Novel Tools and Methods

A New Tool for Quantifying Mouse Facial Expressions

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Abstract

Facial expressions are an increasingly used tool to assess emotional experience and affective state during experimental procedures in animal models. Previous studies have successfully related specific facial features with different positive and negative valence situations, most notably in relation to pain. However, characterizing and interpreting such expressions remains a major challenge. We identified seven easily visualizable facial parameters on mouse profiles, accounting for changes in eye, ear, mouth, snout and face orientation. We monitored their relative position on the face across time and throughout sequences of positive and aversive gustatory and somatosensory stimuli in freely moving mice. Facial parameters successfully captured response profiles to each stimulus and reflected spontaneous movements in response to stimulus valence, as well as contextual elements such as habituation. Notably, eye opening was increased by palatable tastants and innocuous touch, while this parameter was reduced by tasting a bitter solution and by painful stimuli. Mouse ear posture appears to convey a large part of emotional information. Facial expressions accurately depicted welfare and affective state in a time-sensitive manner, successfully correlating time-dependent stimulation. This study is the first to delineate rodent facial expression features in multiple positive valence situations, including in relation to affective touch. We suggest using this facial expression assay might provide mechanistic insights into emotional expression and improve the translational value of experimental studies in rodents on pain and other states.

Key words: mouse grimace scale; mouse profile; optogenetics; positive emotion; somatosensation

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Significance Statement

The existence of distinct mouse facial expressions in painful situations is well established. However, the current methods in use do not offer features both visualizable by a human observer and finely quantifiable. To address this issue, we established seven new facial parameters, directly measurable on mouse profiles, that give insight into the facial regions responsible for changes in expression. This method is completely transparent and can be applied to a wide range of mammals. To complete the spectrum of known facial expressions, we submitted the mice to sequences of pleasant and aversive gustatory and somatosensory stimuli. Broadening the spectrum of experimental contexts amenable to facial expression analysis, including those of positive valence, can improve the translatability of animal emotion studies.

Introduction

Emotions are defined as transient states, experienced and expressed through a multicomponent system, comprising behavioral and physiological responses, as well as a cognitive and a subjective component (Paul et al., 2005; Shuman et al., 2017). Although emotions are relatively easily identified in humans through verbal reports, their existence and identification in nonhuman animals have been debated for many years (de Vere and Kuczaj, 2016).

In particular, the subjective component of any emotional experience is difficult to assess in animals, the verbal production of which remains largely unintelligible for researchers. Nonetheless, observations of neurophysiological changes accompanying exposure to pleasant and aversive stimuli, together with the inclusion of patterns of vocalizations, behavioral and facial displays point toward the existence of a wide range of emotional experiences in laboratory rodents, farm animals and various primates (Désiré et al., 2002; Morris et al., 2008; Makowska and Weary, 2013; Dolensek et al., 2020).

Emotions motivate a large aspect of our behaviors (Berridge, 2018) but can be difficult to recreate in laboratory conditions. The challenge of inducing and identifying them in animal models could contribute to the translational crisis experienced in neuroscience, with a large proportion of physiological, pharmacological or behavioral findings failing to be replicated from the lab to the clinic (Drucker, 2016; Fendt et al., 2020). Most mammals, including humans, can produce facial movements which Darwin had already postulated to be highly conserved between species (Darwin, 1872). Such movements, and their capture in facial expressions are considered the richest source of emotional information (Ekman, 2009). Facial expressions give insight into spontaneous, momentary affect in response to a stimulus or to the memories of its consequences, motivating a physiological and behavioral reaction (Webb et al., 2019). Understanding and interpreting animal emotions is especially relevant to understand social species who have a mutual benefit in communicating and interpreting each other's emotional state.

The potential of facial expressions for emotion decoding was already systematically assessed earlier by (Grill and Norgren, 1978), and they have since successfully been used to assess pain, pleasure, malaise or bitter tasting in several species (Langford et al., 2010; Defensor et al., 2012; Dolensek et al., 2020). Most facial expressions analyses in rodents are based on the pioneer work of Langford and colleagues (Langford et al., 2010) and the development of their Mouse Grimace Scale (MGS). This scale, and those derived, usually focus on three major facial areas: eyes, ears and muzzle; and rely on a three-point rating of facial expressions of pain: 0 "absent," 1 "moderate" or "inconclusive," 2 "obvious." Despite the successful establishment of the MGS, and the subsequent rising interest for animal facial expressions, most of the research in the field focuses on pain responses, and little work has

been done on other negative affective scales (e.g., fear, frustration, anxiety) or positive ones (Mogil et al., 2020).

Positive emotions are notoriously difficult to observe or quantify in animals, compared with negative affective states (Paul et al., 2005). An earlier study found no changes in MGS scores in a positive situation elicited by rat tickling, suggesting that these grimaces might be almost exclusively related to pain (Finlayson et al., 2016). Sucrose tasting, or foods conveying an equivalent hedonic value are the gold standard to evaluate positive affective states. However, this solution is not free of confounds, as tasting per se requires the solicitation of facial muscles, and food reward alone cannot accurately represent the entire range of positive affects. Moreover, defining boundaries between different emotions is challenging (Ekman, 2009), and complex emotions can overlap. Consequently, affective states are regarded as a continuum rather than categorical entities. With regards to these concerns, research still lacks quantitative methods to identify and quantify affective states in animals (Middleton et al., 2021). MGS-based assessments lack fine quantification of facial changes, and newly developed methods using machine-learning algorithms, although powerful, do not provide an understanding of the processes underpinning facial expressions categorization and interpretation. Moreover, the latter may not be easily adaptable to freely behaving animals.

In this study, we propose a new facial expression model based on features both visualizable by a human observer and finely quantifiable for analysis. We compare these features across a range of different stimuli, including two pain models as well as positive and aversive tastants. To extend the range of positive stimuli tested on facial expressions, we include several non-noxious somatosensory stimuli. Gentle, affiliative touch signaled through low-threshold C-fiber mechanoreceptors (C-LTMRs) carries a positive affective value in a wide range of species including social rodents (Cho et al., 2021; Pawling et al., 2017). We analyzed the changes in facial expression over sequences of emotion-inducing stimuli to (1) establish the facial expression associated to each stimulation, (2) determine whether different stimulations differentially affect facial features, and (3) identify the facial features conveying the most emotional valence. Finally, while facial expressions in theory convey emotions in a time-sensitive manner, few studies have exploited the dynamics of expressions changes. Therefore, we monitored facial expressions surrounding brief optogenetic stimulation of CGRP expressing nociceptors. Similarly, we analyzed facial changes over time in a well-established pain assay using formalin, a compound known to elicit two phases of pain behavior separated by an inactive phase, hypothesizing that facial expressions would show a similar pattern.

Materials and Methods

Animals

Five experiments were conducted, including a total of 90 C57BL/6JRj mice from Janvier Laboratories and 16 CGRP; LSL-ReaChR mice bred in house (see details below, Optogenetic stimulation; 62 females (F): 10.8 ± 0.2 weeks old,

20.5 ± 0.2 g; and 44 males (M), 10.5 ± 0.2 weeks old, 26.7 ± 0.4 g on testing). The mice were housed in NexGen IVC system Mouse 500 at 20 ± 2°C, under a 12/12 h light/dark cycle (12L:12D light cycle), lights being on at 7 A.M. Mice included in the first experiment [sucrose-mentholatum-petting (SMP); n = 45] were previously used in an unrelated study without experimental intervention, for 6 d before the experiment, where they were submitted to the same temperature and light cycle. In this previous experiment, groups of five individuals (4 F + 1 M) were housed together in an enriched semi-natural environment. For identification purpose, some of these mice had different patterns shaved on the neck or the back.

All experimental procedures employed in the present experiment were approved by the Local Ethics Committee for Animal Experiments at Linköping University; and in agreement with the European Union council directive 2010/63/EU.

Apparatus

The experimental box was a clear Plexiglas cubicle of dimensions 9 cm long × 5 cm high × 5 cm wide, pierced with holes on the roof to allow for ventilation, and on the side to introduce a pipet containing tastants. The box was cleaned and disinfected between each individual session.

Procedure

Mice were individually introduced to the experimental cubicle where their facial expressions were recorded from the side, using a monochrome Basler ace camera (acA1300-60gm) or a color FLIR camera (Blackfly BFS-U3-23S3C-C) with a 1:1.8/4 mm Basler lens (C125-0418-5M) and the Open Broadcaster Software 27.0.1 (https://obsproject.com/). All experiments took place between 9 A.M. and 13 P.M., during the light phase of the light cycle. Following experiment completion, animals were killed using carbon dioxide asphyxiation and cervical dislocation.

Experiment SMP

Forty-five mice (36 F, 9 M) were included in the SMP experiment consisting of a sucrose-mentholatum-petting (SMP) stimulus sequence in a fixed order, for 5 min each. Each mouse was successively placed in the cubicle, where it was left undisturbed for 5 min to establish a baseline, and then submitted to the sequence. The first stimulus was a 20% sucrose solution (84100, Sigma-Aldrich) introduced by a pipet through the hole pierced in the short side of the cubicle. Tasting was entirely voluntary from the mice. Following sucrose stimulus, a second pipet was introduced containing a bitter solution. This solution was prepared by diluting three drops of anti-bite nail polish (Mentholatum) into 10 ml of water. Finally, the mice were gently petted by the experimenter's finger, from the base of the neck to the lower back, at a velocity averaging 5 cm/s. Among the 45 mice included in this study, 20 (16 F, 4 M) were naive to petting, and 25 had been habituated to it. The habituation protocol consisted in petting the mice 5 min every day, for 6 d before the experiment day. The stimuli-sequence followed a fixed order to optimize taste differentiation, avoid solution contamination

and maximize the accuracy of the elicited emotional state. The bitter stimulus, mentholatum, was given after sucrose as aversive stimuli produce stronger responses than palatable ones. In addition, bitterness tends to be a persistent taste that could have confounded sucrose results. Petting the mice required opening the cubicle, which could cause the mouse to escape, thus it was kept as the last stimulus.

Experiment SQB

Twenty mice (10 F, 10 M) were included in experiment SQB, which consisted of the stimulus sequence sucrose-quinine-brushing in a fixed order, for 5 min each. The same procedure as in experiment SMP was applied. The quinine stimulus was a 0.8 M quinine solution (22630, Sigma-Aldrich). This concentration produced a distinctively less bitter taste than the mentholatum dilution. Brushing was performed by the experimenter with a small paint brush made of synthetic hair (Panduro, 1-cm-wide flat tip), using the same pressure and velocity as in the petting phase of the SMP experiment. Ten mice (5 F, 5 M) were habituated to brushing before the experiment. Habituated mice were individually brushed for 5 min each, every 2 d, for 12 d before the SQB experiment, resulting in six habituation sessions.

Cutaneous touch experiment

Twenty mice (10 F, 10 M) were individually placed into the observation box, where after a 5-min baseline, they were given water to taste through a pipet. Notably, the mice were not water-deprived before experiment. Then, the mice were submitted to a sequence of three cutaneous stimuli in a randomized order. The mice were petted by the experimenter's finger, as in experiment SMP, brushed with the small paint brush as in experiment SQB, and gently poked. Poking consisted of applying the tip of the brush handle on the neck of the mouse, with the same pressure as during brushing and petting, but without motion. Half of the mice had been habituated to the cutaneous stimuli before the experiment, following the same habituation protocol as in experiment SQB.

Formalin test

Twenty mice (10 F, 10 M) went through the formalin test, among which six males and nine females were reused from the SQB experiment. In the first phase of the experiment, the mice were introduced to the observation cubicle, and left undisturbed for 30 min to establish a baseline. Immediately after the end of baseline, each mouse was placed back in its home cage for 5 min to allow for drinking and feeding. Afterwards, the mouse was quickly brought to an anesthesia chamber where it was lightly anesthetized with isoflurane (3.5%). The mouse then received a single 20-μl intradermal injection of either vehicle (saline, 9 mg/ml, n = 10, 5 F, 5 M), or formalin (2% in saline, from Sigma-Aldrich F8775) in the plantar hind paw. When the injection was completed, the mouse was brought back into the cubicle. The observation resumed as soon as the mouse woke up, and carried on for the next 30 min. The interval between the beginning of the anesthesia and the beginning of the second observation phase was <5 min.
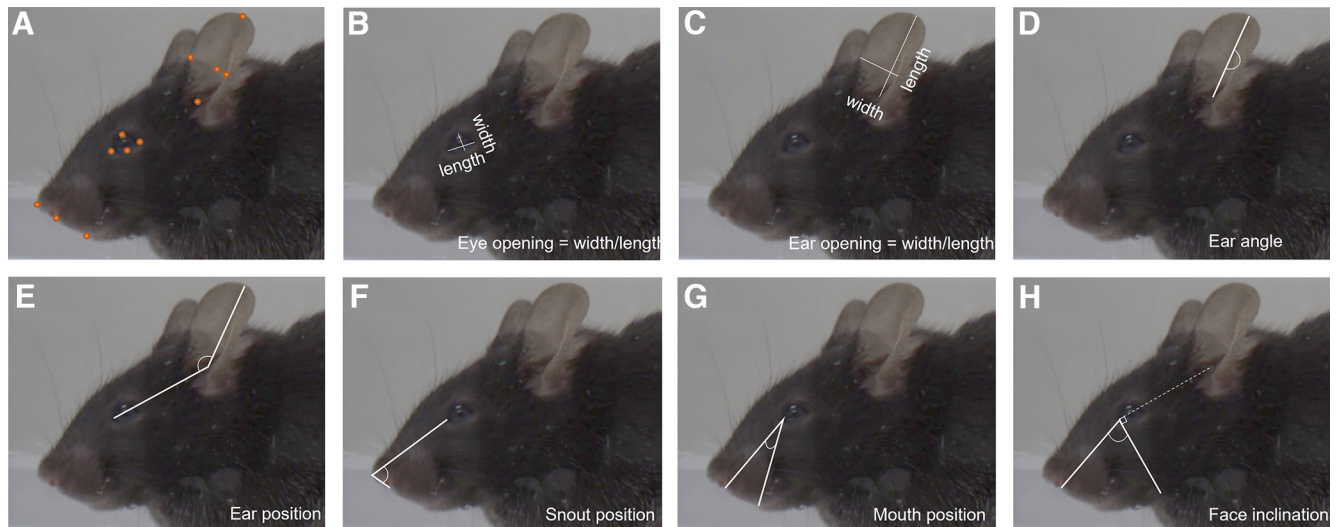
Figure 1. Parameters retained for facial expression analysis. ***A***, Easily visualizable facial points on mouse profile. ***B***, Eye opening. Larger values correspond to a rounder, more open eye, a ratio of 1 corresponded to a perfectly round eye. ***C***, Ear opening. Larger values correspond to the ear being placed side way, a ratio approaching 1 meaning a largely deployed and clearly visible ear pavilion. Smaller values indicate the ear being positioned higher up the head and thus a less visible ear pavilion. ***D***, Ear angle. Decreased values refer to the ear being tilted instead of straight. ***E***, Ear position. Larger values indicate the ear positioned backward. ***F***, Snout position. Smaller values indicate a pointier snout and larger values a rounder one. ***G***, Mouth position. Smaller values correspond to the bottom lip being brought forward. ***H***, Face inclination. This parameter is measured as the angle between lower snout, corner of the eye and ear orifice, to which 90° are subtracted. Smaller values indicate a more convex profile.

Optogenetic stimulation

In order to optogenetically target a broad population of primary afferent nociceptors, we used a recombinase dependent mouse line, Calca^{Cre};R26$^{LSL\_ReaChR}$ which expressed the red-shifted channelrhodopsin ReaChR (Lin et al., 2013) under the control of Cre expressed from the Calca [encoding calcitonin gene-related peptide (CGRP)] locus (referred to as Het mice in the rest of the manuscript). These mice were generated by crossing homozygous Calca^{Cre} mice (#033168, The Jackson Laboratory; Carter et al., 2013) with heterozygous R26$^{LSL\_ReaChR}$ mice. The latter mice were in turn generated by crossing R26$^{LSL\_FSF\_ReaChR-mCitrine}$ mice (#024846, The Jackson Laboratory) with a FlpO deleter mouse strain, B6 ROSA26Flpo (#012930, The Jackson Laboratory; Raymond and Soriano, 2007). As control, we used littermate LSL_ReaChR negative mice (referred to as WT).

Sixteen (5 F, 11 M) mice were included in the experiment, including nine Het (2 F, 7 M) and seven WT controls (3 F, 4 M). The mice were placed in the observation box and left to habituate for 3 min. Optogenetic stimulation was powered by a LED current driver through a 595-nm LED (Doric Lenses). Light was delivered to the base of the neck with an optical fiber patch cord (length 1 m; 0.63 NA; 960 μm in diameter) producing 19.2-mW power at the tip of the fiber; the tip was placed an estimated average distance of 3 mm from the surface of the skin as measured from the video recordings, covering ~25 mm^2 of the skin surface. The mice were stimulated for 3 min at 20 Hz with 10-ms pulses. Following the end of optogenetic stimulation, the mice were left to recover for 3 min. All mice were shaved 24 h before the experiment on the stimulation area at the base of the neck to ensure direct light exposure to the skin.

Facial parameters

The review of rodent facial expression studies in the literature yielded to the identification of facial regions consistently altered by experimental procedures, including the ears, eyes and muzzle (Langford et al., 2010; Finlayson et al., 2016; Andresen et al., 2020; Dolensek et al., 2020). In turn, we identified several easily visualizable points on mouse lateral views, which allow the precise quantification of changes in these areas. Several points of reference were tested to measure mouth-, snout-related and ear-related changes, including a measure of the visible area of the inner ear. Eventually, the reference points used for measuring each facial parameter were selected to maximize the capture of facial changes and limit parameter redundancy. Therefore, following extensive measurements, we retained seven facial parameters that capture quantitative changes associated with facial displays: eye opening, ear opening, ear angle, ear position, mouth position, snout position and face inclination (Fig. 1). Retained parameters included only ratios and angles, to prevent confounds such as camera zoom or individual size.

Data collection

Frame extraction

Screenshots were manually extracted from video clips using VLC media player (https://www.videolan.org/). As many clear profile frames as possible were captured in each video. With regards to the tastants (water, sucrose, mentholatum, quinine), screenshots were captured exclusively when the mice had tasted the solutions, and in the 5 s following it, to avoid for taste fading. Pictures in which the mice were displaying a behavior such as licking the

pipet or self-scratching were excluded, in order not to confound the facial changes elicited by the stimuli with voluntary mechanical movements. For the formalin test, a screenshot was captured every minute along the 30-min baseline and the 30-min experimental phase, resulting in 60 frames per individual.

Facial parameter measurements and interobserver reliability

All selected frames were imported into IC Measure (https://www.theimagingsource.com/), and the defined facial parameters were manually measured on each frame and reported into a master sheet. Measurements were made by five independent observers. All seven facial parameters retained were measured by an experienced observer on an initial selection of 144 frames corresponding to five individuals going through the SMP experiment. Two naive observers then measured the same facial parameters, resulting in good to excellent interobserver reliability assessed by intraclass correlation coefficient (ICC), ranging from 0.801 for ear angle to 0.907 for ear position. Only snout position showed moderate reliability with a coefficient of 0.655. In all other experiments, the observers were blind to the stimulation during measurements.

Data preparation

The measures extracted from all frames related to one individual at baseline or during stimulations were averaged, to obtain a single value per stimulus, per individual. Consequently, an individual data point was based on an average of 4.96 measurements. In addition, for each stimulus for each individual, the proportional change from baseline was calculated with the formula: (value at stimulus – value at baseline)/value at baseline. Animals that belonged to different experiments but had been subjected to the same stimulation were grouped together when assessing the effect of that stimulation. Stimulations were then divided between gustatory tastants, and cutaneous stimuli: poking, petting, brushing. The formalin test and the optogenetic test were analyzed separately.

In the formalin test, response profiles are based on the entire 30 min of baseline or saline injection, as preliminary data inspection showed no changes in facial parameters over time in these cases. To the contrary, formalin is well known to elicit nocifensive responses in a two-phase pattern (Dubuisson and Dennis, 1977). Thus, the first 5 min following injection were used to constitute the early phase of formalin effect, and the 10-min interval between 20 and 30 min following injection was used for the late phase.

For the temporal analysis of facial expressions during the formalin test, the data from the 30-min baseline were averaged into a single reference point. The 30-min phase following injections was separated into six 5-min intervals. Thus, the data from the 5 min corresponding to each interval were averaged to obtain a single value per interval per individual.

Statistical analysis
Control for age, sex, weight, and shaving

First, we explored the link between mouse weight and age on facial parameters at all baseline values using Pearson's correlations. Then, we tested the effect of weight and age on the proportional change from baseline independently for each given stimulus (water, sucrose, quinine, mentholatum, poking, petting, brushing, the first 5 min following formalin injection, 30 min following saline injection, and optogenetic stimulation of Het mice) with Pearson's correlations.

We controlled for sex difference at baseline values, and again on the proportional change from baseline for each given stimulus with t tests. This effect was not investigated during optogenetic stimulation because of the unbalanced sex ratio (2 F vs 7 M).

Finally, we controlled for the effect of shaving patterns on facial parameter values in response to petting during in the SMP experiment, using one-way ANOVAs. The SMP experiment was the only one in which most animals presented a shaving pattern and received a cutaneous stimulus. Therefore, it was irrelevant to control for shaving effect on other experiments.

Changes in facial parameters in response to gustatory and somatosensory stimuli

We proceeded to analyze the responses to drinkable stimuli (water, sucrose, quinine, mentholatum) through one-way ANOVAs followed by Tukey's *post hoc* tests. We did not account for subject repetition as not all animals were given the same stimuli.

Responses to cutaneous stimuli (poking, petting, brushing) and the effect of the habituation protocol on these responses were analyzed with two-way ANOVAs using "habituation" and "stimulus" as factors. Tukey's multiple comparison test was used for *post hoc* analysis.

The effect of optogenetic stimulation on facial parameters was analyzed through two-way ANOVAs with "stimulus" and "genotype" as factors, with repeated measures on the "stimulus" factor, followed by the Tukey's *post hoc* test in case of main effect of the stimulus. In case of significant interaction, the Šidák's multiple comparison test was used.

The temporal changes in facial parameters after saline and formalin injections were assessed using one-way ANOVAs with repeated measures, followed by Dunnett's *post hoc* test with baseline as control.

In order also to account for individual repetition across stimuli, we plotted response profiles to each stimulus using the proportional change in each facial parameter compared with its baseline value. Difference from baseline was analyzed by one-sample t tests, where p-values were adjusted with the Bonferroni correction for the number of comparisons done in the experiment (see figure legends).

Finally, we compiled a master file based on all individual values from frame averages, with respect to each stimulus ($n = 405$ units: baseline = 114; sucrose = 61, mentholatum = 35; water = 18; quinine = 17; poking = 18; petting = 63; brushing = 40; saline = 10; formalin: early phase = 10, late phase = 10; optogenetic = 9). We used principal component analysis (PCA) to assess the use of dimensions reduction and visualize the relative contribution of each facial parameters to the variance of the dataset. We determined with

Horn's parallel analysis the number of components with eigenvalues superior to 1 after adjustment by comparison with a random dataset generated using Monte-Carlo simulation. Then, we visualized stimulus characterization based on facial expression using the uniform manifold approximation and projection (UMAP) algorithm to compute two-dimensional embeddings for each individual mouse average. We used default hyperparameters for our projection, calculating the Euclidean distance between each pair of individual averages, considering a minimum distance of 0.1 between points, and 15 neighbors.

Data analysis was conducted with GraphPad Prism 9 and R 4.1.3, using the irr, ggplot2, tidyverse, PerformanceAnalytics, FactoMineR, factoextra, paran, umap, and scales packages.

Data availability

Data are available on reasonable request.

Results

Age, weight, sex and shaving pattern

First, we tested the influence of weight on absolute values at baseline for each facial parameter, all experiments pooled. We found a moderate correlation between weight and ear position, with heavier mice having ears more backward (Pearson $t_{(119)} = 2.24$, $p = 0.027$, $\rho = 0.20$). Weight also negatively correlated with snout position (Pearson $t_{(119)} = -3.20$, $p = 0.002$, $\rho = -0.28$), the snout being pointier in heavier animals. No other correlations between weight and baseline values were significant (all $ps > 0.073$). We explored correlations between weight and proportional change from baseline for each given stimulus and found a single correlation between mouth position and weight in Het mice during optogenetic stimulation (Pearson $t_{(7)} = -2.70$, $p = 0.031$, $\rho = -0.71$). No other correlation was significant (all $ps > 0.059$).

Then, we tested whether mouse age at the time of the experiment affected absolute values at baseline. Conversely to weight, mouse age was positively correlated with snout position (Pearson $t_{(119)} = 3.32$, $p = 0.001$, $\rho = 0.29$). Age also mildly correlated eye opening at baseline (Pearson $t_{(119)} = -2.50$, $p = 0.014$, $\rho = -0.22$). We found a significant correlation between age and the proportional change from baseline in ear opening during poking (Pearson $t_{(16)} = 3.48$, $p = 0.003$, $\rho = 0.66$) and in eye opening during petting (Pearson $t_{(61)} = 2.30$, $p = 0.025$, $\rho = 0.28$). Other correlations were not significant (all $ps > 0.078$).

Lastly, we controlled for the effect of sex on baseline values and found no significance (all $ps > 0.124$). When looking at proportional changes from baseline for each stimulus, we found a single sex effect showing larger decrease in ear angle in males than in females during poking ($t_{(2.19)} = 13.88$, $p = 0.046$). Other stimuli did not show a sex effect (all $ps > 0.066$).

Weight, age and sex showed inconsistent effects that did not appear meaningful for the present study. Therefore, these factors were not accounted for further in the analyses. Finally, we controlled for the effect of shaving. Shaved patterns did not influence absolute facial parameters values in response to petting during the SMP experiment (all $ps > 0.135$).

Water and a bitter tastant elicit opposite changes in facial parameters

Tastants comparison

The only facial parameter that was not affected by the tastants was ear angle ($F_{(4,211)} = 0.99$, $p = 0.412$). This means that the ear remained consistently straight during exposure to gustatory stimuli. We found a significant effect of the stimuli on eye opening ($F_{(4,211)} = 11.88$, $p < 0.001$), ear opening ($F_{(4,211)} = 9.47$, $p < 0.001$), ear position ($F_{(4,211)} = 19.87$, $p < 0.001$), snout position ($F_{(4,211)} = 2.95$, $p = 0.021$), mouth position ($F_{(4,211)} = 4.39$, $p = 0.002$), and face inclination ($F_{(4,211)} = 17.45$, $p < 0.001$). Tasting familiar water and bitter mentholatum solution elicited the strongest and most opposite facial changes (Fig. 2).

Drinking mentholatum decreased eye opening compared with baseline ($p < 0.001$), as well as water ($p < 0.001$) and sucrose ($p < 0.001$). Drinking water did not modify eye opening in comparison to baseline ($p = 0.054$), but increased it compared with sucrose ($p = 0.045$) and quinine ($p = 0.016$; Fig. 2A). Ear opening was only modified by water drinking, during which it was reduced compared with baseline and all other stimuli (all $ps < 0.005$), corresponding to the ear being placed up on the head (Fig. 2B). Ear position was reduced by all stimuli compared with baseline (all $ps < 0.030$), which corresponds to the ear pointing forward. In addition, sucrose brought the ear further forward than mentholatum did ($p = 0.001$; Fig. 2D). Only water showed an effect on snout position, producing a rounder snout compared with baseline ($p = 0.019$; Fig. 2E). No stimulus caused a mouth position different from baseline (all $ps > 0.149$). However, sucrose and quinine increased mouth position values, positioning the mouth backward, compared with mentholatum ($p = 0.011$ and $p = 0.005$, respectively; Fig. 2F). Finally, drinking water increased face inclination values compared with baseline ($p = 0.005$) producing a flatter face profile, whereas mentholatum decreased them, producing a more convex face, compared with baseline and all other stimuli (all $ps < 0.001$; Fig. 2G). We found no other effects of the tastants on facial parameters (all $ps > 0.054$).

Response profiles to tastants

Responses profiles are summarized in Figure 3. Drinking water proportionally flattened the face ($t_{(17)} = 3.18$, $p = 0.016$) and rounded the snout compared with baseline ($t_{(17)} = 3.34$, $p = 0.012$), while it positioned the ear up and pointing forward ($t_{(17)} = 4.59$, $p = 0.001$ and $t_{(17)} = 6.13$, $p < 0.001$, respectively; Fig. 3A). Similarly, but to a lesser extent, sucrose proportionally flattened the face ($t_{(60)} = 2.54$, $p = 0.027$) and rounded the snout ($t_{(60)} = 2.76$, $p = 0.015$), together with bringing the ear forward ($t_{(60)} = 9.72$, $p < 0.001$; Fig. 3B). The mentholatum solution proportionally made the face more convex ($t_{(34)} = 4.68$, $p < 0.001$), brought the ear forward ($t_{(34)} = 2.96$, $p = 0.011$), closed the eye ($t_{(34)} = 3.88$, $p = 0.001$), and rounded the snout ($t_{(34)} = 3.48$, $p = 0.003$; Fig. 3C). Quinine, which at the concentration used was deemed by the experimenter to be less bitter than the mentholatum solution, showed no significant proportional change from
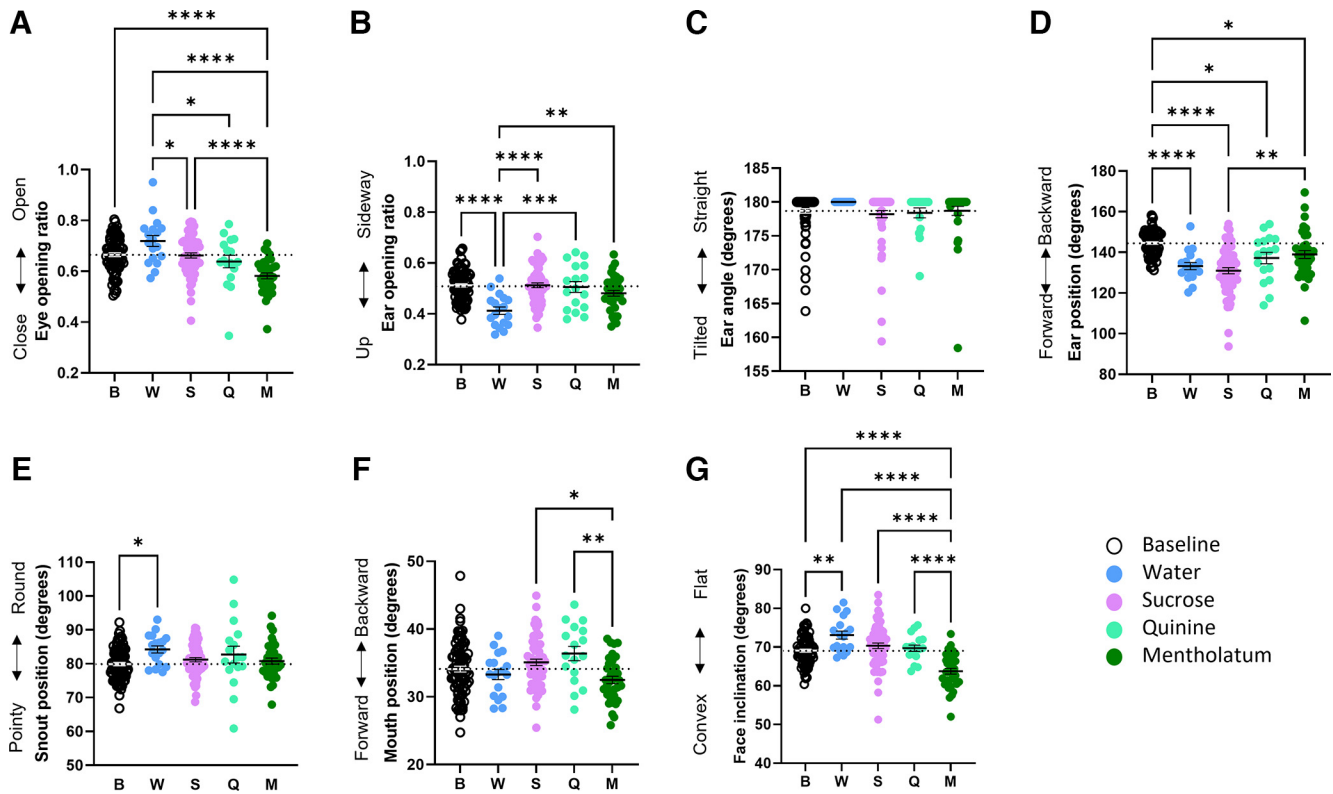
Figure 2. Effect of tastants on facial parameters. **A**, Eye opening ratio. **B**, Ear opening ratio. **C**, Ear angle. **D**, Ear position. **E**, Snout position. **F**, Mouth position. **G**, Face inclination. Data are individual data points. One-way ANOVA followed by Tukey's HSD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Baseline, $n = 85$; water: $n = 18$; sucrose: $n = 61$; quinine: $n = 17$; mentholatum: $n = 35$. A dash line indicates the baseline value.

baseline but induced similar tendencies as mentholatum in eye opening ($t_{(17)} = 2.22$, $p = 0.082$) and ear position ($t_{(17)} = 2.39$, $p = 0.059$; Fig. 3D). Other proportional changes in facial parameters did not significantly differ from baseline (all $ps > 0.136$).

Painful and innocuous somatosensory stimuli differentially affect facial parameters

Response profiles to mechanical somatosensory stimuli

Response profiles are based on pooled data of naive and touch-habituated mice. Poking the mice on the neck produced a tilted ear compared with baseline ($t_{(17)} = 3.66$, $p = 0.006$), associated with an ear placed backwards ($t_{(17)} = 2.79$, $p = 0.038$), a larger eye opening ($t_{(17)} = 3.50$, $p = 0.008$) and a flattened face ($t_{(17)} = 3.79$, $p = 0.003$; Fig. 4A). Petting and brushing the mice in the same area produced a similar response profile as poking, tilting the ear ($t_{(62)} = 8.60$, $p < 0.001$ and $t_{(39)} = 7.89$, $p < 0.001$, respectively), placing it backwards ($t_{(62)} = 8.57$, $p < 0.001$ and $t_{(39)} = 3.50$, $p = 0.004$, respectively) and increasing eye opening ($t_{(62)} = 6.86$, $p < 0.001$ and $t_{(39)} = 3.94$, $p = 0.001$, respectively). The face was also flattened in the case of petting ($t_{(62)} = 2.66$, $p = 0.030$) but not of brushing ($t_{(39)} = 1.58$, $p = 0.364$). Instead, both petting and brushing resulted in the ear being placed on the side of the head (all $ps < 0.001$), a pattern not significant when poking the mice ($t_{(17)} = 2.33$, $p = 0.097$), together with the mouth being positioned

backward too (all $ps < 0.002$), which was also non-significant during poking ($t_{(17)} = 2.53$, $p = 0.065$; Fig. 4B,C). Cutaneous stimuli did not produce other significant proportional changes from baseline in facial parameters (all $ps > 0.364$).

Optogenetic stimulation of the nape of the neck in WT mice only significantly reduced eye opening ($t_{(6)} = 2.99$, $p = 0.024$; Fig. 4D). All other parameters were unaffected (all $ps > 0.059$). To the contrary, in Het mice, optogenetic stimulation reduced eye opening ($t_{(8)} = 3.59$, $p = 0.007$), tilted the ear ($t_{(8)} = 4.82$, $p = 0.001$), brought the ear and the mouth backward (ear position: $t_{(8)} = 9.13$, $p < 0.001$; mouth position: $t_{(8)} = 2.55$, $p = 0.034$), and made the face more convex ($t_{(8)} = 4.00$, $p = 0.004$). Ear opening and snout position were unaffected (all $ps > 0.123$; Fig. 4E).

Mice injected with saline in the hind paw showed no proportional facial change from baseline (all $ps > 0.217$; Fig. 4F). During the first 5 min following formalin injection into the hind paw, we observed a smaller eye opening ($t_{(9)} = 2.77$, $p = 0.022$), the ear being placed sideway and backwards (ear opening: $t_{(9)} = 2.94$, $p = 0.016$; ear position: $t_{(9)} = 3.93$, $p = 0.004$), a pointier snout ($t_{(9)} = 3.94$, $p = 0.003$) and a more convex face ($t_{(9)} = 4.39$, $p = 0.002$; Fig. 4G). During the late phase of the formalin test, three of the five changes observed during the first phase were conserved: the eye was more closed ($t_{(9)} = 5.38$, $p < 0.001$), the ear positioned backward ($t_{(9)} = 3.21$, $p = 0.011$) and the face more convex ($t_{(9)} = 4.68$, $p = 0.001$; Fig. 4H).
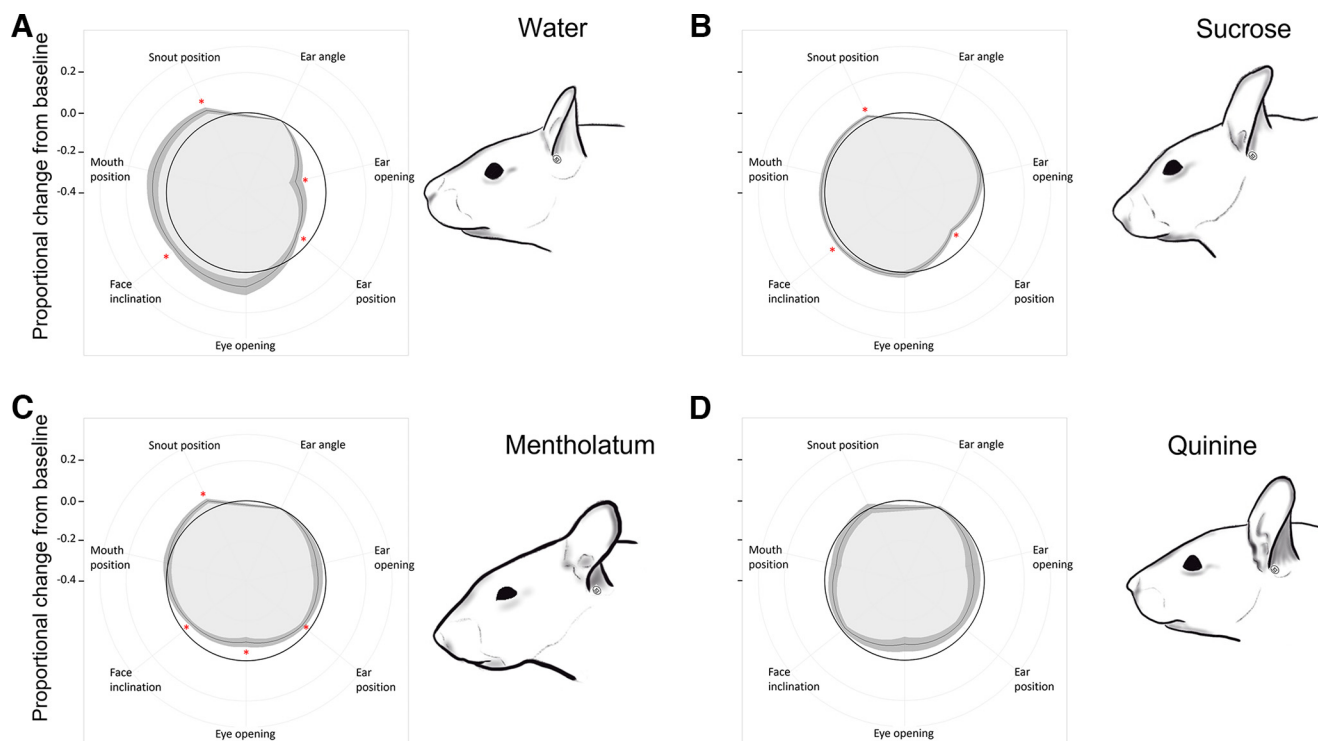
Figure 3. Response profiles to tastants and visual representation of the typical facial expression associated with each. **A**, Response profile to water; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for three comparisons. **B**, Response profile to sucrose; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for two comparisons. **C**, Response profile to mentholatum; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for two comparisons. **D**, Response profile to quinine; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for two comparisons. Data are mean ± SEM. One-sample t tests. Water: $n = 18$; sucrose: $n = 61$; quinine: $n = 17$, mentholatum: $n = 35$. Drawings are based on a quantitatively representative picture among those used for measurements. Drawings by OLM.

Habituation to light touch only modestly modulates facial responses

There was a main effect of cutaneous stimuli on all facial parameters measured but snout position: eye opening ($F_{(3,198)} = 19.69$, $p < 0.001$), ear opening ($F_{(3,198)} = 9.30$, $p < 0.001$), ear angle ($F_{(3,198)} = 33.74$, $p < 0.001$), ear position ($F_{(3,198)} = 24.16$, $p < 0.001$), snout position ($F_{(3,198)} = 2.53$, $p = 0.059$), mouth position ($F_{(3,198)} = 8.40$, $p < 0.001$), and face inclination ($F_{(3,198)} = 5.82$, $p = 0.001$). We found no interaction between habituation and stimuli for any of the parameters (all $ps > 0.077$).

Poking, petting, and brushing the mice all increased eye opening compared with baseline (all $ps < 0.001$; Fig. 5A). The ear was placed sideway during petting ($p = 0.010$) and brushing ($p < 0.001$), but not poking ($p = 0.59$; Fig. 5B). All cutaneous stimuli tilted the ear compared with baseline (all $ps < 0.001$; Fig. 5C). The same pattern was observed on ear position, with all cutaneous stimuli moving the ear backwards (all $ps < 0.012$; Fig. 5D). Petting the mice elicited the most backward ear position, significantly more than during brushing ($p = 0.001$; Fig. 5D). Brushing moved the mouth backward compared with baseline ($p < 0.001$) and to other cutaneous stimuli (poking, $p = 0.009$; petting, $p = 0.001$; Fig. 5F). Finally, poking and brushing the mice, but not petting them ($p = 0.400$), flattened the face compared with baseline (poking-baseline, $p = 0.012$; brushing-baseline, $p = 0.004$; Fig. 5G).

We found a moderate effect of the habituation protocol. Habituation increased the pattern of ear opening caused by cutaneous stimuli ($F_{(1,198)} = 15.98$, $p < 0.001$; Fig. 5B). In parallel, habituation attenuated the ear tilt caused by poking, petting, and brushing ($F_{(1,198)} = 6.10$, $p = 0.014$; Fig. 5C).

Facial expressions convey pain-associated affects in real time

Optogenetic stimulation of CGRP-lineage sensory fibers

We found an interaction between genotype and optogenetic stimulation on ear angle ($F_{(2,28)} = 5.91$. $p = 0.007$), ear position ($F_{(2,28)} = 23.10$, $p < 0.001$), and face inclination parameters ($F_{(2,28)} = 4.78$, $p = 0.016$). Het, but not WT mice, had a tilted ear during stimulation compared with baseline and recovery periods (Het: stimulation-baseline: $p = -0.004$; stimulation-recovery: $p = 0.005$; WT: all $ps > 0.167$; Fig. 6C). Similarly, only Het mice showed a backward ear position during optogenetic stimulation compared with both baseline ($p < 0.001$) and recovery period ($p < 0.001$). Furthermore, Het mice showed a more forward ear position during recovery than at baseline ($p = 0.005$; Fig. 6D). Finally, Het mice had a more convex face profile during optogenetic stimulation compared with baseline ($p = 0.012$) and recovery ($p = 0.010$; Fig. 6G).
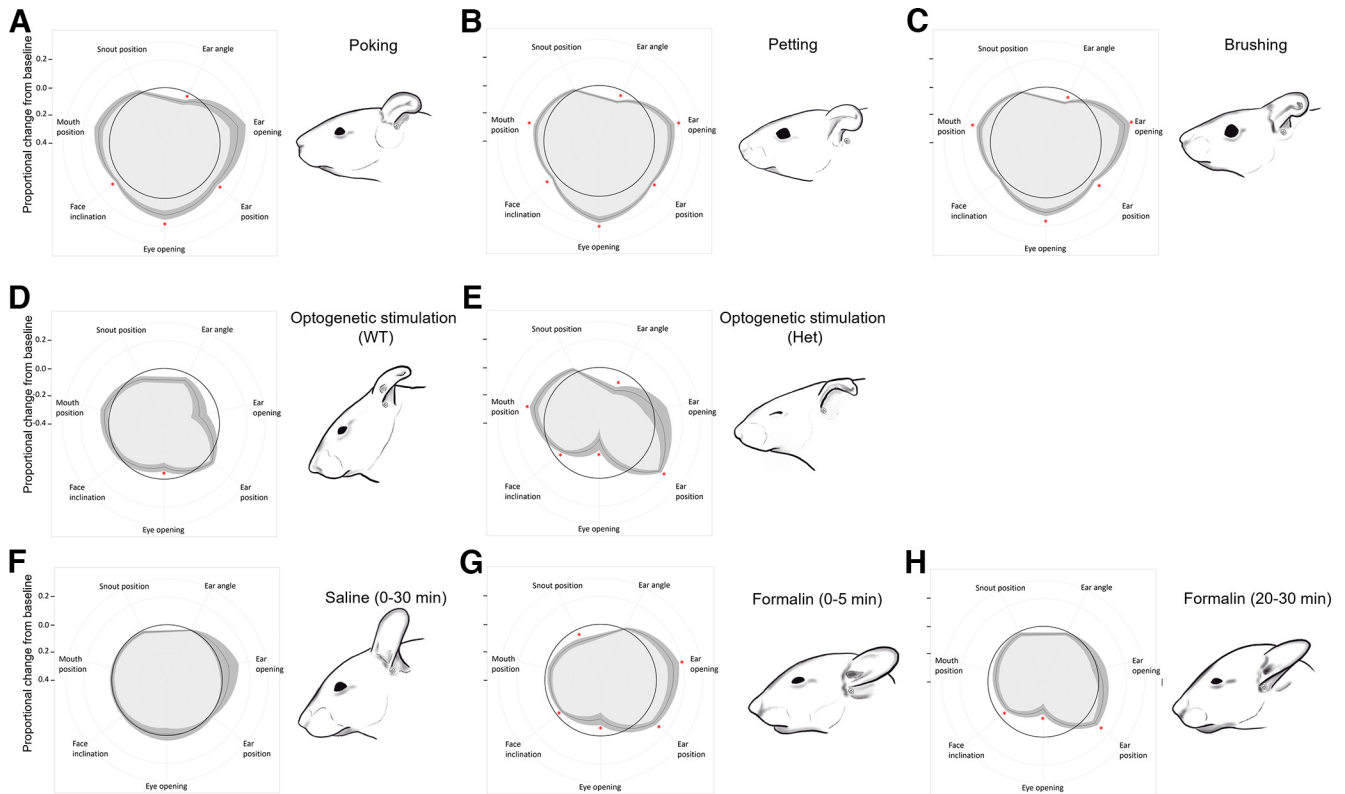
Figure 4. Response profiles to somatosensory stimuli and visual representation of the typical facial expression associated with each. **A**, Poking; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for three comparisons. **B**, Petting; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for three comparisons. **C**, Brushing; *$p < 0.05$, reported values are adjusted with the Bonferroni correction for three comparisons. **D**, Optogenetic stimulation of the neck in WT mice; *$p < 0.05$. **E**, Optogenetic stimulation of the neck in Het mice; *$p < 0.05$. **F**, Saline injection into the hind paw (30-min average); *$p < 0.05$. **G**, Early phase of formalin injection into the hind paw (0–5 min postinjection); *$p < 0.05$. **H**, Late phase of formalin injection into the hind paw (20–30 min postinjection); *$p < 0.05$. Data are mean ± SEM. One-sample t tests. Poking: $n = 18$; petting: $n = 63$; brushing: $n = 40$; optogenetic WT: $n = 7$; optogenetic Het: $n = 9$; saline: $n = 10$; formalin early and late phases: $n = 10$. Drawings are based on a quantitatively representative picture among those used for measurements. Drawings by OLM.
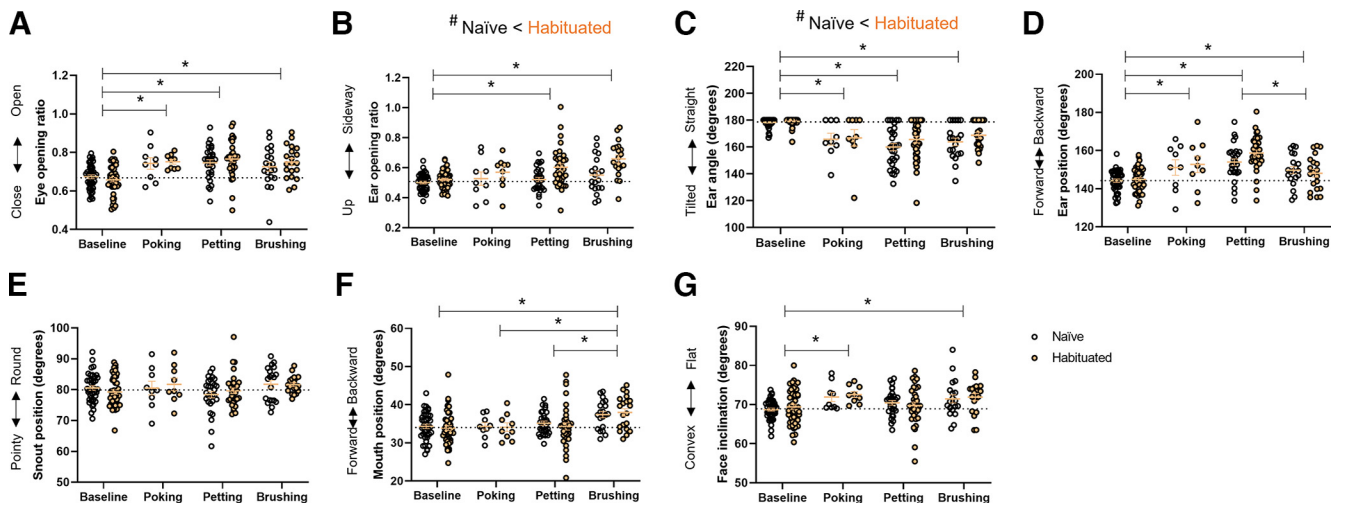

Figure 5. Effect of habituation on cutaneous stimuli. **A**, Eye opening ratio. **B**, Ear opening ratio. **C**, Ear angle. **D**, Ear position. **E**, Snout position. **F**, Mouth position. **G**, Face inclination. Data are individual data points. A dash line indicates the average baseline value. Two-way ANOVA. Stimulus effect: *$p < 0.05$. Main habituation effect: #$p < 0.05$. Baseline: $n = 40$ naive + 45 habituated; poking: $n = 9$ naive + 9 habituated; petting: $n = 29$ naive + 34 habituated; brushing: $n = 20$ naive + 20 habituated.
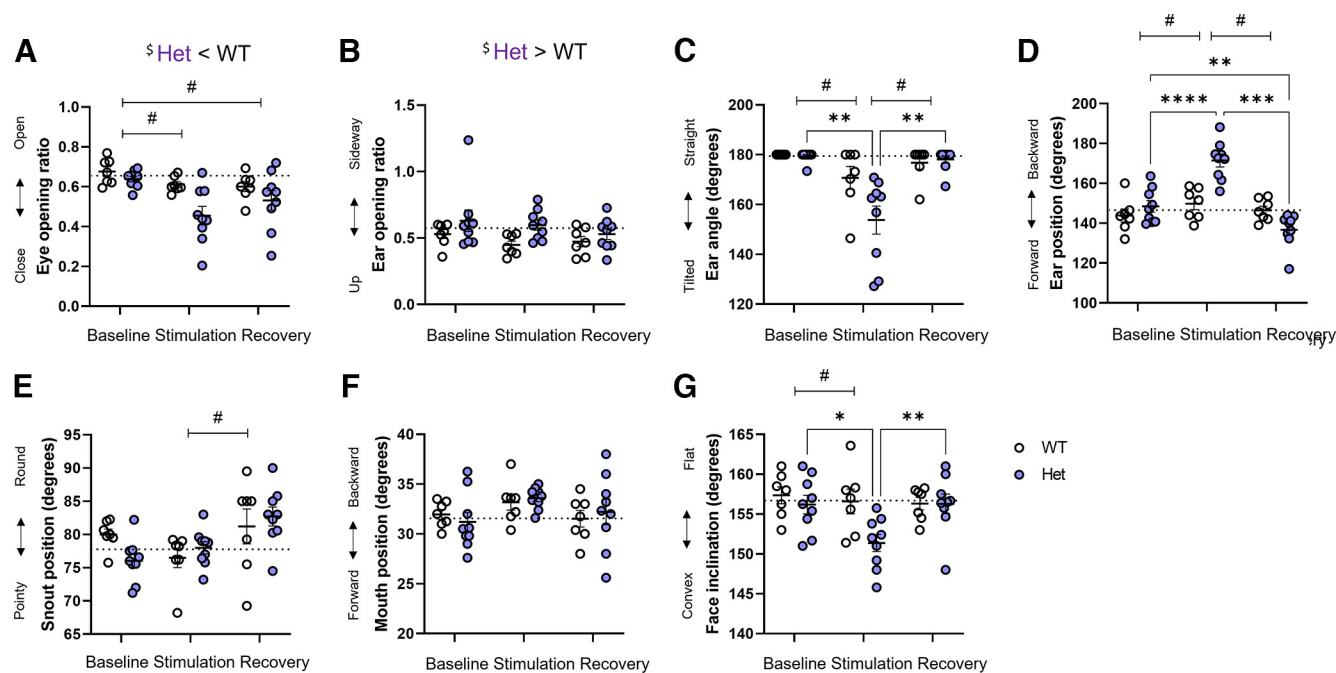
Figure 6. Effect of optogenetic stimulation of the nape of the neck on CGRP;LSL-ReaChR mice. **A**, Eye opening ratio. **B**, Ear opening ratio. **C**, Ear angle. **D**, Ear position. **E**, Snout position. **F**, Mouth position. **G**, Face inclination. Data are individual data points. A dash line indicates the average baseline value. Repeated measures two-way ANOVAs. Interaction between stimulus and genotype: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Main stimulus effect: #$p < 0.05$. Main genotype effect: $$p < 0.05$. WT: $n = 7$; Het: $n = 9$.

These interactions were accompanied by a main effect of the test period on eye opening ($F_{(1.70,23.83)} = 9.11$, $p = 0.002$), ear angle ($F_{(1.26,17.66)} = 21.26$, $p < 0.001$), ear position ($F_{(1.38,19.27)} = 36.68$, $p < 0.001$), snout position ($F_{(1.66,23.24)} = 7.43$, $p = 0.005$), and face inclination ($F_{(1.84,25.74)} = 5.74$, $p = 0.010$). Both Het and WT mice had reduced eye opening during optogenetic stimulation and recovery compared with baseline (stimulation-baseline: $p = 0.003$; recovery-baseline: $p = 0.037$; Fig. 6A). Both had the ear more tilted during stimulation than during baseline ($p = 0.001$) and recovery ($p = 0.002$; Fig. 6C), and the ear more backward during stimulation than during baseline ($p < 0.001$) and recovery ($p = 0.002$; Fig. 6D). The snout appeared pointier during stimulation than recovery ($p = 0.004$; Fig. 6E), and the face more convex during stimulation than at baseline ($p = 0.041$) for both Het and WT mice (Fig. 6G).

We found two simple effects of mouse genotype on facial parameters, showing slightly smaller eye opening in Het than WT ($F_{(1,14)} = 6.25$, $p = 0.025$) and ears placed more sideway in Het than WT mice ($F_{(1,14)} = 4.82$, $p = 0.046$; Fig. 6A,B). We found no other effect of genotype nor of optogenetic test, and no further interaction between these factors (all ps > 0.074).

Formalin test

First, we assessed the temporal dynamics of facial changes across a 30 min period following saline injection into the hind paw compared with a preinjection baseline period. We found very limited effects of saline. There was a main effect of the injection on ear position ($F_{(4.05,36.49)} = 3.25$, $p = 0.022$), but *post hoc* tests did not reach significance (all

ps > 0.072; Fig. 7D). There was also a main effect of saline injection on snout position ($F_{(3.11,27.95)} = 4.93$, $p = 0.007$), with *post hoc* tests revealing that the snout was pointier during the first 5 min following injection than at baseline ($p = 0.043$; Fig. 7E). We found no other effect of saline on facial parameters (all ps > 0.206).

Second, we conducted the same analysis after formalin injection into the hind paw. There was no effect of formalin injection on ear opening, ear angle nor mouth position (all ps > 0.081; Fig. 7B,C,F). However, the formalin assay modified eye opening over time ($F_{(2.6,6,23.90)} = 4.24$, $p = 0.018$). We found a steady reduction in eye opening following formalin injection compared with baseline, from 5 min postinjection to the end of the test (all ps < 0.030; Fig. 7A). Formalin injection temporally affected ear position ($F_{(3.57,32.11)} = 5.24$, $p = 0.003$). The ear moved backward during the first 10 min following injection (all ps < 0.047), and again between 25 and 30 min following injection ($p = 0.044$; Fig. 7D). Similarly to saline, formalin modified snout position ($F_{(3.17,28.54)} = 3.19$, $p = 0.036$). The snout was pointier during the first 5 min following formalin injection compared with baseline ($p = 0.014$; Fig. 7E). Formalin injection strongly modified face inclination ($F_{(3.75,33.79)} = 4.18$, $p = 0.009$). The face was consistently more convex during the first 15 min following formalin injection (all ps < 0.028), and again between 20 and 30 min following it (all ps < 0.017; Fig. 7G).

Facial landscape of emotional stimuli

Facial parameters were reduced by PCA producing a total of seven dimensions. Following Horn's parallel analysis, two dimensions had adjusted eigenvalues >1, cumulatively
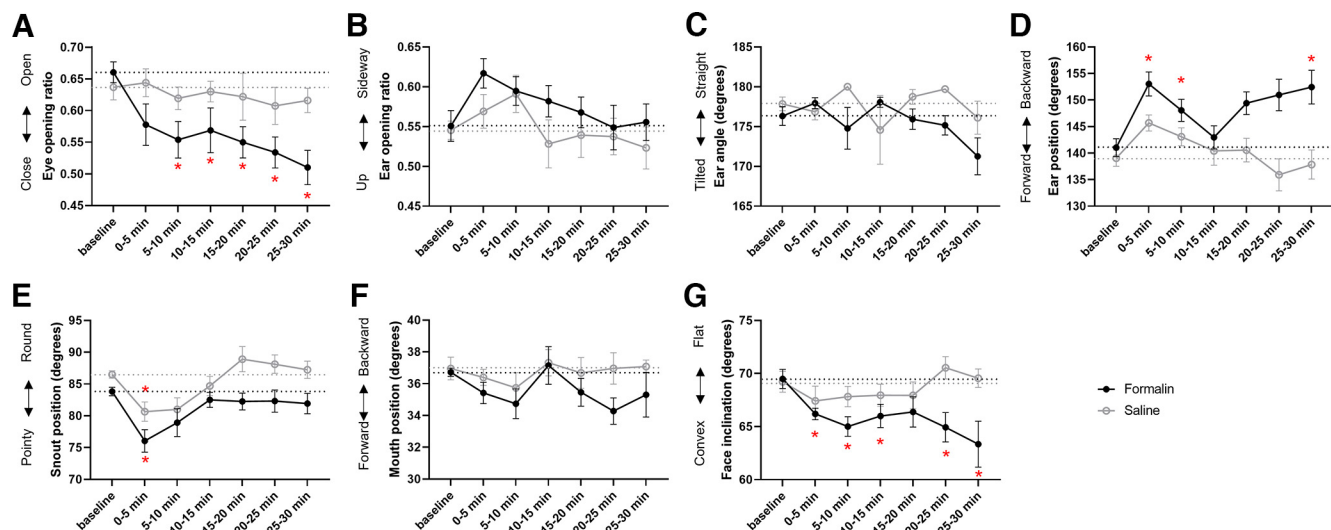
Figure 7. Real-time effect of saline or formalin injections into the hind paw on facial parameters. *A*, Eye opening ratio. *B*, Ear opening ratio. *C*, Ear angle. *D*, Ear position. *E*, Snout position. *F*, Mouth position. *G*, Face inclination. Data are mean ± SEM; *$p < 0.05$, difference from the corresponding baseline. Saline: $n = 10$; formalin: $n = 10$.

explaining 53.32% of the variance. The first dimension, to which contributed mostly face inclination (32.38%), mouth position (25.60%) and eye opening (17.98%), accounted for 28.85% of the variance and can be interpreted as eye and muzzle movements. The second dimension, to which contributed mostly ear position (31.41%) and ear angle (36.04%) explained 24.47% of the variance and can be interpreted as ear-related movement (Fig. 8*A*).

Since the number of dimensions produced by PCA was the same as the initial number of measured facial parameters, and since the two principal components only explained 53.32% of the variance, we used UMAP directly on absolute measured values to visualize clusters of stimuli. The projection based on 405 units made of individual averages in response to each stimulus produced a clear but partly overlapping clusterization. Labeling revealed an aggregation of units related to mechanical cutaneous stimuli (poking, petting, brushing). Optogenetic stimulation clustered in close proximity with many units related to the early and late phases of formalin action after injection. Gustatory stimuli appeared visibly separated, with a distinction between mentholatum and sucrose/water (Fig. 8*B*).

Discussion

Animal emotions have attracted a lot of attention in the context of improving animal welfare and compensating the reductionist bias that has long prevailed in behavioral neuroscience. In this study, the easily visualizable points defined on mouse profiles allowed the fine quantification of facial changes and successfully captured different profiles in response to aversive and pleasurable gustatory and somatosensory stimuli.

Summary of the results

Drinkable solutions, independently from their inherent valence, produced a common ear movement forward.

This might be a confound of the drinking movement despite efforts made to reduce this effect. However, our most salient stimuli, water and mentholatum, oppositely influenced eye opening and face inclination, suggesting that they are reliable indicators of a gustatory stimulus' valence. Quinine's lack of significant effect might be attributed to its comparatively lower bitterness compared with mentholatum.

All effective somatosensory stimuli proportionally positioned the ear more backward than at their respective baseline. We suggest this is a protective reflex of the sensitive ears elicited by any type of somatosensory stimuli. The often observed decrease in ear angle producing an ear tilt might participate in ear protection too. Overall, light somatosensory stimuli including poking, petting, and brushing tended to increased eye opening and face inclination values, all the while painful formalin injections and optogenetic stimulation of CGRP-lineage fibers had opposite effects on these parameters. WT animals showed some degree of response during optogenetic stimulation, mostly related to eye closing, and likely constituted by general light avoidance behavior and the presence of the optic fiber above their neck. Poking the mice with a stationary stimulus elicited a very similar pattern as petting and brushing them at 5 cm/s. Brushing the mice produced the highest facial changes, in particular positioning the mouth more backward. During both petting and brushing, the ears appeared turned toward the side of the head, a pattern further increased by habituation.

Formalin injection modified facial parameters in a clear two-phase pattern, following its well-known two-phase effect on nocifensive behavior. The only common effect between saline and formalin injection were a decrease in snout position values in the first 5 min following injection, which might be a confound of isoflurane anesthesia (Miller et al., 2015; Hohlbaum et al., 2017).
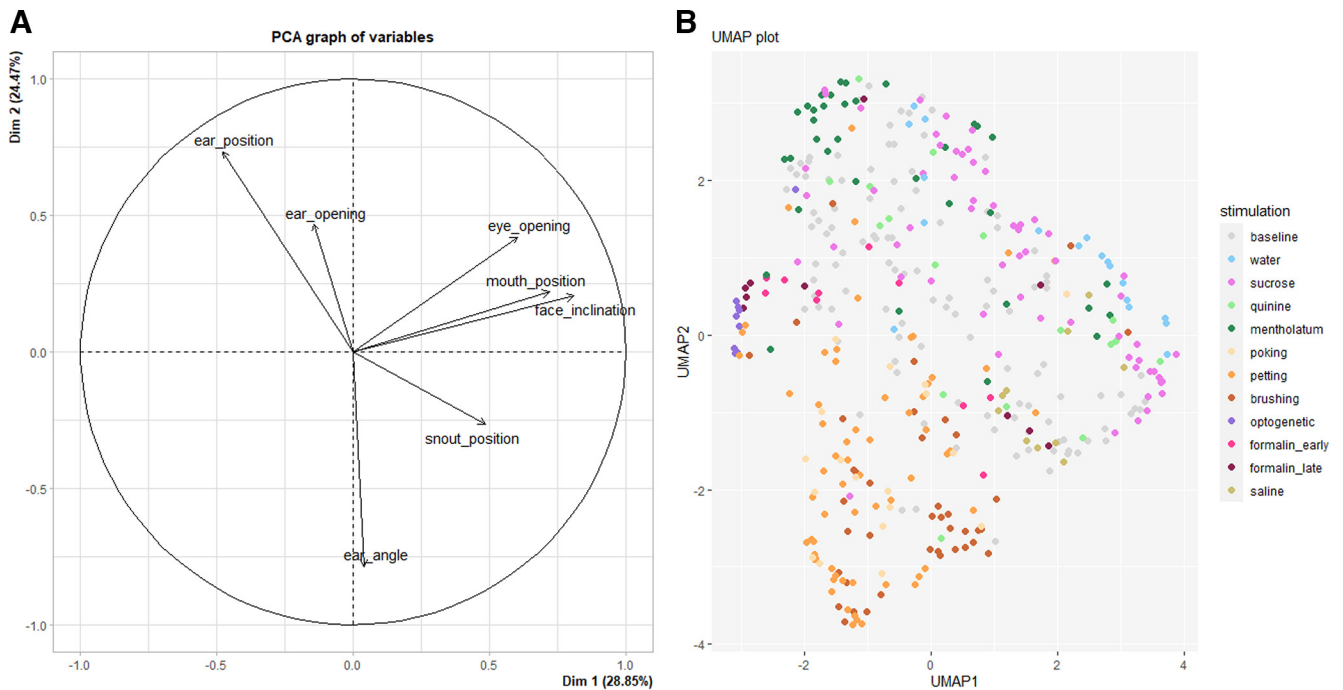
Figure 8. ***A***, Principal component analysis of mouse facial parameters across 11 stimuli (counting early and late formalin phases separately) and their respective baselines. The first component (Dim. 1) is interpreted as Eye and Muzzle movement, and the second (Dim. 2) as ear-related movements. ***B***, Visualization with UMAP of individual average values in response to 10 stimuli and at baseline. $n = 405$ units. Metrics = Euclidean, Neighbors = 15. Minimum distance = 0.1.

Methods validation

The new method developed here proposes to rigorously measure the ratios and angles visible on a mouse profile. This allowed some independence from observer training, as measures were objective, based on easily visualizable points on the face. Calculating proportional changes from baseline ranged from an average -36% to $+22\%$ change in facial parameters in response to the different stimuli, with the most prominent changes usually occurring in eye opening and ear position. The absence of restraint in our study prevented the confound of restraint stress which was shown to increase MGS scores (Senko et al., 2017). Multiple observers scored the present data; all data accurately showed similar patterns across stimuli and across experiments, regardless of the observer. Several sequences of stimuli were used to elicit a range of affective states, along both gustatory and somatosensory modalities. We did observe that the sensory modality stimulated influenced the pattern of facial changes, further pointing the relevance of including several modalities in a stimulation sequence to account for potential confounds.

We included seven facial parameters, which showed some degree of intercorrelation (e.g., proportional change from baseline in ear angle strongly correlated that of ear position, all experiments collapsed: Pearson $t_{(279)} = -10.35$, $p < 0.001$, $\rho = -0.53$, all stimuli collapsed). This is consistent with the fact that some of the angles and ratios measured could have facial points in common. Yet, the first two components produced by PCA retained only 53.32% of the data variance. Moreover, the differential changes observed in facial parameters in response to different stimuli (e.g.,

clear backward movement of the mouth during petting, brushing and optogenetic stimulation absent in response to gustatory stimuli) argues in favor of retaining all seven. Especially, this seems relevant if this method were to be applied to other mammal species in which parameters correlation and mobility might vary because of different facial morphology and musculature.

Salient facial features in the mouse produce robust response profiles

Combining the seven facial parameters, we were able to determine response profiles to each stimulation. Some stimuli elicited similar response profiles, such as petting and brushing the mice at similar pressure and velocity. To the contrary, some facial parameters showed bidirectional changes in response to stimuli at the opposite ends of the pleasantness range. The quantification of facial changes and their proportional comparison to baseline values support the ability for facial expression analysis to detect positive versus negative affects, in combination with the modality stimulated.

The dataset used in this study encompassed more facial parameters than the initial MGS, allowing to precisely determine which facial components contribute to the scores attributed in the MGS (Table 1). Ears are particularly mobile in rodents as well as in other species, and thought to both serve social communication and reflect internal states such as arousal or vigilance (Lecorps and Féron, 2015), fear and aggression (Defensor et al., 2012), pain (Andresen et al., 2020), or possibly positive

Table 1: Correspondence between facial changes identified in the original MGS (Langford et al., 2010) and the method developed in this paper

MGS items	Corresponding facial parameters	Change direction
Orbital tightening	Eye opening	Smaller eye opening
Nose bulge	Snout position	Pointier snout?
	Mouth position	Mouth backward
	Face inclination	More convex face
Cheek bulge	Snout position	Rounder snout?
	Mouth position	Mouth backward
	Face inclination	More convex face
Ear position	Ear opening	Ear to the side
	Ear position	Ear moved backward
	Ear angle	Tilted ear
Whisker change	Snout position	Rounder snout?
	Mouth position	Mouth backward
	Face inclination	More convex face

emotional valence (Descovich et al., 2017). Ear-related parameters were consistently affected by the different stimuli, suggesting that ears might be a critical feature in emotional communication. This is consistent with the large size of the ears relatively to the mouse face that would make them an evolutionary relevant tool for social communication in rodents. This substantially differs from human models in which ear movement is limited, although not absent, and ears themselves relatively small and often concealed under hair. Nevertheless, the parameters defined in this study are applicable to most mammal species. Thus, the conservation of facial movements across species, as well as species-specific salient features could be verified in future studies.

It is intuitive to think that facial changes would be more pronounced in the areas corresponding to the sensory organ detecting the stimulation. Some facial changes were likely not elicited by the subjective experience of a stimulation, but indeed simply a result of facial contact and may serve a protective function (Defensor et al., 2012). However, measures of the muzzle area were also influenced during nongustatory stimuli, and ear parameters changed although formalin was injected into the hind paw. These observations argue in favor of the changes observed being indicators of emotional valence.

Temporal dynamics in the formalin test

Several substances can be used intradermally to induce pain. However, in freely moving mice we found that scratching the injection site might confound results as mice tend to lick their paws following scratching, thus also tasting the injected compound, which could cause oral pain in the case of capsaicin or mustard oil (allyl isothiocyanate). In addition, mustard oil caused burning-like ocular pain in the confined space of the observation cubicle (unpublished observations). In light of these considerations, we used the formalin test to induce pain intradermally. Several facial parameters changed dynamically following formalin injection, correlating with its two-phase action. Such an observation shows that facial expressions, or grimace scales, are useful tools to implement in real time as they accurately reflect instantaneous

changes in internal states. It is also noteworthy that return to baseline levels was almost instantaneous after stopping painful optogenetic stimulation, further strengthening the finding that facial changes inform on spontaneous affects.

In parallel to instantaneous facial changes during the formalin assay, other parameters such as eye opening showed a stable effect across the entire postinjection period, independently from the early and late phases of formalin action. One hypothesis would be that facial expressions reflect both spontaneous responses to specific stimuli such as painful experiences, and the underlying internal states such as unfocused anxiety. Interestingly, and in accordance with previous findings using the MGS (Langford et al., 2010), the effect of formalin on eye opening was not significant during the first phase with respect to absolute values. However, the proportional decrease from baseline in eye opening was significant during both phases.

Limitations

Several previous studies have reported facial asymmetry as an indicator of confusion, poor welfare or pain (Sefcek and King, 2007; Boissy et al., 2011; Gleerup et al., 2015). It is possible that facial asymmetry would have completed these data and further shed light on its relationship to emotional valence. Unfortunately, it was not possible to account for this variable when looking at lateral views. The present stimuli sequences aimed at inducing a range of positive and negative affects. Most importantly, we attempted to develop a new positive stimulus in the form of pleasant touch. Recently, it was shown that male mice developed conditioned place preference in response to gentle stroking of the fur (Cho et al., 2021). In the present study, some of the mice were naive to this type of stimulation and might have expressed fear of the experimenter's fingers or of the brush. Whether we successfully induced pleasantness is unclear. We targeted the nape of the neck, which is stimulated in wild mice during social allogrooming, but experimenter observations suggest that at least some mice exhibited defensive postures. Nevertheless, in the present study, poking, petting, and brushing the mice produced similar response profiles of gradually increasing intensity. These response profiles were largely different from those elicited by optogenetic stimulation of the same zone. In order to prevent fear of the experimenter, we habituated some of the mice to cutaneous stimuli, which resulted in the accentuation of ear opening changes but in a reduction in ear angle. Finally, in our study, ear position varied similarly to the ear angle defined by Finlayson et al. (2016) during rat tickling, which was associated with vocalizations typical of hedonic situations. These elements might signal a positive affect elicited by brushing the mice.

Clustering of stimuli through facial changes was investigating using UMAP. This projection was based on a relatively small sample of 405 units, which might explain the dispersion of the results. Nevertheless, coherent unit aggregations are visible, consistent with the typical face profile elicited by the cutaneous stimuli, and the common facial changes elicited by the gustatory stimuli. Interestingly, baseline units, despite showing a high variability, were well separated from cutaneous stimuli, optogenetic stimulation and

mentholatum. This further highlights the difficulty to establish indicators of positive affects as their features might not be as strongly separated from those of a neutral state. Nevertheless, based on this projection, it appears that the inclusion of new individual units from unknown situations would inform on stimulus modality and valence within that frame. In humans, subjective affect is usually rated in terms of valence and arousal. However, valence itself is ambiguous and cannot be optimally modelled by linear models, even in scale ratings (Mattek et al., 2017). We might add that, in nonverbal animal models, in which we assess purposefully triggered affects through chosen stimuli, the characteristics of the stimuli might also influence valence measurements. The method established in the present study captures detailed changes in facial expression that offers potential to be automated and generalized to multiple species and affective situations.

Prospective machine learning

The future of neuroscience will rely on the use of machine learning. One of the pitfalls of this powerful tool, is that the opacity of this black box approach often hinders human understanding. In our study, we aimed at identifying key facial features involved in facial expression changes and validate their significance in characterizing reactions to several emotional stimuli. We found that even in mice that have a relatively less mobile facial musculature than humans, facial expressions are complex, with some parameters changing similarly in situations of opposed valence. This is not new, as the development of the facial action coding system (Ekman and Friesen, 1978) already identified action units that convey different meaning while showing the same directional change (e.g., lid tightener active in sadness and fear; Hamm et al., 2011). The meaning of a facial expression can therefore only be extracted when looking comprehensively at multiple facial parameters and their interactions, for which machine learning models are highly useful. The facial points used when measuring the hereby defined parameters are easily labelable for a pose estimation softwares like DeepLabCut or SLEAP. The use of machine learning in the future will improve the data collection process and refine emotion identification, while potentially identifying other parameters of interest in freely moving animals, based on the parameters here defined and validated

In conclusion, we report the first case of rodent facial expression analysis with a newly developed method, using several positive, non-noxious stimuli. Our new method uses easily visualizable facial points resulting in seven facial parameters, independent from camera zoom and animal size. The parameters are entirely scalable, conveying meaningful facial information of mouse subjective experience during sequences of emotion-inducing stimuli. The parameters retained are generalizable to most mammals and can be a useful tool to identify overlaps between emotions and their conservation across species. Future studies may focus on the potential for this method to be successfully translated to humans. The present study provides a transparent understanding of the facial regions responsible for the production and perception of facial expressions. These expressions have the potential to reflect spontaneous, stimulus-directed

responses, together with general internal state, as well as stimulus-associated mechanical movements. As such, the sensory modality stimulated and the area of stimulation might influence the severity of facial displays.

Pleasant social touch showed potential for eliciting positive affect, and might be used in the future to mitigate pain or improve resilience from negative experience. Gentle touch delivered by an experimenter may need conditioning to be pleasant to mice, but social touch, and mechanisms such a social buffering have robustly shown rewarding value in social animals. It would be valuable in the future to automatize the capture of mouse facial expressions in a more naturalistic, socially complex model.

Even considering the increasing public concern for animal welfare, and the social imperative for personal development and happiness, positive affects are rarely addressed, and research remains biased toward the study of negative experiences (Webb et al., 2019). This bias leaves out a large panel of affects, not only offering an incomplete view of the issue of mental health, but also distorting the prism of data interpretation. We propose that including positive, ecologically relevant situations, together with improving our understanding of positive affects, will positively impact health research.

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