论著·基础研究

Intradermal administration of formalin to the cheek induces itch as well as pain behaviors in rats

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[Abstract] **Objective**·Formalin is a classic and most widely used algogenic substance, but its itchy effect is not clear. The present study aims to explore the hypothesis that formalin may induce itch as well as pain. **Methods**·Flinching, as well as licking and forelimb wiping of the site of injection were counted as pain responses, whereas biting and hind paw scratching of the cheek were counted as itchy responses. To discriminate formalin-induced sensations in rats, the irritant (saline as control) was injected, and then pain and itchy responses were recorded. **Results**·Intraplantar injection of formalin elicited biphasic behavior responses characterized as flinching, as well as biting or licking of the hind paw without significant gender differences. Following intradermal administration of formalin to the cheek, rats exhibited episodic forelimb wiping of the cheek, representative of pain. No gender difference was noticed for this type of behavior. In addition, episodes of hind paw scratches of the cheek, representative of pruritoceptive responses, also occurred. Interestingly, hind paw scratches appeared to be more pronounced in male than in female rats. **Conclusion**·Intradermal administration of formalin elicits pruritoceptive as well as nociceptive responses in rats.

[Key words] formalin; cheek model; itch; pain

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Chronic pain and itch affect hundreds and millions of patients. According to The International Association For the Study of Pain, pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [1]. Chronic pain is a huge burden to the healthcare system. For example, in the United States alone, at least 100 million adults experience chronic pain annually and the annual economic costs of chronic pain have been estimated as much as 560 billion – 635 billion dollars[2]. Itch is a distinct sensation from pain, albeit also an unpleasant and irritating sensation which triggers a desire to scratch[3]. Chronic itch has a major negative impact on the quality of life and may cause significant mental distress[4-5]. Unfortunately, treatments for both conditions remain inadequate until now.

In order to develop mechanism-based treatments, preclinical models of pain and itch are required. A number of chemicals have been used in animal models to induce pain, such as formalin, acetic acid, carrageenan, capsaicin and complete Freud′s adjuvant, or itch, such as histamine, 5-HT and chloroquine. A major challenge in these models is that animals do not verbally report sensation of pain or itch as humans do. Thence, it is only viable to observe animal behaviors and to speculate the types of sensations they might experience, and this gives rise to ambiguity as to whether a given behavior represents pain or itch [6]. Intraplantar application of formalin is perhaps the most popular rodent model of acute inflammatory pain, in which flinching, as well as biting and licking of the affected hind paw are considered as pain behaviors, with the assumption that maneuvers to alleviate or eliminate the effects of the irritable stimulus or

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discomfort are nociceptive responses[7-10]. However, itch is also an unpleasant sensation that is reflected by behaviors such as biting or scratching of the itchy area to remove irritants[6, 11-13]. So the question is whether behaviors like flinching, biting and licking elicited by intraplantar administration of formalin solely represent pain or might reflect pruritoceptive as well as nociceptive responses instead?

Given the popularity of the formalin model in pain research, it is important to know exactly the sensations induced by formalin. In this study, we seek to determine whether intradermal administration of formalin induces itch as well as pain. To this end, we utilized the cheek model for differentiation of pain and itch behavior responses. The cheek model was first characterized by LaMotte's group in mice and has since been extended to rats[14]. They showed that histamine and capsaicin, known to induce itch and pain respectively in humans, both elicited hind limb scratching behavior after being injected into the nape of mice. In contrast, histamine injected into the cheek evoked hind limb scratching only, but capsaicin evoked forelimb wiping only. Therefore, forelimb wiping and hind limb scratching discriminate pain and itch sensations elicited by chemicals injected into the cheek. We found that administration of formalin to the cheek by intradermal injection elicited episodic forelimb wiping and hind limb scratching as well, suggesting that formalin induces pruritoceptive as well as nociceptive behavior responses.

1 Materials and methods

1.1 Animals

Eight-week-old Sprague Dawley rats of either sex with body masses between 180 g and 230 g were purchased from Shanghai SIPPR-BK laboratory animal Co. Ltd. and were accommodated in the animal facility of Shanghai Jiao Tong University School of Medicine with free access to food and water. All animal procedures were conducted in compliance with the governmental regulations on the use of experimental animals and were approved by the Ethic Committees of Shanghai Jiao Tong University School of Medicine.

1.2 The intraplantar formalin model

Before the experiment, rats were individually placed in the behavioral chambers and were allowed to acclimate for at least half an hour. Following acclimation, 50 μL of 2.5% formalin dissolved in a sterile saline was injected subcutaneously into the plantar surface of the right hind paw using a microsyringe. The rat was immediately returned to the behavioral chamber and its behavior was recorded using a high-definition webcam (Logitech, USA) for one hour. The video was examined off-line and the number of flinches and the duration of biting or licking of the ipsilateral hind paw were counted in each 5-minute block.

1.3 The cheek formalin model

Two days before the experimental procedure, animals were briefly anesthetized with isoflurane and the fur on right cheek was shaved. On the day of experiment, rats were individually placed in the behavioral chambers and were allowed to acclimate for at least half an hour. Following acclimation, formalin (2.5%, 50 μL) or saline (50 μL) was intradermally injected into the right-side cheek using a microsyringe. The rat was returned to the behavioral chamber immediately and recorded using a high-definition webcam for one hour. The video was examined off-line and the number of forelimb wipes and the hind paw scratches of the cheek were counted in each 5-minute block.

1.4 Data analysis

Statistic analysis was performed using Graphpad Prism 7. Values are expressed as $\bar{x} \pm se$. Unpaired Student's t-test was used to compare the means between two groups. For multiple comparisons, One-Way Analysis of Variance (ANOVA) with the two-stage step-up method was employed. $P<0.05$ was considered as indicating significant differences.

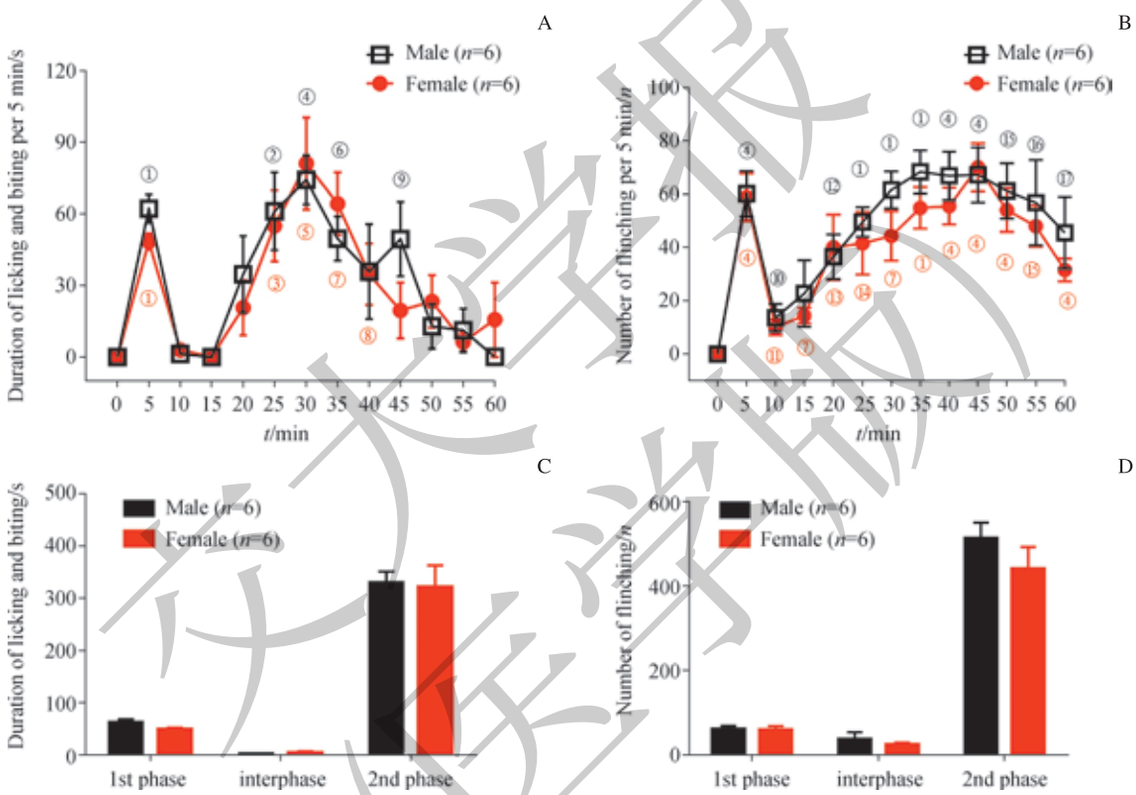
2 Results

2.1 Intraplantar injection of formalin induced flinching, licking or biting in male and female rats

We first replicated the classic intraplantar formalin model of acute inflammatory pain in male and female SD rats. Following subcutaneous injection of formalin into the plantar surface of the right hind paw, rats exhibited significant behavioral responses characterized by lifting the right hind limb (flinching) and licking or biting the paw injected with formalin. In agreement with the literature[15-17], quantitative

analysis revealed a biphasic pattern of behavior responses (Figure 1): the 1st phase (0—5 min post-injection) of vigorous flinching and licking/biting followed by a relatively quiescent period (5—15 min post-injection, interphase), and then the 2nd phase of vigorous behaviors that lasted for approximately 45 minutes (15—60 min post-injection). Notably, there was no significant gender difference in the behavior responses to intraplantar formalin.

Previous studies reported that hind paw injection of algogenic agents elicited paw-licking [6,18-21] whilst itchy pathological lesions evoked hind paw-biting in mice[12,18,22]. Thus licking and biting the injured sites might represent discrete sensations, pain/nociception and itch/pruritoception, respectively. The afore-mentioned behaviors (flinching and licking/biting) would suggest that rats might be experiencing both pain and itch following formalin injection. However, it was not easy to distinguish whether the rat was licking or biting the paw following formalin injection. Therefore, additional models are necessary in order to determine whether formalin induces itch as well as pain.



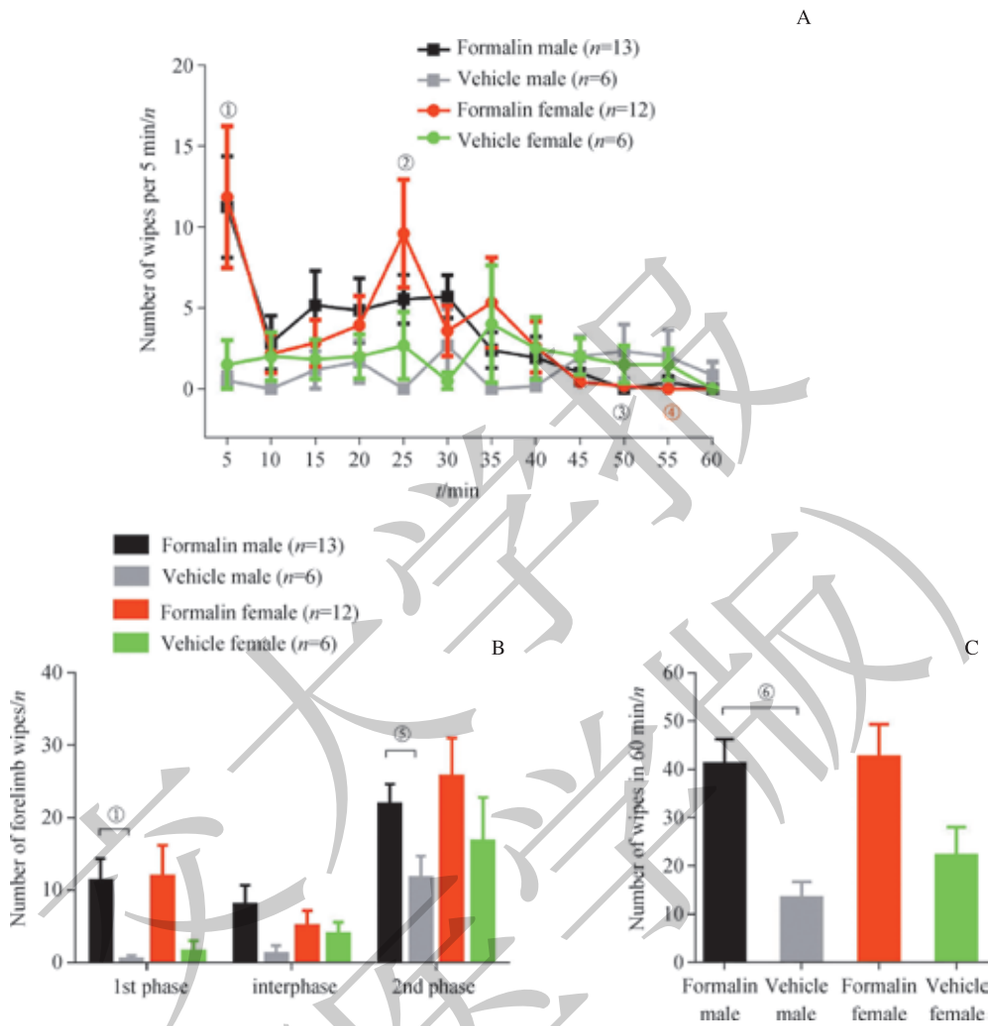
Note: A. The duration of ipsilateral biting or licking episodes every 5 min following intraplantar injection of formalin. B. The number of ipsilateral flinching every 5 min following formalin injection. C and D show the behaviors in the different phases (1st, 2nd and interphase) following intraplantar formalin injection. ① P=0.000, ② P=0.013, ③ P=0.014, ④ P=0.001, ⑤ P=0.008, ⑥ P=0.003, ⑦ P=0.005, ⑧ P=0.044, ⑨ P=0.025, ⑩ P=0.047, ⑪ P=0.019, ⑫ P=0.007, ⑬ P=0.023, ⑭ P=0.017, ⑮ P=0.002, ⑯ P=0.016, ⑰ P=0.020, compared with each baseline.

Fig 1 Intraplantar administration of formalin elicited biphasic behavior responses characterized by flinching the affected limb and licking or biting the affected paw

2.2 Intradermal administration of formalin to the cheek elicited hindlimb scratching as well as forelimb wiping in male and female rats

The cheek model is an established model for the discrimination of pain and itch responses in rodents [14, 23-24]. Hence, we utilized this model to test the possibility that formalin induces itch in addition to pain. Indeed, following intradermal injection of formalin into the cheek, rats responded with episodic hindlimb scratching as well as forelimb wiping of the cheek, indicative of both itch and pain sensations. It was also noted that forelimb wiping was quite frequent but scratching of the site of injection was relatively sparse. Only forelimb wiping of the site of injection was counted as pain responses. Quantitative analysis of the counts of forelimb wiping of the injection site suggested a biphasic pattern of the pain responses (Figure 2): frequent forelimb wiping in

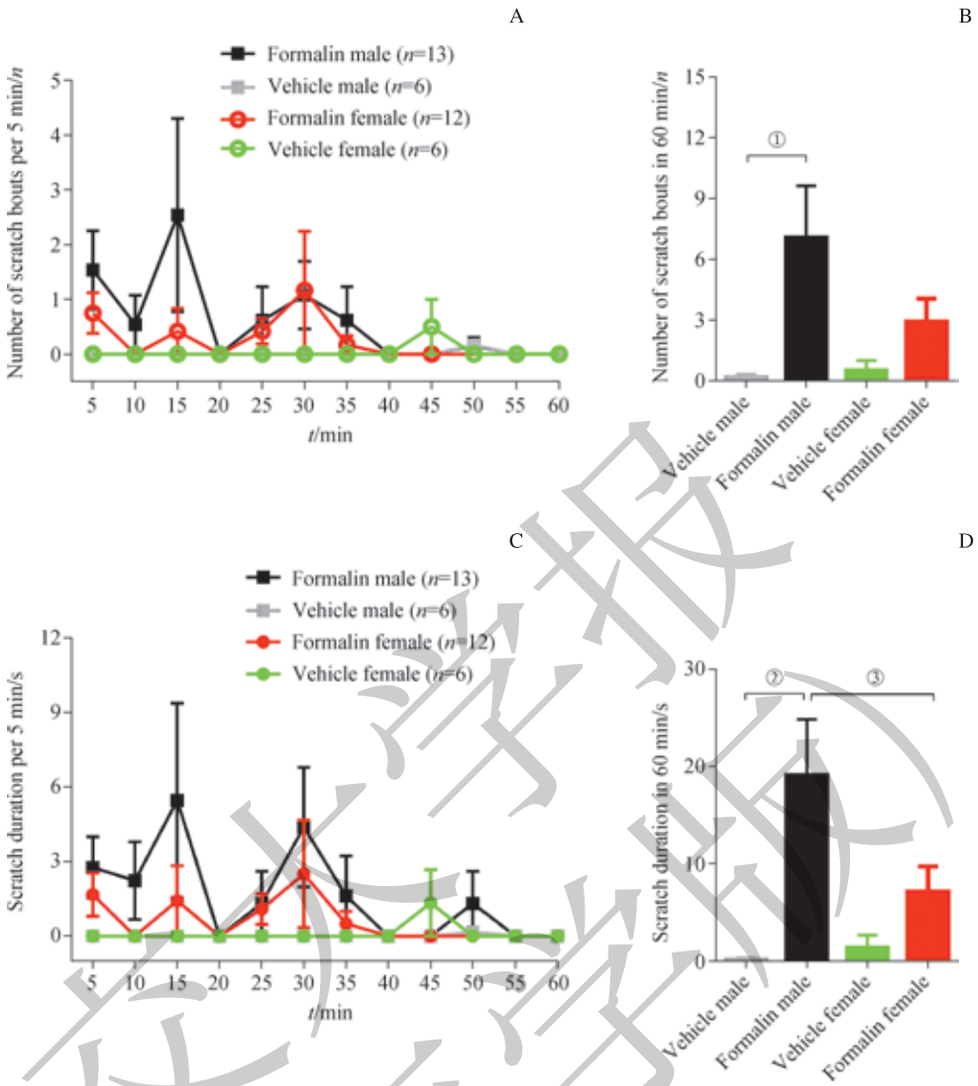
the first 5 min (1st phase) followed by a relatively quiescent period (5−15 min, interphase) and then another (2nd) phase of increased forelimb wiping, similarly to the biphasic behavior responses seen in the intraplantar formalin model. Again, no gender differences were noticed in formalin-induced forelimb wiping.



Note: A. The number of forelimb wipes of the injection site every 5 min following intradermal injection of formalin or vehicle into the right-side cheek. B. The counts of forelimb wipes of the injection site in the 1st (0−5 min) phase, 2nd (15−60 min) phase and the interphase (5−15 min) of the nociceptive response to formalin injection. C. The total counts of formalin/vehicle-induced forelimb wiping of the injection site during the 60 min recording period. ① P=0.035, ② P=0.026, ③ P=0.048, ⑤ P=0.047, ⑥ P=0.003, Formalin male *vs* Vehicle male; ④ P=0.036, Formalin female *vs* Vehicle female.

Fig 2 Forelimb wiping (nociception) evoked by intradermal injection of formalin into the cheek in rats with no gender difference

Figure 3 shows the quantitative analysis of the hindlimb scratching following intradermal injection of formalin into the cheek. Following intradermal injection of vehicle, there was hardly any bout of hindlimb scratching. However, following injection of formalin, rats exhibited episodic scratching of the cheek with the hindlimb. This behavior occurred primarily in the first 30 min and subsided in about an hour. Interestingly, in contrast to the lack of gender differences in forelimb wiping, male rats appeared to scratch more vigorously than female rats.



Note: A. The number of scratching bouts every 5 min following intradermal injection of formalin or vehicle into the cheek. B. The total numbers of scratching bouts over the one hour period following administration of formalin or vehicle to the cheek. C. The duration of hindlimb scratching every 5 min after injection of formalin or vehicle. D. The total duration of scratching episodes following injection of formalin or vehicle. ① P= 0.031, ② P= 0.026, Formalin male *vs* Vehicle male; ③ P= 0.049, Formalin male *vs* Formalin female.

Fig 3 Hindlimb scratching (pruritoception) evoked by intradermal injection of formalin into the cheek in rats with pronounced response in the male

3 Discussion

Intraplantar formalin is one of the most commonly used preclinical model of acute inflammatory pain. However, there has been a lack of evidence that formalin solely induces pain. In this investigation, the possibility that formalin induces pruritoception as well as nociception has been explored by observing the behavior responses to intradermal injection of formalin into the cheek in male and female rats. Our results showed that formalin elicited hindlimb scratching as well as forelimb wiping of the cheek. No gender differences were noticed for the forelimb wiping responses. However, hindlimb scratching was more vigorous in male than in female rats following formalin injection into the cheek. These results support the notion that formalin induces both pain and itch. The gender differences in formalin-induced hindlimb scratching suggest that sex hormones might play a role in modulation of formalin-induced itch.

Formalin is a strong irritant that causes acute inflammation of the tissue exposed to it. Intraplantar injection of formalin has been a very popular model of acute inflammatory pain. Typically, rodents displayed biphasic behavior responses including lifting the ipsilateral limb and licking or biting the paw following intraplantar formalin injection. The 1st

phase of vigorous flinching and licking/biting which occurs immediately after formalin injection has been attributed to the direct stimulation of nerve endings by formalin, whereas the delayed (2nd) phase of the behavior responses has been attributed to an inflammatory hyperalgesia associated with peripheral and central sensitization[25]. However, there have been studies indicating that licking and biting of the sites of injury may respectively reflect pain and itch behaviors. For example, intradermal injection of pruritogens into hairy skin of the calf of the hindlimb elicited biting, whereas algogens elicited licking behaviors [7]. Similarly, hind paw injection of 5-HT elicited paw-biting which could be reduced by the opioid antagonist, naltrexone, whilst capsaicin elicited licking behavior which could be reduced by opioid agonist morphine[12,21]. Mice exhibited spontaneous biting in a model of dry skin of the hind paws[22]. The behavior that responses to intraplantar formalin-flinching, licking and biting, would suggest that formalin induces itch as well as pain, although these behaviors have generally been deemed as nociceptive responses.

In the present investigation, we replicated the biphasic behavior responses to intraplantar formalin in rats. However, we noticed that to distinguish licking or biting was a challenge. In rodents, biting is characterized by high-frequency and low-excursion head movements with contact of the incisors with the injured skin, just like scratching with the teeth, whereas licking is a lower frequency and longer excursion motion of the tongue protrusion[21]. However, we found that even with high-definition video recording and careful visual inspection at a slow playback speed, quantitative analysis of licking or biting was a tedious task and accuracy can hardly be guaranteed.

To better determine whether formalin induces itch in addition to pain, we chose to observe the behavior responses to intradermal injection of formalin into the cheek. Previous studies have shown that intradermal injection of pruritogens (*e.g.*, histamine) into the cheek of mice induced hind paw-scratching, whereas injection of algogens (*e.g.*, capsaicin) elicited forelimb-wiping of the injection site only[14]. Furthermore, hindlimb scratching induced by pruritogens was attenuated by the μ-opioid antagonist naltrexone but not morphine, whilst algogen-evoked forelimb wiping was reduced by the μ-opioid agonist morphine respectively[23-24]. Hence the cheek model is a useful behavioral test that discriminates itch and pain. We found that following intradermal injection of formalin into the cheek, rats responded with frequent forelimb wiping accompanied by episodic hindlimb scratching. Forelimb wiping appeared to occur in a biphasic manner, akin to the biphasic pattern of the behavior response to intraplantar formalin, whilst hindlimb scratching occurred monotonically, being more frequent initially and subsiding within an hour. These data strongly suggest that formalin induces itch as well as pain.

An interesting finding in the cheek model was that formalin-induced hindlimb scratching appeared to be more pronounced in male than in female rats, whilst no gender differences were noticed in formalin-induced forelimb wiping. Gender related differences in pain or itch have been extensively reported and have been attributed to modulation by gonad hormones, but the exact mechanisms remained unresolved [26-31]. Our observation suggests that formalin-induced itch might be inhibited by female hormones, which warrants further investigation.

In summary, pain and itch are both unpleasant but distinct sensory experiences. In addition, pain and itch are generally antagonistic, in that painful stimuli inhibits itch sensation and morphine inhibits pain but on the contrary elicits or enhances itch. This signifies the importance of distinguishing the type of sensation that experimental subjects are experiencing in preclinical models. The intraplantar formalin model is an extensively-used rodent model of acute inflammatory pain. Our results suggest that formalin is not solely algogenic but induces itch as well. This aspect is worthy of consideration in the interpretation of observations in this model.

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