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USE OF RAT GRIMACE SCALE IN PAIN RESEARCH

Since its introduction in 2011, the Rat Grimace Scale (RGS) has proven to be a valuable tool for pain assessment in preclinical studies. This scale includes the assessment of four functional units to quantify pain responses in rats. By observing and assessing changes such as orbital tightening, nose/cheek flattening, ear changes, whisker change researchers can objectively assess the severity of pain experienced by animals. In our article, we offer an overview of a various studies applying this method across surgical and postoperative pain models, inflammatory pain models, orthodontic and orofacial pain models, neuropathic pain models, and others. This study specifically investigates the effectiveness of the RGS in assessing pain in rats, with a particular focus on a nitroglycerin-induced migraine model. The article underscores the humane and non-invasive nature of the RGS, aligning with current ethical standards in animal research. Additionally, it explores the potential application of the RGS across diverse disciplines, including neuroscience, pharmacology, and veterinary medicine. The study also addresses limitations and biases in the current pain assessment methods, proposing future research directions to enhance accuracy and ethical practices in animal pain research. The development of an automated RGS system capable of identifying facial action units in rat images and predicting RGS scores has become imperative to to reduce the labor intensity of the image acquisition and scoring process.

Key words: rat grimace scale, pain, migraine, preclinical study.

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Ауырсынуды зерттеу кезінде егеуқұйрықтардың Бет-әлпеті Шкаласын қолдану

2011 жылы енгізілгеннен бастап, Егеуқұйрықтардың Бет-әлпеті Шкаласы (ЕБШ) клиникаға дейінгі зерттеулердегі ауырсынуды бағалаудың құнды құралына айналды. Бұл шкала егеуқұйрықтардағы ауырсыну реакцияларын сандық бағалау үшін төрт функционалды бірлікті бағалауды қамтиды. Орбиталық тарылу, мұрын мен щектің тегістелуі, құлақ пен мұрттың өзгеруі сияқты өзгерістерді бақылау және бағалау арқылы зерттеушілер жануарлардың ауырсынуының ауырлығын объективті түрде бағалай алады. Біздің мақалада біз бұл әдіс хирургиялық және операциядан кейінгі ауырсыну үлгілерінде, қабыну ауруы үлгілерінде, ортодонтиялық және ауыз-бет ауруы үлгілерінде, невропатиялық ауырсыну үлгілерінде және т.б. қолданылған әртүрлі зерттеулерге шолу жасаймыз. Зерттеу егеуқұйрықтардағы ауырсынуды бағалауда ЕБШ тиімділігін көрсетеді, нитроглицеринмен индукцияланған мигрень үлгісіне ерекше назар аударылады. Жануарларды зерттеуде қазіргі этикалық стандарттарға сәйкес келетін гуманитарлық, инвазивті емес әдіс ретінде ЕБШ рөлі атап өтіледі. Нейроғылым, фармакология және ветеринарияны қоса алғанда, әртүрлі пәндерде ЕБШ қолдану әлеуеті зерттеледі. Зерттеу ауырсынуды бағалаудың қолданыстағы әдістеріндегі шектеулерді қарастырады, ауырсынуды зерттеу саласындағы дәлдікті жақсарту және этикалық ережелерді сақтау үшін болашақ зерттеулердің бағыттарын ұсынады. Егеуқұйрық кескіндеріндегі функционалдық бірліктерді анықтауға және ЕБШ бағалауларын болжауға қабілетті автоматтандырылған ЕБШ жүйесін әзірлеу кескіндерді алу және бағалау процесінің күрделілігін азайту үшін қажет болды.

Түйін сөздер: егеуқұйрықтардың бет-әлпеті шкаласы, ауырсыну, мигрень, клиникаға дейінгі зерттеу.

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Использование Шкалы Гримас крысы в исследованиях боли

С момента своего внедрения в 2011 году Шкала Гримасы Крыс (ШГК) стала ценным инструментом для оценки боли в преклинических исследованиях. Эта шкала включает оценку четырех функциональных единиц для количественной оценки болевых реакций у крыс. Наблюдая и оценивая такие изменения как орбитальное сужение, уплощение носа и щек, изменение ушей и усов, исследователи могут объективно оценить тяжесть боли, которую испытывают животные. В нашей статье мы предоставляем обзор разнообразных исследований, в которых данный метод применялся в хирургических и послеоперационных моделях боли, воспалительных моделях боли, ортодонтических и орофациальных моделях боли, невропатических моделях боли и других. В исследовании демонстрируется эффективность ШГК при оценке боли у крыс, особое внимание уделяется модели мигрени, индуцированной нитроглицерином. В статье подчеркивается роль ШГК как гуманного, неинвазивного метода, соответствующего современным этическим стандартам в исследованиях на животных. Кроме того, изучается потенциал применения ШГК в различных дисциплинах, включая нейронауку, фармакологию и ветеринарию. Исследование также рассматривает ограничения в существующих методах оценки боли, предлагает направления будущих исследований для повышения точности и соблюдения этических норм в области исследований боли. Разработка автоматизированной системы ШГК, способной идентифицировать функциональные единицы на изображениях крыс и прогнозировать оценки ШГК, стала необходимой для уменьшения трудоемкости процесса получения изображений и их оценки.

Ключевые слова: шкала гримасы крыс, боль, мигрень, преклинические исследования.

Abbreviations

RGS – rat grimace scale, NTG – nitroglycerin

Introduction

It is imperative to employ a diverse range of methods for assessing pain in animal models. This necessity arises from the fact that animals lack the ability to provide feedback on their pain in the manner humans do during a medical examination. A similar challenge is encountered in studies conducted on non-verbal patients, such as children who have not yet learnt to speak. In such cases, pain assessment is often relies on analyses of facial characteristics. Drawing on Darwin's observation that animals exhibit changes in facial expressions analogous to humans when expressing emotion [1], a mouse grimace scale [2] was developed in 2010. Subsequently, in the following year, a similar rat grimace scale was introduced to evaluate the pain state of rats [3] This progression was logical, considering that the number of studies utilizing rats in pain research has consistently outnumbered those employing mouse models (Figure 1).

The rat grimace scale is utilized to assess rat head images based on four distinctive features [3]:

1. Orbital Tightening: This involves the narrowing of the orbital area, manifesting either as (partial or complete) eye closure or eye "squeezing";
2. Nose/Cheek Flattening: This feature observes the reduction inbulging of the nose and cheek, eventually leading to the absence absence of the crease between the cheek and whisker pads;
3. Ear Changes: Ears exhibit folding, curling and angling forwards or outwards, resulting in a pointed shape, and the space between the ears may appear wider;
4. Whisker Change: Vibrissae move forward (away from the face) from the baseline position, tending to bunch and giving the appearance of whiskers standing on end.

The manifestation of these characteristics varies in relation to the presence and severity of pain, as depicted in Figure 2. Each of the four functional units is assigned a score on a 3-point scale: 0 indicates no change, 1 indicates moderate change, and 2 indicates marked change.

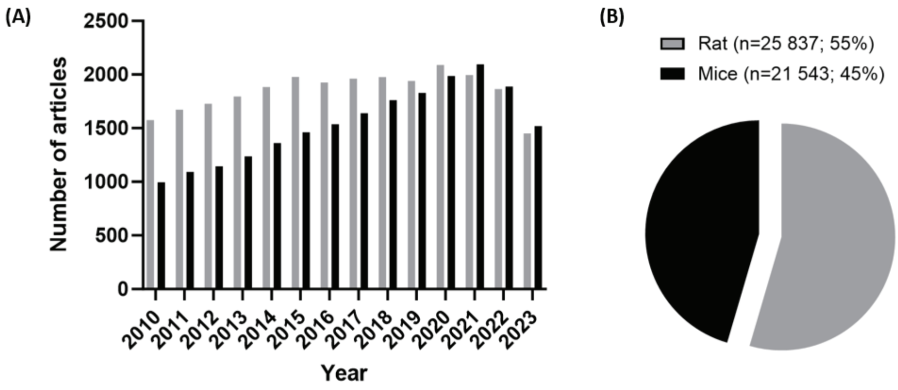


Figure 1 – Number of articles in the PubMed database for the keywords “pain” and “rat” or “pain” and “mice” for 2010-2023. (A) Articles by year ; (B) Total number for the period.

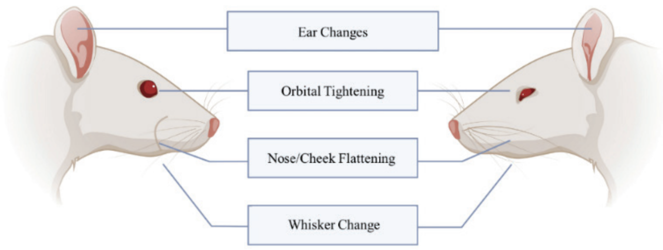


Figure 2 – Comparison of rat grimaces without pain (left) and with pain (right).

A comprehensive search of the PubMed database was conducted, covering articles up to October 23, 2023, using the keywords “rat grimace scale” and “pain” The aim was to analyze the utilization of the scale in various pain models, assess its suitability for gauging pain status, and determine the optimal timing for obtaining photographs post-surgery or injection. The article selection workflow is outlined in Figure 3.

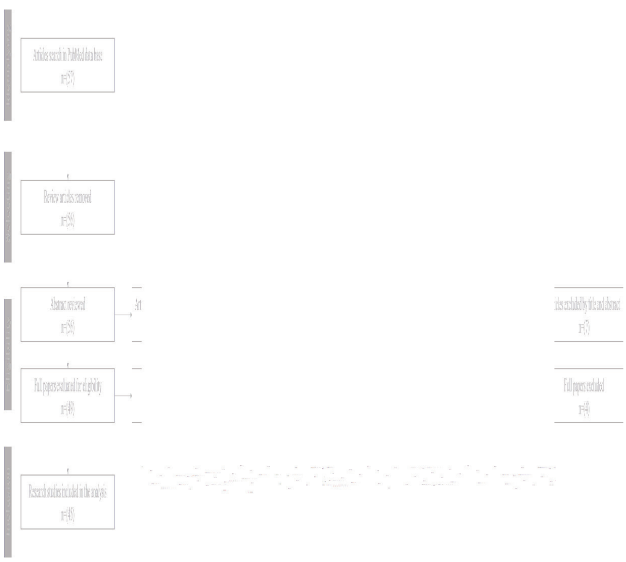


Figure 3 – Scheme for selecting articles for analysis.

The application of the rat grimace scale extends to various pain models, which can be broadly categorizedinto five main types: surgical and postoperative pain models, inflammatory pain models, orthodontic and orofacial pain models, neuropathic pain models and others (Table 1).

Table 1 – Studies that have used RGS to assess different pain models

Type of Pain	Pain Model	Reference	Was RGS sensitive in the model? (the time of the significant increase if mentioned)
Surgical and postoperative pain models	Laparotomy	Sotocinal *et al.* (2011) [3]	Yes, there was a significant increase until 6 hours, with the highest level observed at 6 hours
		Chi *et al.* (2013) [4]	Yes, there was a significant increase until 8 hours, with the highest level observed at 4 hours
		Kawano *et al.* (2014) [5]	Yes, there was a significant increase until 8 hours, with the highest level observed at 4 hours
		Waite *et al.* (2015) [6]	Yes
		Thomas *et al.* (2016) [7]	Yes, there was a significant increase until 8 hours
		Gao *et al.* (2017) [8]	Yes
		Guo *et al.* (2017) [9]	Yes, all surgical groups developed significant acute pain starting 2 h postoperatively and recovered to baseline level 12 h after surgery
		Klune *et al.* (2019) [10]	Yes, with the highest level observed at 0.5 hour post-operatively
		Koyama *et al.* (2019) [11]	Yes, with the highest level observed at 2, 4, 6 hours after inhalation period
	Implantation surgery	Oliver *et al.* (2014) [12]	Yes, there was a significant increase until 8 hours, with the highest level observed at 4 hours
	Incisional pain	De Rantere *et al.* (2016) [13]	Yes, there was a significant increase at 6 hours and 9 hours
		Kawano *et al.* (2016) [14]	Yes, there was a significant increase at 2 hours
		Yamanaka *et al.* (2017) [15]	Yes, there was a significant increase at 2 hours post incision
	Laminectomy	Schneider *et al.* (2017) [16]	Yes, animals exhibited a significant increase in average grimace score 20 sec after the acetone application
		Krishnan *et al.* (2019) [17]	Yes, presence of pain was evident until Day 7 in the conventionally spinal cord injured group
		Harikrishnan *et al.* (2021) [18]	Yes
		Pal *et al.* (2022) [19]	Yes, all rats exhibited pain, which diminished by 10 days and stayed low
		Semita *et al.* (2023) [20]	Yes
	Craniotomy model	Tsaousi *et al.* (2022) [21]	Yes

Table continuation

Type of Pain	Pain Model	Reference	Was RGS sensitive in the model? (the time of the significant increase if mentioned)
Inflammatory pain models	Complete Freund's adjuvant (CFA) model	Sotocinal *et al.* (2011) [3]	Yes, there was a significant increase until 24 hours, with the highest level observed at 6 hours
		Asgar *et al.* (2015) [22]	Yes, the RGS score was significantly increased from the baseline at 6 hour, day 1 (the highest) and day 3 and returned to the baseline level by day 7
		De Rantere *et al.* (2016) [13]	Yes, the highest increase was at 4 hours
	Kaolin/carrageenan carrageenan model	Sotocinal *et al.* (2011) [3]	Yes, there was a significant increase at 3 hours
		De Rantere *et al.* (2016) [13]	Yes, there was a significant increase at 6 hours and 9 hours
		Leung *et al.* (2016) [23]	Yes, there was a peak in carrageenan-induced pain at 6 hours
		Leung *et al.* (2019) [24]	Yes, with an expected peak in carrageenan-induced pain at 6 hours
	Lipopolysaccharide-induced dental pulp inflammation	Nurhapsari *et al.* (2023) [25]	Yes
	Experimental Acute Rhinosinusitis	Lovrenčić *et al.* (2020) [26]	Yes, within 40 minutes after injection
Orthodontic and orofacial pain models	Tooth Movement	Liao *et al.* (2014) [27]	Yes, there was a significant increase on day 1 and day 3
		Long *et al.* (2015) [28]	Yes, it started to increase on day 1, peaked on day 3, maintained at the increased level on day 5 and day 7, and returned to baseline level on day 14
		Gao *et al.* (2016) [29]	Yes, RGS scores were significantly higher on day 1, day 3, day 5, and day 7
		Guo *et al.* (2019) [30]	Yes, there was a significant increase until 7 days, with the highest level observed on day 3
	Jaw loading	Sperry *et al.* (2018) [31]	Yes, there was a significant increase until 7 days, with the highest level observed on day 5
	Force-induced pain	Thammanichanon *et al.* (2021) [32]	Yes, there was a significant increase until 3 days
	Orofacial Pain	Long *et al.* (2017) [33]	Yes, the pain levels were the highest on days 1 and 3
Neuropathic pain models	Cervical Radiculopathy	Philips *et al.* (2017) [34]	Yes, RGS values were the highest at 6 hours after cervical nerve root compression
	Trigeminal neuropathic pain	Akintola *et al.* (2017) [35]	Yes, RGS scores 10 days 27 days after CCI were higher in experimental group

Table continuation

Type of Pain	Pain Model	Reference	Was RGS sensitive in the model? (the time of the significant increase if mentioned)
Other pain models	Burn model	Goder *et al.* (2021) [36]	Yes
		Goder *et al.* (2022) [37]	Yes
	Model of intracerebral hemorrhage	Saine *et al.* (2016) [38]	Yes
		Wilkinson *et al.* (2020) [39]	Yes
	Model of Chemotherapy-Induced Mucositis	George *et al.* (2019) [40]	No, RGS lacked the sensitivity to successfully discriminate pain in this model
	Model of fibromyalgia	Nagakura *et al.* (2019) [41]	Yes, a significant increase in the RGS score, which was sustained for 2 weeks or more after the induction of fibromyalgia-like state by reserpine injection
	Model of fibromyalgia Traumatic Injury	Tanei *et al.* (2020) [42]	Yes, RGS score for reserpine-induced fibromyalgia-like rats was significantly higher compared to that for control group
		Uddin *et al.* (2019) [43]	Yes
	Traumatic Injury NGF-Induced Trunk Mechanical Hyperalgesia	Kudsi *et al.* (2023) [44]	Yes
		Reed *et al.* (2020) [45]	Yes, RGS scores increases reached significance on Day 5 + 4 h
	Temporomandibular joint osteoarthritis chronic pain	Liu *et al.* (2022) [46]	Yes
	Endometriosis model	Chen *et al.* (2024) [47]	Yes

In terms of the number of scorers, vary in their approach, ranging from a single experienced scorer to several researchers trained in the use of the scale but lacking prior experienceexperience in image assessment. It is crucial to highlight that these scorers analyzed the images blindly, without information about the groups or conditions of the study. Data analysis commonly involves calculatingthe mean values of each scorers' scores for each of the four functional units, followed by averaging these values across all assessors. This method ensures the generation of reliable and objective results, minimizing the potential impact of subjective assessments by individual scorers. In cases involving multiple assessors,statistical measures such as Cronbach'salpha are often employed. Ponterotto and Ruckdeschel [48] proposed that alpha values exceeding 0.75 are excellent for scales with a small number of items. Two methods are employed for obtainingimages of rats: video recording of rats over a period of a specified period, followed by photo extraction, and direct photography of rats. It's worth noting that both frontal and profile images of rats can be used for grimace estimation. Following photography, the images undergo processing, including randomization and the removal of removing all identifying features, before being presented to assessors for evaluation.

The RGS has emerged as a valuable tool for pain assessment in laboratory rats, offering several advantages over other methods. Most of the methods assess pain by evaluating responses to nociceptive stimuli like mechanical, thermal, and chemical reactions or overall changes in behavior such as weight loss and socialization pattern changes. Although these tests offer valuable insights into the well-being of rats, conventional evaluations of pain responses face challenges in establishing a direct correlation between behavior and the actual pain experience. Many of these tests primarily monitor secondary responses to pain, such as withdrawal latency from a stimulus, rather than directly capturing the subjective pain experience itself.

The RGS measures characteristic changes in facial expressions associated with pain, providing a non-invasive means of pain evaluation. This method has been shown to be sensitive in different pain models, demonstrating its its versatility and effectiveness in different pain contexts. Compared to other pain assessment tools, the RGS has been noted for its ability to capture spontaneous pain, representing the animal's affective response to pain [32]. This is a crucial advantage, as the RGS's remarkable ability to measure spontaneous pain, particularly in conditions like migraine characterized by this type of pain, holds immense potential for successfully translating basic science findings into clinically relevant applications.

The RGS provides ethical advantages in the assessment of pain in laboratory rats. By offering a non-invasive method for quantifying pain through facial expressions, the RGS reduces the need for invasive procedures or distressing manipulations that may be involved in other pain assessment methods. This aligns with the ethical principle of minimizing harm and distress to research animals, as the RGS allows for pain assessment without subjecting the animals to additional stress or discomfort.

The primary goal of this study was to investigate the potential of the RGS as a tool for pain assessment across diverse scientific research contexts. Subsequently, the secondary aim was to reassess the effectiveness of the RGS in evaluating pain in rats, with a specific focus on a model of nitroglycerin-induced migraines. Lastly, the tertiary objective aimed to advocate for the integration of technological advancements in pain assessment methods.

Materials and Methods

Ethical approval.

All experiments conducted in this study received ethical approval from the local Ethics Committee of Al-Farabi Kazakh National University located in Almaty, Republic of Kazakhstan. Approval was granted with the permission number IRB-377 on 24 February 2022.

Animals and their housing.

The study utilized sexually mature male white laboratory mongrel rats born and bred in the educational and scientific laboratory base of Al-Farabi Kazakh National University. Animals were housed under natural light conditions at a temperature of 21-22°C, in cages with hard floor and soft bedding. Food and water were provided to the animals *ad libitum*. Prior to behavioral tests, animals underwent a one-week acclimatization period and were familiarized with the test equipment and procedures. All tests were conducted between 8:00 to 14:00.

Migraine model.

To induce and chronicize migraine, five intraperitoneal injections of nitroglycerin at a dose of 10 mg/kg were administered every other day for ten days, using an insulin syringe with a 30G needle. Animals in the control group received injections of 0.9% saline solution in the same volume The injected volume was determined by measuring the weight of the animals on the day of the study. Following injection, animals were placed in recovery cages and observed for several minutes.

Rat grimace scale.

The rat grimace scale was employed to quantify pain. Photographs were manually taken 30 minutes after each of the five injections. Evaluation was based on the image that best depicted orbital tightening, based on a 3-point scale (0 – no change, 1 – moderate change, 2 – obvious change). Two independent investigators, unaware of the groups, performed these evaluations.

Methods of statistical processing of data.

Data were statistically analyzed using GraphPad Prism version 10.0.3 and SPSS 29.0 software. Results are presented as mean values (MEAN) with standard error (SEM). Two-way ANOVA was employed to determine the statistical significance of the behavioural results, with significance defined as $P<0.05$.

Results and Discussion

Image collection

A total of 60 images (see Figure 4A), gathered from 12 rats on each of the 5 days during the induction of migraine episodes, were selected for analysis. The scores were averaged across the two raters to derive an overall pain score for each rat.

Reliability assessment

Reliability varied based on the action unit being rated. The overall inter-rater reliability, as measured by was Cronbach's alpha, was 0.89 (see Figure 3B).

Grimace scores and stability

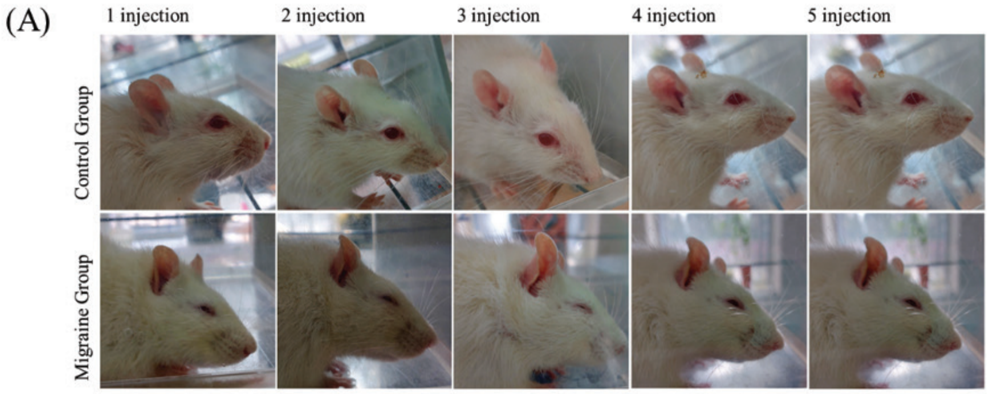
Grimace scores were consistently higher in the NTG group, remaining relatively stable over the course of the 5 test sessions when compared to the control group (refer to Figure 3C).

ANOVA results

A two-way ANOVA conducted on this dataset revealed substantial main effects for Treatment [$F(1,10) = 63.00$, $p < 0.0001$] and the Treatment x Test Session interaction [$F(4,40) = 2.755$, $p = 0.0409$]. However, the Test Session [$F(2.970,29.70) = 2.741$, $p = 0.0613$] did not reach significance.

In the context of migraine research, the RGS proves to be a valuable tool for evaluating pain in various pain models, demonstrating consistent patterns in response to nitroglycerin across multiple migraine episodes. Among the four functional units comprising the Rat Grimace Scale, orbital tightening emerges as the most observable and quantifiable in this rodent migraine model. The data reveal the highest inter-rater agreement in assessing orbital tightening. According to the Harris *et al.* [49], orbital tightening may represent a behavioral manifestation of acute hyperalgesia related to migraine photosensitivity. On the other hand, Yamamoto *et al*. study in 2016 [50] offers evidence supporting the idea that nausea can influence the eye action unit.

However, there are several critical aspects surrounding the application of grimace scales in rats that should be considered. Notably, in both the original study and subsequent investigations, no sex differences were identified in grimace responses among rats. However, a notable observation is that the majority of grimace studies in rats appear to have been conducted in a singular sex, signifying a potential gap in our understanding of sex-specific responses. Furthermore, external environmental factors, such as the presence of observers, have been shown to influence grimace responses in rodents. The study by Sorge *et al.* suggests that the gender of the observer can affect grimace scores, with significant decreases recorded in the presence of male observers, indicating a potential physiological stress response [51]. This finding prompts questions about the potential confounding effects of external stimuli on grimace scales and underscores the necessity for careful consideration of environmental factors in experimental design.



(B)

	Cronbach α
RGS overall	0,893
Orbital tightening	0,965
Nose or cheek flattening	0,693
Ear position	0,790
Whisker change	0,690

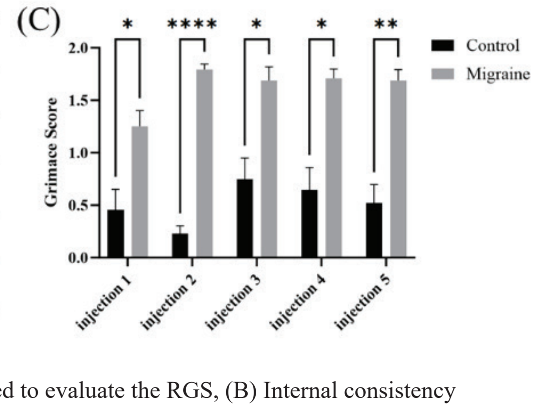


Figure 4 – (A) Representative images used to evaluate the RGS, (B) Internal consistency of scoring (Cronbach α) among 2 blinded raters, (C) RGS across 5 test sessions. Values represent mean±SEM. *Indicates $p<0.05$, ** indicates $p<0.01$, **** indicates $p<0.0001$. Sample size was n=6 per group.

While grimace scales offer valuable insights into pain assessment in rats, the discussion underscores the need for a comprehensive exploration of factors influencing grimace responses, including sex differences and external environmental stimuli.

Conclusion

In conclusion, the field of rat grimace scale in biomedical research holds immense promise, with the potential to transform both preclinical pain research and veterinary pain management. Despite a decade of investigation, widespread adoption has been hindered by labor-intensive methodologies involving manual image extraction and scoring. However, recent advancements, particularly the integration of Artificial Intelligence (AI) and object recognition technologies, offer exciting possibilities for automation and efficiency.

The future trajectory of grimace scales is poised to benefit significantly from AI, streamlining tasks such as image selection, parameter randomization, data reassembly, and statistical processing. AI systems have the potential to excel in key areas, including selecting optimal photographs, standardizing images, and conducting measurements. This technological evolution not only enhances efficiency but also opens avenues for large-scale implementation, addressing practical challenges associated with dealing with substantial numbers of animals.

Furthermore, the success demonstrated in mice, as evidenced by Tuttle *et al.* [52] convolutional neural network achieving a remarkable accuracy of approximately 93%, underscores the viability and reliability of machine learning and AI in grimace recognition. As these technologies mature, they hold the promise of making rat grimace scale scoring a routine outcome measure through facility-automated systems, addressing public concerns about ethical decision-making in biomedical research. The integration of AI is not just a technological advancement but a pivotal step toward unlocking the full transformative potential of grimace scales in advancing pain research and management.

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