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INVESTIGATIVE REPORT

Opioid Modulation of Facial Itch- and Pain-related Responses and Grooming Behavior in Rats

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Intradermal facial injections of pruritogens or algogens elicit distinct behavioral hindlimb scratch or forelimb wiping responses in rodents. We systematically investigated the parameters and opioid modulation of these evoked behaviors and spontaneous facial grooming in rats. Serotonin (5-HT) elicited hindlimb scratch bouts with few wipes. Scratching was attenuated by the μ-opiate antagonist naltrexone but not morphine. In contrast, cheek injection of mustard oil (allyl-isothiocyanate (AITC)) elicited ipsilateral forelimb wipes but little hindlimb scratching. AITC-evoked wiping was significantly attenuated by morphine but not naltrexone. Spontaneous facial grooming by the forepaws was attenuated by naltrexone, whereas morphine did not affect grooming behavior before or after cheek injections of 5-HT or AITC. These data validate that the rodent "cheek" model discriminates between itch- and pain-related behaviors. Naltrexone sensitivity of facial grooming and 5-HT-evoked scratching suggests a common functionality. Forelimb wipes may represent a nocifensive response akin to rubbing an injury to relieve pain. ***Key words: itch; pain; grooming; naltrexone; rat; opioid.***

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Itch and pain are unpleasant sensations that signal the occurrence of a potentially dangerous stimulus and initiate appropriate responses to remove or avoid it. Chronic itch or pain are often symptomatic of skin conditions, nerve injury, systemic disease and many other conditions, and represent a large socioeconomic burden to society (1, 2). Knowledge about basic mechanisms and treatment for itch have lagged those of pain, and novel treatment modalities are needed for the many types of chronic itch that are not relieved by antihistamines (3, 4). Because of the causal relationship between itch and scratching, animal models to investigate itch have assessed scratching behavior directed to a site of pruritic stimulation (5). It was recently reported that injection of pruritogens in the cheek of mice (6, 7) or rats (8) elicits bouts of hindlimb scratching directed to the stimulus, while algogens such as capsaicin or allyl-isothiocyanate (AITC; pungent chemical in mustard oil) elicit singular wiping motions of the ipsilateral forepaw across the cheek. This model thus appears to distinguish between pruritogenic and algogenic stimuli. Moreover, it is an improvement on the traditional model in which stimuli are delivered to the nape of the neck of rodents, for which only one response – hindlimb scratching – is available due to limitations of limb accessibility. In mice, pruritogen-evoked hindlimb scratch bouts and algogen-evoked forelimb wipes were differentially reduced by μ-opioid antagonists or the μ-agonist morphine, respectively (7). This further supports the ability of the model to distinguish between itch and pain, since the μ-antagonist naloxone reduces itch (but not pain) sensation in humans (9) whereas morphine is well-known to suppress pain while inducing or enhancing itch (10). The ability of morphine to induce itch was recently reported to involve the MOR1-D opioid receptor isoform that is co-expressed with gastrin releasing peptide receptor in a subset of spinal neurons; morphine induces heterodimerization leading to excitation of this group of neurons that is thought to signal itch and initiate scratching (11).

In the present study, we wished to further validate the "cheek" model in the Sprague-Dawley rat, a species commonly used in mechanistic studies of itch and pain. We hypothesized that pruritogen-evoked hindlimb scratching and algogen-evoked forelimb wiping would be differentially modulated by opioids as in the mouse. We also systematically investigated parameters of scratching as well as spontaneous facial grooming behavior and their modulation by opioids.

MATERIAL AND METHODS

The study was approved by the University of California, Davis Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats (weight range: 322–625 g, Simonsen and Charles River) were singly-housed on a normal light cycle and had *ad libitum* access to food and water.

The methods for intradermal (id) cheek injections were similar to those described previously (8). Experiments were conducted during the same daily time period to reduce circadian effects. Rats were first habituated to a Plexiglas recording arena on a clear surface through which animals were videotaped from below. For formal testing, rats received one of the following pretreatments: (a) intraperitoneal (ip) injection of vehicle (1:1:1:17 of DMSO:ethanol:Tween-80:saline in a volume based on body weight), (b) ip morphine (3 mg/kg), or (c) ip naltrexone (5 mg/

kg). Rats were either placed into the recording arena for habituation 15 min after ip injection (and videotaped for 15 min), or were habituated for 30 min first followed by the ip injection and placement in the recording arena (only for experiments examining naltrexone effects on normal facial grooming behavior). Rats only tested for normal facial grooming behavior (no id injection) were then recorded for one h post-ip injection. After habituation in all other groups, the rats were removed from the testing arena and received an id cheek injection (10 µl) of either 5-HT (5-hydroxytryptamine HCl; 1%=47 mM, in sterile 0.9% saline; Sigma, St. Louis, MO) or AITC (mustard oil; 10%=1 M, in 7% Tween 80/saline; Sigma) into one previously-shaved cheek using a 30-gauge hypodermic needle connected via PE 50 tubing to a Hamilton microsyringe. The injection was verified visually as a small wheal. The rat was then immediately placed back into the recording arena and videotaped for an additional 60 min.

Videotapes were subsequently reviewed by observers who were blinded as to treatment. The following behavioral responses were scored in 5-min intervals over the 15- or 30-min habituation period and the 60-min testing period following id/ip injection: (*i*) number of bouts of hindpaw scratches directed to the injected cheek (off-site scratches, such as ears, were excluded), (*ii*) number of scratch bouts occurring in a sequence/series, (*iii*) duration of each hindpaw scratch bout, (*iv*) number of ipsilateral forelimb wipes directed to the injected cheek, (*v*) number of bouts of facial grooming behavior, which consisted of discrete episodes of head- or face-washing by the forepaws, and (*vi*) duration of each facial grooming bout. We only scored facial grooming and did not consider licking, scratching and other grooming behaviors directed to the lower body.

Data from the behavioral testing are expressed as mean ± SEM. Data displayed in line graphs represent the total amount of specified behavior quantified per 5-min bin either during the baseline period (up through 0 min) or during the testing period (up through 60 min), and were analyzed by repeated measures ANOVA. When Mauchly's Test of Sphericity indicated significance, the Greenhouse-Geisser correction factor was applied to the interaction term of all repeated factors. When a significant interaction was present, 1-way ANOVAs were performed for each time point with Tukey post-hocs as needed if more than two groups were being compared. When repeated measures indicated a significant group effect, Tukey post-hocs were performed as indicated. The data displayed in bar graphs were analyzed via 1-way ANOVAs and Tukey post-hocs as needed; these values represent the total amount of the specified behavior quantified over the entire 60-min testing period.

On the figures, each symbol designates a unique significance as described in the figure legends. Symbols set to the side of a line graph next to a bracket designate a significant group effect, while symbols above specific time points indicate the post-hoc results of a significant interaction.

Statistics were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA) and SPSS 8.0 (IBM, Chicago, USA).

RESULTS

Opioid modulation of 5-HT and AITC-evoked scratching and wiping

There were low levels of spontaneous hindlimb scratch bouts (0.24 ± 0.1 [SEM] bouts/15 min) or ipsilateral forelimb wipes (0.1 ± 0.07). Intradermal cheek microinjection of 5-HT elicited many hindlimb scratch bouts and few ipsilateral forelimb wipes (Fig. 1A, left-hand bars), as previously reported (8). 5-HT-evoked scratching commenced within the first 5-min bin, peaked at 10–20 min and persisted out to 55 min (Fig. 1B). In contrast, AITC elicited many singular, ipsilateral forelimb wipes of short duration, directed from caudal to rostral across the cheek, with very few hindlimb scratch bouts (Fig. 1C). AITC-evoked wiping peaked at 10–25 min and then declined (Fig. 1D).

Pretreatment with naltrexone resulted in a significant attenuation of 5-HT-evoked hindlimb scratch bouts (Fig. 1A), with delayed onset and reduced number of scratch bouts vs. time (Fig. 1B). Naltrexone did not significantly affect the number of AITC-evoked forelimb wipes (Fig. 1C).

Pretreatment with morphine did not significantly affect 5-HT-evoked hindlimb scratching vs. vehicle controls (Fig. 1A). However, morphine significantly attenuated AITC-evoked forelimb wipes vs. vehicle controls (Fig. 1C), as manifested by an initial increase in wipe counts that quickly declined to zero (Fig. 1D).

Parametric analysis of 5-HT-evoked hindlimb scratching and opioid modulation

Previous studies reported that the within-bout scratch frequency was constant (8–10 Hz) and did not vary as a function of time or 5-HT concentration (8, 12). We therefore focused on number and length of individual scratch bouts, and cumulative time spent scratching, as measures of itch intensity. The total cumulative time spent scratching was reduced by naltrexone but not morphine (Fig. 2A). The total cumulative time spent scratching following morphine pretreatment was slightly but not significantly greater compared to vehicle pretreatment (Fig. 2A), and the mean number of scratch bouts was virtually identical (Fig. 1A), such that the mean duration of individual scratch bouts after morphine (2.87 s) was similar to that after naltrexone (2.89 s) and slightly but not significantly different compared to vehicle controls (2.45 s), in agreement with previous reports (8, 12, 13).

Individual scratch bout durations usually ranged from 0.5–6 s following vehicle pretreatment. Following morphine, a small number of scratch bouts were longer (15–21 s), such that the mean maximal scratch bout duration was significantly greater (10 s ± 2.2 SEM) compared to vehicle (5.3 s ± 0.5 SEM) or naltrexone pretreatment (4.6 s ± 0.7 SEM). Minimum scratch bout lengths (0.5–1 s) did not vary significantly across treatment groups.

Scratch bouts terminated when the animal licked the hindpaw and/or returned it to the floor. Often rats paused between scratch bouts (usually to lick the hindpaw), and then resumed scratching without placing the hindpaw down; we called this sequence of scratch bouts a series. The mean numbers of scratch series were significantly reduced by naltrexone but not morphine (Fig. 2C), and exhibited time courses (Fig. 2D) similar to those obser-

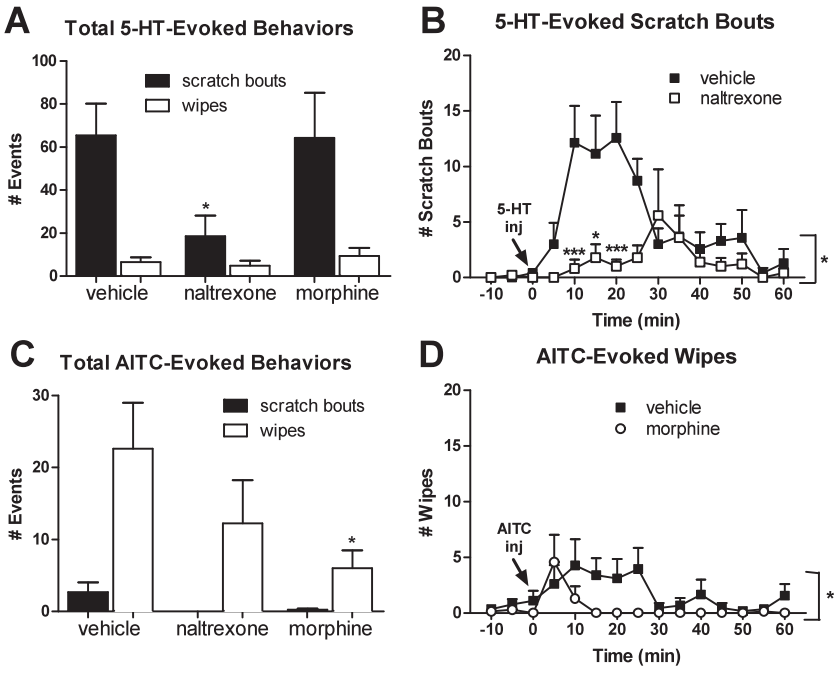

Fig. 1. Opioid modulation of behavioral responses to cheek injection of 5-HT or AITC. A) Bar graph plots total mean number of hindlimb scratch bouts (*black bars*) and ipsilateral forelimb wipes (*white bars*) following cheek injection of 5-HT, under conditions of vehicle, naltrexone (5 mg/kg intraperitoneal (ip)) or morphine pretreatment (3 mg/kg ip). B) Time course of 5-HT-evoked scratching. Vehicle (■) or naltrexone (□) was injected ip at time –30. At time 0, 5-HT was injected in the cheek intradermally. Graph plots mean counts of scratch bouts in 5-min bins. C) As in A for AITC-evoked scratch bouts and wipes. D) As in B for AITC-evoked wipes. Data are mean ± SEM (n = 7–12/group). *significant difference compared to vehicle; bracket indicates overall group effect (*$p \leq 0.05$, ***$p \leq 0.001$).

ved for the number of scratch bouts and cumulative time spent scratching. The mean duration of scratch series following vehicle pretreatment was 10.1 ± 1.8 s (SEM) and did not change significantly following pretreatment with naltrexone or morphine.

Facial grooming behavior

Spontaneous hindlimb scratch bouts and forelimb wipes directed to the face were infrequent prior to cheek microinjections, but facial grooming with the forepaws was common. Facial grooming (also referred to as face or head washing) occurred in discrete bouts as the initial component of a general cephalocaudal grooming sequence common to rodents. The occurrence of bouts of facial grooming was not affected by cheek microinjection of 5-HT (Fig. 3A). Fig. 3B plots the number of bouts of grooming in 5-min bins vs. time to show that this measure was not significantly affected following 5-HT which was injected at time 0. Rats receiving cheek AITC preceded by vehicle exhibited a similar overall number of grooming bouts (Fig. 4A). Immediately after cheek injection of AITC, there was a small but significant increase in grooming that quickly returned to baseline levels (Fig. 4B). In animals pretreated with vehicle, the time spent grooming was not significantly

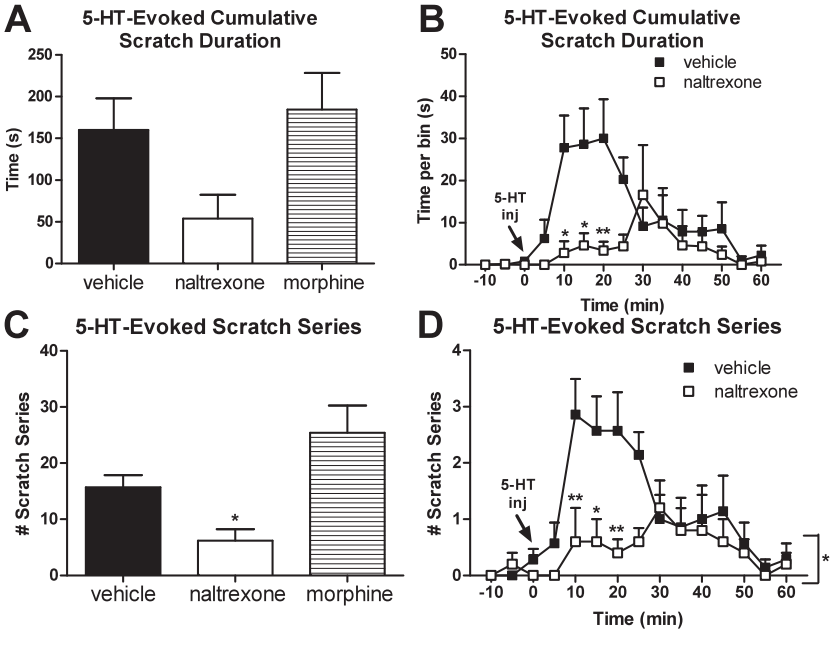

Fig. 2. Opioid modulation of 5-HT-evoked scratching. A) Bar graph plots total mean cumulative duration of hindlimb scratch bouts following cheek injection of 5-HT, under conditions of vehicle (*black bar*), naltrexone (5 mg/kg intraperitoneal (ip), *white bar*) or morphine pretreatment (3 mg/kg ip, *hatched bar*). B) Time course of 5-HT-evoked cumulative scratch time. Vehicle (■) or naltrexone (□) was injected ip at time –30. At time 0, 5-HT was injected intradermally in the cheek. Graph plots mean sum of scratch time in 5-min bins. C) As in A for 5-HT-evoked scratch series. D) As in B for 5-HT-evoked scratch series. Data are mean ± SEM (n = 7–12/group). *significant difference compared to vehicle; bracket indicates overall group effect (*$p \leq 0.05$, **$p \leq 0.01$).

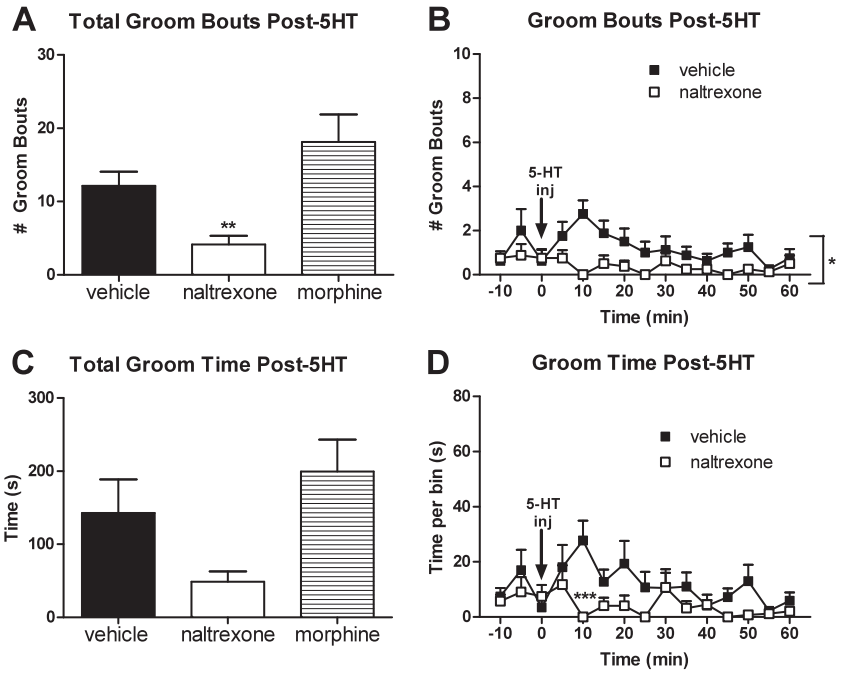

Fig. 3. Opioid modulation of facial grooming: 5-HT. A) Bar graph plots total mean number of bouts of facial grooming behavior after cheek injection of 5-HT, under conditions of vehicle (*black bar*), naltrexone (5 mg/kg intraperitoneal (ip), *white bar*) or morphine pretreatment (3 mg/kg ip, hatched bar). B) Time course of occurrence of bouts of grooming. Vehicle (■) or naltrexone (□) was injected ip at time –30. At time 0, 5-HT was injected intradermally in the cheek. Graph plots mean counts of grooming bouts in 5-min bins. C) As in A for total mean cumulative duration of facial grooming behavior. D) As in B for total cumulative time spent grooming, counted in 5-min bins. Data are mean ± SEM (n = 7–12/group). *significant difference compared to vehicle; bracket indicates overall group effect (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

affected following cheek injection of 5-HT (Fig. 3D) or AITC (Fig. 4D).

In rats receiving cheek injections of 5-HT (Fig. 3A) or AITC (Fig. 4A), pretreatment with naltrexone significantly attenuated grooming bouts vs. vehicle pretreatment. Naltrexone also significantly reduced the cumulative time spent grooming in animals receiving cheek injection of AITC (Fig. 4C) with a similar trend in animals receiving cheek injection of 5-HT (Fig. 3C). After id injection of 5-HT or AITC at time 0, naltrexone-pretreated rats displayed a significant reduction in number of grooming bouts and time spent grooming compared to vehicle (Fig. 3B, D and 4B, D). Pretreatment with morphine had no significant effect on the total number of grooming bouts or cumulative time spent grooming (Figs 3A, C and 4A, C).

To determine if naltrexone depresses facial grooming per se, we compared the effects of ip naltrexone or vehicle injection in the absence of id cheek microinjections. Figs. 5A and C show that the total number of facial grooming bouts, and cumulative time spent grooming, were significantly attenuated by naltrexone. Figs. 5B and D show the time course of grooming behavior prior to and after ip injection of vehicle or

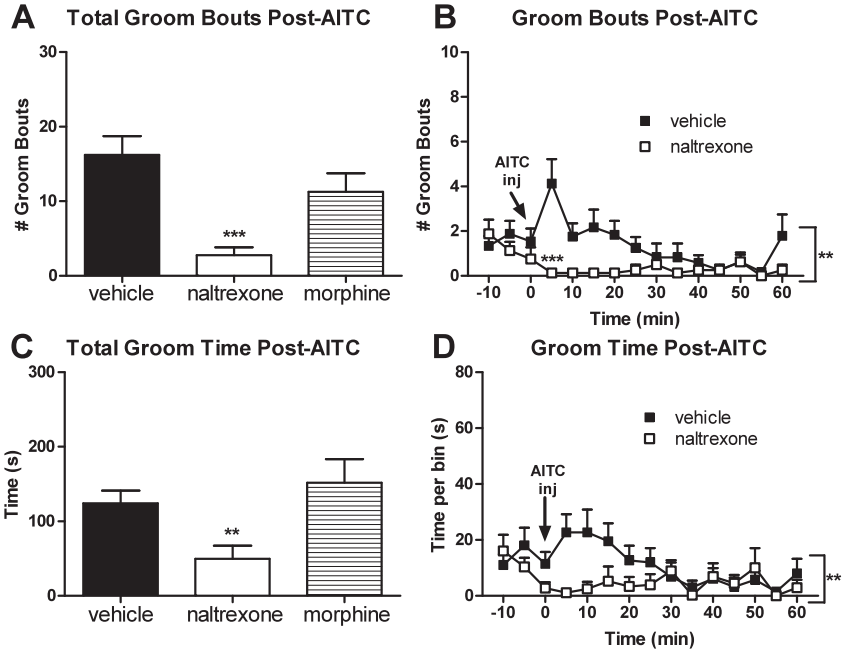

Fig. 4. Opioid modulation of facial grooming: AITC. A) Bar graph plots total mean number of bouts of facial grooming behavior after cheek injection of AITC, under conditions of vehicle (*black bar*), naltrexone (5 mg/kg intraperitoneal (ip), *white bar*) or morphine pretreatment (3 mg/kg ip, *hatched bar*). B) Time course of occurrence of bouts of grooming. Vehicle (■) or naltrexone (□) was injected ip at time –30. At time 0, AITC was injected intradermally in the cheek. Graph plots mean counts of grooming bouts in 5-min bins. C) As in A for total mean cumulative duration of facial grooming behavior. D) As in B for total cumulative time spent grooming, counted in 5-min bins. Data are mean ± SEM (n = 7–12/group). *significant difference compared to vehicle; bracket indicates overall group effect (**$p \leq 0.01$, ***$p \leq 0.001$).

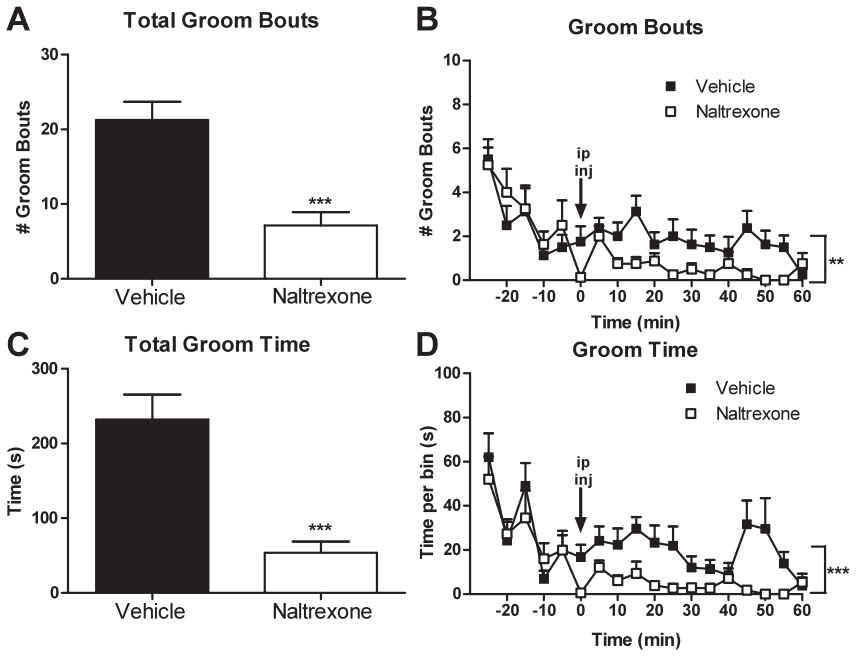

Fig. 5. Opioid modulation of normal facial grooming. A) Bar graph plots total mean number of bouts of facial grooming behavior after ip injection of vehicle (*black bar*) or naltrexone (5 mg/kg intraperitoneal (ip), *white bar*). B) Time course of occurrence of bouts of grooming. Vehicle (■) or naltrexone (□) was injected ip at time 0. Graph plots mean counts of grooming bouts in 5-min bins. C) As in A for total mean cumulative duration of facial grooming behavior. D) As in B for total cumulative time spent grooming, counted in 5-min bins. Data are mean ± SEM ($n = 8$). *significant difference compared to vehicle; bracket indicates overall group effect (**$p \leq 0.01$, ***$p \leq 0.001$).

naltrexone at time 0. Facial grooming decreased over the first 15 min to reach a fairly steady level prior to the injection. In animals receiving vehicle injection, the grooming behavior continued at a fairly constant level over the ensuing 60 min (Fig. 5B, D). In contrast, animals receiving naltrexone exhibited a further reduction in facial grooming behavior to very low levels that were significantly different from the vehicle-treated group (Fig. 5B, D).

DISCUSSION

The present results confirm previous reports that id cheek injections of pruritogens or algogens differentially elicit hindlimb scratch bouts or ipsilateral forelimb wipes in mice (6, 7) and Sprague-Dawley rats (8). 5-HT-evoked facial scratching was attenuated by naltrexone but not morphine, whereas AITC-evoked wiping was attenuated by morphine but not naltrexone. This confirms our recent observations in mice (7) and further supports the contention that scratching and wiping reflect itch- and pain-related behaviors in this rodent model.

5-HT elicited bouts of hindlimb scratching directed to the cheek injection site. The number of bouts, total cumulative duration of scratching, and number of scratch series (successive scratch bouts interrupted by a pause but without placing the hindpaw down), each appear to be a robust measure of itch magnitude. Each of these parameters of scratching had a similar time course post-5-HT, and was significantly attenuated by the μ-opioid antagonist naltrexone. The number and cumulative duration of scratch bouts were previously shown to be proportional to the dose of 5-HT (8, 12, 14). The mean duration of scratch bouts was presently observed to be ~2–3 sec, consistent with previous studies (8, 12). Each scratch bout consisted of rhythmic scratch motions at a constant frequency of ~8–10 Hz (8, 12). Neither the bout duration nor within-bout frequency varied significantly as a function of 5-HT concentration (8, 12), although we presently observed a tendency for increased duration of scratch bouts following morphine pretreatment.

In Sprague-Dawley rats, 5-HT is the most effective inducer of hindlimb scratching (8, 15). Chloroquine was also shown to differentially elicit scratching but not wiping in the cheek model (8). In mice, a variety of pruritogens elicit hindlimb scratching of the cheek, including 5-HT, histamine, agonists of protease-activated receptors PAR-2 and -4, and chloroquine (7). Overall, the number of scratch bouts (and series) and total time spent scratching appear to be robust endpoints for measuring the magnitude of itch-related behavioral responses elicited by id injection of pruritogens in the cheek of rodents.

In contrast to 5-HT, AITC elicited ipsilateral forelimb wipes but very few hindlimb scratch bouts. Wipes consisted of singular caudal-to-rostral movements of the inner aspect of the forepaw across the injected cheek lasting approximately 0.3 sec. The number of wipes, but not wipe duration, varied with AITC dose (8) and was presently shown to be significantly reduced by morphine but not naltrexone. In mice, AITC, capsaicin and bradykinin each elicited wiping but not hindlimb scratching, with capsaicin-evoked wiping being attenuated by morphine (but not naltrexone) in a dose-related manner (7). These data suggest that the number of wipes elicited by cheek injection of algogens is a valid measure of pain-related behavior in rodents.

We presently observed that pretreatment with naltrexone suppressed facial grooming by the forepaws.

Facial grooming was significantly reduced for at least one h following id cheek injection of AITC or 5-HT. In vehicle-treated animals, cheek injection of 5-HT did not result in any significant change in facial grooming over time and AITC only elicited a transient increase in facial grooming, suggesting that naltrexone reduced facial grooming regardless of whether the cheek was injected with an algogen or pruritogen. To verify this, we observed that systemic injection of naltrexone significantly reduced spontaneous facial grooming behavior (Fig. 5). Previous studies have reported that μ-opioid antagonists suppress rat grooming behavior. Systemic naloxone reduced excessive grooming behavior elicited by intracerebroventricular injection of bombesin in rats (16) or by administration of an enkephalinase inhibitor (17). Interestingly, in control animals (without excessive grooming) naloxone also significantly reduced overall spontaneously-occurring grooming behavior but this was attributed primarily to decreased body grooming, while scratching and head-washing were not affected (16). To our knowledge, there are otherwise few if any studies that have examined the effect of μ-antagonists on specific components of grooming behavior. Our present data appear to be the first to show that facial grooming behavior is significantly attenuated by naltrexone.

It is interesting that both pruritogen-evoked facial scratching by the hindlimb, and spontaneous forelimb grooming of the face, were attenuated by naltrexone but not morphine. Spontaneous scratching directed to a site of chronic dry skin was also significantly attenuated by naltrexone (18). The sensitivity of both spontaneous facial grooming behavior and evoked hindlimb scratching to suppression by naltrexone suggests a common function presumably serving to remove stimuli from the skin. However, the incidental scratching associated with grooming behavior may subserve a more general hygienic function, while pruritogen-evoked scratching represents a more urgent nocifensive response. Moreover, spontaneous grooming also increased with the stress associated with handling and transporting rats to a novel environment, and was attenuated by naloxone in a dose-dependent manner (19). Our data showing that naltrexone attenuated spontaneous facial grooming in otherwise-untreated animals (Fig. 5) is consistent with the possibility that grooming represents an opioid-dependent stress response that is not necessarily related to itch sensation.

In contrast, algogen-evoked wiping of the face by the forepaw was attenuated by morphine but not naltrexone. These data suggest that ipsilateral forelimb wipes represent a nocifensive response, possibly akin to rubbing a site of injury to relieve pain. We speculate that forelimb wiping directed to the face is unlikely to be a component of normal grooming, since it is not sensitive to μ-antagonists and occurs very infrequently in the absence of a noxious facial stimulus.

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