler et al. 2012). The baseline RGS scores of our study range from 0.32 to 0.57, and the baseline of other studies range from 0.1 to 0.67 (Sotocinal et al. 2011, Oliver et al. 2014, Liao et al. 2014, Whittaker et al. 2014, De Rantere et al. 2015). Only one paper uses equal numbers of male rats and female rats and their baseline scores range from 0.2-0.4 (Sotocinal et al. 2011). There are two male-only studies, the first one with the same baseline range as the Sotocinal study (De Rantere et al. 2015). The other one does not mentioned the actual RGS score, but set baseline RGS as 0 with all data subtracted 0.1, so we assume their baseline is 0.1 (Liao et al. 2014). One female-only study did not mentioned the actual value of RGS scores either. However, the article proposed an “analgesic intervention score” from the RGS system and defined values less than 0.67 as “non painful” (Oliver et al. 2014). The baseline value from their group is assumed to be 0.67. It is interesting to notice that the RGS score of the male rats tend to have lower value than the female rats. Though there are limited studies or data to discuss the differences of RGS scores between the female rats and the male rats, gender may be one issue we have to consider in RGS scores.

De Rantere compared the evoked withdrawal responses with RGS scoring for rats (De Rantere et al.2015). Male rats were given a chemical injection or a plantar incision. The peak of pain was the same for mechanical reflex testing and RGS. But the withdrawal threshold remained at a lower level after RGS values returned to baseline levels. It was concluded that RGS scores showed pain caused by inflammatory reaction, while the mechanical reflex testing reflected the response to an external insult to the inflammed tissue. The results of De Rantere support the results from our study. From the literature, the mechanism of nociceptive pain is know to be different from inflammatory pain (Woolf 2010). This may account for the RGS score returning to baseline levels sooner than the value of the withdrawal threshold.
The mechanical stimulation can only detect the actual or potential tissue damage but the emotional component. The head withdrawal action might just the avoidance to the stimuli, not the actual withdrawal reflexes reflect. From previous literature, we know facial expression of nonhuman animals are able to tell the emotion (Darwin 1872, Langford et al. 2010). Pain is defined as, “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey et al. 1994). Evoked pain assessment method need to contact nonhuman animal directly, the communication between human and nonhuman animal might alter the result. RGS is a non-evoked pain assessment tool that reduce the chance that interaction between human and nonhuman animal. It is more objective way to evaluated the pain. The downside of the RGS system is that workers need to spend much more time than evoked pain assessment. Extra works need to be done after finishing video recording. We may know more about the pain when combining the two behavior testing systems together.

From the results, the RGS seems to be a useful tool to detect mechanically-induced TMJ pain in rats. The advantage of the RGS is in avoiding any interference of providers with subjects and reducing stress-induced analgesia (Sorge et al. 2014). Although it might not be a useful tool in the clinical situation, the RGS is a good way to quantify the ongoing acute pain in open-jaw rat model. There has some limitations. This is a pilot study to transfer the RGS to the TMJ pain model in rats, there has some limitations. First, we only include female rats in this study. Male rats might have different RGS scores in the TMJ pain model. Second, this study only has a small sample size (n=23). The small sample size means the statistical power is low, which might reduced the chance to detect the true effect (Button et al. 2013). Also, the effect of naproxen in this study is not obvious. Additional studies are required to increase the dosage of naproxen as well.
Conclusions

In this study, pain is evaluated by mechanical reflex testing and RGS after mechanical TMJ loading. Also, analgesic interventions were used to detect changes in RGS scoring. Both reflex testing and RGS scoring can detect pain in rats subjected to TMJ loading. RGS may help us to detect changes earlier than mechanical reflex testing. From this study, we can say that orofacial pain can be detected by RGS, which may provide a useful new method to evaluate TMJ pain.
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