Ongoing research to understand the mechanism behind pain is heavily dependent on animal testing. However, unlike humans, animal subjects cannot directly communicate with researchers to express the degree of pain they are experiencing. Therefore, measuring the presence of pain in animal studies is based on behavioral tests. The use of arbitrary values for determining the presence of pain in animal studies is an oversimplification of a complex and cortically dependent process.

The purpose of the present study was to identify a statistically supported latency time indicator that can be used as an accurate index for hyperalgesia to thermal stimuli in Sprague-Dawley rats subjected to T9 contusive spinal cord injury (SCI).

A statistical analysis of latency of withdrawal from stimulus-mediated spinal reflex in 979 Sprague-Dawley rats that had been subjected to a T9 contusive SCI was performed.

This is a retrospective review of a large research database derived from a series of studies performed evaluating thermal hyperalgesia in rats after SCI. Sprague-Dawley rats underwent a T9 contusive SCI and were tested for withdrawal latency from a heat stimulus. Assessment was done preinjury and on Postinjury Days 21, 28, 35, and 42 of the chronic phase of injury via a plantar withdrawal test.

The baseline test results of the 979 rats showed a significant resemblance to the normal distribution. The observed change in withdrawal showed mean latency drops of 0.42 second (standard error of the mean (SEM), 0.18; p = .026), 0.57 second (SEM, 0.19; p = .004), 0.63 second (SEM, 0.19; p = .002), and 0.69 second (SEM, 0.19; p = .0003). The standard deviation from the mean at all four postsurgical assessments was between 2.8 and 2.9 seconds.

Interpretation of withdrawal latency times as a marker for thermal hyperalgesia must be based on an appreciation for the normal distribution of pain scores. Recognizing that withdrawal latency is normally distributed both before and after injury allows for rational assignment of animals to groups designated as hyperalgesic and nonhyperalgesic. Two point nine seconds faster than the mean latency time is a statistically reliable indicator of thermal hyperalgesia in Sprague-Dawley rats subjected to contusive SCI. Repeated testing of animals to establish the presence or absence of thermal hyperalgesia beyond 21 days is not necessary in the absence of intervention.

Keywords: Spinal cord injury; Thermal hyperalgesia; Plantar test; Spinal reflex; Withdrawal latency time; Statistical analysis; Neuropathic pain
Introduction

Neuropathic pain is defined as secondary to a lesion or dysfunction of the nervous system [1]. Damage to either the peripheral or the central nervous system is a well-defined cause of such pain [2,3]. Neuropathic pain as a result of spinal cord injury (SCI) is a significant clinical problem, affecting up to 80% of SCI patients [4,5] (National Spinal Cord Injury Statistic Center 2006). Neuropathic pain is often a significant detriment to quality of life in patients with SCI [6,7]. Better understanding of the mechanism behind neuropathic pain through studying animal models of pain is central to the development of effective therapies. However, direct assessment of the subjective sensation of pain in animal models is not straightforward.

Thermal hyperalgesia (TH) and mechanical allodynia are commonly used indicators for the presence of pain in animal models [8]. Thermal hyperalgesia testing developed by Hargreaves et al. [9] assesses an animal’s withdrawal of a hind paw to a thermal noxious stimulus. We have used this method of pain assessment after a contusive SCI in a rat model in a variety of studies since 2004. In some of our previous work, we have defined a decreased withdrawal latency of greater than 2 seconds compared with baseline values to be indicative of the development of TH. This value was selected based on previous studies investigating peripheral nerve injuries in which a reliable and consistent change in withdrawal latency was elicited through ligation of the sciatic nerve [10]. Others and we have questioned whether this value is too arbitrary, given our observed variability in withdrawal latency time and the variability on pain response in human patients with SCI. In patients with SCI, there is significant variability in the degree of neuropathic pain with nearly identical injury [11,12]. Our laboratory and others have resorted to reporting differences between treatment and control groups as statistically significant (or not), irrespective of the characteristics of individual animals within the groups or clinical importance of the magnitude of difference [13,14]. Therefore, we examined the response of animal subjects to thermal stimulation through a retrospective review of a large prospectively collected database of animals injured in a series of experimental studies.

Materials and methods

Information regarding every animal operated on in our laboratory has been entered into a common database, and information regarding all assessments performed on each animal has been recorded. All studies were approved by the local institutional animal care and control committee. All animals were treated in accordance with published National Institute of Health standards. Data derived from animals subjected to experimental treatments were included up to the point that they received the treatment. No new surgery was performed for this analysis.

Contusive SCI

To induce contusive SCI in rats, we used the MASCIS Impactor protocol as described previously [15–20]. Briefly, adult male Sprague-Dawley rats (250–300 g) were anesthetized with gaseous isoflurane in oxygen (5% for induction and 3% for maintenance) throughout the duration of surgery. A T9 laminectomy was performed under aseptic conditions without disrupting the dura mater. Stabilizing vertebral clamps were placed at T8 and T10, and the animal was positioned in the MASCIS Impactor (Model II; WM Keck Center for Collaborative Neuroscience, Rutgers University, Piscataway, NJ, USA). The spinal cord was injured by releasing a 10-g rod (2.5 mm diameter) from a height of 12.5 mm. Bupivacaine (Sensorcaine-MPF 0.25%, 0.20 mL; Fresenius Kabi USA, LLC, Lake Zurich, IL, USA) was administered subcutaneously as a local anesthetic and the wound was closed in layers. Throughout the procedure, body temperature was maintained at 37°C with a constant temperature heating pad. The animals were then returned to their cages after recovering from anesthesia. Animals underwent daily manual bladder expression until bladder control was reestablished, and each animal received cefalexin antibiotic (0.10 mL; 330 mg/mL in saline) for 7 days after injury.

Basso, Beattie, and Bresnahan field locomotion test

Animals underwent an open-field locomotor test on Postinjury Days 2, 7, 14, 21, 28, 35, and 42. Once animals showed consistent forelimb and hind limb coordination (scoring 15 in the Basso, Beattie, and Bresnahan locomotor rating scale) [21], they were tested for TH.

Plantar test

Baseline reaction time was measured for 979 male Sprague-Dawley rats (250–300 g). Three sets of left and right hind paw reaction latencies to the thermal noxious stimulus were collected by placing an animal inside the Plexiglas apparatus (Plantar Test; Ugo Basile, Comerio VA, Italy). A movable focused beam of radiant heat was applied under the sole of one hind paw (Plantar Test, Biological Research Apparatus; Ugo Basile). When the animal retracts its paw from contact with the beam, a photocell turns off the heat, and the latency time is automatically recorded with a built-in timer. The strength of stimulation is 60 IR and is adjusted to produce baseline latencies of 8 to 10 seconds (typically 45°C–47°C). Animals were first acclimatized in the apparatus for 30 minutes before any measurements were taken for consistency. Each measurement was taken with the intermission of 5 minutes in between the measures. Testers were blinded to the animal’s experimental group. No measurements aside from the behavioral baseline and locomotion tests were taken before 21 days in our study, and all rats were sacrificed after 42 days.
Digitalization and database establishment

Data were available from all animal subjects tested for neuropathic pain between 2004 and 2012. We assigned a unique identification number to every rat. The data set was constructed to represent an average reaction time from each rat at Postinjury Days 21, 27, 35, and 42. Baseline data from rats that did not complete postsurgical testing because of euthanasia (for cord harvesting or due to medical complications) were not included in the analysis. Similarly, data from animals treated with experimental therapies were not included once the therapies began. As a result, we were able to construct a database containing a total of 979 male and female rats (n = 979). For each subject, latency time between the baseline and each Postsurgical Days 21, 27, 35, and 42 was calculated. This Δt value was then used to identify the occurrence and degree of TH response in rats with SCI by generating a histogram with a class interval of 1-second difference, ranging from 0 to 20 seconds. We then combined the cohorts of animals at four postsurgical days to represent the response-time distribution of total population. All animals that showed reduction in latency time during the postinjury behavior test were categorized as exhibiting TH pain behavior. The data collected for these rats were used to generate a linear model representing the correlation between the baseline data.

Statistical analysis

All data are given as 95% confidence interval. Statistical significance was assessed by Q-Q and Anderson-Darling tests followed by Student t test, one-way, or two-way analysis of variance (ANOVA) paired t test for differences in means of the subpopulation. Statistical analysis was derived using the R 64-bit statistic analysis program (R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism, version 6.00 for Mac OS (GraphPad Software, San Diego, CA, USA).

Results

The baseline test results of the 979 rats showed a significant resemblance to the normal distribution generated using the same data (average baseline response time, 10.3 seconds; standard deviation, 2.3). Eight hundred two of 979 rats demonstrated a response between 8 and 13 seconds (Fig. 1). Anderson-Darling test showed that the population of rats before a surgical SCI showed no bias and was normally distributed.

A paired t test between baseline data and each postsurgical time frame was calculated using data from 202 animals that were assessed at all time frames (Fig. 2). The postsurgical TH data at Days 21, 28, 32, and 45 showed mean latency drops of 0.42 second (standard error of the mean [SEM], 0.18; p = .026), 0.57 second (SEM, 0.19; p = .004), 0.63 second (SEM, 0.19; p = .002), and 0.69 second (SEM, 0.19; p = .0003), respectively. The standard deviation at each postsurgical interval was similar to one another, ranging between approximately 2.7 and 2.9 seconds. We began with 979 rats at baseline. Group sizes vary in the postsurgical assessment groups because of protocol variations between studies and cord harvest for histologic and other studies. The bar graphs for the total SCI population at each time frame and the Anderson-Darling test (data not shown) supported the normal distribution for all four subgroups.

To explore the possible correlation between the baseline value and the postinjury behavior measures, two-way ANOVA linearization test was performed for all subpopulations. For Day 21 population, the linear model showed an intercept of −3.29 (standard error, 0.43) with p value less than $2.2 \times 10^{-16}$ ($R^2$=0.38), with a residual standard error of 1.529 on 284 degrees of freedom. On Day 28, the population resulted in a slope of 0.47 (standard error, 0.038) and an intercept of −2.55 (standard error = 0.43) with p value less than $2.2 \times 10^{-16}$ ($R^2$=0.33), with a residual standard error of 1.506 on 298 degrees of freedom. The Day 35 measurement group against the baseline data showed a slope of 0.50 (standard error, 0.049) and an intercept of −2.81 (standard error, 0.55) with p value less than $2.2 \times 10^{-16}$ ($R^2$=0.31), with a residual standard error of 1.556 on 223 degrees of freedom. Finally, remaining rats tested on Day 42 against the baseline behavior had a linear model with a slope of 0.51 (standard error, 0.07) and an intercept of −2.85 (standard error, 0.76) with p value less than $2.727 \times 10^{-11}$ ($R^2$=0.27), with a residual standard error of 1.499 on 133 degrees of freedom.

Discussion

In each cohort of animals that were subjected to plantar test, the change in latency time (Δt) showed that approximately 75% of the rats that underwent SCI exhibited a drop in withdrawal latency time at Day 21 (Fig. 3). These data are identical to the literature value of mechanical allodynia model in which 75% of the Sprague-Dawley rats with hemisection SCI developed mechanical allodynia [22]. Thermal hyperalgesia is established within 21 days after injury in Sprague-Dawley rats. When we compared a mean value of each postsurgical population and performed a paired t test between the groups with the largest difference in mean value (Days 21 and 28), the resulting p value was insignificant. This indicates that the mean value of the whole population in their postcontusive SCI latency change was insignificant (p > .05). This implies that the detection of TH is not time dependent after Day 21. It also indicates that the degree of induced contusive SCI in the population was uniform, and the postsurgical care for the surviving rats was carried out effectively, further ensuring that the error range of latency measures due to the random degree of surgical
contusive SCI is low. Two limitations of the study are as follows. First, because we did not assess for TH before Day 21, it is possible that TH existed before Day 21. However, because of motor deficits, testing before Day 21 is problematic with this level of injury. Second, we did not test animals beyond 42 days, so we cannot comment on the long-term durability of the TH. Thermal hyperalgesia has been reported to spontaneously resolve months and years after SCI [23].

Our data indicate that animals with SCI with adequate motor function for testing will withdraw from a thermal stimulus as described previously between 5 and 12 seconds. All four postcontusive SCI surgery groups showed resemblance of Gaussian distribution and had 2.91-second mean standard deviation of latency drop. This indicates that the 2.9-second standard deviation is a statistically driven accurate indicator of distinguishing the presence of TH in animals with SCI.

The two-way ANOVA test between baseline and degree of change in latency time after contusive SCI indicates that there is a correlation between these two measures. This linear model cannot predict the outcome of a postsurgical time with precision ($R^2$ values ranging between 0.27 and 0.35). However, the large sample size and the consistency of the behavior test result support the hypothesis that rats with lower initial thermal sensitivity (high baseline reaction
time) tend to develop a greater change in reaction time after the injury. The numerical mean for each population after contusive SCI was nearly identical at all four different postsurgical days. The scatter about the linear model of the surviving rat population shows highly concentrated distribution near the linear model at Day 21 but shows gradual scattering as time progresses. This shows that the correlation between innate baseline and postsurgical latency diminishes over time beyond 35 days. This may indicate that the correlation is ephemeral; however, it is also likely that the scattering effect is caused by the decreasing total sample size due to natural mortality and continuous harvesting of the rats.

These findings indicate that the interpretation of withdrawal latency times as a marker for TH to be performed with caution. An arbitrary cutoff to define a binary “pain” or “no pain” state is not appropriate. The detection model should be based on an appreciation for the normal distribution of pain scores and a correction for baseline latency values for each animal. The latency time value of a hind paw reflex test found in our study could be used as one of the indicating variables in developing a comparative pain scale to quantify the degree of posttraumatic TH development in Sprague-Dawley rats. For example, the 25% of animals that did not develop latency changes may be considered TH free. The remaining 75% should be considered as having some degree of TH, and the results should be interpreted accordingly. Using the data from this article, we may attempt to develop a sliding scale used to assign values based on the absolute latency shift corrected for the individual animal’s baseline latency value. The use of such a scale would allow a more complete description of the response of an individual animal to experimental treatment and would also allow standardization of group characteristics for population-based experiments.

In summary, we described the characteristic pattern of thermal withdrawal latency change in Sprague-Dawley rats subjected to a contusive SCI when tested using the Harreaves apparatus. This information will allow more rational and streamlined study design and better interpretation of therapeutic effects.

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