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An automated flinch detecting system for use in the formalin nociceptive bioassay

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Yaksh, Tony L., George Ozaki, Damon McCumber, Michael Rathbun, Camilla Svensson, Shelle Malkmus, and Michael C. Yaksh. An automated flinch detecting system for use in the formalin nociceptive bioassay. *J Appl Physiol* 90: 2386–2402, 2001.—The biphasic display of paw-flinch behavior in the rat after injection of formalin into the dorsum of the hind paw is used for the screening of anti-hyperalgesic agents. Described and characterized here is a less labor-intensive system for counting flinch activity by detecting movement of a small metal band placed on the formalin-injected paw. A signal is generated as the band breaks the electromagnetic field of a loop antenna located under the rat and processed through an algorithm that determines flinch activity using *1*) amplitude, *2*) zero-voltage crossing, and *3*) signal duration. Flinches are summed and stored over a selected collection interval throughout the assay for later analysis. Studies have validated the measures with respect to *1*) system stability over time; *2*) system-to-"practiced observer" correlation on flinch detection, $r^2 = 0.94$; *3*) system variables including time of day, sex, age, and body weight; and *4*) 50% effective dose values similar to those previously reported for intrathecal morphine and the NMDA antagonist MK-801.

formalin test; spinal sensitization; pain models; flinching behavior

THE ESCAPE RESPONSE OR AGITATION evoked by a transient, strong stimulus attests to there being a close relationship between stimulus intensity, peripheral afferent discharge, and magnitude of the pain state as defined by response latency and magnitude. There are situations, however, in which the magnitude of the response to pain may exceed what would normally be anticipated, given the magnitude of the physical stimulus and the afferent traffic generated by that stimulus (31, 45, 47). These situations are loosely considered as reflecting a state of hyperalgesia, possibly arising from sensitization of the peripheral terminal and/or a central facilitation.

Several preclinical models have been developed that may reflect the significance played by such facilitation on behavior. The common characteristic found in these models is the injury that is induced and its causing of the sensory axon to produce a persistent discharge. A frequently used method of producing injury in the rat is the subcutaneous injection of a small volume of irritant such as formalin into its hind paw. Typically, after the formalin injection, the rat displays a biphasic (phase I and phase II) incidence of flinching (rapid paw shaking) and licking of the injected paw (18, 42, 43). The behavioral syndrome produced by the injection of formalin into the paw has been widely used to define the pharmacology of systems that regulate facilitated processing. The "formalin test" has evolved into a widely used tool in the screening of analgesic and anti-hyperalgesic drugs (45).

An important limitation of this behavioral model is its labor-intensive nature regarding data collection and the time required to train observers in its reliable implementation. Several automated systems have been proposed to facilitate data collection. One approach has been to employ strain gauges to measure mass movements of a rat in a confined cylindrical cage (21). A second model involves a video system that employs a pattern recognition algorithm (22). Although of merit, these approaches are limited in that each only indirectly measures movement of the injected paw. To address this limitation, we devised a system that assesses only the movement of the injected paw. As described below, this approach involves placing a metal band on the injected paw and detecting the movement of that band with a localized low-strength sinusoidal electromagnetic field. This paper describes the system and outlines studies used in defining its ability to measure the behavior of interest.

METHODS

Description of the System

The detection device consists of a pair of electromagnetic coils: one serving as a transmitter and the other as receiver. When current passes through the transmitter coil, an electromagnetic field in the 6- to 8-kHz range is generated with a signal strength on the order of 5–8 mW. Eddie currents are set up in ferrous or nonferrous metals within this field, and it is movement of the metal in the field that is detected. Loca-

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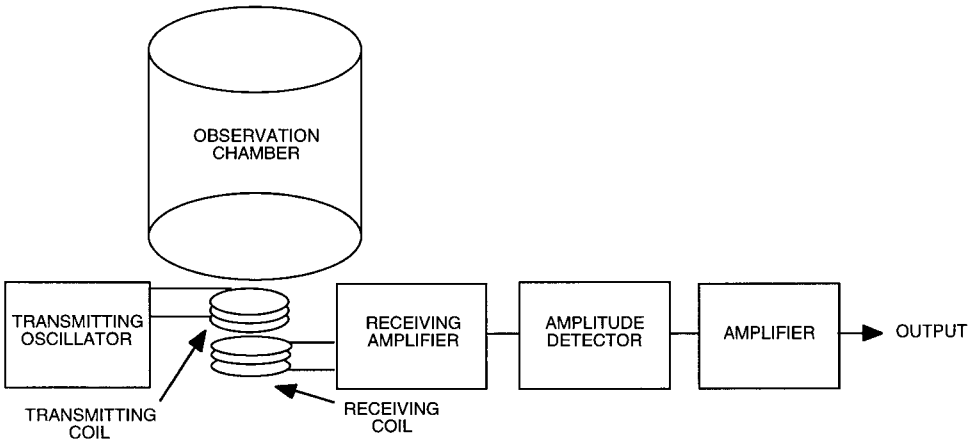

Fig. 1. *Top*: block diagram of the detection unit and its functional elements. *Bottom*: raw signal tracing over 2 min of an animal displaying flinch behavior, with arrows added where the processing algorithm detected flinches.

tion, size, electrical conductivity, and magnetic permeability are factors that determine how well movement of the metal is detected. The receiver output is amplified, filtered, and digitized for analysis (see Fig. 1). The spatial displacement of the injected paw within the electromagnetic field is detected using a small metal collar placed on the paw. During testing, the animal is placed into a cylindrical Plexiglas container (15 cm diameter $\times$ 30.5 cm height) mounted above the transmitter-receiver coil assembly, which is contained within a plastic enclosure. The cylinder ensures that the rat will remain inside the electromagnetic field generated by the coil without the added stress of being in restraint. As shown in Fig. 2, the system includes independent detection units and permits concurrent testing of four rats.

Modeling of Physical System Parameters

Several parameters of the electromagnetic field and pawband interaction were initially modeled to understand operating characteristics of the flinch detection system with the aim of defining the contributions of *1*) collar permeability, *2*) a complete or "O"-shaped collar configuration vs. a partial or "C"-shaped collar configuration, and 3) relationship of collar mass and sinusoidal frequency (*f*).

Modeling assumptions. The following modeling assumptions were made to define the effects of the several variables on system performance.

1) Whereas the collar may be variably located and oriented during normal study conditions, for the purpose of this evaluation, the collar was assumed to be centered with respect to the transmitter and receiver coils, thus simplifying the model to an axisymmetrical representation of the collar and coils. The coil motion was limited to movement along the coil axis in a vertical direction (see Fig. 3).

2) The collar was considered to be permeable, and the representative values were assumed to range from 100 to 1,000 (low-grade ferrous) or 100,000 (permaloy). It was as-

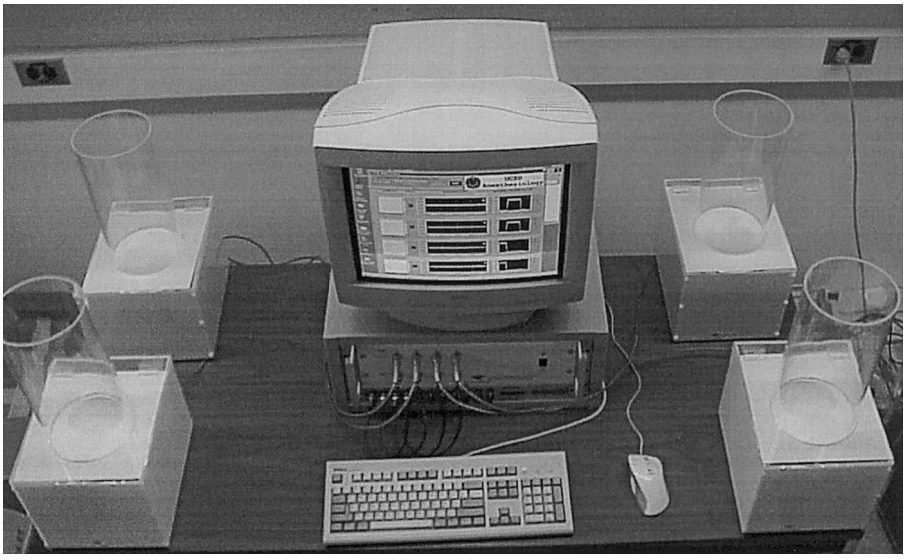

Fig. 2. The 4 detection units in which rats are placed for flinch test and the analog input and signal processing module. The computer used in flinch detection, display, and analysis is located below the testing table.

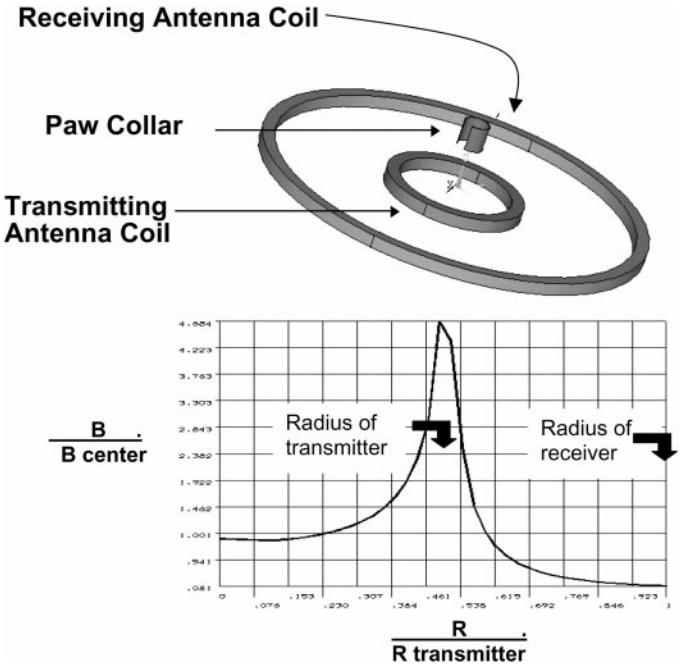

Fig. 3. *Top*: modeled transmitter (inner) and receiver (outer) coil configuration and modeled position of the rat paw collar. *Bottom*: graph displays the variation of the magnitude of the magnetic field when in phase with the transmitter current across the bore of the coils using a fixed-paw collar configuration. The *y*-axis presents the dimensionless ratio of B (magnetic flux density at current location along the radius from coil center) divided by B center (magnetic flux density generated at the coil center). The *x*-axis shows the ratio of flux measured at the specified location along the radius (R) divided by the radius of the receiver (outer) coil (R transmitter). Where the *x*-axis ratio is 1.0, the measurement is over the receiver coil. In the graph displayed, the transmitter coil is configured to be one-half of the radius of the receiver coil.

sumed that the system contained only materials that could be represented by a single permeability value.

3) Given the dimensions of the coils, the magnitude, and the *f* of the voltage (e.g., wavelength relative to coil diameter), the capacitive effect was considered to make a negligible contribution.

4) Motion of the collar was assumed to be limited to height range between 0.35 and 0.45 in. above and perpendicular to the coils and to be periodic, with a period of motion (between 1 and 20 Hz, see below). It is important to note that the field sinusoidal *f* was chosen to be large compared with the target motion (6–9 kHz). The extreme positions of 0.35 and 0.45 in. were selected for this modeling to correspond to the elevation extremes of the rat's paw above the coils. Even though the collar is changing its position, this assumption permits the motion of the collar to be treated as a steady-state condition. Whereas the direction of motion and coil dimensions will affect the receiver output, the effects due to the collar material, collar thickness, and the open vs. intact configuration of the collar can be identified by using a simplified model.

5) For modeling purposes, antenna coils were considered to be of a fixed-loop design and constructed of small strands to permit minimal eddy current.

6) The properties of the paw collar were varied in terms of its configuration (C or O), thickness, and material permeability. Collar thickness was of interest as it is related to collar weight and the ability of the animal to move freely. Magnetic permeability was also considered to be a significant parameter as it governed whether or not collars were to be made of specialized materials. These factors affect the distribution of the current generated in the collar and its magnetic flux (ϕ). Collar configuration was important, as it relates to construction and mounting and distribution of the currents generated within the collar by the transmitter coil. A continuous circle tends to collect maximum current flow along its outer diameter, whereas an incomplete circle (the arc) current distribution loops around the entire surface perimeter, as shown in Fig. 4.

7) The electromotive force (EMF; in V) appearing across the receiver coil is the time derivative of ϕ (in Wb), being linked with the receiver coil of N turns. Because the supply signal is sinusoidal at a f (in Hz), the EMF across the receiver coil was computed by the formula $2\pi fN\phi$, where $f = 8$ kHz. By using the assumption that the paw motion can be treated as a steady-state problem, the maximum and minimum collar elevations were examined. Electromagnetic analysis, employing the sinusoidal assumption, was performed for each position of the collar. Two solutions were produced: first, an imaginary solution that represented the electromagnetic field 90° out of phase with the input signal; and, second, the real solution where the electromagnetic field was in phase with the input signal. The peak EMF was computed by combining the EMF for each solution using the square root of the sum of the squares. The effect of collar motion is represented by calculating the differences of EMF at the two extremes of collar position. Calculations were carried out using the commercially available software ANSYS, employing the finite element method to represent the electromagnetic field. Guidelines for analysis of sinusoidal-varying fields are provided with the software.

Modeling results. The modeling reflects the analysis of four combinations from two collar thicknesses, 0.05 and 0.005 in., and two collar configurations, open and closed circumference. These four cases were evaluated using a variety of magnetic permeabilities of the collar (100–100,000) and are summarized in Table 1. The results are normalized by a single case. The values shown represent the relative behavior of the system with respect to alterations in collar design. It is understood that EMF can also be altered by the number of turns in the receiver coil or the transmitter coil and that larger paw motions would result in larger values, but the trend, shown by these results, would not be altered.

As noted above, the data presented in Table 1 reflect the analysis relevant to the paw collar being in the center of the antenna coil. The relative contribution of the several variables would, however, be the same if it was assessed in any part of the field. To determine the EMF generated across the

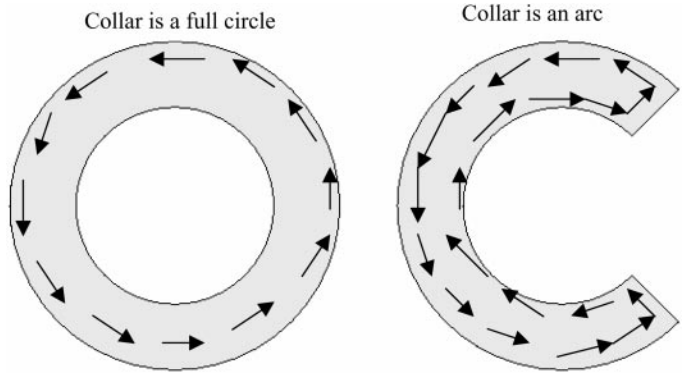

Fig. 4. Representation of current flow occurring with open- and closed-collar configurations.

Table 1. *Summary of collar modeling results for voltage signals generated by movement of paw collar in electromagnetic field generated by a surrogate flinch detection device*

Permeability	*Case 1,* 0.05 in., O shape	*Case 2,* 0.05 in., C shape	*Case 3,* 0.005 in., O shape	*Case 4,* 0.005 in., C shape
100	0.07	0.09	0.06	0.06
150	0.11	0.13	0.09	0.09
175	0.79	0.90	0.64	0.64
250	0.86	0.95	0.64	0.64
500	0.88	0.98	0.67	0.71
1,000	0.95	1.00	0.69	0.76
100,000	1.05	1.05	0.76	0.76

C and O, shape of collars; 0.05 and 0.005 in., thickness of collar. All electromotive force receiver values are normalized to *case 2,* permeability of 1,000.

field, the analysis was carried out for a standard coil across its radius, where the transmitter coil is constructed to be one-half the radius of the receiver coil. A typical variation of the normalized field across the bore of the transmitter and antenna is shown in Fig. 3. Values larger than unity correspond to the ϕ density (B) being larger than the ϕ density at the centerline of the coils. This variation is normally observed for such coil configurations. Larger flux densities lead to larger values of EMF because the ϕ is the area integral of the flux density over the bore of the antenna. Collar motion nearer the transmitter radius thus produces larger EMF. Outside the receiver coil, the field is observed to decay rapidly to a level at which a response would not be recorded, regardless of the collar design.

Modeling conclusions. This analysis provides several conclusions that were of concern during system optimization.

1) The most significant factor was metal permeability. Typical permeability for standard iron is ~1,000. It should also be noted that, if collar cross-sectional area is reduced, permeability would be further reduced. The significant drop in the values is at a permeability level of 150 and corresponds to the physical condition of a collar, where current cannot be concentrated at its surface. Additional sensitivity could be derived from the use of materials with high permeability and/or with increased thickness. Whereas *cases 3* and *4* (Table 1) show a 20–30% decrease in the relative performance, it must be remembered that the mass of the thinner collar is only 10% of that of the thicker collar.

2) The analysis confirms that use of an open collar does not result in major degradation of system performance.

3) The use of higher permeability materials (>1,000) reduces concern with collar geometry.

4) The relationship between *f* of sinusoidal current flow and generated EMF is linear; thus increasing energy will increase signal and system sensitivity.

5) Modeling the cross-sectional field profile makes clear that the field intensity peaks over the antenna coil. The area to which the animal is restrained should thus be centered over this radius. Importantly, profiling emphasizes the rapid tailing of the flux density as one extends beyond the radius of the receiver coil. This serves to limit the external influences of the field. This, plus the low-*f* energy signal produced by this system reduces the likelihood of EMF interference with other electronic devices.

6) For any given transmitter *f* and energy level, the flux density will be increased by reducing the relative diameter of the transmitter and receiver loops. Thus the magnitude of the EMF generated for a given paw movement and collar parameter can be increased if enhanced sensitivity is required (e.g., as with a smaller collar or smaller paw displacement).

Signal Analysis

Signal conditioning. The analog signal obtained from the sensing coil is filtered at 3 Hz and amplified before being digitized at a sampling rate of 1,000 Hz using 12-bit resolution. Besides the flinch behavior that occurs during the testing process, any general displacement of the limb, such as during ambulating and/or grooming activity, also generates signals that need to be addressed. To minimize the interference caused by nonflinch movement, the digitized signal is subjected to real-time analysis with the use of a software (LabView) algorithm to pick out a flinch from other paw movement.

Signal detection. Two approaches were considered in selection of triggering algorithm. The first employed a "power-*f*" analysis, in which spectral analysis of the limb's flinching movement revealed a characteristic *f* at ~8 Hz. The peak in the power spectrum was found, however, to be very broad, with significant components observed as low as 1 Hz and as high as 20 Hz. The breadth of the flinch bandwidth caused the algorithm to be judged not sufficiently discriminatory at differentiating flinching behavior from regular paw movement.

The second approach was a "zero-crossing interval-peak height" analysis based on estimating the amplitude of the signal over a sliding time window. The range of the voltages (maximum and minimum differences) over a moving 128-ms interval was calculated to produce a continuous output waveform. This secondary or range waveform contained jagged peaks that correlated well with the flinch transients found in the acquired waveform. It was then smoothed by using a linear convolution filter (a 128-ms nonweighted moving average filter), and the smoothed range waveform was examined in real time by a peak-detection algorithm set to pick out spikes of ~300-ms duration and amplitudes of >0.5 V. Each peak thus detected was counted as a paw flinch. This algorithm was observed to produce flinch reports that correlated well with data obtained from animals in which concurrent scoring by trained observers was obtained (see below).

Data Collection

Signal events, which meet the criteria of a flinch, as explained above, are captured and summed by time interval (normally set at 1-min increments) over the course of the study. An example of a typical "real-time" signal showing flinch behavior is displayed in Fig. 1. Signals are collected and stored in master raw data spreadsheets by animal according to its study and animal identifier. The software (Labview and VisualBasic) presents the data for four animals simultaneously on the screen and includes for each animal *1*) the study and animal identifiers, including animal numbers, treatment codes, dates, and other information relevant to the study; *2*) a window displaying the previous 2 s of digitized signal with markers indicating any flinch detection activity and a count of the flinches within that window; and *3*) a line graph of each animal's flinch count by sampling interval (1 min) since initiation of testing. A typical view of the computer screen during testing of four animals is shown in Fig. 5.

Preparation of Animal

A soft metal band (10 mm wide × 27 mm long, shaped into a C, and weighing ~0.5 g) is placed on the hind paw of the

Fig. 5. The screen present during concurrent data collection in 4 rats. The view, for each animal, from *left* to *right* is *1*) study and animal; *2*) small green virtual on/off switch that initiates and ceases data collection; *3*) smoothed range waveform signal of previous 2 s with indicators for flinch activity and flinch count for that interval; and *4*) minute-by-minute flinch count sums over completed portion of the study for each animal.

animal being tested. The open part of the C is positioned at the top of the paw with the arms of the C gently compressed to form a bracelet around the paw (see Fig. 6). Retention of the band is enhanced by applying a small amount of adhesive (cyanoacrylate, Elmers, Columbus, OH). The size and weight of the band are sufficiently small so as not to hinder the animal's normal movement. Animals are allowed to accommodate in individual Plexiglas chambers for 1 h before being moved to a test chamber. Just before the animal's placement into the test chamber, it is briefly restrained in a cloth towel, and irritant (typically 5% formalin, in volumes of 50 μl) is injected into the dorsum of the banded paw. Data collection is initiated after the animal is placed inside the test chamber.

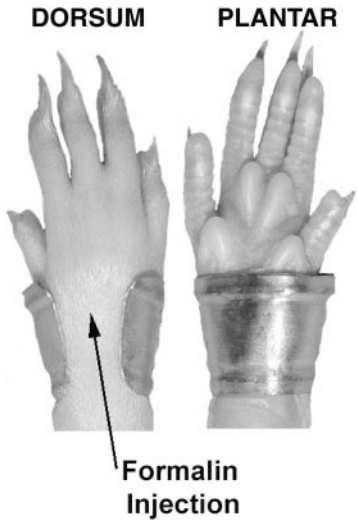

Fig. 6. The paw band and the typical placement on the left hind paw.

Drug Delivery

To examine the effects of drugs on flinching behavior, rats received intraperitoneal or intrathecal injections of the drug. Intraperitoneal drugs were delivered in volumes of 0.5 ml/kg. Intrathecal injections were done in rats that had been previously implanted with chronic intrathecal catheters (see below) and using drug volumes of 10 μl followed by a 10-μl flush using vehicle.

Drugs and Chemicals

The drugs delivered intrathecally and systemically were morphine sulfate (Malinkrodt) and MK-801 (RBI) dissolved in physiological saline (0.9% NaCl wt/vol). The formalin solutions were prepared by diluting formaldehyde (Formalde-Fresh 20%; Fisher Scientific, Fair Lawn, NJ) with physiological saline (0.9% NaCl wt/vol). Solutions were prepared fresh daily.

Data Analysis

Primary data management. Each animal's flinch count value over an interval of time (usually 1 min), for the duration of the study (usually 60 min), forms the data set used in all subsequent analyses. These data are averaged to characterize similar groups of animals by means and SD. For graphic display of data, flinches per minute are normally presented, but the mean of the flinches observed over 5-min time periods (12 per hour) has been used to clarify the graph when a number of groups are being displayed for comparison of time effect. For statistical comparison, the total number of flinches observed during any selected time period (phases I, II, IIA, and IIB) is calculated by accumulating each individual animal's flinches over that time period and averaging the group. Spreadsheet software (EXCEL) used in conjunction

with the data-collection software is constructed so as to allow the user to select up to four phases within the study's time frame for making a phase-related analysis, including phase cumulative flinch averages, SDs, and SE values, and calculates percent maximum possible effect (%MPE), group means, SDs, and SE values when a group of control animals is available. Dose-response curves are based on the calculated %MPE. The flinch time course has frequently been divided into two principal phases: phase I (0–9 min) and phase II (10–60 min). In this facility, it been observed that different drug effects may arise between early and late phase II; therefore, additional analyses occur at phase IIA (10–40 min) and phase IIB (41–60 min) (23). For statistical comparisons, ANOVA is normally carried out among treatment groups for each of the four phases, with statistically significance triggering post hoc test comparisons. Control groups normally consisted of a minimum of five rats run in temporal proximity to the drug groups.

Dose-response analysis. Dose-response slopes and values of effective dose in 50% of animals (ED_{50}) with 95% confidence intervals are calculated by using a least squares linear regression analysis carried out on percent inhibition of the cumulative flinching number for each phase (37). The percent inhibition is computed by dividing the response of each animal in a given group by the respective vehicle control group and multiplying by 100.

Statistical power. The minimum difference between the flinch count number measured in a test group and that measured in a control group, and determined to be statistically different, was calculated. The calculations were based on data obtained by using independent control groups, while assuming different power estimates in the 0.7–0.9 range and fixed group sizes ($n = 8$) and where power is defined as 1, the probability of a type II error being committed. Data were calculated as described elsewhere (52).

System measurement stability. A program was initiated for estimating measurement expectations from the paw-flinch system under control conditions and to document long-term process reliability. Data were collected at regular intervals over a period of 7 wk for the purpose of preparing control charts to answer the following quality control issues: *1*) what is the statistical distribution on which we can define the process as being "in control," in other words, stable and distributed normally?; and *2*) does the process show itself to be "capable," that is, does it meet assigned specification limits? Control and specification parameters (upper and lower control limits) were defined at study outset to be 3 SDs of the generated flinch count distribution for phases I and II. Analysis was done using the quality control tools found in Statview version 5.0 (SAS Institute, Cary, NC).

The animals were acclimated and then treated only with formalin injection just before the start of the 60-min data collection period. For each phase, the following values were calculated: *1*) the Xbar chart center line value, which is an estimate of the process average and is computed by averaging subgroup averages; *2*) the Xbar chart control limits, which identify the distribution range within which the process can be considered in control and are computed as three times the estimate of the process SD divided by the square root of the subgroup size ($n = 4$); *3*) the S chart center line value, which is an estimate of the process variation computed by multiplying an unbiasing constant based on subgroup size ($n = 4$) by the process SD estimate; *4*) the S chart control limits, which identify the distribution range within which process variation can be considered in control and are computed as three times the estimate of the process SD times an unbiasing constant based on the subgroup size ($n = 4$); *5*) the percent upper and lower specification limits, which indicate the number of times the measured values exceeded either the upper or lower control limits and are displayed as a percentage of the total number of measured values; and *6*) the capability index (Cp), an indication of whether the process is in control and can stay within the limits specified as necessary for test relevance and calculated by dividing the upper and lower specification difference by six times the process SD estimate. Upper and lower specification limits were calculated by phase as the mean of all tested animals $\pm 3 \times$ SD of the tested population, where, if the lower specification limit was <0, that limit became 0. A Cp value >1.33 means that a very small number (6 out of 100,000) of the formalin tests do not fall within the limits considered relevant to the normal testing process (e.g., $Cp < 1.0$, process not capable; $Cp = 1.0$, process marginally capable; $Cp > 1.0$, process is capable).

Animals

Rats were male or female Sprague-Dawley, Holtzman (Indianapolis, IN). Unless otherwise stated, the typical weight and age of these animals were 275–300 g and 100–120 days, respectively. All animals were given a minimum acclimation period of 3 days on site before being entered into a study. For those studies requiring the drug to be delivered spinally, each rat was implanted with a chronic intrathecal catheter. The catheter, constructed of polyethylene tubing (PE-10), was implanted under halothane anesthesia by insertion intrathecally through the cisternal membrane and being passed 8.5 cm caudally to the rostral edge of the lumbar enlargement. It was then externalized percutaneously at the top of the head for access during drug delivery (49). Animals receiving intrathecal catheter implants were allowed 4–5 days of recovery before testing. No animal showing neural deficit or behavior abnormality was used in the studies. All animals were euthanized immediately after completion of testing.

Study Objectives

Testing for "time of day" and test chamber effects on flinch behavior. Adult groups of male rats (325–375 g) were tested in the morning (0800–1000) or in the afternoon (1500–1700) over 5 consecutive days.

Testing for process long-term stability. Adult groups (4 rats/group) of male rats (325–375 g) were tested for flinching behavior over 8 wk, with separate groups being examined at 3- to 4-day intervals during this period. Adult groups (4 rats/group) of male rats (325–375 g) were tested for flinching behavior at three groups per week for 4 wk to evaluate process control and capability using statistical process control analysis. All studies were carried out between 0800 and 1000.

Testing for body weight effects on flinch behavior. Groups of adult male Holtzman Sprague-Dawley rats weighing either 100–125 g (small), 300–350 g (medium), or 400–450 g (large) were tested for flinching behavior. No test, control article, or vehicle was given.

Test of intrathecal catheterization effect on flinching behavior. Groups of rats were prepared with lumbar intrathecal catheters. After a 5-day recovery period, unimplanted animals of comparable weights (325–350 g) were tested for formalin response.

Test for intrathecal and intraperitoneal drugs on flinch behavior. Groups of rats received intrathecal or intraperitoneal injections of morphine sulfate or MK-801. Dose-response curves of each agent were generated to relate drug effect to paw flinch and for comparison with previously reported data.

Test of correlation between computer and human observer in detecting flinch behavior. A trained observer counted flinches each minute for 60 min at the same time as the automated device performed its data logging. A rat was tested once in the morning for 6 days.

RESULTS

Formalin-induced Behavioral Response

The injection of formalin into the dorsum of one hind paw of 100 male Sprague-Dawley rats over a period of 10 mo showed a reliable biphasic flinching of the injected paw, with peak flinch rates during phases I and II being on the order of 50 and 37 flinches/min. Figure 7 presents the time course of flinch responses, the mean cumulative flinches observed by phase [phase I: 203 ± 9 (SE); II: 1,058 ± 39; IIA: 792 ± 27; and IIB: 266 ± 16], and the statistical distribution of flinching in phases I and II. Analysis of this distribution (Kolmogorov-Smirnov) indicates that each phase was distributed normally ($P > 0.9999$).

System Stability and Reliability

Separate groups of four male Holtzman Sprague-Dawley rats were run in the morning and afternoon on 5 consecutive days. The following summarizes comparisons of the data.

Comparison among test chambers. There was no difference in the scores for phase I, II, IIA, or IIB

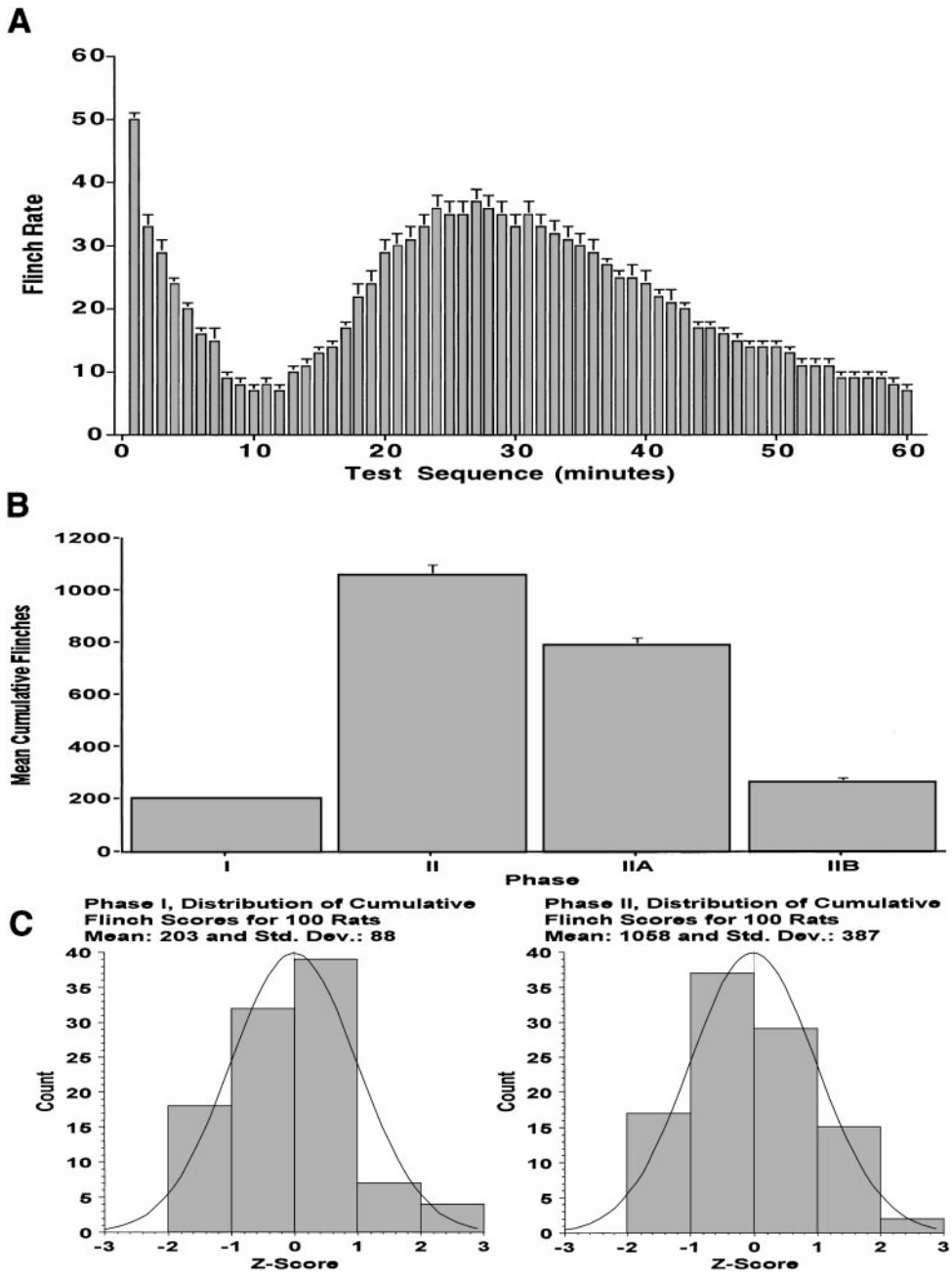

Fig. 7. *A*: average (means +SE) time effect curve for flinching behavior expressed as mean flinches for 100 rats. *B*: mean cumulative flinches (+SE) observed during phase I (0–9 min), phase II (10–40 min), phase IIA (10–40 min), and phase IIB (41–60 min). *C*: frequency of flinching counts for phases I and II displayed as a standardized distribution (Z scores).

Table 2. *Cumulative flinching behavior by test chamber and phase*

Group, Test Chamber	*n*	Phase I	Phase II	Phase IIA	Phase IIB
1	10	93 ± 47	794 ± 332	584 ± 215	210 ± 156
2	10	140 ± 35	740 ± 192	548 ± 134	192 ± 91
3	10	151 ± 64	692 ± 331	508 ± 208	184 ± 149
4	10	131 ± 65	737 ± 162	542 ± 107	195 ± 79

Values are means ± SD; *n*, no. of animals.

generated in each of the four chambers when examined over the 5 days of AM and PM testing (Table 2). There were no differences across test chambers for any phase (one-way ANOVA, $P > 0.1$–0.97).

Comparison of flinching behaviors measured over 5-day intervals. Examination of flinching behavior over 5 consecutive days by phase revealed no systematic differences for phase I, II, IIA, or IIB (Table 3). There were no differences across days for any phase (one-way ANOVA, $P > 0.07$–0.32).

Paw-flinch system stability measurements. Table 4 shows the results of the initial data analysis taken on selected days using four animals per day over a period of 7 wk. For both phase I and phase II, the following values were calculated (Fig. 8): *1*) the Xbar chart center line value, an estimate of the process average; *2*) the Xbar chart control limits, which identify the distribution range within the process; *3*) the S chart center line value, an estimate of the process variation; *4*) the S chart control limits, which identify the distribution range within which process variation can be considered in control; *5*) the percent upper and lower specification limits indicating the number of times the measured values exceeded either upper or lower control limits and displayed as a percentage of the total number of tests run; and *6*) the Cp, an indication of whether the formalin testing process was in control and capable of staying within the limits specified.

Characterization of the Variables Influencing the Formalin Response

Morning vs. afternoon test periods. Examination of flinching behavior in the morning (0800–1000) and in the afternoon (1500–1700) over 5 days of testing showed no difference between AM and PM for all four phases (Table 5). There were no differences across time of day for any phase (one-way ANOVA, $P > 0.10$–0.99).

Effects of body weight on flinching behavior. Three groups of rats differing in body weight (125–175, 300–350, and 400–450 g) were examined for flinching behavior. No difference in flinch response was observed in phases I, II, and IIA (one-way ANOVA, $P > 0.05$). A weight-related difference was seen in the flinching behavior for phase IIB (one-way ANOVA, $P < 0.05$) (Table 6).

Effects of sex on flinching behavior. Sex differences in flinching behavior were tested using 125- to 150-g Holtzman Sprague-Dawley rats. No difference in flinch response was observed in phases I, II, and IIA (one-way ANOVA, $P > 0.05$). A sex-related difference was seen in the flinching behavior for phase IIB (one-way ANOVA, $P < 0.05$) (Table 7).

Effect of formalin concentrations. The injection of saline into the paw resulted in a modest incidence of flinching behavior, whereas formalin concentrations from 0.5 to 5.0%/50 μl resulted in a concentration-related increase in flinching behavior measured in all phases and compared with saline (one-way ANOVA, $P < 0.002$) (Table 8). Post hoc analysis among groups disclosed phase and concentration differences in flinch response (Table 9).

Effects of Intrathecal Catheterization on Flinching Behavior

No statistically significant differences were observed in flinch response among animal groups with and without intrathecal catheters over all four study phases (one-way ANOVA, $P > 0.2$) (see Table 10).

Comparison Between Computer and Human Observer

On different days, six male Holtzman Sprague-Dawley rats (300–350 g) were injected with 50 μl of 5% formalin, and an experienced human observer counted flinching behavior concurrent with signal acquisition and flinch detection by computer. Phase I counts were observed to be lower for the human observer, but total counts remained close between the two methods (Fig. 9). A scattergram plot of simultaneously acquired data revealed good correlation ($r^2 = 0.94$).

Table 3. *Cumulative flinching observed over 5 consecutive days by phase*

Group	*n*	Phase I	Phase II	Phase IIA	Phase IIB
Day 1	8	133 ± 56	660 ± 175	522 ± 106	138 ± 106
Day 2	8	103 ± 59	620 ± 187	449 ± 93	171 ± 111
Day 3	8	150 ± 60	889 ± 401	658 ± 234	231 ± 169
Day 4	8	149 ± 61	860 ± 175	609 ± 139	251 ± 73
Day 5	8	109 ± 42	676 ± 208	491 ± 171	186 ± 110

Values are means ± SD; *n*, no. of animals.

Table 4. *Paw flinch system stability summary*

Group	Xbar Center Value	Xbar Control Limits	S Center Value	S Control Limits	USL	LSL	Percentage Greater Than USL	Percentage Less Than LSL	Cp
Phase I	257	±96	59	±74	456	50	0.0	0.0	1.064
Phase II	1,298	±447	274	±348	2,186	353	0.0	0.0	1.026

USL and LSL, upper and lower specification limits, respectively; Cp, capability index. See text for details.

Effects of Systemic and Spinal Drugs on Formalin-evoked Flinching Behavior

Morphine. Intrathecal and systemic injection of morphine (μ-opioid agonist) resulted in a potent dose-dependent reduction of all phases of the flinching response (Fig. 10). The ED_{50} values were calculated by using least squares linear regression analysis of the dose effect %MPE curves (Table 11).

MK-801. Intrathecal and systemic injection of MK-801 (a noncompetitive NMDA-receptor antagonist) resulted in a potent dose-dependent reduction in phase II, but not phase I, of the flinching response (Fig. 11). The ED_{50} values were calculated by using a least squares linear regression analysis of the dose effect (%MPE) curves (Table 11).

Power Analysis

The difference in flinch counts necessary to show statistical significance was determined by using the following assumptions: *1*) two groups being compared with eight animals per group, *2*) a two-tailed analysis, *3*) a 0.05 level of rejection, and *4*) a reasonably high probability (70–90%) of detecting true group mean differences. Determinations were based on mean and SD averages derived from five different eight-animal formalin control studies (Table 12).

DISCUSSION

Injection of formalin into the paw leads to a biphasic flinching behavior with the magnitude of the behavior positively covaried with the concentration of formalin.

Formalin-evoked Flinching: Supraspinally Organized Complex Behavior

From the perspective of a model of nociceptive transmission, an important question is whether the formalin-evoked flinching behavior reflects an endpoint that is mediated through the exaggeration of spinal traffic

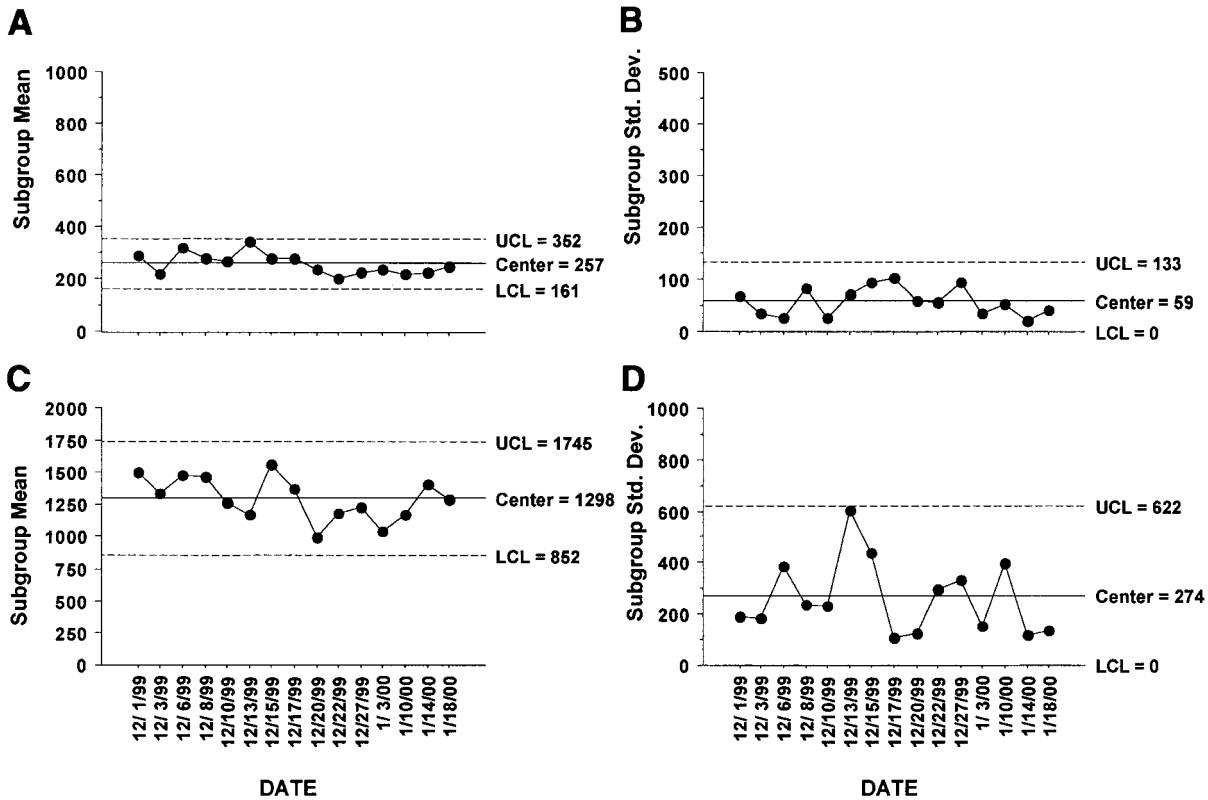

Fig. 8. Control charts. *A* and *C*: Xbar line chart provides information about variation between the subgroup means over time by displaying individual group mean flinch counts plotted along its time (date) axis. *B* and *D*: S line chart plots subgroup SD along the time axis, providing information about variation within the subgroups. *A* and *B*: phase I; *C* and *D*: phase II. Calculated upper (UCL) and lower control limit (LCL) and average (center) lines have been added.

Table 5. *Cumulative flinching behavior as a function of testing time by phase*

Group	*n*	Phase I	Phase II	Phase IIA	Phase IIB
AM	20	129 ± 55	684 ± 210	506 ± 141	179 ± 103
PM	20	129 ± 60	797 ± 293	586 ± 186	212 ± 134

Values are means ± SD; *n*, no. of animals. AM and PM, morning and afternoon examinations, respectively.

to supraspinal centers, or does the biphasic (phases I and II) flinching behavior represent an exaggerated spinal reflex? Several observations are relevant. First, injection of formalin induces a variety of complex, unconditioned behaviors, such as licking and guarding of the injected paw, which are reflective of a higher order motor organization. Second, spinal transections markedly reduce phase I flexion extension of the formalin-injected hind paw, and phase II is abolished (42). Third, in addition to the paw withdrawal, formalin injection induces biphasic autonomic (cardiovascular) and supraspinally mediated hormonal responses (29, 38, 51). Fourth, assessment of the firing patterns of dorsal horn wide dynamic range neurons, many of which are projection neurons, has shown that comparable formalin injections result in a similar biphasic activation pattern (15, 33) and an increase in the expression of c-Fos in dorsal horn neurons (1). Jointly, these findings support the argument that phase II behaviors reflect augmented responses to spinofugal traffic and not simply an augmented reflex.

Role of Small Afferents in Formalin-evoked Flinching

The flinch response is evoked and maintained by persistent small afferent input. Two lines of evidence support this hypothesized mechanism: *1*) treatment with the C-fiber neurotoxin capsaicin reduces the response to an irritant injected into the paw, suggesting a role for small afferents (17, 29, 46); and *2*) local anesthetic blockade of the afferent input during phase II reduces dorsal horn neuron activity (15) and halts flinching and grooming (13, 39) during phase I and phase II. In other words, all flinching behavior (phase I and phase II) requires ongoing afferent traffic.

Mechanisms Underlying the Biphasic Components of the Flinching Behavior

Measurement of the firing pattern of small sensory afferents evoked by formalin injected into their receptive fields reveals an acute burst of activity that persists for several minutes. This initial discharge is followed by a persistent low level of afferent activity in slow (small diameter) and fast (large diameter) sensory afferents (32). Temporally, the initial intense flinching (phase I; 0–10 min) correlates with the initial afferent barrage, whereas the prominent second phase of flinching (phase II; 20–60 min) corresponds with the interval when there is a relatively modest, but nonzero, level of afferent input. An important question is, given that there is reduced afferent traffic during phase II (32), what is the origin of the prominent flinching that is observed during phase II? We believe that the system is unexpectedly complex, involving several potential mechanisms, all of which may contribute to this second phase.

Peripheral components. As noted above, based on local anesthetic blockade, ongoing afferent traffic is essential for dorsal horn neuron activity and the delayed onset of behavior during the second phase after formalin. Peripheral inflammation generated by formalin can initiate activity, perhaps in populations of small cutaneous afferents that are not normally active and have the ability to strongly drive dorsal horn neurons ("silent nociceptors") (19). This provides a peripheral mechanism that would initiate enhanced neuronal activity and flinching behavior in the face of an apparent reduction in overall afferent traffic during the second phase (13).

Central components. Persistent small afferent input evokes a facilitation of spinal nociceptive processing (14, 44). Accordingly, it is reasonable to conclude that intradermal formalin may initiate such a cascade, leading to a state of central facilitation that is maintained during the low level of afferent traffic in phase II. Such a facilitated state, combined with a low level of ongoing afferent traffic, would provide a mechanism for the observed high level of flinching during phase II (14). Two observations support this role of a central sensitization: *1*) delivery of agents believed to suppress small afferent input (e.g., opiates) reduces the phase II response, even when their action is limited to the interval of phase I (3, 9) [but see studies with systemic opiates (40)]; and *2*) delivery of classes of agents that do not block acute excitation or acute pain behavior but block afferent-evoked spinal facilitation, such as NMDA antagonists, cyclooxygenase inhibitor, and nitric oxide synthase inhibitors, diminishes the second

Table 6. *Cumulative flinching behavior as a function of body weight by phase*

Group, Body Weight	*n*	Phase I*	Phase II*	Phase IIA*	Phase IIB†
125–175 g	8	193 ± 45	768 ± 208	686 ± 174	81 ± 69
300–350 g	8	133 ± 56	660 ± 175	522 ± 106	138 ± 106
400–450 g	8	232 ± 60	905 ± 196	689 ± 162	216 ± 96

Values are means ± SD; *n*, no. of animals. *$P > 0.05$ and †$P < 0.05$, one-way ANOVA.

Table 7. *Cumulative flinching behavior as a function of gender by phase*

Group	*n*	Phase I*	Phase II*	Phase IIA*	Phase IIB†
Female	7	161 ± 83	865 ± 262	675 ± 168	191 ± 111
Male	8	193 ± 45	768 ± 208	686 ± 174	81 ± 69

Values are means ± SD; *n*, no. of animals. *$P > 0.05$ and †$P < 0.05$, one-way ANOVA.

phase of the formalin-evoked behavior (see Ref. 45 for references).

Interactive contributions. We believe that it is likely that changes in both afferent traffic (peripheral) and spinal processing (central sensitization) components contribute to the observed formalin-induced behavioral states. As noted, sensory afferent recordings have emphasized that formalin injection leads to ongoing activity in large and small afferents. Large afferent activity (e.g., light touch) is not typically associated with the initiation of a pain state. However, after local injury or small afferent activation, an exaggerated response of dorsal horn neurons to high-intensity stimuli applied to the site and to low-intensity tactile input applied adjacent to the injury site is detected (see Ref. 14). These phenomena have the behavioral parallel of 1° hyperalgesia and 2° tactile allodynia (8, 28). Pharmacological studies have shown that the 1° hyperalgesia has a well-defined peripheral component, whereas the 2° tactile allodynia is initiated but not sustained by the primary injury input. We thus hypothesize that the second-phase pain behavior may arise from *1*) an ongoing central sensitization initiated and maintained by small afferent input, and *2*) the afferent traffic coming in from small afferents (normally high threshold, nociceptors) and, perhaps of equal importance, from large afferents (low threshold, mechanoreceptors) that can induce pain behavior when there is a central sensitization.

Manual Formalin Testing

The evident utility of the formalin response suggests that it serves as a robust model for screening antihyperalgesic agents. A limitation is the time and training required to perform the test. In previously published work, the primary behavioral index has been the counting of the number of flinches in which 1-min flinching epochs are counted every 5 or 10 min over a 60-min observation interval. This spacing of observations permits a single technician to follow several rats concurrently. Other methods, such as the counting of time spent in multiple behaviors (12, 42), might provide additional sensitivity; however, the complexity of such an analysis places limitations on the implementation of the model. First, it reduces the number of animals that can be followed concurrently. Second, the need for observer vigilance means that, with repeated testing during the workday, the drift in operator reliability can surely be anticipated. It is important to note that, in work published on the method, such practical issues as reliability among observers over time are not discussed. Third, the use of indexes that involve judgments on weight bearing or appropriate licking behavior requires that the observer undergo extensive training and some form of technique validation to prove that needed skills have been acquired before it is considered that the data produced are useable. Again, most investigators do not generally discuss these issues. Wheeler-Aceto and Cowan (42) specifically cited the simple counting of formalin flinches as reducing the degree of interobserver variation. Coderre et al. (12) emphasized the degree of interobserver reliability, but, again, this is likely a best case analysis unencumbered by drug- or behavior-related perturbation.

Automated Systems

Given the limitations associated with manual assessment of formalin-evoked flinching, efforts have been made to develop automated assessment systems. The systems described include one by Jett and Michelson (21) that involves measurement of mass shifts secondary to the movement of a rat in a confined cylindrical cage. The use of a complex filtering algorithm is said to reduce the contribution of whole body movement from those arising from the movement of the much smaller mass of the hind paw. This model has been employed in several reports (7, 29). Another model utilizes a video camera system and relies on pattern recognition. The algorithm employed is not described, but it appears to depend on the symmetry of the animal (22). In neither case can the movement of

Table 8. *Cumulative flinching behavior as a function of injection dose by phase*

Group	*n*	Phase I	Phase II	Phase IIA	Phase IIB
No injection	8	92 ± 56	146 ± 102	92 ± 48	54 ± 79
Saline	12	109 ± 75	302 ± 175	201 ± 123	102 ± 73
Formalin, %					
0.5	12	127 ± 66	400 ± 185	301 ± 169	100 ± 79
1.0	12	171 ± 55	613 ± 265	495 ± 211	118 ± 100
2.5	12	214 ± 75	898 ± 266	662 ± 210	236 ± 148
5.0	12	230 ± 84	811 ± 194	659 ± 131	152 ± 88

Values are means ± SD; *n*, no. of animals.

Table 9. *Post hoc group comparisons of formalin injection concentrations by phase*

Group Comparisons	Phase I	Phase II	Phase IIA	Phase IIB
No injection vs. saline				
No injection vs. formalin, 0.5%				
No injection vs. formalin, 1.0%		S	S	
No injection vs. formalin, 2.5%	S	S	S	S
No injection vs. formalin, 5.0%	S	S	S	
Saline vs. formalin, 0.5%				
Saline vs. formalin, 1.0%	S	S		
Saline vs. formalin, 2.5%	S	S	S	S
Saline vs. formalin, 5.0%	S	S	S	
Formalin, 0.5% vs. formalin, 1.0%				
Formalin, 0.5% vs. formalin, 2.5%	S	S	S	S
Formalin, 0.5% vs. formalin, 5.0%	S	S	S	
Formalin, 1.0% vs. formalin, 2.5%		S		
Formalin, 1.0% vs. formalin, 5.0%				
Formalin, 2.5% vs. formalin, 5.0%				

S, significant difference between groups ($P < 0.05$), Tukey-Kramer post hoc test.

the injected paw be defined with certainty. In contrast, the model presented here addresses this issue directly.

Modeling of the Detector System

It was our aim at the outset to minimize radio-frequency interference and the interaction of the coil fields with the surrounding environment. Collar size was configured to be unencumbering to the rat and constructed of a metal with sufficient permitivity to generate a useful signal. Modeling of the electromagnetic field and collar interaction was undertaken to determine a workable system configuration, and a configuration was defined that uses an open collar to produce a useful signal within a relatively localized field at the *f* range of 6–8 kHz. Computational analysis indicates that increased field strengths can be achieved by using higher coil voltage or by using a fixed signal strength and *1*) metals of higher permitivity, *2*) a higher sinusoidal *f*, and/or *3*) increasing field density by decreasing the field coil size. In recent work, we have confirmed these predictions and have developed a system variation that effectively measures paw flinching in the mouse (T. L. Yaksh, G. Ozaki, and S. Malkmus, unpublished observations).

Validation of the Automated Formalin System

The two methods used here to determine system relevance obtained human and system flinch counts concurrently and compared flinch-count data obtained from previous drug studies with similar new studies using the automated system.

As indicated, concurrent paw-flinch assessment by a trained human observer and by the automated system demonstrated good correlation. There was a tendency for the automatic flinch counts in phase I to be higher than those obtained by the observer, but this is believed to reflect the inability of the human observer to follow the rapid flinching that is evident during that phase.

With regard to the activity of drugs known to alter flinching behavior, morphine given intrathecally and systemically produced a dose-dependent reduction in all phases of the response to formalin. These results are consistent with the mechanism of action of morphine, in which, at the spinal level, μ-opiate receptors are believed to diminish the excitability of input associated with the activation of small, primary afferents (45). The ED_{50} values obtained from the automated assessment correspond closely with the ED_{50} values reported using human scoring. Intrathecal delivery of an NMDA-receptor blocker, MK-801, has been shown to block phase II of the response to formalin and, to a lesser degree, phase I in a dose-dependent manner. Interestingly, whereas the intrathecal response curve was clearly monotonic, there was an apparent increase in flinching behavior at the lowest doses after systemic delivery of the drug. Examination of the animals during the study indeed confirmed that there was enhanced activity leading to increases in flinch count. The reason for this biphasic component is not known, but it is appreciated that the noncompetitive NMDA antagonists may have stimulatory properties that might reflect a phencyclidine-like action. Such effects may be accounted for if the systemically delivered drug is getting into the brain (as compared with a spinal action).

Assessment of System Reliability

In any assay system, baseline stability over time is a primary concern. Baseline flinching behavior was as-

Table 10. *Cumulative flinching behavior as a function of catheter by phase*

Group	*n*	Phase I	Phase II	Phase IIA	Phase IIB
Catheterized	12	153 ± 42	701 ± 409	445 ± 202	255 ± 226
Not catheterized	12	129 ± 57	741 ± 258	546 ± 168	195 ± 119

Values are means ± SD; *n*, no. of animals.

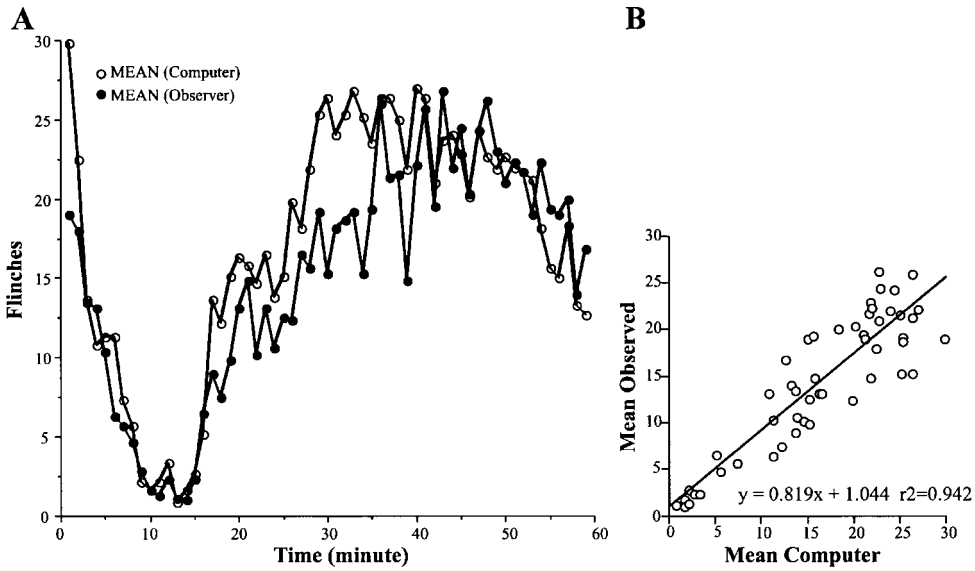

Fig. 9. Time-effect plot, comparing flinches per minute as determined by a trained human observer and by computer (*A*), and a scattergram (*B*) showing linear regression between the concurrent human and computer flinch detection of 6 rats.

sessed in separate studies over 5-day, 7-wk, and 11-mo time increments. Over these intervals, there were no statistically significant shifts in baseline activity in standard, untreated animals.

A more definite approach is to undertake a statistical process analysis. Using statistical process control analysis, we specifically focused on the 7-wk period. We sought to determine whether, over the 7-wk time in-

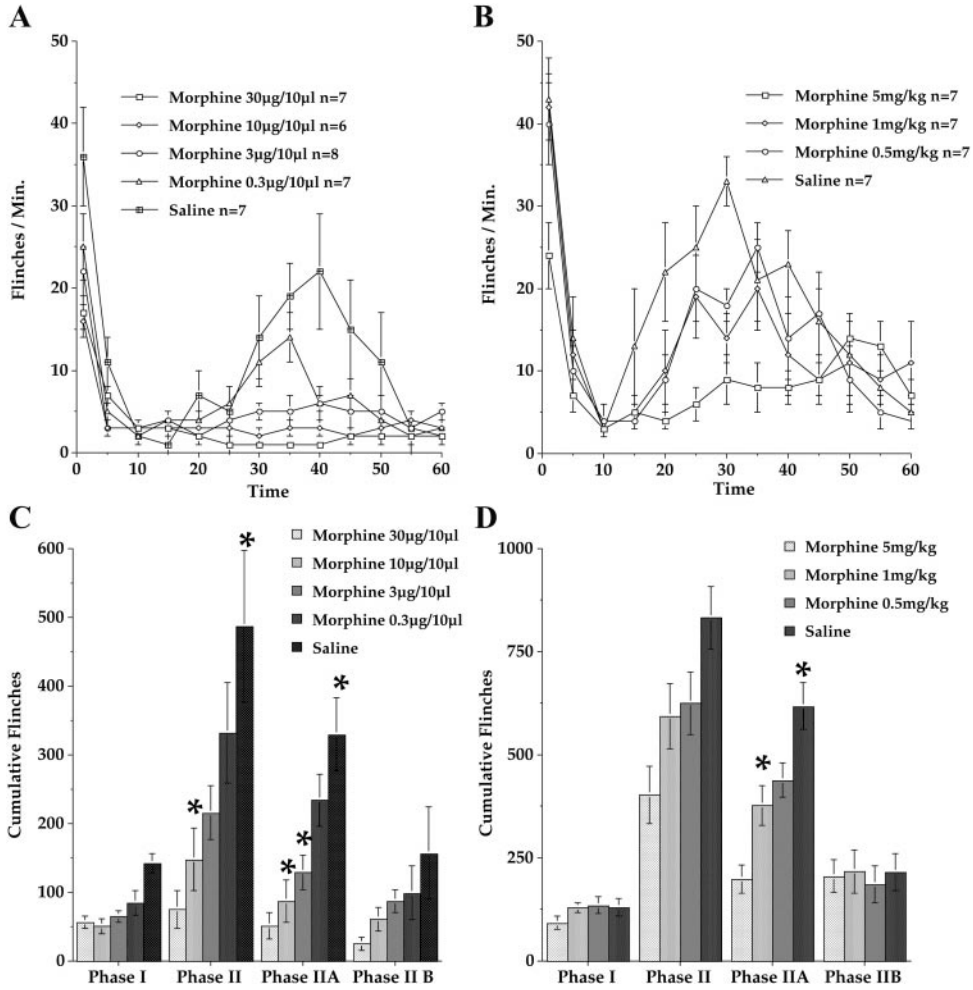

Fig. 10. Time-effect curves (means ± SD) for vehicle, intrathecal (*A*), and intraperitoneal (*B*) morphine on formalin-induced flinching in the rat. Cumulative flinches, by dose, are presented by phase for intrathecal morphine (*C*) and for intraperitoneal morphine (*D*). $*P < 0.05$, by one-way ANOVA across dose.

Table 11. *ED_{50} values for the effects of systemic morphine, intrathecal morphine, and MK-801 on flinching behavior*

Group	Phase II	Phase IIA
Intrathecal morphine, μg	1.6(0.6–4.7)	1.5(0.6–3.6)
Intraperitoneal morphine, mg	4.7(7,888–0.003)	1.8(1.1–2.9)
Intrathecal MK-801, μg	9.5(87–1.0)	7.3(43–1.2)

Values are means with 95% confidence intervals in parentheses. Data for intraperitoneal MK-801 were not calculated from dose-effect curves due to lack of monotonic sequence. ED_{50}, effective dose in 50% of animals.

crement, the process, measurement of paw flinches, was in control (i.e., paw-flinch measurement varied only within the limits of a selected statistical distribution) and capable (i.e., a high percentage of the measurements taken fell within previously determined specification limits). A process that is in control, or stable, can be defined by a normal distribution of the observations obtained over time. One measure of process control is the Cp. This measure defines how satisfactory a process is at meeting the requirements placed on it. A Cp value of >1.00 means that only a small number of the formalin tests would not fall within the limits considered relevant to the normal testing process. Conversely, a Cp < 1.0 indicates that a significant number of observations fell outside of specified distribution. In the present studies, Cp values for both phases I and II over a 7-wk interval were >1.000 and thus in control and capable.

Although seldom used in behavioral system studies, these types of analyses can *1*) bolster confidence in a testing process, *2*) help to define its sensitivity limits, and *3*) aid in the detection of transient or systematic changes before they affect study results.

Variables Influence the Formalin Response

Several variables may impact the magnitude of the flinching behavior. With the use of the automated assessment systems, selected variables were examined.

Behavioral indexes. Injection of formalin into the paw results in a variety of spontaneous behaviors including flinching of the paw, licking of the paw, lifting of the paw from the surface of the chamber, changes in weight bearing, and vocalization. Assessment of the intensity of a behavior induced by formalin injection has typically involved one or a combination of measures, including the measurement of duration and/or *f* for one or more of the known behaviors (2, 12, 18, 42). In most cases, as formalin concentration increases or as the dose of an analgesic agent (e.g., morphine) decreases, there is a corresponding increase in the assessed index (see references in Table 13). Several authors have demonstrated that weighted measures

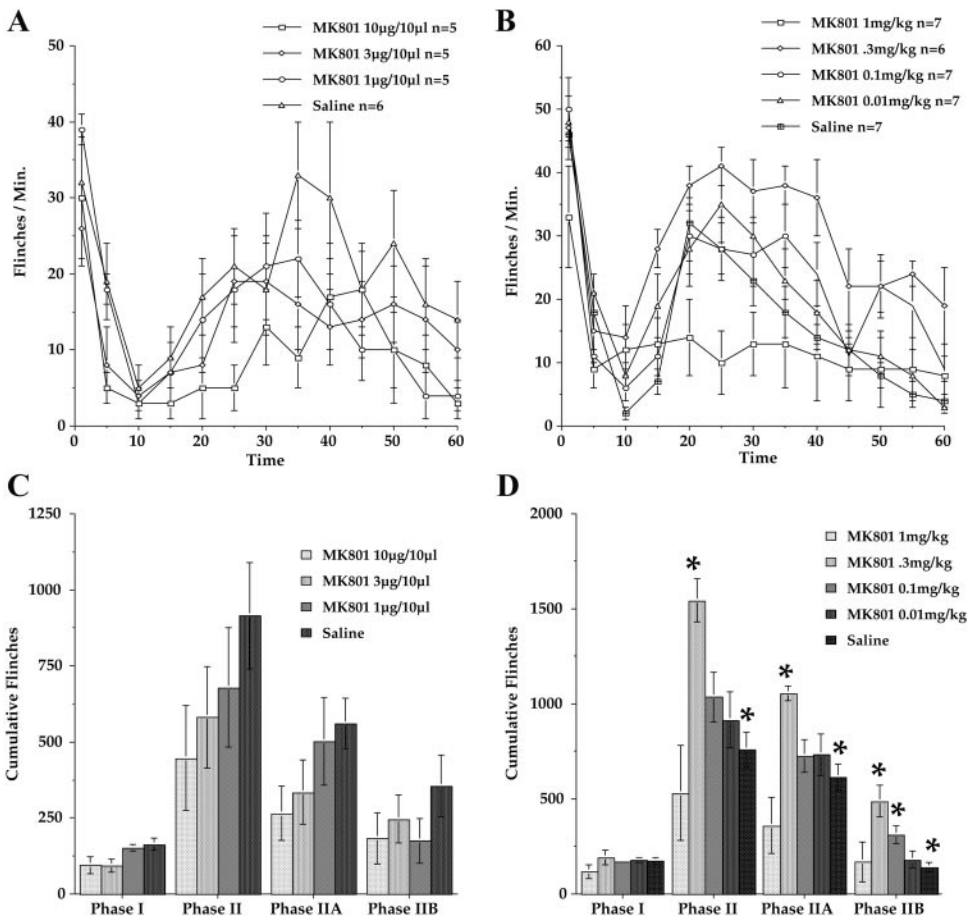

Fig. 11. Time-effect curves (means ± SD) for vehicle and intrathecal (*A*) and intraperitoneal (*B*) MK-801 on formalin-induced flinching in the rat. Cumulative flinches, by dose, are presented by phase for intrathecal MK-801 (*C*) and for intraperitoneal MK-801 (*D*).*$P < 0.05$, by one-way ANOVA across dose.

Table 12. *Test group mean values with the power (70–90% probability) of showing statistical significance from control means, by phase, and expressed as a percentage of the control mean and as a range of applicable values*

Group	Control Mean	Control SD	Difference,* as Percentage and Bounds, for 70% Power	Difference, as Percentage and Bounds, for 80% Power	Difference, as Percentage and Bounds, for 90% Power
Phase I	129	56	42% 75 > X > 183	47% 69 > X > 189	52% 62 > X > 196
Phase II	741	229	30% 521 > X > 961	33% 496 > X > 986	37% 466 > X > 1016
Phase IIA	546	149	26% 403 > X > 689	29% 387 > X > 705	33% 367 > X > 725
Phase IIB	195	114	56% 85 > X > 305	63% 73 > X > 317	70% 58 > X > 332

Power is 1, probability of committing a type II error. X, mean. $n = 8$; $P = 0.05$, two-tailed. *Minimum detectable difference analysis from Ref. 52.

(e.g., time of licking and flicking) may provide a more robust index with a greater dynamic response at lower formalin concentrations than other simple indexes such as flinching. Choice of indexes may be influenced by the time required to complete a test and the level of training required to achieve reliability.

Concentration of formalin. The current literature indicates that increasing formalin concentration, typically over a range of 0.5 to 5%, leads to a more intense and progressively longer lasting flinch response. Assessment of the intensity of several pain behaviors has shown that, as formalin concentration rises, the magnitude or incidence of the measured behaviors, blood pressure, or flinching increases (2, 5, 11, 12, 39, 41). In the present studies, we observed a plateau effect such that the maximum was observed at 2.5%. These results, obtained with automated flinch counting, are in accord with previous work using weighted behavioral measures and blood pressure (11, 12, 39).

Rat strain. The present studies were done with Sprague-Dawley Holtzman strains. There are few systematic studies describing strain differences with regard to formalin response. A variety of rat strains have been used, including Sprague-Dawley Harlan strain (25) and Sprague-Dawley Holtzman strain (see Ref. 16), Wistar (2), and Long Evans (12). Taylor and colleagues (39) noted that the magnitude of the blood pressure response observed during phase I was similar in Sprague-Dawley rats obtained from two suppliers (Charles Rivers and Bantin Kingman) but that the latter displayed a significantly reduced phase II response.

Body weight and age. Body weight varies directly with age. In the present work, young (110–125 g) rats flinched less than larger, older rats (400–450 g) of the same strain.

Sex. There appear to be few differences in flinching behavior as a function of sex. In our work, when matched for size, females and males of a single strain showed comparable flinching behaviors, with the exception of the late component (phase IIB), in which females showed a statistically greater response than males. The significance of this modest difference is not known.

Catheterization. The surgical placement of a spinal catheter had no significant effect on flinching behavior compared with the unimplanted rat.

Pharmacology of the Formalin Test

The pharmacology of the formalin test has been a subject of considerable investigation. Table 13 summarizes a number of families of agents, which have been examined for their effects on phase II of the formalin test, after systemic and intrathecal delivery. Agents may be functionally considered in two classes: those that will completely reduce phase II (the facilitated component) and those that appear to exert a significant, but limited, plateau effect. To date, all agents

Table 13. *Effects of intrathecal and systemically delivered pharmacological classes of agents on Phase II of the formalin test in the rat*

Drug	Intrathecal	Systemic
Agonists		
μ-Opiate	25	42
∂-Opiate	25	
κ-Opiate	25	42
α_2 Adrenergic	25	
Adenosine A_1	25	
Nicotinic		6
$GABA_A$	16	
$GABA_B$	16	
Benzodiazepine	16	
Gabapentin	34	36
Antagonists		
NMDA antagonist	10	20
Glycine site antagonist		20
Kainate antagonist		35
AMPA antagonist	10	20
NK_1 antagonist	50	21
COX inhibitor	24	24
EP antagonists	25	
NOS inhibitor	26	
Sodium channel blocker		4
N-type Ca channel blocker	27	
Cholinesterase inhibitor	30	

Nos. are reference nos. NK_1, neurokinin-1; COX, cyclooxygenase; NOS, nitric oxide synthase.

that are effective for acute pain processing fall into the first category and include opiate and α_2 agents. Agents that fall in the second category include, for example, NMDA and neurokinin-1-receptor antagonists, adenosine A_1 agonists, $GABA_{A/B}$ agonists, and cyclooxygenase inhibitors (47, 48). In the present studies, we have demonstrated that the automatic assessment of flinching behavior provides data that are both qualitatively and quantitatively similar to data that have been previously reported by human observers.

Conclusions

The current literature suggests that the formalin test reflects a neural substrate that involves the generation and support of a facilitated state of processing. This behavior observed during phase II appears to arise out of the initial barrage of afferent traffic and continuous low-level input found in a formalin-induced injury.

The response of this system is such that it appears to reveal a pharmacology that is considered to reflect facilitated states of processing.

An automated sensing system has been developed that counts the spatial displacement of the injected hind paw. This system has been shown to describe a response count and distribution that resembles that obtained with manual counting systems.

The limitations of this approach are that it only counts paw flinching and does not assess time spent in other behaviors deemed to be representative of the animal's pain behavior. Nevertheless, based on sensitivity to formalin concentrations, duration of behavioral responses, and response to drugs, there appear to be no distinguishable differences between simple flinch analysis and a weighted behavioral score.

The strength of the system lies in the ability to screen large numbers of animals on a daily basis, absence of operator fatigue, a likely increase in reliability over repeated tests, and reduced training time.

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