Comprehensive analysis of neurobehavior associated with histomorphological alterations in a chronic constrictive nerve injury model through use of the CatWalk XT system

Laboratory investigation

Chien-Yi Chiang, M.S.,1 Meei-Ling Sheu, Ph.D.,1 Fu-Chou Cheng, Ph.D.,4 Chun-Jung Chen, Ph.D.,4 Hong-Lin Su, Ph.D.,2 Jason Sheehan, M.D., Ph.D.,5 and Hung-Chuan Pan, M.D., Ph.D.1,3,6

1Institute of Biomedical Sciences and 2Institute of Life Sciences, National Chung-Hsing University; Departments of 3Neurosurgery and 4Education and Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan; 5Department of Neurological Surgery, University of Virginia Health System, Charlottesville, Virginia; and 6Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan

Object. Neuropathic pain is debilitating, and when chronic, it significantly affects the patient physically, psychologically, and socially. The neurobehavior of animals used as a model for chronic constriction injury seems analogous to the neurobehavior of humans with neuropathic pain. However, no data depicting the severity of histomorphological alterations of the nervous system associated with graded changes in neurobehavior are available. To determine the severity of histomorphological alteration related to neurobehavior, the authors created a model of chronic constrictive injury of varying intensity in rats and used the CatWalk XT system to evaluate neurobehavior.

Methods. A total of 60 Sprague-Dawley rats, weighing 250–300 g each, were randomly assigned to 1 of 5 groups that would receive sham surgery or 1, 2, 3, or 4 ligatures of 3-0 chromic gut loosely ligated around the left sciatic nerve. Neurobehavior was assessed by CatWalk XT, thermal hyperalgesia, and mechanical allodynia before injury and periodically after injury. The nerve tissue from skin to dorsal spinal cord was obtained for histomorphological analysis 1 week after injury, and brain evoked potentials were analyzed 4 weeks after injury.

Results. Significant differences in expression of nerve growth factor existed in skin, and the differences were associated with the intensity of nerve injury. After injury, expression of cluster of differentiation 68 and tumor necrosis factor–α was increased, and expression of S100 protein in the middle of the injured nerve was decreased. Increased expression of synaptophysin in the dorsal root ganglion and dorsal spinal cord correlated with the intensity of injury. The amplitude of sensory evoked potential increased with greater severity of nerve damage. Mechanical allodynia and thermal hyperalgesia did not differ significantly among treatment groups at various time points. CatWalk XT gait analysis indicated significant differences for print areas, maximum contact maximum intensity, stand phase, swing phase, single stance, and regular index, with sham and/or intragroup comparisons.

Conclusions. Histomorphological and electrophysiological alterations were associated with severity of nerve damage. Subtle neurobehavioral differences were detected by the CatWalk XT system but not by mechanical allodynia or thermal hyperalgesia. Thus, the CatWalk XT system should be a useful tool for monitoring changes in neuropathic pain, especially subtle alterations.

Key Words • CatWalk XT • neuropathic pain • mechanical allodynia • thermal hyperalgesia • chronic constrictive injury • peripheral nerve • rat

Abbreviations used in this paper: CCI = chronic constriction injury; CD68 = cluster of differentiation 68; NGF = nerve growth factor; TNF-α = tumor necrosis factor–α.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction of the nervous system. It arises as a direct consequence of a lesion or disease affecting the somatosensory system. Epidemiologists estimate that up to 8% of the general population experience chronic pain associated with neuropathic features, which significantly affects their lives physically, psychologically, and socially. Because of its very complex nature, neuropathic pain responds poorly to traditional analgesics such as antiinflammatory and opiate drugs. Thus, it remains quite a substantial challenge for both clinicians and scientists.

For investigation of the mechanisms of neuropathic pain, several established animal models are available.
Analysis of neurobehavior by CatWalk XT system

The neuropathic pain models usually share alterations in hindlimb cutaneous sensory thresholds after partial injury of a peripheral nerve. The most frequently used models are chronic constriction injury (CCI) of the sciatic nerve, partial sciatic nerve ligation, and spinal nerve ligation. The animal model of CCI represents an advance in the study of neuropathic pain because the location of loose chromic gut ligatures on the rat sciatic nerve results in behavior that seems analogous to that of humans with neuropathic pain. Obstacles to establishing stable and appropriate animal models for neuropathic pain remain because of rodents’ lower thresholds to heat, cooling, and mechanical stimuli. Despite CCI yielding highly consistent neurobehavior, the method of conducting the nerve injury, including the number of ligatures, is still not determined. Thus, the histomorphological alterations from the skin to the brain associated with numbers of ligatures and the correlation of histomorphological changes with neurobehavioral changes have yet to be well defined.

The novel, automated CatWalk gait analysis system has the advantage of controlling speed of locomotion and automated data acquisition. CatWalk gait analysis detects dynamic and static gait parameters. These parameters include base of support, stride length, box length, box width, maximum area, print area, mean intensity, stance duration, swing duration, regularity index, and phase lags. The CatWalk system has been used to assess static and dynamic gait parameters in a variety of central and peripheral nerve injury models. Vrinten and Hamers described a possible approach that used the CatWalk system for quantifying mechanical allodynia in a rat CCI model for neuropathic pain; they found a strong correlation between von Frey mechanical withdrawal thresholds and parameters obtained from the CatWalk gait analysis.

Although some limited studies describe the association between traditional assessment methods such as the von Frey and the CatWalk systems, the neurophysiological changes and molecular mechanism of alteration in the microenvironment around injury sites remain unclear. Furthermore, the severity of nerve injury associated with the neurobehavior detected by the CatWalk system is unknown. In this study, we examined the histomorphological alterations from skin to sensory cortex by using different intensities of CCI (1–4 ligatures) and correlated the neurobehavior of mechanic allodynia and thermal hyperalgesia with different intensities of nerve damage. Moreover, we correlated the multiple parameters of the CatWalk XT system with histomorphological alterations and inflammatory responses from skin to brain cortex as well as the electrophysiologic alterations in the brain cortex.

Methods

Animal Model

A total of 60 Sprague-Dawley rats, each weighing 250–300 g, were used in this study; permission for their use was obtained from the Ethics Committee of the Taichung Veterans General Hospital. Food and water were provided ad libitum before and after the experiments. The animals were kept in a temperature-controlled environment at 20°C, and they were exposed to alternating light and dark cycles of 12 hours. All animals were treated and cared for in accordance with the guidelines recommended by the Ethics Committee of Taichung Veterans General Hospital.

These animals were randomly assigned to 1 of 5 groups (6 in each group) for a total of 30 animals that would receive sham surgery or 1, 2, 3, or 4 ligatures of 3-0 chromic gut loosely ligated around the left sciatic nerve without changing the morphology of the nerve. The other 30 rats were used for determination of histomorphology.

Before creation of the CCI model in the rats, anesthesia was induced with 4% isoflurane and maintained with 1%–2% isoflurane. Constriction was created 1 cm above the left sciatic nerve trication, as described previously, with 1-mm intervals between each suture. The surgical wound was closed with 4-0 silk sutures in layers, and the animals were allowed to recover.

These animals were subjected to behavioral testing such as mechanical allodynia, thermal hyperalgesia, and CatWalk XT system gait analysis 3 days before injury for baseline measurements and then weekly until the end of the experiment. After the above experiments, the same animals were used to complete the evoked potential analysis 1 month after CCI. The other 30 rats (6 per group) were used for determination of histomorphology such as synaptophysin, tumor necrosis factor–α (TNF-α), cluster of differentiation 68 (CD68), and nerve growth factor (NGF) from skin to brain.

Mechanical Allodynia and Thermal Hyperalgesia

A technician blinded to treatment group evaluated the thermal hyperalgesia and mechanical allodynia. During mechanical allodynia testing, a rat was placed on a customized platform that was fixed in a transparent acrylic chamber with dimensions of 20 × 20 × 20 cm. The customized platform was 20 × 20 cm and made of 5-mm-thick acrylic. It contained 2-mm-diameter holes in a 5-mm grid of perpendicular rows throughout the entire area of the platform. A trial consisted of applying a von Frey hair (Touch-Test Sensory Evaluator, North Coast Medical, Inc.) to the hind paw 5 times at 5-second intervals or until the hind paw was placed appropriately on the platform. If hind paw withdrawal did not occur during 5 applications of a hair, the next larger hair was applied. When the hind paw was withdrawn from a particular hair either 4 or 5 times out of the 5 applications, the value of that hair in grams was considered to be the withdrawal threshold.

Thermal hyperalgesia was tested according to the previous study of the hot plate test (TSE Systems). The paw withdrawal latency, defined as the time from the rat touching the 52°C hot plate to withdrawing the paw, was recorded with a timer. To prevent tissue damage, we used a maximal cutoff time of 20 seconds.

CatWalk Automated Quantitative Gait Analysis

The method for conducting the CatWalk gait analysis has been described. In brief, the CatWalk XT system comes with a high-speed digital camera with a sample rate of 100 frames per second. The video camera transforms each scene (the area in front of the lens) into a digital image (an image composed of discrete pixels of digital brightness values). The digital images are trans-
ferred to a computer through an Ethernet connection. The brightness of a pixel depends on the amount of light received from such an area by the camera. The Illuminated Footprint enables detection of intensity differences between animals’ paws.

The 3D footprint intensity tab plots on a 3D chart the print intensity of the 4 paws for each individual frame in which the paws contact the glass plate. The intensities vary from 0 to 225, and they are represented by different colors. A 3D chart can be rotated in all directions. Quantitative analysis of the data from the CatWalk XT system includes the following parameters: step sequence distribution, regularity index, print area, duration of swing and stance phases, and maximum contact maximum intensity (maximum intensity at the maximum contact of a paw).

**Electrophysiologic Analysis**

One month after CCI injury, the sensorimotor evoked potential and conduction latency were investigated just before each animal was euthanized. In brief, after the rats were anesthetized, 2 recording electrodes were fixed on the dural surface of the somatosensory area (3 mm lateral and 2 mm posterior to the bregma) bilaterally 1 day before euthanasia. Electrical stimulation was conducted through an active stimulating electrode placed over the sciatic nerve 1 cm proximal to the injury area. The conduction latency and evoked potential were determined over the recorded electrode of the skull and a reference needle 20 mm from the recorded electrode. The stimulation intensity and filtration range were 20 mA and 20–2000 Hz, respectively. To adjust for the effect of anesthesia, we converted the data of conduction latency and evoked potential to a ratio of the injured side divided by the normal side.

**Immunohistochemical Analysis**

Animals were anesthetized and perfused with phosphate-buffered saline, followed by a fixative solution containing 4% paraformaldehyde. Brain (brain cortex and hippocampus), spinal cord dorsal horn, dorsal root ganglia (bilateral L-4 to L-6 dorsal root ganglia), sciatic nerves (middle of the crushed site), and foot skin (hindlimb paw skin) were resected and placed in 4% paraformaldehyde for 4 hours and then transferred to 30% sucrose at 4°C overnight. The samples were subsequently embedded in Tissue-Tek O.C.T. Compound (Sakura) and rapidly frozen.

Serial 8-µm-thick sections of skin, sciatic nerve, dorsal root ganglion, dorsal spinal cord, and brain were cut on a cryostat and mounted on Superfrost Plus slides (MenzelGlaser). The sections were then subjected to immunohistochemical examination with antibodies against NGF (Chemicon, 1:300 dilution), CD68 (Chemicon, 1:200 dilution), S100 protein (Neomarkers, 1:400 dilution), synaptophysin (Abcam, 1:200 dilution), and TNF-α (Abcam, 1:300 dilution) for the detection of inflammatory cells, Schwann cells, small neurosecretory vesicles, and inflammatory cytokines, respectively. The immunoreactive signals were visualized by using goat anti–mouse Immunoglobulin G (fluorescein isothiocyanate, Jackson; 1:200 dilution) and anti–mouse Immunoglobulin G (rhodamine, Jackson; 1:200 dilution). Six tissue specimens in each group were cut into 8-µm-thick sections and stained with antibody. The areas occupied by the stained tissues were highlighted and measured for each section of sciatic nerve (n = 6 per group) and expressed in terms of the density (number/mm²) of all the resected tissue.

**Statistical Analysis**

The Student t-test and repeated-measures ANOVA were used to compare the experimental results among groups. The statistical analyses were conducted using SPSS software version 12. The data are presented as a mean ± standard error. A p value < 0.05 was considered significant.

**Results**

**Severity of Histomorphological Alteration in the Nerve System Associated With Intensity of the Nerve Injury**

Expression of NGF in axons of the epidermis and dermis was associated with the severity of the neuropathic pain. The highest expression of NGF in innervated skin was present in the 4-ligature (ring) group, and its expression was gradually increased with higher numbers of ligature rings (Fig. 1). The NGF expression level was significantly higher in the 4-ring group than in the sham (p < 0.001), 1-ring (p < 0.001), 2-ring (p < 0.001), and 3-ring (p < 0.001) groups. The NGF expression level in the 4-ring group was 1.66-fold higher than that in the 1-ring group (p < 0.01) and more than 11-fold higher than that in the sham group (p < 0.001). However, no significant differences in NGF expression were found between the 1-ring and the 2-ring groups and between the 2-ring and 3-ring groups.

Myelinating Schwann cells can be visualized immunohistochemically by using an antibody against S100 protein, which was reciprocally associated with the expression of inflammatory cytokines and inflammatory cell deposition such as TNF-α and CD68 (Fig. 2). In this study, we found that 4 rings caused significantly less expression of S100 protein than did 1 ring (p < 0.01) to 2 rings (p < 0.05). The S100 protein expression level in the sham group was 1.2-fold higher than that in the 1-ring group (p < 0.05) and more than 3.88-fold higher than that in the 4-ring group (p < 0.001). The density of expression correlated with the number of ligatures. The trends of decreased S100 protein expression were reciprocal with the increased TNF-α expression and CD68 deposition (Fig. 2). The TNF-α expression level in the 4-ring group was 6-fold higher than that in the 1-ring group (p < 0.001). The CD68 expression level in the 4-ring group was 2-fold higher than that in the 1-ring group (p < 0.01) and more than 30-fold higher than that in the sham group (p < 0.001). No significant difference in CD68 expression was found between the 2-ring and 3-ring groups.

Synaptophysin expression within the dorsal root ganglion was highly correlated with the severity of neuropathic pain. Synaptophysin was highly expressed in the 4-ring group and significantly increased compared with the 1-ring, 2-ring, and 3-ring groups (p < 0.001 each) (Fig. 3). The synaptophysin expression level in the 4-ring group was more than 8-fold higher than that in the 1-ring group (p < 0.001) and the sham group (p < 0.001).

Increased aberrant innervation within the dorsal column also reflected the severity of neuropathic pain.
Increased synaptophysin and TNF-α within the dorsal column were highly expressed in the 4-ring groups. The intensity was associated with the number of ring ligatures (Fig. 4). The synaptophysin expression level in the 4-ring group was 3.5-fold higher than that in the 1-ring group (p < 0.01) and more than 10-fold higher than that in the sham group (p < 0.001). The TNF-α expression level in the 4-ring group was more than 2-fold higher than that in the 1-ring and 2-ring groups (p < 0.001 each). No significant differences were found for the TNF-α expression between the 1-ring and 2-ring groups or between the 3-ring and 4-ring groups.

Synaptophysin expression and TNF-α expression within the brain hippocampus (CA3) and somatosensory cortex reflect the brain plasticity response to CCI injury. Increased synaptophysin expression within the sensorimotor strip indicates the responsiveness to peripheral nerve neuropathic injury. In this study, high levels of synaptophysin and TNF-α expression were noted in the 4-ring group compared with the 1-ring, 2-ring, and 3-ring groups (Fig. 5). The synaptophysin expression level of the hippocampus in the 4-ring group was 2-fold higher than that in the 1-ring group (p < 0.01) and more than 5-fold higher than that in the sham group (p < 0.001). The TNF-α expression level in the 4-ring group was 3-fold higher than that in the 1-ring group (p < 0.001) and 1.7-fold and 1.2-fold higher than that in the 2-ring (p < 0.01) and 3-ring groups (p < 0.05), respectively. Significant differences were found for TNF-α and synaptophysin expression within the somatosensory cortex between the 1-ring and 2-ring groups and between the 2-ring and 3-ring groups. However, there was no significant difference in expression of synaptophysin within the hippocampus between the 1-ring and 2-ring groups.

**Mechanical Allodynia and Thermal Hyperalgesia**

Mechanical allodynia and thermal hyperalgesia are the typical representations of neuropathic pain. Chronic constriction injury of different intensities caused a long-lasting increase in mechanical allodynia and thermal sensitivity. The effect of CCI on mechanical allodynia was seen as early as 3 days postoperatively and persisted for at least 28 days after the operation (Fig. 7; Tables 1–5). During preoperative testing, none of the animals responded to the largest von Frey filament tested (21.97 g) on either hind paw. After chronic constriction of the sciatic nerve, mechanical allodynia developed, as demonstrated by a large decrease in the withdrawal threshold of the hind paw with the constricted nerve 1 week after the operation to 7.28% (7.28%–8.01%) of preoperative thresholds, and this decrease persisted for more than 1 month. The effect of CCI on thermal sensitivity was that the most grievous pain was seen at 7 days after the operation and persisted for at least 28 days. However, there were no significant differences in thermal sensitivity between the different ring groups.
ferences in mechanical allodynia or thermal hyperalgesia among the different groups at various time points.

**Automatic CatWalk Gait Analysis**

CatWalk XT gait analysis indicated that the variables (print area, maximum contact maximum intensity, stand phase, swing phase, single stance, and regularity index) differed significantly according to the number of rings (Fig. 8; Tables 1–5). Before surgery, the predominant step pattern was alternate (forelimb and contralateral hindlimb in sequence). After placement of the ligatures, the main step pattern persisted, but other patterns also occurred. Indicative of these changes in step patterns, the regularity index changed, indicating significant loss of interlimb coordination in CCI animals. The decreasing regularity index correlated with the number of ligatures.

Data analysis was performed with a threshold value of 31.0 arbitrary units (range 0–255); that is, all pixels brighter than 31.0 were used. In our data, the mean (± SD) baseline intensity of the left hind paw was 244.02 ± 5.71 arbitrary units. Two weeks after CCI, the intensity was significantly reduced to 88.61% of the preoperative value (p < 0.01). The print area for the 4-ring group was 21.4%–25.2% lower than that for 1-ring group (F = 7.49, p < 0.01) and the sham group (F = 13.6, p < 0.01).

Before CCI, the duration of the stance phase was 0.39 ± 0.04 seconds. Two weeks after the surgery, the duration of the stance phase of the CCI paw was significantly reduced to 58.97% of preoperative values (F = 7.46, p < 0.01). There was also a very high degree of correlation between this parameter and the number of ligatures. The swing phase duration was a fraction of 0.10 ± 0.01 of the total step duration (almost 3 steps). Two weeks after CCI, the swing phase increased to 212.62% of the preoperative value (F = 7.936, p < 0.01). The duration of stance and the swing phase in the contralateral paw differed significantly between groups with different numbers of ligatures. In the CatWalk XT system, single stance is the part in the step cycle of a hind paw where the contralateral hind paw does not touch the glass plate. Before CCI, the duration of the stance phase was 0.11 ± 0.01 seconds. One week after the operation, the duration for the stance phase for the CCI paw was significantly reduced to 79.14% of its preoperative value (F = 17.39, p < 0.01). Duration of the single-stance phase in the contralateral paw differed significantly for the groups with different numbers of ligatures.

**Fig. 2.** A: Depiction of S100 protein, TNF-α, and CD68 over the distal end of the nerve associated with the different number of ligature rings. Bar = 200 μm. B: Quantitative analysis of expression level of S100 protein (upper), TNF-α (center), and CD68 (lower) in the different intensities of CCI injury. See Immunohistochemical Analysis for description of staining. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 3. A: Depiction of synaptophysin (red) over the dorsal root ganglia associated with the number of ligature rings. Bar length = 100 μm. B: Quantitative analysis of synaptophysin associated with the number of rings. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 4. A: Depiction of synaptophysin and TNF-α over the dorsal spinal cord associated with the number of ligature rings. Bar length = 100 μm. B: Quantitative analysis for expression level of synaptophysin and TNF-α associated with the number of ligature rings. See Immunohistochemical Analysis for description of staining. *p < 0.05; **p < 0.01; ***p < 0.001.
Motor Function Assessment

The number of rings required to effectuate a chronic nerve injury model remains the subject of debate. In this study, our motor functional assessment included the sciatic function index, muscle compound action potential, and muscle weight; these data are shown in Table 6. These data showed a significant difference between sham and the injured groups (1 ring to 4 rings), but there was no appreciable difference among the experimental groups. Hence, between the 1-ring and 4-ring groups, the variation in number of rings translated to little alteration in motor function within the CCI model.

Correlation Between Parameters of the CatWalk Gait Analysis and Histomorphological Alteration

We retrieved 6 CatWalk parameters (print area, maximum contact maximum intensity, stand phase, swing phase, single stance, regularity index) and conducted a correlation analysis related to NGF, S100 protein, CD68, synaptophysin, and TNF-α (Table 7). The NGF expression in the paw skin was highly correlated with 6 CatWalk parameters, with R² values from 0.8641 (p = 0.002) to 0.9546 (p = 0.011). The expression of CD68 and S100 protein in injured nerve was highly correlated with various CatWalk parameters. The expression of synaptophysin from dorsal root ganglion to somatosensory cortex showed a significant correlation with various CatWalk parameters. Trends
for TNF-α were also very similar to trends for synapto-physin.

On the basis of the CatWalk gait analysis, we found that alternation in CatWalk parameters such as intensity of printed area, maximum contact maximum intensity, stand phase, swing phase, single stance, and regularity index exhibited significant differences between rats with the various numbers of rings. This alteration was in line with the histomorphological alteration and associated inflammatory response as well as neural remodeling markers.

Discussion

In this study, different numbers of ligatures caused various degrees of nerve damage in terms of neural architecture, inflammatory response, and electrophysiology. Less severe nerve damage alters the histomorphology only in the peripheral nervous system, but severe damage causes significant change in brain cortex remodeling. These alterations in histomorphology within the nerve system were correlated with various animal neurobehavioral responses. The CatWalk XT system detected subtle

### TABLE 1: Parameters of the CatWalk XT system and neurobehavior 3 days before CCI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical allodynia (g)</td>
<td>20.5 ± 5.5</td>
<td>22.33 ± 5.19</td>
<td>22.33 ± 5.19</td>
<td>20.5 ± 5.5</td>
<td>24.17 ± 4.10</td>
</tr>
<tr>
<td>thermal hyperalgesia (sec)</td>
<td>11.17 ± 1.67</td>
<td>10 ± 0.82</td>
<td>11.67 ± 0.47</td>
<td>10.5 ± 1.26</td>
<td>10.83 ± 1.46</td>
</tr>
<tr>
<td>printed area (cm²)</td>
<td>1.72 ± 0.13</td>
<td>1.73 ± 0.20</td>
<td>1.72 ± 0.14</td>
<td>1.78 ± 0.13</td>
<td>1.84 ± 0.09</td>
</tr>
<tr>
<td>maximum contact maximum intensity</td>
<td>248.13 ± 3.24</td>
<td>244.31 ± 7.06</td>
<td>241.49 ± 4.26</td>
<td>240.7 ± 8.65</td>
<td>245.45 ± 5.32</td>
</tr>
<tr>
<td>stand phase (sec)</td>
<td>0.4 ± 0.05</td>
<td>0.38 ± 0.04</td>
<td>0.38 ± 0.03</td>
<td>0.39 ± 0.05</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>swing phase (sec)</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>single stance (sec)</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>regular index (%)</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
</tr>
</tbody>
</table>

![Fig. 7. Plots of mechanical allodynia and thermal hyperalgesia associated with time and intensity of injury. A: Plot of mechanical allodynia for different numbers of rings as a function of time. B: Plot of thermal hyperalgesia for different numbers of rings and different time points. *p < 0.05; **p < 0.01; ***p < 0.001.](image-url)
neurobehavior alterations in intensity of nerve damage at different time points. However, these subtle changes were not determined by mechanical allodynia and thermal hyperalgesia. The CatWalk XT system proved to be a powerful tool for detecting subtle changes in the nerve system and should be helpful for testing the clinical benefits of pharmacological targets for treating neuropathic pain.

Mechanical allodynia and thermal hyperalgesia are the typical representations of neuropathic pain. In a previous study, in the presence of chronic neuropathic pain the inflammatory and algesic mediators predominated, resulting in painful hypersensitivity after nervous system damage. In this study, the escalated nerve damage was related to the number of ligatures in the CCI model. An increased number of ligatures corresponded with an increased inflammatory response and sprouting of aberrant nerves. When either severe or mild injury has occurred, mechanical allodynia or thermal hyperalgesia can detect the trend of neuropathic pain. However, in our model, the subtle alterations in severity of nerve damage did not yield demonstrable differences in mechanical allodynia and thermal hyperalgesia. However, the CatWalk XT system not only detected the trend in intensity of neuropathic pain, but it also measured the subtle alterations in neurobehavior associated with mild histomorphological changes. Hence, the CatWalk XT system seems to be a useful test for detecting the severity of neuropathic pain.

Peripheral nerve injury, a model of neuropathic pain, reportedly increases spontaneous activity in primary afferent neurons, thus inducing a secondary, enduring increase in excitability of the sensory circuit in the spinal dorsal horn. The excitability is associated with a series of synaptic plasticity such as long-term potentiation of synaptic transmission, which might be related to the synthesis of more presynaptic vesicle protein, synaptophysin. Several studies that used synaptophysin to quantify the number of terminals during neuroanatomical remodeling found that an increase within the ipsilateral dorsal horn of a CCI model highly correlated with the severity of neuropathic pain behaviors. In our study, the increased expression of synaptophysin within the dorsal root ganglion and dorsal spinal cord correlated with severity of damage and mirrored alterations of neurobehavior.

Within a peripheral nerve and dorsal root ganglion, there is a resident population of macrophages. In a peripheral nerve, the resident macrophages are assisted with clearance of cellular debris by an influx of hematogenous macrophages. Hyperalgesia is delayed in mice that exhibit a genetic defect that postpones the recruitment of hematogenous macrophages; this finding suggests that macrophages might contribute to pain resulting from nerve injury. This phenomenon was confirmed by demonstration that depletion of macrophages in nerve injury models alleviated neuropathic hyperalgesia. In addition, in a model of neuropathic pain, the development of mechanic allodynia was totally abrogated in mice lacking the chemokine receptor CCR2, which is involved in monocyte recruitment. Macrophages also invade the dorsal root ganglion after a peripheral lesion has been created and might contribute to neuropathic pain by releasing excitatory agents that generate ectopic activity in sensory neurons. Macrophages secrete prostaglandins, including prostaglandins E2 and I2, which directly sensitize primary afferent neurons. Other algesic mediators released by macrophages that contribute to neuropathic pain are reactive oxygen species, TNF-α, interleukin-1,

### TABLE 2: Parameters of the CatWalk XT system and neurobehavior 7 days after CCI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical allodynia (g)</td>
<td>22.33 ± 5.19</td>
<td>1.63 ± 0.39</td>
<td>1.6 ± 0.28</td>
<td>1.8 ± 0.28</td>
<td>1.63 ± 0.39</td>
</tr>
<tr>
<td>thermal hyperalgesia (sec)</td>
<td>10.5 ± 1.12</td>
<td>5.33 ± 0.47</td>
<td>5.17 ± 0.37</td>
<td>5.83 ± 0.37</td>
<td>4.67 ± 0.47</td>
</tr>
<tr>
<td>printed area (cm²)</td>
<td>1.76 ± 0.11</td>
<td>1.46 ± 0.1</td>
<td>1.29 ± 0.09</td>
<td>0.82 ± 0.10</td>
<td>0.65 ± 0.12</td>
</tr>
<tr>
<td>maximum contact maximum intensity</td>
<td>249.41 ± 3.15</td>
<td>235.5 ± 5.12</td>
<td>220.92 ± 8.32</td>
<td>211.79 ± 6.40</td>
<td>213.85 ± 8.52</td>
</tr>
<tr>
<td>stand phase (sec)</td>
<td>0.35 ± 0.04</td>
<td>0.28 ± 0.02</td>
<td>0.26 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>swing phase (sec)</td>
<td>0.11 ± 0.01</td>
<td>0.16 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>single stance (sec)</td>
<td>0.11 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>regular index (%)</td>
<td>100 ± 0.00</td>
<td>82.19 ± 2.95</td>
<td>82.41 ± 2.89</td>
<td>80.72 ± 1.59</td>
<td>75.33 ± 2.20</td>
</tr>
</tbody>
</table>

### TABLE 3: Parameters of the CatWalk XT system and neurobehavior 14 days after CCI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical allodynia (g)</td>
<td>22.33 ± 5.19</td>
<td>2.23 ± 0.82</td>
<td>2.57 ± 1.04</td>
<td>2.47 ± 1.11</td>
<td>1.63 ± 0.39</td>
</tr>
<tr>
<td>thermal hyperalgesia (sec)</td>
<td>10.5 ± 1.61</td>
<td>6.33 ± 0.75</td>
<td>6.5 ± 0.76</td>
<td>5.5 ± 0.50</td>
<td>5.67 ± 0.75</td>
</tr>
<tr>
<td>printed area (cm²)</td>
<td>1.75 ± 0.17</td>
<td>1.49 ± 0.20</td>
<td>1.28 ± 0.15</td>
<td>0.43 ± 0.28</td>
<td>0.38 ± 0.22</td>
</tr>
<tr>
<td>maximum contact maximum intensity</td>
<td>244.10 ± 5.96</td>
<td>222.34 ± 6.66</td>
<td>222.4 ± 7.19</td>
<td>214.57 ± 2.84</td>
<td>205.57 ± 3.91</td>
</tr>
<tr>
<td>stand phase (sec)</td>
<td>0.37 ± 0.04</td>
<td>0.27 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.23 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>swing phase (sec)</td>
<td>0.10 ± 0.01</td>
<td>0.15 ± 0.04</td>
<td>0.23 ± 0.07</td>
<td>0.24 ± 0.02</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>single stance (sec)</td>
<td>0.12 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>regular index (%)</td>
<td>100 ± 0.00</td>
<td>84.71 ± 1.20</td>
<td>83.38 ± 2.47</td>
<td>81.73 ± 1.80</td>
<td>78.57 ± 1.60</td>
</tr>
</tbody>
</table>
Analysis of neurobehavior by CatWalk XT system

### TABLE 4: Parameters of the CatWalk XT system and neurobehavior 21 days after CCI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical allodynia (g)</td>
<td>24.17 ± 4.10</td>
<td>5 ± 1.00</td>
<td>5 ± 1.00</td>
<td>3.67 ± 1.37</td>
<td>1.8 ± 0.28</td>
</tr>
<tr>
<td>thermal hyperalgesia (sec)</td>
<td>10.33 ± 1.37</td>
<td>7.67 ± 0.47</td>
<td>7.33 ± 0.47</td>
<td>7.33 ± 0.75</td>
<td>6.00 ± 1.15</td>
</tr>
<tr>
<td>printed area (cm²)</td>
<td>1.74 ± 0.14</td>
<td>1.54 ± 0.10</td>
<td>1.27 ± 0.12</td>
<td>0.77 ± 0.09</td>
<td>0.63 ± 0.07</td>
</tr>
<tr>
<td>maximum contact maximum intensity</td>
<td>241.77 ± 6.43</td>
<td>224.41 ± 3.51</td>
<td>218.46 ± 4.38</td>
<td>218.20 ± 3.36</td>
<td>207.65 ± 1.81</td>
</tr>
<tr>
<td>stand phase (sec)</td>
<td>0.36 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>0.25 ± 0.03</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>swing phase (sec)</td>
<td>0.11 ± 0.004</td>
<td>0.15 ± 0.03</td>
<td>0.16 ± 0.04</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>single stance (sec)</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.003</td>
<td>0.10 ± 0.002</td>
<td>0.09 ± 0.004</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>regular index (%)</td>
<td>100 ± 0.00</td>
<td>90.99 ± 3.44</td>
<td>88.75 ± 4.78</td>
<td>83.36 ± 2.30</td>
<td>81.09 ± 2.85</td>
</tr>
</tbody>
</table>

and interleukin-6. Injury of the sciatic nerve leads to upregulation of TNF-α and its receptors within the nerve. This upregulation is found mainly in Schwann cells and endothelial cells. Topical application of TNF-α to a restricted portion of the sciatic nerve leads to ectopic firing of Aδ and C fibers, and intraneural injection of TNF-α into the sciatic nerve of rats at a physiological dose induces thermal hyperalgesia and mechan al allodynia. In our study, differing severities of nerve damage caused an upregulation of expression of macrophage deposition and of TNF-α. The upregulation of macrophage deposition and TNF-α is compatible with increased severity of neuropathic pain.

It is now recognized that NGF plays a significant role in nociception by sensitizing nociceptors in the peripheral nervous system. NGF contributes to the development and maintenance of neuropathic pain in animal models. In rats with a CCI of the sciatic nerve, the onset of hyperalgesia was delayed by application of antisera to NGF at the site of injury. Sprouting was also observed from the saphenous nerve in the CCI model. Sprouting of collateral fibers from the sensory axon in the skin into a denervated area after nerve crush injury has been described. This sprouting occurs around 10 days postoperatively, but the degree of sprouting is not proportional to the degree of hyperalgesia after chronic sciatic nerve sectioning. These results indicate that collateral sprouting is unlikely to contribute significantly to the painful behavior seen in the CCI model. The sprouting was effectively blocked by the administration of anti-NGF, and it is, therefore, likely that a local release of NGF from sources within the skin (for example, keratinocytes, immune cells) is responsible for axon sprouting under these circumstances.

In our study, increased expression of NGF in skin paralleled the increased severity of nerve damage. The alteration in expression of NGF in skin correlated with development of neuropathic pain.

Recent evidence suggests that sensory dysfunction caused by nerve injury should be attributable to not only the functional, cellular, and biochemical events occurring in the peripheral nervous system but also to functional and anatomical changes in the cerebral cortex. It is well documented that peripheral nerve injury in rodents can lead to expanding representation of the neighboring cortex of the peripheral region within the affected hemisphere. In addition, the alteration of evoked potential in the brain cortex and dorsal spinal cord substantially reflect nerve system plasticity and response to nerve damage. In our study, we found that the graded increases of evoked potential amplitude were highly correlated with the intensity of nerve damage, and these changes in the evoked potentials paralleled increased expression of synaptophysin within the brain somatosensory system. In addition, the increased errors of the regularity index and step sequence detected by the CatWalk gait analysis were highly correlated with the alteration in brain somatosensory cortex. This finding further suggests that the CatWalk XT system may serve as a useful tool for assessing the alteration in somatosensory neuronal plasticity in the setting of neuropathic pain.

The CatWalk XT provides a high-speed camera with a sample rate of 100 frames per second, and it had sharp delineation of footsteps with a combination of green light in the glass plate and red light in the illuminated ceiling. The CatWalk XT gait analysis can detect changes in dynamic and static gait parameters. These parameters included base of support, stride length, box length, box width, maximum

### TABLE 5: Parameters of the CatWalk XT system and neurobehavior 28 days after CCI

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
</tr>
</thead>
<tbody>
<tr>
<td>mechanical allodynia (g)</td>
<td>24.17 ± 4.10</td>
<td>9 ± 1.00</td>
<td>7.67 ± 1.80</td>
<td>5.33 ± 1.49</td>
<td>4.33 ± 1.37</td>
</tr>
<tr>
<td>thermal hyperalgesia (sec)</td>
<td>10.5 ± 0.96</td>
<td>8.83 ± 0.69</td>
<td>8.5 ± 0.5</td>
<td>8.17 ± 0.69</td>
<td>8.17 ± 0.90</td>
</tr>
<tr>
<td>printed area (cm²)</td>
<td>1.69 ± 0.15</td>
<td>1.55 ± 0.12</td>
<td>1.62 ± 0.14</td>
<td>0.85 ± 0.07</td>
<td>0.74 ± 0.09</td>
</tr>
<tr>
<td>maximum contact maximum intensity</td>
<td>242.10 ± 5.94</td>
<td>233.04 ± 6.82</td>
<td>231.04 ± 1.78</td>
<td>222.17 ± 5.55</td>
<td>216.33 ± 3.09</td>
</tr>
<tr>
<td>stand phase (sec)</td>
<td>0.37 ± 0.03</td>
<td>0.33 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.26 ± 0.03</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>swing phase (sec)</td>
<td>0.11 ± 0.01</td>
<td>0.13 ± 0.04</td>
<td>0.18 ± 0.02</td>
<td>0.21 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>single stance (sec)</td>
<td>0.11 ± 0.004</td>
<td>0.10 ± 0.002</td>
<td>0.10 ± 0.002</td>
<td>0.09 ± 0.002</td>
<td>0.09 ± 0.001</td>
</tr>
<tr>
<td>regular index (%)</td>
<td>100 ± 0.00</td>
<td>92.32 ± 2.98</td>
<td>90.49 ± 3.00</td>
<td>85.41 ± 1.39</td>
<td>84.73 ± 1.82</td>
</tr>
</tbody>
</table>
In our study, varying numbers of ligatures produced different intensities of nerve damage, which were in line with alterations detected by the Cat Walk analysis. Gait parameters that were altered with the severity of nerve damage included base of support, stride length, box length, box width, maximum area, print area, mean intensity, stance duration, and swing duration. However, the interpretation of the regularity index and step sequence remains a subject of debate. Vrinten and Hamers reported that there was no significant alteration of regularity index and step sequence in a CCI model. This discrepancy with our findings could be explained by differences in the experimental design and/or the force of ligations. In addition, we found that alterations of the regularity index and step sequence were highly correlated with histomorphological changes and associated cytokine (TNF-α) and plasticity marker (synaptophysin) expression and somatosensory evoked potential. Based on the histomorphological and electrical physiological changes, the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham Surgery</th>
<th>1 Ring</th>
<th>2 Rings</th>
<th>3 Rings</th>
<th>4 Rings</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFI</td>
<td>−3.6 ± 12</td>
<td>−68.1 ± 10.8</td>
<td>−77.4 ± 4.9</td>
<td>−81.9 ± 7.9</td>
<td>−85.9 ± 6.9</td>
<td>0.47</td>
</tr>
<tr>
<td>MW (%)</td>
<td>98.1 ± 7.2</td>
<td>78.2 ± 4.3</td>
<td>75.3 ± 8.2</td>
<td>71.4 ± 5.5</td>
<td>69.5 ± 9.1</td>
<td>0.57</td>
</tr>
<tr>
<td>CMAP (%)</td>
<td>53.5 ± 5.86</td>
<td>43.6 ± 4.70</td>
<td>40.5 ± 5.2</td>
<td>38 ± 4.4</td>
<td>37.6 ± 6.7</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* CMAP = compound muscle action potential presented as ratio of left/right; MW = muscle weight presented as ratio of left/right; SFI = sciatic functional index.
regularity index and step sequence were highly correlated with the alteration in central neuronal remodeling during CCI.

Conclusions

Different intensities of nerve damage caused histomorphological changes from the skin to brain cortex. The neurobehavior alteration led to mechanical allodynia and thermal hyperalgesia. The allodynia and hyperalgesia were directly related to the severity of the nerve damage. The subtle alterations in the nerve damage were coupled with a significant difference in neurobehavior detected by the CatWalk XT system. The increased brain plasticity reflected an increased evoked potential and was related to changes in step sequence and regularity index. The CatWalk XT gait system provided better detection power with gait analysis than did mechanical allodynia and thermal hyperalgesia testing. The parameters of the CatWalk XT system also were correlated with the expression of synaptophysin and TNF-α of somatosensory cortex in the setting of CCI.

Acknowledgments

We thank Mrs. Shu-Zhen Lai and Miss Mu-Jung Liu for preparation of the manuscript and the Biostatistics Task Force of Taichung Veterans General Hospital for their assistance with statistical analysis.

Disclosure

This study was supported by grants from Taichung Veterans General Hospital, Providence University (TCVGH-NCHU987611), and the National Science Council (NSC-99-2314-B-075A-001-MY2), Taiwan.

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Pan, Sheehan. Acquisition of data: Chiang, Chen, Su. Analysis and interpretation of data: Chiang, Sheu, Chen, Su. Drafting the article: Chiang. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Pan. Administrative/technical/material support: Pan, Sheu. Study supervision: Cheng.

References

13. Deumens R, Jaken RJ, Marcus MA, Joosten EA: The Cat-
18. George A, Buehl A, Sommer C: Tumor necrosis factor receptor 1 and 2 proteins are differentially regulated during Wallerian degeneration of mouse sciatic nerve. Exp Neurol 192:163–166, 2005

C. Y. Chiang et al.