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Learned Avoidance from noxious mechanical Simulation but not Threshold Semmes Weinstein filament Stimulation after Nerve Injury in Rats

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Abstract

Noxious mechanical stimulation evokes a complex and sustained hyperalgesic motor response after peripheral nerve injury that contrasts with a brief and simple withdrawal that is seen after noxious stimulation in control animals or after threshold punctate mechanical stimulation by the von Frey technique. To test which of these behaviors indicate pain, the aversiveness of the experience associated with each was determined using a passive avoidance test in rats following sciatic nerve ligation or skin incision alone. After 18d, step-down latency was measured during 9 sequential trials at 10min intervals. At each trial, rats received either no stimulus, needle stimuli, or threshold Semmes Weinstein (SW) filament stimuli after stepping down. Reactions were either a hyperalgesic response or a brief reflexive withdrawal. In SNL animals, needle stimulation produced substantial learned avoidance when animals showed hyperalgesic responses, but produced minimal prolonged latency in SNL animals that showed only simple withdrawal responses. No learned avoidance developed using threshold SW testing in SNL animals. These findings show that needle stimulation is aversive in rats responding with hyperalgesic behavior. In contrast, SW stimulation, as well as needle stimulation that produced mere withdrawal, is minimally aversive.

Keywords

neuropathic pain; hyperalgesia; sciatic nerve ligation; dorsal root ganglion; rat

Perspective

The validity of measures of pain in animals is open to question. We demonstrated that needle stimulation is aversive in rats that respond with hyperalgesic-type behavior, and is therefore a valid indicator of pain. Stimulation by SW is minimally aversive, and is a problematic indicator of pain.

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Numerous rodent models have been devised to emulate inflammatory and neuropathic components of human pain conditions.^{2,7,12,14,18,21,31} Regardless of the model, it is critical to confirm that pain is created in order for the findings to have relevance and potential translational value. Defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience²³, pain itself cannot be determined in animals, so secondary indicators are used, typically behavioral responses to sensory activation. Numerous such tests have been devised, but the most commonly used methods involve determining thresholds for initiating motor behavior in response to tactile or thermal stimulation. Simple withdrawal movements that may include a component of spinal cord reflex are the basis for the tail-flick, von Frey test, Randall-Selitto, and Hargreaves tests.^{1,5,7-8,12,32}

Theoretical considerations have raised doubts about the use of reflex withdrawal as a proxy for painful experience.^{13,20} For instance, nonpainful sensations such as itch and tickle may be highly motivating, movements may occur with arousal and upon becoming aware of contact with a moving object, and reflex movements may occur without pain. A clear example of the latter is the routine occurrence of movements induced by surgical manipulations during adequate general anesthesia, during which unpleasant experiences are impossible. These observations are paralleled by withdrawal responses from plantar heat stimulation in anesthetized rats.³⁵ The use of threshold determination likewise has limitations. Specifically, painful clinical neuropathy is typically associated with elevated rather than reduced sensory thresholds,³⁰ and painful experiences rarely involve stimulation near threshold levels.

We have previously demonstrated that simple paw withdrawal from tactile stimulation similar to von Frey fibers, specifically using Semmes Weinstein (SW) filaments, fails to distinguish rats with peripheral nerve injury from control rats, whereas a complex, sustained response involving lifting, shaking and grooming (hyperalgesic response) after fully nociceptive mechanical stimulation with a needle is expressed in neuropathic animals but not in control animals.¹³ We have also shown that important shifts in cellular phenotype, including depressed neuronal resting cytoplasmic Ca^{2+} level, loss of activity-induced Ca^{2+} transients, K^+ current depression, and kinase signaling, as well as ganglionic glial activation, evolve preferentially in animals that demonstrate this hyperalgesic behavior after peripheral nerve injury compared to those that show withdrawal only.^{9-10,15-16,37} We now seek to further probe the relevance of these tests to animal pain by equating the unpleasantness of pain with an aversive quality, in accordance with Zimmermann's³⁶ application of the IASP definition of pain to animals as "an aversive sensory experience caused by actual or potential injury that ... results in learned avoidance behavior..." Based upon the strategy that an aversive stimulus provides reinforcement for avoidance learning, the passive avoidance paradigm has been used as a means of comparing motivational aspects of painful stimulation in animals.^{19,22,25-27,34} We therefore employed a passive avoidance technique to determine the aversiveness of needle stimulation that is fully capable of activating nociceptive pathways, hereafter referred to as a nociceptive stimulus. Although mechanical threshold determination with SW is commonly accepted as a standard measure of pain, its aversiveness has not been tested. Therefore, we additionally examined passive avoidance with this form of mechanical stimulation. We further examined the influence of nerve injury on the ability of these stimuli to progressively change behavior.

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic, Hudson, NY) were housed individually in a room maintained at $22 \pm 0.5^\circ\text{C}$ and constant humidity ($60 \pm 15\%$) with an alternating 12-h light-dark cycle. Food and water were available *ad libitum* throughout the experiments. Each animal was used in only one protocol. All animal procedures conformed to the guidelines of, and were approved by, the Animal Care and Use Committee of the Medical College of Wisconsin.

Animals weighed 160-180 g at the time of surgery and weighed 250-290 g at the time of sensory testing.

Surgery

Animals were randomly assigned to an SNL group or a control group that received sham surgery. Animals within a cohort that arrived at the laboratory together were divided between the surgery groups. After surgery, the animals were housed individually under pre-surgical conditions. For SNL^{13,18}, rats were anesthetized with 2-3% Forane (isoflurane, Baxter Healthcare Corp., Deerfield, IL) in oxygen, the back was shaved, and the right lumbar paravertebral region was exposed. After subperiosteal removal of the sixth lumbar transverse process, both the right fifth and the sixth spinal nerves were tightly ligated with 6-0 silk suture and transected distal to the ligature. To minimize non-neural injury, no muscle was removed, muscles and intertransverse fascia were incised only at the site of the two ligations, and articular processes were not removed. The lumbar fascia was closed with 4-0 resorbable polyglactin suture, and skin was closed with four staples. Control animals receiving sham surgery had only anesthesia, a lumbar skin incision, and skin closure.

Passive avoidance test

To determine the aversiveness of the experience associated with different stimuli and behaviors, the acquisition of learned avoidance was measured using a step-down passive-avoidance test.²⁶ The protocol began on day after surgery, at which time behavioral changes from SNL are established.¹³ Animals rested in the experimental environment for 1 h seventeen before each of two testing sessions. During testing, the rat was placed on a platform (10.3 cm in diameter and 2.5 cm in height) located in a 30 × 28 × 28 cm clear plastic enclosure with a ¼-in wire grid floor. Latency to step down from the platform was measured (cutoff 150s), and the rat was taken back to its cage immediately. This procedure was repeated in each rat for 9 learning trials at 10min intervals.

On the first day of the protocol, no stimuli were imposed in order to provide initial habituation to the environment and handling. Rats that spent more than 30s on the platform during this phase and the baseline measurement on the second day of the protocol were considered not to be neutral in step-down preference and were excluded from further study (less than 20% of total rats). On the second day of the protocol, upon stepping down from the platform, the rat received either no stimuli, needle stimulation, or SW stimulation at each of the 9 trials. Stimuli were given after stepping down of the platform starting from trial 0. Rats were removed from the apparatus after each trial, each rat received only one type of stimulation, and each rat was used in only one protocol. If the rat stayed on the platform after the cutoff time (150s) in a trial, it would be returned to its cage without any stimuli for that trial. The nature of the injury was not explicitly made known to the examiner. However, the postural consequences of SNL treatment make a fully blinded study impossible.

Conditioning stimulation

Mechanical stimulation of the right paw upon stepping down from the platform was produced by one of two techniques. In the first, stimulation was performed with a 22-gauge spinal anesthesia needle with a Quincke tip, applied to the center of the right paw with enough force to indent the skin but not to puncture it. Touches were applied three times at 2s intervals.

Alternatively, mechanical stimulation was applied by SW fibers (Smith and Nephew Inc., Germantown, WI) to the center of the right paw. Care was taken to approach the skin slowly to standardize the force-time relation during stimulation. Contact was made for 1s with a force just adequate to bend the fiber. Eight fibers with different forces were used (0.40, 1.19, 2.05, 3.63, 5.5, 8.65, 15.0, 29.0 g).^{6,17-18-24} An up-and-down method to identify the 50% response

threshold was employed for animal pain behavior evaluation⁴ after the training session on day one. A suprathreshold force or the second higher forces above the threshold was used as the stimulating force for the following day experiments. In the experiment with suprathreshold force, each animal received 3 stimuli at a 2s interval right after stepping down starting from trial 0. In order to achieve the withdrawal activity as that of needle stimulation, in the experiment with the second higher force above threshold, each animals also received 3 stimuli at a 2s interval right after stepping down from the platform but a fourth stimulus was given if no 3 withdrawals were achieved in the first three stimuli.

Categorization of response type

Responses to mechanical stimulation were classified into one of two types, either a brisk simple withdrawal that appeared as a reflexive flinch, or a complex hyperalgesia-type response, in which the paw was held in the air for a second or more, accompanied by shaking, grooming, licking, or chewing of the paw.¹³ Rats that demonstrated a hyperalgesia-type response after one or more stimulations were classified as hyperalgesic and the others as nonhyperalgesic.

Statistical analysis

One-way analysis of variance (ANOVA) with repeated measures (trial 1 through 9) was used to test if step-down latency of an individual group was affected over the course of the repeated trials, indicating acquisition of place avoidance. Non-parametric analysis was performed using Kruskal-Wallis ANOVA followed by Dunn's multiple comparison post-hoc test (Fig.1 and 3) or the Mann-Whitney test (Fig. 2) for determining differences between groups for the 9th trial.

A second form of analysis was performed in which each individual rat's acquisition of learned avoidance was characterized. To measure the total learning across the 9 sequential trials, the relationship of step down latency (after subtracting baseline latency) *versus* trial number for each rat was smoothed by fitting a 3rd order polynomial regression, and the value of the fit at the 9th trial was taken as a representation of net learning over the 9 trials. The goodness of this fit (R^2) was also tabulated for each animal, and the step down latency at the 9th trial was compared between injury groups and sensory testing method using parametric analysis with a two-way ANOVA (Table 1A) or one-way ANOVA (Table 1B). Statistical software was used to perform the analyses (Prism version 4.1, GraphPad Software, Inc., San Diego, CA). Step-down latencies are presented as mean $\pm$ SEM, and $P < 0.05$ was considered significant.

Results

Animals tolerated the testing procedure without apparent stress, showing routine exploring behavior both on and off the platform.

Needle stimulation reinforces avoidance learning

Every needle application in all animals produced a withdrawal or hyperalgesia response, indicating sensory events occurred with each touch and that postsurgical anesthesia was not a factor influencing development of learned avoidance in the various groups. Without stimulation, control rats ($n = 8$) showed no change in step-down latency across trials, although SNL ($n = 9$) rats did develop an increase in latency (Fig. 1).

With needle stimulation, control rats ($n = 8$) developed no increase in latency across trials (Fig. 1). In contrast, SNL rats with needle stimulation ($n = 16$) developed a prolongation in latency (Fig. 1). The step-down latency at 9th trial showed a significant difference between groups ($P < 0.05$) and identified a greater prolongation of latency with needle stimulation than with no stimulation in sham animals ($P < 0.05$).

All needle touches produced either a simple withdrawal or a hyperalgesia response. In control animals, needle stimulation produced only withdrawal responses. In SNL animals, some ($n = 7$) failed to show any hyperalgesic responses with needle stimulation despite anatomically confirmed correct SNL. Separate evaluation of these non-hyperalgesic animals and the other hyperalgesic animals ($n=9$) revealed a clear-cut difference in the pattern of avoidance acquisition (Fig. 2). Both hyperalgesic and non-hyperalgesic SNL animals developed a prolonged step-down latency during the trial sequence. However, the prolongation for the hyperalgesic animals (69.8 ± 18.8 s at the 9th trial) was much greater than for the non-hyperalgesic animals (11.3 ± 2.7 s; $P<0.05$). In 3 SNL animals, the 150s cutoff was reached in a total of 13 trials, and the animals were not stimulated.

SW stimulation fails to reinforce avoidance learning

Three techniques were used in separate populations of rats to test the influence of SW stimulation on place avoidance. We first used a standard up-down technique of SW fiber application to produce punctate mechanical stimulation just above and below the threshold for withdrawal, with the weakest fiber applied first.⁴ Stimulation of both control ($n = 8$) and SNL rats ($n = 14$) producing 3 withdrawals at each trial produced no change in latency across trials, even in animals classified as hyperalgesic ($n=7$; data not shown). A weakness of this approach is the variable delay between the step-down event and the first SW stimulation that provoked a withdrawal, thus potentially weakening the avoidance learning.

To address this limitation, we altered the stimulation protocol to resemble the timing of stimulation of the needle application. Specifically, we determined the 50% withdrawal threshold on day one after the training session by the standard up-down method,⁴ and on the experiment day, the fiber just above the threshold was applied three times at 2s intervals (Fig. 3A). There was no change in latency across trials in SNL rats ($P = 0.4275$; $n=12$), while control rats had a borderline increase ($P = 0.05$; $n=9$). There was no difference in step-down latency between groups at the 9th trial ($P = 0.1363$). However, the average paw-withdrawal numbers per trial was only 0.8 for control and 1.3 for SNL rats. No animal reached the 150s cutoff during the trials.

Because of the low response rate using a SW fiber just above threshold, we repeated the experiment with separate animals using a fiber two increments above the threshold (Fig. 3B). In control rats ($n = 8$), this produced a borderline increase in latency across trials ($P = 0.04$) and no for SNL rats ($P = 0.06$). There was no significant difference of the step-down latency between groups at the 9th trial ($P = 0.07$). The average paw-withdrawal numbers per trial with this method was 2.6 for control and 2.7 for SNL rats, closely resembling the stimulus pattern with needle.

SW stimulation produced only a simple withdrawal response in both control and SNL animals, except for 2 SNL rats in each group that showed hyperalgesic responses to SW stimulation.

Secondary analysis by characterization of individual subjects

To further examine the effects of injury and stimulation on acquisition of learned avoidance, we applied a different analysis strategy to the data described above, in which the behavioral change over the 9 sequential trials was characterized for each animal subject. Despite the naturally high variability of animal behavior in a repeated measures format, progression of learning in these groups could be readily characterized by the polynomial fit with an average R^2 of 0.72 in groups which latency increased at least 10s over the 9 trials. No influence of injury was found, but a significant main effect of stimulation type was found ($P < 0.05$) without differences in post-hoc paired comparisons (Table 1, A). A separate analysis in which responses to needle were considered separately in hyperalgesic and non-hyperalgesic SNL animals (Table

1, B) showed a higher final latency in needle-stimulated hyperalgesic SNL rats than in non-hyperalgesic and control groups. Unstimulated animals were not included in this analysis since they could not be categorized as hyperalgesic or not.

Discussion

The unpleasant aspect of pain is key to its importance to human experience. Suitable animal testing must therefore incorporate this feature in order to claim validity. It is important to distinguish innate responses based upon spinal or spino-bulbospinal reflexes from operant responses composed of complex learned motor actions.³³ In the present study, we have taken advantage of the ability of aversive experience to propel the learning of avoidance behavior in order to compare stimuli used for pain evaluation in animals. We have found that needle stimulation results in acquisition of learned avoidance following SNL, and this behavioral shift is selective for animals that respond to a needle with a hyperalgesia-type motor event. Stimulation with SW filaments produces fewer hyperalgesia-type responses and no behavioral changes. From these observations, we conclude that needle application is aversive after nerve injury, and that hyperalgesia-type motor behavior after needle stimulation accompanies a particularly aversive experience that is common in a subset of animals after SNL. However, application of SW filaments is minimally aversive, and therefore has uncertain value as a means to identify pain in animals.

Two inconsistencies in our data deserve consideration. After SNL, animals developed prolonged latency for descending from the platform in the absence of experimental stimulation, although this does not take place in control animals. It is possible that standing on the wire grid is aversive in comparison to standing on the smooth metal surface of the platform in animals with neuropathy,²⁸ which may produce an avoidance for the wire surface. However, this is a small effect, there was no similar effect during SW stimulation, and comparison of final latencies at the 9th trial showed no differences between sham and SNL groups with SW stimulation or no stimulation.

A second inconsistency is the failure of animals performing hyperalgesic type of motor response to acquire learned avoidance when the stimulation is provided by SW filaments. This may be due to the lower rate of these events during SW stimulation compared to needle stimulation. Alternatively, there may be a variety of forms of the hyperalgesic response, and the experience accompanying them is not uniform. We made no attempt to characterize subtypes of this behavior. Finally, it is possible that the experience associated with the hyperalgesia event is not the critical element in acquisition of learned avoidance in these animals, but rather these animals may have a generally more painful experience with stimuli, and the occurrence of hyperalgesic motor responses serves as a marker of this sensitivity. If this is the case, failure to acquire learned avoidance during SW stimulation may be due to the low intensity of this stimulation applied at the threshold for initiating a response, whereas the fully nociceptive needle stimulus is sufficiently intense to produce learned avoidance in this hyperalgesia subgroup.

SW monofilaments are a widely used standard method for testing mechanical sensory threshold. However, problems of tip geometry, force generation, inconsistency in use, and experimenter bias suggest that monofilament stimulators are not ideal for quantitative sensory testing.³ It has also been shown that withdrawal threshold measurement is complex and depends on the various parameters of the stimulus profile, therefore complicating the concept of threshold.¹¹ The key finding of our study is the aversiveness of nociceptive stimulation when it is accompanied by a complex, sustained hyperalgesia response. Because of the risk of a type-I error, we performed a second conservative analysis characterizing each animal's learning pattern, which confirmed this finding. Our data also indicate that needle stimulation followed

by only a brief flinch-like withdrawal is minimally aversive in control animals and a subset of nerve-injured rats. While this is counterintuitive from human experience, it is unsafe to assume that rats and humans have comparable sensory experiences, and teleological explanations could be based on the different sensory environments of the two species.

While threshold testing with SW filaments is believed to have good predictive ability, our findings indicate that there is no aversive experience associated with this form of testing in normal animals or after peripheral nerve injury. This is likely due to the low intensity of stimulation created by SW testing as performed conventionally, and by us in this study, at mechanical threshold such that this stimulus is not adequately noxious to be aversive. It is possible, however, that in other models such as skin incision or paw inflammation, liminal SW stimulation could provide an aversive influence. Also, repeated and persistent application of a very stiff SW filament may be successful in producing learned avoidance.¹⁹ Nonetheless, the present findings indicate that a truly nociceptive stimulus such as provided by a needle touch is necessary to result in learned avoidance, supporting its use in pain testing after nerve injury. Additionally, with this mode of stimulation, the subgroup of animals having an aversive experience is selectively identified by their sustained, complex motor behavior that we have termed a hyperalgesia response.

Pain is a complex phenomenon that includes both sensory-discriminative and affective-motivational components, which are readily dissociable.²⁹ Simple withdrawal responses from tactile or thermal stimuli may be triggered by spinal cord reflexes and need not reflect an unpleasant experience, as is familiar from events of daily life. Because of the impossibility of directly knowing the experience of animal subjects, it is highly desirable that behavioral proxies used in animal pain research be tested to determine if they are accompanied by aversive motivation.

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Fig. 1. Effect of needle stimulation on step-down latency in control and SNL rats

Different groups of control and SNL rats ($n = 8-16$) received 3 needle touches immediately after stepping down from a platform, or no stimulation (No Stim.), on 9 consecutive trials at 10 min intervals. Effect of trials for each group was tested by one-way ANOVA with repeated measure; ††† $P < 0.001$. Difference between groups at the 9th trial was tested by Kruskal-Wallis ANOVA; * $P < 0.05$. Mean $\pm$ SEM.

Fig. 2. Step-down latency after needle stimulation of SNL rats considering hyperalgesic and non-hyperalgesic animals separately

SNL rats with needle stimuli from Fig.1 are classified into separate groups of animals that either responded at least once to needle touch with a sustained licking, lifting and grooming response (hyperalgesic, $n = 9$) or produced only brief reflex withdrawals (non-hyperalgesic, $n = 7$). Rats received 3 needle touches immediately after stepping down from a platform, or no stimulation (No Stim.), on 9 consecutive trials at 10 min intervals. Effect of trials for each group was tested by one-way ANOVA with repeated measure; †† $P < 0.01$, ††† $P < 0.001$. Difference between groups at the 9th trial was tested by Mann-Whitney test; * $P < 0.05$. Mean $\pm$ SEM.

Fig. 3. Effect of Semmes Weinstein filament stimulation on step-down latency in control and SNL rats

Different groups of control and SNL rats ($n = 7-12$) received suprathreshold Semmes Weinstein filament with 3 touches (A) or the 2nd higher force above threshold with 3 or 4 touches (B) immediately after stepping down from a platform, or no stimulation (No Stim.), on 9 consecutive trials at 10 min intervals. Effect of trials for each group was tested by one-way ANOVA with repeated measure; ††† $P < 0.001$, † $P < 0.05$. Difference between groups at the 9th trial was tested by Kruskal-Wallis ANOVA. Groups with no stimulus are shared with Figure 1. Mean $\pm$ SEM.

Table 1
Effect of stimulation and injury on place avoidance

A. SNL animals considered as single groups

	Sham Group			SNL Group		
	Final Latency (s)	R ²	N	Final Latency (s)	R ²	N
No Stimuli	2.7±3.7	0.44±0.10	8	14.7±5.5	0.62±0.06	9
Needle	12.9±5.4	0.58±0.10	8	41.4±12.9	0.65±0.06	16
Von Frey Fiber ^a	7.5±4.6	0.49±0.10	9	3.4±4.1	0.40±0.06	12
Von Frey Fiber ^b	3.9±3.6	0.43±0.09	8	1.7±4.6	0.72±0.05	7

B. SNL animals separated by presence or absence of hyperalgesic response

	Sham Group			SNL Group		
	non-Hyperalgesic			Hyperalgesic		
	Final Latency (s)	R ²	N	Final Latency (s)	R ²	N
Needle	12.9±5.4 [*]	0.58±0.10	8	6.9±3.2 ^{**}	0.71±0.05	7
				68.2±18.6	0.61±0.10	9

For each rat, a 3rd order polynomial was fit to the relationship of normalized latency (baseline = 0) vs. trial for each rat, and the value of the fit at the 9th trial was used to estimate the passive avoidance effect (Final latency). Data are presented as mean ± SEM. Table A: significant differences were found in stimulation (P < 0.05) factor but not interaction and injury type by two-way ANOVA followed by Bonferroni's test. Table B: *P < 0.05, **P < 0.01 compared with hyperalgesic group by one-way ANOVA followed by Bonferroni's test. SNL = sciatic nerve ligation; R² = goodness of fit; N = number of rats in each group; SEM = standard error of mean.

^a Animals received stimulus of von Frey fiber just over the 50% threshold with 3 stimuli.

^b Animals received stimulus of von Frey fiber 2 number higher than the 50% threshold with 3 or 4 stimuli.