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Neuromuscular Alterations After Ankle Sprains: An Animal Model to Establish Causal Links After Injury

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Context: The mechanisms that contribute to the development of chronic ankle instability are not understood. Investigators have developed a hypothetical model in which neuromuscular alterations that stem from damaged ankle ligaments are thought to affect periarticular and proximal muscle activity. However, the retrospective nature of these studies does not allow a causal link to be established.

Objective: To assess temporal alterations in the activity of 2 periarticular muscles of the rat ankle and 2 proximal muscles of the rat hind limb after an ankle sprain.

Design: Controlled laboratory study.

Setting: Laboratory.

Patients or Other Participants: Five healthy adult male Long Evans rats (age = 16 weeks, mass = 400.0 ± 13.5 g).

Intervention(s): Indwelling fine-wire electromyography (EMG) electrodes were implanted surgically into the biceps femoris, medial gastrocnemius, vastus lateralis, and tibialis anterior muscles of the rats. We recorded baseline EMG measurements while the rats walked on a motor-driven treadmill and then induced a closed lateral ankle sprain by overextending the lateral ankle ligaments. After ankle sprain, the rats were placed on the treadmill every 24 hours for 7 days, and we recorded postspair EMG data.

Main Outcome Measure(s): Onset time of muscle activity, phase duration, sample entropy, and minimal detectable change (MDC) were assessed and compared with baseline using 2-tailed dependent t tests.

Results: Compared with baseline, delayed onset time of muscle activity was exhibited in the biceps femoris (baseline = -16.7 ± 54.0 milliseconds [ms]) on day 0 (5.2 ± 64.1 ms; t4 = -4.655, P = .043) and tibialis anterior (baseline = 307.0 ± 64.2 ms) muscles on day 3 (362.5 ± 55.9 ms; t4 = -5.427, P = .03) and day 6 (357.3 ± 39.6 ms; t4 = -3.802, P = .02). Longer phase durations were observed for the vastus lateralis (baseline = 321.9 ± 92.6 ms) on day 3 (401.3 ± 101.2 ms; t3 = -4.001, P = .03), day 4 (404.1 ± 93.0 ms; t3 = -3.320, P = .048), and day 5 (364.6 ± 105.2 ms; t3 = -3.963, P = .03) and for the tibialis anterior (baseline = 103.9 ± 16.4 ms) on day 4 (154.9 ± 7.8 ms; t3 = -4.331, P = .050) and day 6 (141.9 ± 16.2 ms; t3 = -3.441, P = .03). After sprain, greater sample entropy was found for the vastus lateralis (baseline = 0.7 ± 0.3) on day 6 (0.9 ± 0.4; t4 = -3.481, P = .03) and day 7 (0.9 ± 0.3; t3 = -2.637, P = .050) and for the tibialis anterior (baseline = 0.6 ± 0.4) on day 4 (0.9 ± 0.5; t4 = -3.224, P = .03). The MDC analysis revealed increased sample entropy values for the vastus lateralis and tibialis anterior.

Conclusions: Manually inducing an ankle sprain in a rat by overextending the lateral ankle ligaments altered the complexity of muscle-activation patterns, and the alterations exceeded the MDC of the baseline data.

Key Words: entropy, inhibition, ankle injury

Key Points

- Manually inducing an ankle sprain in a rat by overextending the lateral ankle ligaments altered the complexity of muscle-activation patterns in the vastus lateralis and tibialis anterior beyond the minimal detectable change of the baseline data.
- Not all rats behaved similarly, suggesting that subtle variations in the extent of tissue damage may play a role in muscle coordination.
- Pain associated with the ligamentous disruption may be a mechanism for these alterations.
- Continued exploration of this model is warranted to determine how it compares with changes after ligament transection and to explore the natural recovery of muscle function after ankle sprain.
- These observations provide new insights into the recognition, rehabilitation, and prevention of ankle sprains in humans.

Ankle sprains are the most common lower extremity injury associated with physical activity and sports.1 Approximately 11 ankle sprains occur for every 1000 exposures.2 Researchers3,4 have reported that 30% to 80% of individuals who sustain a lateral ankle sprain develop chronic ankle instability (CAI). This condition is characterized by persistent lateral ankle instability, leading to repetitive sprains, and is thought to result from functional or mechanical ankle instability.5 Accordingly, these seemingly innocuous lateral ankle sprains are common;
affect a large percentage of the active population; and often lead to long-term impairments in function, recurrent injury, and chronic symptoms.6

The mechanisms that contribute to the development of CAI are not fully understood. Based on retrospective laboratory observations, investigators have noted diminished muscle activation,7,8 prolonged muscle response to perturbation,9 and altered onset of muscle activity after ankle sprains.10 Furthermore, researchers11 have reported bilateral hamstrings inhibition and ipsilateral quadriceps facilitation, providing evidence that differences in muscle activity occur both proximal to and at the ankle joint postsprain. Based on the available literature, investigators6,9,11 have proposed a model in which damage to the lateral ankle ligaments results in neuromuscular alterations that are not limited to the ankle joint and often affect proximal structures. However, a major limitation to their work is that, due to the retrospective nature of these studies, no causal link can be established between the hypothetical model and the observable differences found. To establish a causal link, a prospective study design is required.

The optimal prospective study design to enable the systematic determination of factors that contribute to CAI would include an evaluation of individuals before and after ankle sprain. Based on the required time and difficulty associated with performing these systematic investigations of neuromuscular alterations before and after lower extremity injury, this design is currently not feasible in humans. However, longitudinal, live-animal experimental designs circumvent this concern and allow the application of systematic perturbations to the ankle joint to quantify the neuromuscular consequences of ankle sprain to selected muscles periarticular or distal to the ankle joint. Importantly, the lateral ligaments of the rat ankle have anatomic locations and functions similar to those in humans (Figure 1),15 so the rat ankle is a useful model for studying neuromuscular adaptations after ankle injury. Whereas rat models have been well established for exercise adaptations,16 osteoarthritis,17 and massage,18 a longitudinal rat ankle-sprain model has not been used. However, 2 rat ankle-sprain models have been validated as joint arthralgia models and used in analgesia and pain research.19 Therefore, the purpose of our study was to assess temporal alterations in the muscle activity of 2 periarticular muscles of the rat ankle and 2 proximal muscles of the rat hind limb after an ankle sprain. We evaluated the biceps femoris (BF), medial gastrocnemius (MG), vastus lateralis (VL), and tibialis anterior (TA) muscles, as these muscles have been found to display alterations in humans with ankle injuries7,8,10,11 and the peripheral nervous system in rats is considered a suitable model for studying the structure and function of neural alterations in humans.20,21 Furthermore, by studying both periarticular and proximal muscles, we could evaluate the effect of mechanical deformation of the lateral ankle ligament on the response of muscles with different neural innervations.21 We hypothesized that the ankle sprain would result in immediate alterations in both periarticular and proximal muscle activation compared with preinjury data and that the observed alterations would continue for 7 days after joint injury.

METHODS

Study Design and Subjects

With the approval of the Institutional Animal Care and Use Committee at the University of Kentucky, we conducted this pilot study using 5 healthy adult male Long Evans rats (age = 16 weeks, mass = 400.0 ± 13.5 g) that were obtained from Harlan Laboratories, Indianapolis, Indiana. All animals were housed in individual cages within the Division of Laboratory Animal Resources and allowed food and water ad libitum for the duration of the study.

Surgical Instrumentation

Anesthesia was induced using an induction chamber with 5% isoflurane and 1 L/min of oxygen and maintained via a nose cone with 2% isoflurane and 500 mL of oxygen. After the animals reached the surgical plane of anesthesia, we made an incision on the anterolateral portion of the left hind limb and used blunt dissection to identify the BF, MG, VL,
and TA muscles. After identifying the muscles, we implanted custom-fabricated, indwelling electromyography (EMG) electrodes constructed of Teflon-coated, 10-strand, stainless steel wire (model AS-631; Cooner Wire, Chatsworth, CA) in line with the muscle fibers using a small, curved surgical needle. Wires were routed through a subcutaneous tunnel along the spine to the base of the skull, and a 1-cm sagittal-plane incision was made along the coronal suture just anterior to the lambdoid suture. We exposed and cleaned the top of the skull and drilled 4 holes into its outer dense layer. We secured four 4-mm self-taping, stainless-steel bone screws (Fine Science Tools, Foster City, CA) in the holes and a 10-pin connector header (Digi-Key Corporation, Thief River Falls, MN) to the top of the skull using layers of dental cement (Excel Formula Dental Material; St George Technology, Inc, Wilmington, NC) bonded to the 4 screws. The 2 leads of each EMG electrode were soldered to 2 corresponding and opposite pins of the head connector. After attaching all EMG leads and 1 ground electrode, we covered all exposed pins with a final layer of dental cement, closed all incisions using 4-0 Vicryl (Ethicon, Inc, Somerville, NJ) subcuticular sutures, and allowed the rats to recover on a heated pad. Rats were given a 0.02 mg/kg subcutaneous injection of buprenorphine immediately after surgery and additional buprenorphine injections every 8 hours thereafter for 48 hours.

Data Collection and Ankle Sprain

Rats were allowed to recover in their cages for 1 week (Figure 2) after EMG-implantation surgery. Next, baseline EMG data were collected during level-ground walking. To accomplish this, we placed the rats in the first lane of a motor-driven treadmill (EXER 3/6 treadmill; Columbus Instruments, Columbus, OH). A custom-fabricated cable was secured to the head connector, attached to the leads of the EMG data-acquisition system (1400A myoMuscle; Noraxon Inc, Scottsdale, AZ), and synchronized with a high-speed video camera (myoVideo HS, myoSync; Noraxon Inc; Figure 3). On baseline day 1 (7 days after EMG implantation), the treadmill speed was set to 10 m/min to introduce the rats to walking before slowly increasing the speed to 16 m/min. When the rats were accustomed to walking at 16 m/min, we acquired baseline pre–ankle-sprain EMG data at a rate of 2000 Hz and synchronized the EMG signals with the high-speed video data sampling at 100 Hz.

After collecting 2 days of baseline data, we placed the rats under anesthesia as described above and induced a mild lateral ankle sprain to the left hind limb as described by Koo et al. Briefly, the ankle joint was moved repeatedly into inversion and plantar flexion for 4 minutes, causing an overextension of the lateral ankle ligaments. When we could move the joint into 180° of inversion and plantar flexion, we considered its condition to replicate a mild lateral ankle sprain. Immediately after ankle sprain, the rat was placed on the treadmill and allowed to recover from anesthesia. We determined that rats had recovered from anesthesia when they could ambulate freely on the treadmill and walk at a pace of 16 m/min. When the rats recovered from anesthesia, post–ankle-sprain EMG data were collected (16 m/min pace). After data collection, rats were disconnected from the EMG system and placed in their cages. Data collection occurred every 24 hours for the 7 days of recovery.
next 7 days, as most humans with first-time ankle sprains return to activity within 1 week after injury. Although compared with humans, rats have an accelerated life span, we chose this time frame because these data would provide a preliminary view of the neuromuscular adaptations that occur during a clinically relevant period. Immediately after the final data collection, rats were euthanized with an overdose of pentobarbital sodium, and their muscles were visually inspected for potential damage and dissected to confirm that electrode placement had remained intact for the duration of the study. Furthermore, we visually inspected the lateral ankle ligaments postmortem to confirm that ligament damage was induced.

**Data Reduction**

Each day, multiple step-cycles were recorded during treadmill walking over 1 minute, and 5 consecutive step-cycles were chosen from the midpoint of walking. One step-cycle was defined as the instrumented hind limb performing an entire swing-and-stance phase. Using the high-speed video camera, an investigator (L.K.L.) marked the moment when the paw lost ground contact (paw off) and the moment when the paw regained ground contact (paw on). In general, a visible limp was seen on day 0 (immediately postsprain) and typically rectified by day 2 postsprain in most rats. Given the low-level activation in some muscles, automated analyses were not reliable. Hence, EMG data were left as raw signals (ie, not processed), events were inspected visually by the same trained examiner (L.K.L.), and events were confirmed by a second trained examiner (T.A.B.) who was blinded to the trials to reduce the possibility of bias. To establish the onset of muscle activity, the examiner selected the point when EMG activity began (ie, substantial departure from baseline), which coincided with paw on. Phase durations, which were the times in milliseconds when the muscle was deemed active, were identified as the time from onset of muscle activity to muscle offset, which was defined as the time at which muscle activity returned to baseline.

In addition to identifying alterations in muscle timing (eg, onset of muscle activity and phase durations), an analysis of sample entropy was applied to the data to assess the variability in EMG signaling postsprain. Sample entropy is a mathematical algorithm that measures chaos or the lack of inherent variability in the locomotor system, the EMG data are left as raw signals to calculate the sample entropy. We analyzed the EMG sample entropy of each rat over the 5 step-cycles from each day with the following inputs: series length (m) of 2 data points and window (r) of 0.2 times the standard deviation of individual time-series data.

**Statistical Analysis**

To determine if our system could reliably collect baseline EMG data and to ensure that no differences existed in baseline data, we compared baseline day 1 muscle-activity onset time, phase duration, and sample entropy with baseline day 2 using 2-tailed dependent-samples t tests (Table). When we determined that no difference was present in baseline values, all post–ankle-sprain data (muscle-activity onset time, phase duration, and sample entropy) were compared with baseline day 2 (eg, baseline day closest to ankle sprain) using 2-tailed dependent-samples t tests to detect alterations in muscle activity in response to the acute lateral ankle sprain. In addition, given that sample entropy is a relatively novel method of quantifying changes within EMG signals, we compared 2 days of baseline data across all 5 rats to establish intersession reliability and calculated intraclass correlation coefficient (ICC [2,3]) estimates for each muscle. From the ICC, the standard error of the measure (SEM) was established for each muscle. To identify the meaningfulness of any potential changes due to ankle sprain, we calculated change scores from the pooled baseline days to each individual postsprain assessment. Any changes that were outside the minimal detectable change (MDC) were considered to be the effect of the manually induced ankle sprain. The MDC was defined as $\pm SEM \times \sqrt{2}$ and lasted at least 2 consecutive days. The $\alpha$ level was set a priori at .05, and values $\leq .05$ were considered different. All statistical tests were performed using SPSS software (version 22.0; IBM Corp, Armonk, NY).

**RESULTS**

**Baseline Data**

No differences in baseline days 1 and 2 were found for EMG muscle-activity–onset time, phase duration, or
sample entropy ($t$ range $= -1.597$ to $1.622$, $P$ range $= .11$ to .95), indicating that our system could detect consistent values of EMG muscle signaling in our rat model before ankle sprain.

### Muscle-Activity Onset and Phase Duration

The BF and TA muscles demonstrated alterations in muscle-activity–onset time postsprain. Specifically, the BF muscle exhibited delayed activity-onset time on day 0 postsprain compared with baseline data ($t_4 = -.4655$, $P = .043$; Table 2). The TA muscle also displayed delayed activity-onset time on day 3 ($t_4 = -5.427$, $P = .03$) and day 6 ($t_4 = -3.802$, $P = .02$) postsprain compared with baseline (Table). Whereas other muscles visually displayed delayed muscle-activity–onset time postsprain, we did not observe differences, likely due to high standard deviations (Table). The BF and MG muscles exhibited very low activation levels that precluded quantification of muscle-activity onset time on several days postsprain (Table). Specifically, these muscles demonstrated nonspecific bursting patterns with amplitudes of muscle activity that did not depart from baseline (Figure 4).

The VL and TA muscles demonstrated longer phase durations postsprain. Specifically, the VL muscle demonstrated longer phase durations than baseline on day 3 ($t_3 = -4.001$, $P = .03$), day 4 ($t_3 = -3.320$, $P = .048$), and day 5 ($t_3 = -3.963$, $P = .03$) postsprain. The TA muscle demonstrated longer phase duration on day 4 ($t_3 = -3.331$, $P = .050$) and day 6 ($t_3 = -3.441$, $P = .03$) postsprain. Again, the BF and MG muscles exhibited very low activation levels that precluded phase-duration quantification on several days postsprain (Table). Whereas no alterations in phase durations were observed for the BF or MG muscles ($P > .05$; Table), it appeared that, on days that were quantifiable, these muscles displayed longer phase durations postsprain than at baseline.

### Sample Entropy

We observed greater sample entropy postsprain for the VL and TA muscles, indicating that erratic motor control was present in these muscles postsprain. Specifically, the VL muscle demonstrated greater sample entropy on day 6 ($t_4 = -3.481$, $P = .03$) and day 7 ($t_4 = -2.637$, $P = .050$) postsprain, and the TA muscle demonstrated greater sample entropy on day 4 ($t_3 = -3.224$, $P = .03$) postsprain. Whereas different, sample entropy values across all muscles tended to be elevated postsprain (Table) through the conclusion of the experiment (day 7 postsprain), suggesting that widespread alterations in motor control may occur after ankle sprain. No differences in sample entropy were noted for the BF or MG muscles ($P > .05$; Table).

### Sample Entropy and Its Responsiveness

The VL (ICC [2,3] = 0.99, SEM = 0.04) had the highest intersession reliability estimates and the smallest SEM, followed by the TA (ICC [2,3] = 0.93, MDC = 0.17), BF (ICC [2,3] = 0.68, MDC = 1.25), and MG (ICC [2,3] = 0.45, MDC = 0.48). The MDC responsiveness analysis revealed that, for the VL, 3 of the 5 rats displayed increased sample entropy values for at least 2 consecutive days, whereas the
other 2 showed no change beyond the MDC (Figure 5). For the TA, 2 rats demonstrated increased complexity beyond the MDC across at least 2 consecutive days for days 1 to 5, but 1 of the 2 displayed substantially reduced sample entropy at days 6 and 7. The other 3 rats did not display any pattern beyond the MDC. The BF and MG responsiveness analyses revealed that none of the rats had any changes beyond the MDC due to an ankle sprain.

**DISCUSSION**

The purpose of our investigation was to explore the effects of an ankle sprain on muscle behavior in both the periarticular and proximal regions of the rat hind limb. We hypothesized that deformation of the lateral ankle would lead to substantial alterations in the EMG profiles. The most important finding was that manually introducing damage to the lateral ankle ligaments via a repeated inversion force substantially altered the EMG profiles of muscles surrounding and proximal to the joint. Specifically, the VL and the TA demonstrated the greatest alterations in both longer phase durations and higher activation complexity as measured by sample entropy.

The manual ankle-sprain model that we used resulted in damage to but not complete disruption of the calcaneo-fibular ligament (CFL; Figure 6), which likely represented a grade I ankle sprain. This observation confirmed that the repeated bouts of inversion and plantar flexion disrupted the major ligamentous stabilizer of the ankle. In addition, on visual inspection postmortem, we found no additional damage to the bones or tendons around the ankle. The goal of previous investigators who used this model was to elicit pain, especially with gait. The increased pain due to ankle sprains in these previous studies was confirmed by examining the amount of weight-bearing force on the affected limb and the quality of vocalizations during gait. In our study, the increased sample entropy beyond the MDC in the VL and TA may have been due to increased ankle pain. This observation provides strong evidence for the sample entropy alterations found in humans with patellofemoral pain and medial tibial stress syndrome. Rathleff et al reported that individuals with patellofemoral pain had greater sample entropy of the VL during stair descent than healthy control participants. Rathleff et al also noted that individuals with medial tibial stress syndrome had greater sample entropy of the TA than did healthy control participants. In both studies, participants with pathologic conditions reported active pain at their respective injured sites. Given that these investigations were retrospective, no causal link could be established between pain and increased muscle-activity sample entropy. However, the prospective design of our study enabled us to begin to build that link. Whereas we did not directly measure the amount of pain the rats were experiencing, this ankle-sprain model was developed specifically to induce pain. Therefore, increased sample entropy during muscular activation may be a good marker for the presence of pain.

The ankle sprain resulted in changes within the muscles that are not innervated by the same nerve. Given that the VL and TA are innervated by different nerves within the lumbarosacral plexus, the ankle sprain may have affected multiple spinal levels rather than only those directly related to innervating the structures around the ankle joint. This observation supports the report of Bullock-Saxton et al, who observed muscular alterations proximal to the ankle in human patients who sustained a severe ankle sprain.
compared with healthy control participants. The damage to the ligamentous receptors due to sprain potentially resulted in an altered pattern of muscle behavior that impaired the control of both periarticular and proximal muscle function. Importantly, this link between sensory and motor pathways was the original hypothesis for the development of CAI. Freeman first proposed that recurrent ankle instability resulted from deafferentation of the lateral ankle ligaments due to sprain. These episodes of “giving way” were attributed to the inability of the muscles to appropriately coordinate the control of the entire lower limb. Since the original hypothesis of Freeman, Wikstrom et al proposed that ankle sprains affect the entire sensorimotor system rather than only the periarticular muscles that surround the ankle. The results of our study support the hypothesis that ankle health plays a critical role in the behavior and coordination of the muscles within the lower extremity.

Whereas we observed changes beyond the MDC in the TA and VL, we did not find substantial changes in the BF or MG. The overextension of the lateral ligaments produced changes within the CFL but not complete disruption (Figure 6). Currently, it is unclear whether full disruption would affect the lower extremity more than incomplete disruption. In a study of the effect of ankle sprain in mice, Wikstrom et al surgically transected the anterior talofibular ligament (ATFL), CFL, or both, and followed the mice over 1 year to explore changes in coordination and physical activity. They reported that transecting both the ATFL and CFL had a substantially greater negative effect on sensorimotor function in these mice. One year after the initial surgery, the mice with ATFL and CFL transection displayed greater balance deficits and decreased physical activity than healthy mice or mice with isolated CFL transection. From these results, it appears that, as the number of involved ligaments increases, the effect on the sensorimotor system increases. Surgical transection of both the CFL and ATFL in rats may have a greater effect on more muscles of the lower extremity than a closed injury due to overextension of the ligaments.

In our study, not all rats responded similarly to the ankle sprain. The sample entropy increases were found in only 3 rats, and the changes within the phase duration and onset of muscle activity were not consistent across days (Table). This observation may be attributed to the extent of damage imposed by the manual ankle-sprain method. Whereas we did not provide a quantitative analysis of the amount of damage to the CFL, the extent of damage may not have been sufficient in 2 of the rats to elicit the substantial changes found in the other 3. Our goal was to recreate an ankle sprain that a human would experience. In doing so, we could not standardize the amount of damage to the ligaments, which is a limitation compared with the surgical-transection model of ankle injury, because the amount of ligamentous damage in the model we used could be related to the extent of muscular alterations. Similarly, given the nature of the closed model of ankle injury, it seems plausible that the mechanical overload of the joint could also have damaged the periarticular muscles. Although we did not find any obvious muscle damage on visual inspection postmortem, we did not microscopically evaluate the supporting musculature to ensure that this did not occur. Despite this limitation, we
contend that, when humans experience an ankle injury, it remains unclear if damage is restricted to the ligaments. Therefore, despite the potential variance in the extent of ligament injury achieved via the closed model of ankle injury compared with the transection model, the closed model is more clinically translatable because the injury occurs due to mechanical overload in humans. Furthermore, we used a closed model because it does not surgically violate the joint capsule, which is known to lead to altered neuromuscular activity and does not occur during human ankle sprain. In the future, we aim to investigate the alterations in muscle function due to the manual-overextension method compared with surgical transection of the ligaments in a greater number of rodents. By doing so, we want to determine if a direct relationship exists between the extent of tissue damage and magnitude of change in muscle function. This information would have direct implications for the care and rehabilitation of ankle sprains in humans.

Our study had limitations. Given that not all of the rats responded consistently to the ankle sprain, it is unclear whether the changes found can be attributed directly to the extent of damage to the CFL. Similarly, it is unclear whether the muscular alterations were due to pain, the damage to the ligamentous receptors, or some other underlying factor. However, we have established that manually inducing an ankle sprain in a rat potentially offers insight into lower extremity coordination, specifically as it relates to the complexity of muscle-activation patterns in both the periartricular and proximal muscles.

CONCLUSIONS

Manually inducing an ankle sprain in a rat via overextension of the lateral ankle ligaments appeared to alter the complexity of muscle-activation patterns in the VL and TA. These alterations exceeded the MDC in the baseline data, which served to establish healthy behavior. Not all rats behaved similarly, which suggests that subtle variations in the extent of tissue damage may play a role in muscle coordination. Pain associated with the ligamentous disruption may be a mechanism for these alterations. Continued exploration of this model is warranted to determine the changes when compared with ligament transection and to explore the natural recovery of muscle function after ankle sprain. From this information, new insights can be gained into the recognition, rehabilitation, and prevention of ankle sprains in humans.

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