Video Article

Automated Gait Analysis in Mice with Chronic Constriction Injury

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URL: https://www.jove.com/video/56402
DOI: doi:10.3791/56402

Keywords: Neuroscience, Issue 128, Gait analysis, mechanical allodynia, von Frey test, neuropathic pain, mice, pain

Date Published: 10/17/2017

Citation: Kang, D.W., Choi, J.G., Moon, J.Y., Kang, S.Y., Ryu, Y., Park, J.B., Kim, H.W. Automated Gait Analysis in Mice with Chronic Constriction Injury. *J. Vis. Exp.* (128), e56402, doi:10.3791/56402 (2017).

Abstract

The von Frey test is a classical method that has been widely used to examine the sensory function of neuropathic pain animals. However, it has some disadvantages such as subjective data and the requirement of a skilled, experienced experimenter. To date, a variety of modifications have improved the von Frey method, but it still has a few limitations. Recent reports have suggested that gait analysis produces more accurate and objective data from the neuropathic animals. This protocol demonstrates how to perform the automated gait analysis to determine the degree of neuropathic pain in mice. After several days of acclimation, the mice were allowed to walk freely on the glass floor to illuminate footprints. Then, quantification of the footprints and gait were performed through video clips with automatic analysis of various walking parameters, such as area of paw print, swing time, angle of paw, *etc.*

The main purpose of this study is to describe the methodology of automated gait analysis and briefly compare it with data from the classical sensory test using von Frey filament.

Video Link

The video component of this article can be found at https://www.jove.com/video/56402/

Introduction

Pathological changes of nervous system induced by trauma, metabolic dysfunction, inflammation, infection, ischemia, or autoimmune disease occasionally result in neuropathic pain, which is defined as a pain arising as a direct consequence of a lesion or disease affecting the somatosensory system[1]. Neuropathic pain is usually unbearable and unfortunately, conventional analgesics generally do not produce sufficient pain relief[2]. A major feature of neuropathic pain contains spontaneous and stimulation-evoked (*i.e.*, allodynia and hyperalgesia) pains. Allodynia is a nociceptive response that occurs to normally non-painful stimuli, such as light touch or warm stimulation. Hyperalgesia indicates an enhanced pain response to noxious mechanical and/or thermal stimuli[3]. Although these two symptoms both critically impair the patient's quality of life, mechanical allodynia evoked by gentle tactile stimulation is the most aggravating symptom because soft contact is difficult to avoid in everyday life.

To investigate the underlying mechanism and new analgesics for the treatment of neuropathic pain, the precise measurement of the pain response is essential. Numerous neuropathic pain animal models have developed nociceptive responses on the hind paw area because of its high accessibility[4,5,6,7]. Thus, most pain response assessments have been performed on the plantar or dorsal surface of the hind paw by applying mechanical stimuli using special instruments, such as von Frey filaments. One of the most frequently used method is the up and down method described by Dixon[8] and the later modified versions[9,10]. However, very skilled, experienced experimenters are required to perform the von Frey test and the results may be subjective.

The automated gait analysis system can investigate neurological and neuromuscular disorders by measuring various parameters of walking in freely moving rodents. In a variety of nerve injury animal models, the degree of nociception and the antinociceptive effect of several treatments can be evaluated without adding a pain stimulation[11,12,13,14]. This analysis system can detect static and dynamic gait parameters, such as: paw print area (the area of the complete paw print that contacts with the floor), paw intensity (the average intensity of the contacting paw area), stride length (the distance between successive placements of the same paw), stance phase (the duration of ground contact for a single hind paw), step sequence (the order in which the four paws are placed on the floor), swing (the duration of the swing phase), and swing speed (computed from stride length and swing duration and expressed as pixels per second). This paper demonstrates the use an analysis system and provides a brief comparison of data with the von Frey test using chronic constriction injury (CCI) neuropathic mice.

Protocol

All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee at the Chungnam National University (Daejeon, South Korea).

1. Induction of CCI on Sciatic Nerve

1. **House male ICR mice weighing 20 - 25 g under a 12 h light/dark cycle at controlled room temperature (maintained at 20 - 25 °C) and humidity (40 - 60%), with free access to food and water. Allow an acclimation period for mice in the animal care room of at least 1 week before surgery.**
    1. During housing, observe the appearance and behavior of the mice and do not use mice showing abnormal locomotor activity.
    NOTE: One day prior to the CCI surgery, measure the mechanical sensitivity of the hind paw by applying von Frey filaments and perform the automated gait analysis to obtain normal baseline values. Then, assign mice randomly to control and experimental groups.

2. **On the day of surgery, anesthetize mice by injecting 2,2,2-tribromoethanol (250 mg/kg, intraperitoneal (IP)).**
NOTE: Wear personal protective equipment such as surgical gown, gloves, and mask.
    1. Weigh 2.5 g of 2,2,2-tribromoethanol and add 2-methyl-2-buthanol to the final volume of 5 mL. Keep the solution away from light (*e.g.*, use a dark container or wrap in foil).
    2. Allow reagents to dissolve completely by heating at 40 °C and stirring for 10-30 min.
    3. Add distilled water to the final volume of 200 mL and stir until mixed well.
    4. Store aliquots at 4 °C and keep in the dark. After two weeks, the anesthetic should be replaced with new aliquots.
    5. Inject a volume of 20 µL per 1 g body weight. For example, if the body weight of the mouse is 25 g, give 500 µL of solution. The typical duration of this anesthesia is 1 h.
    NOTE: This solution will become toxic when exposed to light and/or heat so be careful to avoid exposure to light and heat. An IP injection route is highly recommended.

3. When the mouse falls into deep anesthesia, place it on the operating table with the dorsal side up (*i.e.*, prone position) and sterilize the outer mid-thigh area of the right side with a 70% alcohol swab.
NOTE: Confirm deep anesthesia by checking the lack of response to pinch or pressure stimulus on the hind paws or tail.
4. **Induce the CCI on the right sciatic nerve of the mouse.**
    1. Make an incision on the skin around the mid-thigh area of 1.0 - 1.5 cm length with a scalpel blade.
    2. Bluntly dissect the thigh muscles with the micro-mosquito hemostat to expose the sciatic nerve.
    3. Ligate the exposed sciatic nerve 3 times with 1.0 - 1.5 mm intervals using a 4-0 chromic catgut suture.
    NOTE: Make loose ligations by tying off the sciatic nerve until mild shaking appears on the ipsilateral hind paw. The sham group of mice received the same surgical opening under similar conditions except without nerve ligation.

5. Close the surgical opening with 3 - 4 simple interrupted sutures using 5-0 silk and sterilize with povidone iodine for disinfection of the outer area of surgery.
6. After surgery, place the mouse in a clean cage on a heating pad. When animals are recovered from anesthesia, return them to their home cage.
NOTE: In this study, antibiotics were not used. The CCI+GBP group received IP gabapentin (GBP) at a dose of 50 mg/kg once daily as a positive control.

2. Measurement of Mechanical Allodynia (von Frey Test)

NOTE: Assess the frequency of the withdrawal response to the mechanical stimuli by using 1 g of von Frey filament to the plantar surface of the ipsilateral hind paw.

1. On each test day, bring mice to the behavioral test room and acclimate the mice in their own home cage for at least 30 min before the test.
NOTE: Wear personal protective equipment such as surgical gown, gloves, and mask.
2. Put the mouse in a transparent acrylic box on the metal mesh floor and acclimate the mouse for 30 min.
3. Gently apply 1 g of von Frey filament to the plantar surface of the hind paw until the filament bends.
4. Apply filament stimulation to the ipsilateral hind paw 10 times with at least 10 s intervals and record the result.
NOTE: In this study, the number of paw withdrawal responses from 10 trials are shown as percentage of paw withdrawal frequency (PWF, %). The von Frey test was performed every two days after CCI surgery.

3. Performing Automated Gait Analysis

NOTE: The gait analysis system visualizes each paw print while the animal is walking and analyzes automatically various gait parameters, such as paw print, paw intensity, stride length, stance phase, step sequence, swing, swing speed, *etc.* In this study, we showed paw print area and single stance after converting the data into the percentage change between contralateral left versus ipsilateral right hind paw. Thus, a result of 50% indicates that the size of the paw print and the duration of paw area in contact with floor, between the left and right sides are same. In addition, lower data values approaching 0% indicate that both the size and duration of contact are decreased in the ipsilateral hind paw as compared to the contralateral side (see panel B of **Figure 2** and **Figure 3**).

1. For acclimation, keep the mice within the gait analysis device for 10 min once a day beginning 5 days before the CCI surgery.
NOTE: Gait analysis test including acclimation should be performed in the dark.

2. **Bring the mice to the test room for gait analysis and acclimate in their home cage for at least 30 min before the test.**
    1. In the setup tab of the program menu, set the "walkway length" to 30 cm, and set the "maximum run duration" to 5 s and the "maximum run variation" to 50%.
    2. Select a registered camera from the 'setup' tab of the 'program' menu.
    3. Select "open acquisition" from the 'acquire' tab of the 'program' menu.
    4. Following the status message, click the 'snap background' button to take a background picture of the empty (*i.e.*, blank) walkway.
3. Click the 'Start acquisition' button and place a mouse on the walkway; the recording starts automatically according to the movement of the mouse.
   NOTE: When the mouse clearly walks across the walkway, the program automatically classifies this move as "Compliant run" with a green icon. If the software failed to detect the foot print, the experimenter will see a red icon and must repeat the recording. At least five successful compliant runs are needed for analysis.
4. After the test, select 'classify runs' from the acquire tab of the 'program' menu.
5. Select the runs for analysis and click the 'auto classify' button.
   NOTE: After classification, all statistic parameters are automatically saved and the experimenter can find the results by clicking "view run statistics" on the analyze menu.

Representative Results

We have performed the von Frey test and automated gait analysis in CCI mice until 10 days after CCI surgery. For statistical analysis, repeated measures of two-way analysis of variance (ANOVA) determines the overall effects, and Dunnett's *post-hoc* analysis was performed to determine the p-value among the experimental groups.

The results shown in **Figure 1** indicate the time course of the classical von Frey test data. The PWF (%) of CCI mice was increased 2 days after CCI surgery and sustained for 10 days as compared with the Sham group. In the CCI positive control group, GBP (50 mg/kg, IP, once daily for 10 days starts 1 day after surgery) significantly alleviated CCI-enhanced PWF. In the Sham group of panel A, PWF (%) was 8.00 ± 5.83 on the timepoint pre-surgery (Pre), 6.00 ± 2.45 on day 2, 4.00 ± 2.45 on day 4, 2.00 ± 2.00 on day 6, 4.00 ± 22.45 on day 8, and 6.00 ± 2.45 on day 10. In the CCI group, PWF (%) was 2.86 ± 1.84 on Pre, 61.43 ± 5.95 on day 2, 68.57 ± 4.59 on day 4, 72.86 ± 5.22 on day 6, 75.71 ± 6.49 on day 8, and 75.71 ± 3.69 on day 10. In CCI+GBP group, PWF (%) was 2.50 ± 1.43 on Pre, 57.50 ± 5.22 on day 2, 50.00 ± 4.04 on day 4, 45.00 ± 4.81 on day 6, 46.25 ± 5.65 on day 8, and 47.50 ± 4.88 on day 10. In panel B, the area under curve (AUC) was 23.00 ± 6.00 in Sham, 317.86 ± 17.04 in CCI, and 223.75 ± 17.05 in CCI+GBP group.

The results shown in **Figure 2** indicate a time course change of the paw print area after CCI surgery. Data are shown as the percent change of the paw print area between the left and right hind paws. If the paw print area is the same at the left and right sides in a normal condition, the data will be 50%. CCI significantly reduced paw print area of the ipsilateral hind paw 2 days after surgery and was maintained for 10 days. GBP remarkably recovered the reduced paw print area. In the Sham group of panel B, the paw print area (%) was 50.13 ± 2.13 on Pre, 50.30 ± 1.45 on day 2, 53.24 ± 0.80 on day 4, 50.30 ± 1.68 on day 6, 52.08 ± 1.79 on day 8, and 49.66 ± 2.24 on day 10. In the CCI group, the paw print area (%) was 50.71 ± 3.17 on Pre, 0 ± 0 on day 2, 0 ± 0 on day 4, 8.86 ± 3.27 on day 6, 6.6 ± 3.20 on day 8, and 10.30 ± 5.60 on day 10. In the CCI +GBP group, the paw print area (%) was 55.59 ± 2.01 on Pre, 4.65±4.17 on day 2, 15.18±5.57 on day 4, 20.20 ± 4.00 on day 6, 26.01 ± 5.53 on day 8, and 28.40 ± 6.04 on day 10.

The results shown in **Figure 3** indicate the time course change of a single stance after CCI surgery. Data were calculated and shown as the percent change (similar to the paw print area data). CCI reduced the single stance of the ipsilateral side and GBP significantly recovered it. In the Sham group of panel B, the single stance (%) was 49.31 ± 2.15 on Pre, 50.71 ± 0.67 on day 2, 50.76 ± 0.44 on day 4, 50.60 ± 1.11 on day 6, 51.50 ± 0.96 on day 8, and 49.00 ± 2.35 on day 10. In the CCI group, the single stance (%) was 50.36 ± 3.17 on Pre, 0 ± 0 on day 2, 0 ± 0 on day 4, 11.5 ± 3.25 on day 6, 13.61 ± 5.04 on day 8, and 12.94 ± 6.40 on day 10. In the CCI+GBP group, the single stance (%) was 52.35 ± 0.91 on Pre, 5.44 ± 4.87 on day 2, 18.66 ± 4.33 on day 4, 25.48 ± 4.10 on day 6, 30.26 ± 2.17 on day 8, and 32.24 ± 4.95 on day 10.

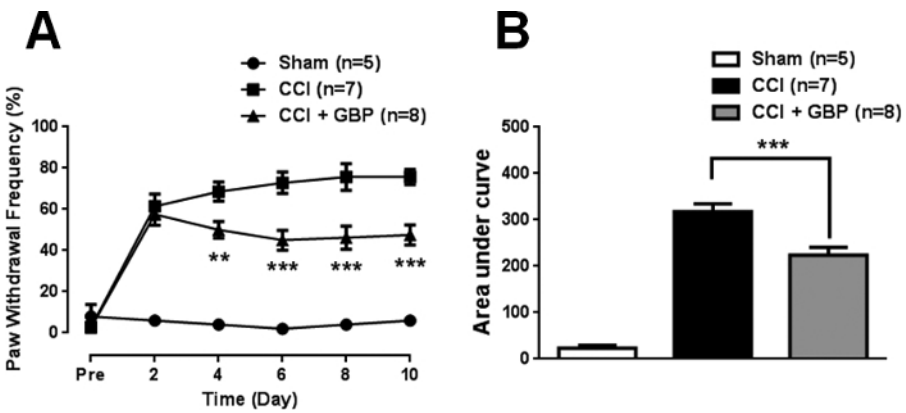

Figure 1: Mechanical allodynia assessed by von Frey test in CCI mice. (**A**) The paw withdrawal frequency (PWF, %) in the von Frey test was increased 2 days after CCI surgery and sustained for 10 days as compared with the Sham group. Gabapentin (GBP, 50 mg/kg, IP) significantly reversed the CCI-induced mechanical allodynia. (**B**) Accumulated data of PWF are shown as area under curve. **p <0.01 and ***p <0.001 versus CCI group. Error bars indicate mean ± SD. Please click here to view a larger version of this figure.

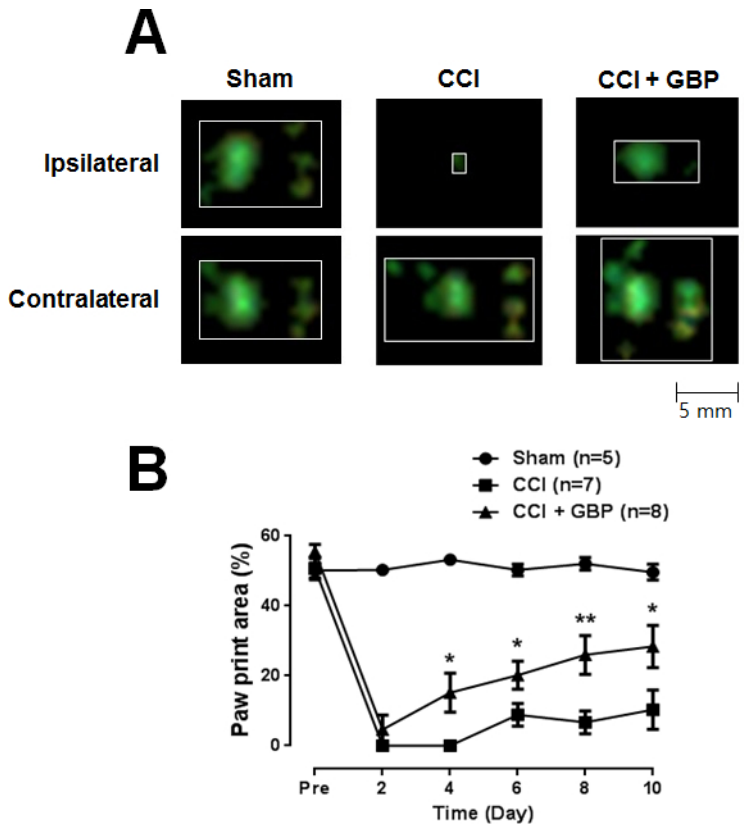

Figure 2: Paw print area by automated gait analysis in CCI mice. (**A**) Captured images of hind paws from the groups of Sham, CCI, and CCI+GBP (gabapentin, 50 mg/kg, IP) by analysis software. White rectangles are indicators of hind paw detected by the analysis system. Note the smallest paw size of CCI and this reduction was partly recovered by GBP treatment. (**B**) Time course of the paw print area (%). Data are calculated as the percent change of print area between the left and right hind paw (*e.g.*, 50% indicates the same paw print areas in the left and right sides). *p <0.05 and **p <0.01 versus CCI group. Error bars indicate mean ± SD.

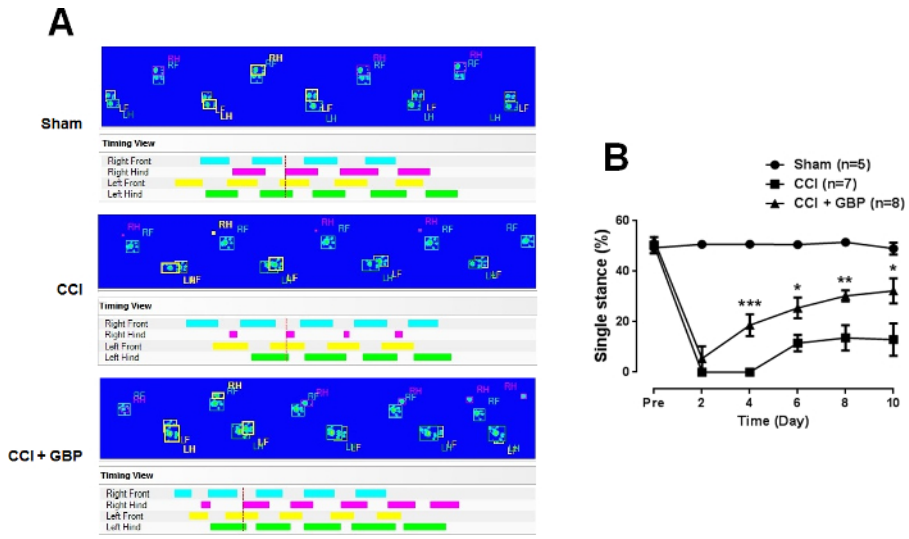

Figure 3: Single stance by automated gait analysis in CCI mice. (**A**) Captured and converted images of single stance highlighted by different colors for each paw. Note that the pink trace for the ipsilateral hind paw is shortened in the CCI group and gabapentin (GBP, 50 mg/kg, IP) significantly recovered it similarly to the Sham level. (**B**) Time course of single stance (%). Data are summarized as a line graph after calculating percent change of single stance between the left and right hind paw (*e.g.*, 50% indicates the same single stance in the left and right sides). *p <0.05, **p <0.01 and ***p <0.001 versus CCI group. Error bars indicate mean ± SD. Please click here to view a larger version of this figure.

Discussion

Currently, measurement of mechanical allodynia by using von Frey filaments is the most widely used method in pain animal models to demonstrate tactile hypersensitivity. As animal models for neuropathic pain have continued to be developed, the methodology of assessment for

sensory function has been also improved[8,9,10,15]. In those reports, it has been suggested these modifications can provide a more accurate, fast, and user-friendly replacement for older methods. However, data from those methods still may be limited by its subjectivity and the requirement of skill or experience to perform the method. In addition, restraining the animals cannot be avoided when performing the von Frey test, and therefore restraint-related stress of the animals may affect the pain response results.

The automated gait analysis system automatically measures various parameters of animal walking (*e.g.*, total area of paw contact to the floor, angle of paw print, stance time, *etc.*), and therefore the experimenter can use more objective data for analysis[12,13,14,16,17,18]. Furthermore, this method can be performed without any stimulation; thus, it is less-invasive and data from the freely moving rodent can be obtained. As shown in results of this study, the foot print size and stance time of the ipsilateral hind paw are reduced by CCI surgery and recovered by GBP treatment; similar results are also found in the von Frey test. Consistent with previous observations[14], results of gait analysis in neuropathic pain animal models have a high degree of positive correlation with that from the von Frey test, indicating that the CCI-related changed parameters may reflect the degree of mechanical allodynia, one of the most irritating symptoms of neuropathic pain.

Here, we provide a method on how to operate the automated gait analysis system in detail and a brief comparison of data with the classical von Frey test in CCI neuropathic mice. In conclusion, the automated gait analysis may be the most improved accessible tool for the study of neuropathic pain because of it is user-friendly, objective, and sensitive.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This research was supported by Chungnam National University, Korea Institute of Oriental Medicine (KIOM) and a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C0007).

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