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The tail pigmentation pattern of C57BL/6J mice affects nociception/pain quantification in the tail flick test

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Abstract

The heat radiant tail flick test is commonly used to quantify nociception and pain levels. Likewise, the C57BL/6J strain of mice is frequently used in pain-related studies as transgenic mice are often backcrossed onto this background. C57BL/6J mice naturally develop non-pigmented patches of variable length on the distal part of the tail that could conceivably modify the response latency in tail flick assays. Here we find that these non-pigmented regions, in a position-independent manner, significantly increase the response latency in the heat radiant tail flick assay, but not the warm water immersion test. This finding demonstrates that the extent of pigmentation, and not other potential variables between pigmented and non-pigmented skin, affects radiant heat tail-flick latency, and should be considered in the design of pain-related studies using mice with variable tail pigmentation.

Keywords

pain quantification; nociception; heat radiant tail flick assay; C57BL/6J mice; tail pigmentation; non-pigmented patch

Introduction

The heat radiant tail flick assay, developed by D'Amour and Smith in the 1940s (D'Amour and Smith, 1941), is a commonly used experimental model for thermo-pain quantification, in which a rodent tail is exposed to a light source (radiant heat) and the latency of tail withdrawal from the heat source is recorded and analyzed. Depending on different experimental settings, the tail-flick technique can be used to determine the basal nociception level, the analgesic effectiveness of pharmacological agents, and tolerance formation. The tail-flick assay remains a valuable method due to its simplicity, reproducibility, its relatively low variation, and the minimal requirement for apparatus. Moreover, a unique feature of tail-flick vs. other thermo-pain quantification methods, such as hot plate or Hargreaves, is that a spinally mediated simple reflex is primarily, if not exclusively, involved to produce the end-point detection of a flicking tail (Gebhart and Ossipov, 1986). Thus, the absence of complex behavior (e.g. paw-licking in hot-plate test) renders the tail-flick method advantageous because it avoids the confounding effects arising from scoring or interpreting more complicated behaviors. In some cases, tail-flick test can even be carried out in lightly anesthetized animals (Ness and Gebhart, 1986).

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Since the mouse tail is the subject of the assay, it is conceivable that physiological or anatomical characteristics of the tail could influence the tail flick results. C57BL/6J mice, which are characterized by non-agouti (black) hair and dark pigmentation on the dorsal portion of the tail, are a commonly used inbred strain in pain-related studies. We observed that a substantial portion of inbred C57BL/6J mice (~1/3 population, independent of sex) develop light non-pigmented patches on the dorsal part of their tail (Fig. 1A). These non-pigmented patches tend to occur on the distal half of the tail, are often found at multiple loci, and have lengths varying from ~2-3 mm to several cm. Since the tail-flick test employs radiant heat as the noxious stimulation and since the photo-absorption properties and radiant heat conduction of the target could potentially be modulated by the extent of tail pigmentation, we wanted to determine whether these lightly pigmented areas could affect tail flick response latencies.

Results

As an initial test of this hypothesis, we first collected 36 naïve male C57BL/6J mice (ranging from 2-6 months in age) and conducted tail-flick assays using a model 33 tail flick analgesia meter (IITC Life Science Inc., Woodland Hills, CA) with the beam intensity set at 4.0. All mice were habituated for 30 minutes in the procedure room prior to testing. During the tail flick test, mice were wrapped with a soft paper towel with the whole tail length exposed, and handheld with appropriate strength. Ten of these initial 36 mice bore at least one non-pigmented patch larger than 5mm, which is the approximate diameter of the focused radiant light spot. In a preliminary test, the 26 mice with a completely pigmented tail were subjected to the tail flick assay with the light focused 15-20mm to the tail tip (Bannon and Malmberg, 2007), while the ten mice with non-pigmented tail patches, which were randomly distributed in the distal half of the tail, were tested with the light beam deliberately projected onto these light patches. When the beam was focused on the non-pigmented patch, there was a pronounced and significant increase in baseline tail-flick latency compared to the pigmented tail control (10.7 ± 2.6 vs. 6.0 ± 1.1 sec, $p < 0.0001$ by two-tailed t-test, mean $\pm$ SD). To test whether the tail pigmentation pattern also serves as a determinant for other thermo-nociception quantification methods that involve the mouse tail, the same mice were rested overnight and then subjected to a warm water test (tail-immersion test), in which the caudal half of the tail was dipped into a circulating water bath maintained at 47.0°C (Janssen et al., 1963). In this test, the control group and the depigmentation group gave indistinguishable tail-flick latencies (8.3 ± 2.6 vs. 7.6 ± 2.1 sec, mean $\pm$ SD).

To confirm the results of this preliminary study and rule out a potential confound raised by the varying distance of patch location, we collected another cohort of 50 male C57BL/6J mice, among which 21 mice possessed at least one non-pigmented patch larger than 5mm. With the same experimental design described above, each mouse with a non-pigmented patch at a specific distance was paired with a littermate completely lacking light tail patches. For each animal in the pair, the light beam was focused so that the distance from the area illuminated to the tail tip was identical. The results from the initial test were confirmed in this experiment with the patch distances matched, as shown in Fig. 1B. Thus, the beam focused on the non-pigmented patch produced a significantly longer latency in comparison with the distance-matched pigmented area of the littermate pair (10.1 ± 1.8 vs. 5.8 ± 0.7 sec, $p < 10^{-12}$ by two-tailed t-test, mean $\pm$ SD). Again, the pigmentation pattern had no effect in the warm water test (8.5 ± 1.9 vs. 8.6 ± 2.1 sec, $p = 0.86$ by two-tailed t-test, mean $\pm$ SD). The similarity in the tail immersion test strongly suggests that pigmentation does not contribute significantly to contact type heat conduction as it does to radiant type heat conduction.

By linear regression analysis, we also found that there was no correlation ($r^2 = 0.007$) between the position of the light patch illuminated and the tail flick response latency in mice containing light patches, at least when the beam was focused on a spot 4mm to 24mm from the tail tip

(Fig. 1C). This interval corresponds to the segment typically measured as suggested by a widely accepted tail flick protocol ($\sim 15 \pm 10$ mm from the tail tip) (Bannon and Malmberg, 2007). Also, there was no correlation between the location of the beam focus and response latency over the same distance range in a fully pigmented tail ($r^2=0.01$, Fig. 1C). This lack of correlation between light beam distance and response latency suggests a certain degree of flexibility when choosing the spot of heat exposure in tail flick test, which renders the avoidance of the light patches more valid and practical.

To further confirm whether the non-pigmented patch alone causes the increase in tail flick latency, we then analyzed 11 male C57BL/6J mice containing a light tail patch using two consecutive tail flick measurements. The radiant light was first focused on a non-pigmented patch (>5 mm in length) and then on an adjacent pigmented segment 20 minutes later. As shown in Fig. 1D, this internally controlled experiment demonstrated that the non-pigmented patch again shows a longer latency and indicates that the pigment density is the only causative factor for the increase in pain-threshold (9.5 ± 1.7 vs. 6.0 ± 0.8 sec, $p < 0.0001$ by two-tailed t-test, mean $\pm$ SD).

In addition, as a final method of confirmation, we wanted to test whether pigment artificially applied on the non-pigmented patch would alter the latency reading. For this purpose, we collected two cohorts of male C57BL/6J, patched and fully pigmented, respectively, and performed an initial tail-flick test with the light beam distance matched. As above, the fully pigmented group showed a significantly shorter response latency. We then painted both the patch in the non-pigmented group and a distance-matched segment in the fully pigmented group with a black Sharpie® fine point permanent marker (Sanford, Bellwood, IL), and measured the tail flick latency with light focused on the painted area after 20 minutes rest. There were two results of interest (Fig. 1E). First, after the tail painting, no difference was detected between the patched group and fully pigmented group. Second, the response latency was significantly reduced in both groups compared to baseline readings prior to marking. These data provide direct evidence that the differences in tail pigmentation lead to the tail flick response difference between the patched and fully pigmented mice and also indicate that additional, artificial pigment can itself alter the response latency.

Discussion

A potential link between tail pigmentation and tail flick latency has been suggested by some prior reported data. For example, it was proposed that the baseline difference in tail flick latencies between C57BL/6 and DBA/2J mice might be due to the different tail color, which was tested indirectly by tail painting (Vetulani et al., 1988). However, the issue of whether the pigmentation pattern on a single animal could influence tail flick latency in an inbred strain has not been previously addressed. Interestingly, Lariviere et al. (Lariviere et al., 2002) performed baseline tail flick and warm water tests in 10 inbred strains of mice (all “J” strains), and showed that agouti and non-agouti black mouse strains with darker tail pigmentation (C57BL/6, C57BL/10, C58, C3H/He, SM) generally had lower baseline tail flick latencies than albino strains (129P3, A, AKR, BALB/c, RIIS), while these strain differences were much less apparent when the warm water test was used. Lariviere et al. interpreted this pattern as primarily a hereditary effect reflected as an inbred strain difference. Our studies from analysis of pigmented and non-pigmented tail regions of C57BL/6J mice indicate that the amount of tail pigmentation could also have contributed substantially to the strain-dependent tail flick phenotypes observed by Lariviere et al. in addition to more general nociceptive genetic differences. Importantly, the disparate results seen here from the pigment-dependent tail flick test and pigment-independent warm water test make it highly unlikely that other potential differences between pigmented and non-pigmented regions, such as differences in skin

thickness, local vascularization and/or thermo-nociceptor density, account for the difference in radiant heat response.

A general guideline for the radiant heat tail flick test recommends that the light beam be focused on a point ~15mm to the tail tip and then ~10mm up and down for re-testing (Bannon and Malmberg, 2007). Using this criterion, the light beam could easily at least partially overlap a non-pigmented patch (Fig. 1A). In the study reported here, we have provided evidence that the areas of depigmentation on C57BL/6J mice should be noted and considered when performing heat-radiant tail-flick assays in order to avoid potentially misleading measurements. Investigators should be aware that including part of a non-pigmented patch in the illuminated region would tend to increase the tail flick latency reading. Also, analysis of a mutant genes of interest using mice of mixed genetic background with variable tail pigmentation due to coat color alleles present in different lines of ES cells (e.g. 129/C57 outbred) can give rise to erroneous interpretation.

In summary, two major conclusions arising from these data apply to pain/nociception studies using the tail-flick technique: (i). For tail-flick assays using the C57BL/6 inbred strain, the non-pigmented patch on the tail can and should be avoided for heat radiation by simply moving the beam to an adjacent pigmented segment, since patch location appears to contribute little, if any, variability to the tail flick latency reading. (ii). It is not advisable to compare tail-flick readings in a population with significantly varying tail pigmentation/color. If this situation is unavoidable, tail color matching to ensure equivalent tail pigmentation in each group is necessary, while other methods such as the warm water test could alternatively be used.

Acknowledgments

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Fig 1.

(A) Representative tail pigmentation patterns generally seen in C57BL/6J mice include a fully pigmented tail (1) and tails with varying patterns of non-pigmentation as indicated by black arrows (2-4); an albino mouse tail is shown as a reference (5).

(B) Analysis of response latencies using the tail flick and warm water tests. C57BL/6J male mice were divided into a fully pigmented tail group and a group with non-pigmented patch (area > 5 mm in length). Mice containing a non-pigmented patch were paired with a fully pigmented littermate and the tail flick latency at a non-pigmented patch and a corresponding segment distance-matched (to the tail tip) in the fully pigmented tail were measured, respectively. Each group was subjected to both tail flick and warm water tests. Mice with a

non-pigmented tail region showed significantly higher tail flick latency than the control group with a fully pigmented tail when the radiant light was projected specifically on the non-pigmented patch. These two groups do not exhibit any difference in the warm water test. Data are graphed as mean $\pm$ SEM. ***, $p < 10^{-12}$ by two-tailed t-test.

(C) Linear regression analysis between the location of the light beam and tail flick latency, based on a dot-plot conversion of the tail flick data in panel B. Depending on the patch position (ranging from 4-24mm to the tail tip for N=21), the light beam was aimed at the light patches of mixed pigment tails and distance-matched to fully pigmented control animals. Within the distance range tested, the regression analysis indicates that there is no correlation between the patch position and tail flick latency in mice bearing light patches, nor does it support a correlation between distance and response latency in mice with fully pigmented tails.

(D) Two tandem measurements of heat radiant tail flick assay were carried out on male C57BL/6J mice with at least one depigmented patch larger than 5mm in length. Radiant light was first focused on the depigmented patch and then on the immediate adjacent pigmented area 20 minutes later. Data are graphed as the response latencies of a depigmented segment vs. pigmented segment on the same experimental animal. When the beam was focused on the non-pigmented patch, tail flick latency was significantly increased. Data are mean $\pm$ SEM. ***, $p < 0.0001$.

(E) Two cohorts of patched and fully pigmented male C57BL/6J mice were tested by tail flick with distance match, painted by black marker at the patch and at the same distance in the fully pigmented group, and then re-tested 20 minutes later. The response difference disappeared after the tail painting, while the latencies of both groups were significantly reduced after tail painting. Data are mean $\pm$ SEM, *, $p < 0.05$.