Olfactory stimulation Inhibits Nociceptive Signal Processing at the Input Stage of the Central Trigeminal System

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Abstract—The spinal trigeminal nucleus caudalis (SpVc) in the mammalian brainstem serves a pivotal function in pain processing. As the main relay center for nociceptive signals, SpVc conducts pain-related signals from various regions of the head toward higher levels of central processing such as the thalamus. SpVc also receives modulatory signals from other brain areas, which can alleviate the perception of headache. We studied the impact of olfactory co-stimulation on pain-related behavior and SpVc neural activity in mice. Using the TRPA1 agonist allyl isothiocyanate (AITC) as noxious stimulus, we quantified the aversive response and the perceived pain intensity by evaluating explorative running and the mouse grimace scale, respectively. We found that the floral odors phenylethyl alcohol (PEA) and lavender oil mitigated the aversive response to AITC. Consistent with this finding, a newly developed, automated quantification of c-Fos expression in SpVc revealed that co-stimulation with PEA or lavender profoundly reduced network activity in the presence of AITC. These results demonstrated a substantial analgesic potential of odor stimulation in the trigeminal system and provide an explanation for the palliative effect of odors in the treatment of headache. © 2021 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: trigeminal system, pain, modulation, odorants, cross-modal, c-Fos.

INTRODUCTION

Non-nociceptive stimuli can profoundly influence the perception of pain intensity and pain quality, a phenomenon for which a mechanistic framework was first described in the “gate theory of pain” for the modulation of nociceptive signal processing by innocuous touch (Melzack and Wall, 1965). According to this concept, non-nociceptive Aβ afferents of touch receptors activate inhibitory interneurons in the spinal cord dorsal horn, thus “closing the gate” for nociceptive signal flow and suppressing pain perception. In addition to touch, various other sensory modalities were also reported to reduce pain perception, including cooling (Proudfoot et al., 2006), olfaction (Bartolo et al., 2013; Tashiro et al., 2016; Gossrau et al., 2020; Higa et al., 2021; Kashiwadani et al., 2021), taste (Kakeda et al., 2010), as well as vision and hearing (Bascour-Sandoval et al., 2019). However, in contrast to the well-established inhibitory circuitry that forms the underpinning of the spinal gate control by touch (Comitato and Bardoni, 2021), only little is known about neural pathways involved in modulatory and analgesic effects of other senses. Such information is particularly scarce for the trigeminal nociceptive system, although substantial evidence supports the notion that innocuous stimuli do indeed have the potential to interfere with various forms of trigeminal pain (Sjostrand et al., 2010; Dussor and Cao, 2016; Lotsch et al., 2016; Chichorro et al., 2017; Sandri et al., 2021). Indeed, studies of cross-modal interactions between the nasal trigeminal and olfactory systems in human subjects have clearly demonstrated a close but complicated interplay between both systems in the analysis of chemical stimuli (Brand, 2006; Frasnelli et al., 2007; Bensafi et al., 2008; Lotsch et al., 2016; Pellegrino et al., 2017; Hummel and Frasnelli, 2019; Tremblay and Frasnelli, 2021). While it is generally accepted that olfactory stimuli have the potential to impinge on trigeminal signaling, it is difficult to identify where exactly this cross-talk occurs. A recent study argues strongly against peripheral interaction in the nose (Maurer et al., 2019), but functional brain imaging studies from thalamic and cortical areas suggest that the two systems modulate each others central signal processing (Boyle et al., 2007; Albrecht et al., 2010). In the present study, we look for the initial point of convergence, the first stage where modulation of trigeminal activity by olfactory stimuli can be detected.
One reason for the dearth of detailed neurophysiological data in this field is the lack of established animal models, which are required to correlate functional and structural aspects of interactions between innocuous and noxious signals in the trigeminal system. Such models would have to produce a graded neuronal and behavioral response to noxious stimulation in the absence and presence of non-noxious co-stimulation, and should permit analysis of the neural networks involved. In the present paper, we demonstrate that the quantitative evaluation of c-Fos expression within the murine trigeminal ganglion that form the three divisions mandibular (V2) and maxillary (V3) resident in the trigeminal ganglion that form the three divisions mandibular (V2) and maxillary (V3) of the trigeminal ganglion and the subnucleus spinal trigeminal nucleus pars caudalis (SpVc) fulfills the requirements for such a model. The brainstem subnucleus SpVc serves as an initial relay center for noxious signals in the trigeminal system. Such models asapivotal hub at the onset of noxious signal processing in the central trigeminal network, and its integrative function permits analysis of the neural networks involved. In the present paper, we demonstrate that the quantitative evaluation of c-Fos expression within the murine trigeminal ganglion that form the three divisions mandibular (V2) and maxillary (V3) resident in the trigeminal ganglion and the subnucleus spinal trigeminal nucleus pars caudalis (SpVc) fulfills the requirements for such a model. The brainstem subnucleus SpVc serves as an initial relay center for noxious signals in the trigeminal system. Thus, SpVc receives noxious input via neurons resident in the trigeminal ganglion that form the three branches of the trigeminal sensory system, the ophthalmic (V1), mandibular (V2) and maxillary (V3) divisions (Bereiter et al., 2000; Sessle, 2000). At the same time, SpVc neurons are subject to control by higher brain levels, including cortical regions (Malmierca et al., 2012; Castro et al., 2017; Kobayashi and Nakaya, 2020), midbrain centers (Yin et al., 2009; Yin et al., 2011; Nguyen et al., 2019) and the hypothalamus (Abdallah et al., 2013; Abdallah et al., 2015). Moreover, SpVc is interconnected with the other subnuclei of the spinal trigeminal nucleus, the subnuclei interpolaris (SpVi) and oralis (SpVo) (Li et al., 1999; Han et al., 2008). Current evidence clearly shows that SpVc operates as a pivotal hub at the onset of noxious signal processing in the central trigeminal network, and its integrative function renders it a prime candidate for cross-modal effects in the pain pathway.

A suitable approach to assess changes of neural activity in the SpVc is to monitor activity-induced expression of the proto-oncogene c-fos within SpVc neurons. This method has been successfully applied in a number of studies to examine both sensory responses and modulatory effects in rodent SpVc (Anton et al., 1991; Ebersberger et al., 1995; Bereiter, 1997; Cutrer et al., 1999; Mitsikostas et al., 1999; Takeda et al., 1999; Mitsikostas and Sanchez del Rio, 2001; Ro et al., 2003), and was employed in the present study to find out whether innocuous olfactory stimulation could affect noxious signal processing. To this end, aversive, noxious stimuli were paired with innocuous olfactory stimuli, and the response of SpVc neurons under both conditions was compared. Quantitative analysis of c-Fos expression in SpVc was used to measure the impact of olfactory co-stimulation on noxious signal processing at the first stage of the central pain pathway. Our results demonstrate a pronounced analgesic effect of olfactory co-stimulation and extend the concept of pain modulation by innocuous sensory stimuli to the trigeminal system.

EXPERIMENTAL PROCEDURES

Animals
Male, eleven week old C57BL/6N mice (Janvier Labs, France) were used for all stimulation experiments. We focused on young, male animals because studies exploring novel aspects of olfaction require uniform responses to olfactory stimulation, as unaffected as possible by the estrous cycle and social signaling. For anatomical illustrations, SNS\textsubscript{Cre} mice were kindly provided by Prof. Rohini Kuner (Heidelberg University, Heidelberg, Germany). These C57BL/6 mice express Cre-recombinase under the control of the Snc10a gene promoter in cells expressing the TTX-resistant, voltage-gated sodium channel Na\textsubscript{v}1.8 (Agarwal et al., 2004). Upon crossing SNS\textsubscript{Cre} mice with a Rosa\textsubscript{26}LSLt\textsubscript{tdTomato} line, tdTomato expression is observed in the F1 generation of heterozygous SNS\textsubscript{tdTomato} mice used in the present study. Mice were kept in the Interdisciplinary Neurobehavioral Core (INBC, Heidelberg, Germany) under standardized conditions and sacrificed by inhalation of isoflurane (Baxter Healthcare Corporation, USA). All experiments were approved by the Regierungspräsidium Karlsruhe, Germany, and were carried out in compliance with the Animal Welfare Guidelines as stipulated by the Federal Republic of Germany (approval numbers T-04/18, A-16/14, G-35-9185-81/G-104/16).

Trigeminal stimulation
Exposure of test mice to chemical stimuli was conducted at the INBC under standardized conditions. Mice were kept in individually ventilated cages (IVC; 39.1 cm × 19.9 cm × 16 cm) to avoid exposure to environmental odors for a total of seven days before the start of experiments. During this acclimatization period, free access to water and food was ensured. On the stimulation day, mice were kept in the laboratory in their IVC home cage for 1 hour before starting the experiments. Allyl isothiocyanate (AITC, Sigma-Aldrich, Munich, Germany, #377430; vapor pressure at 20 °C: 5.21 hPa) was chosen as an established trigeminal stimulus in rodents (Anton et al., 1991; Bereiter, 1997; Takeda et al., 1999; Ro et al., 2003), whereas phenethyl alcohol (PEA, Sigma-Aldrich, Munich, Germany, #W285811; vapor pressure at 20 °C: 0.08 hPa) was used as pure olfactory stimulus that does not induce activity in the trigeminal ethmoid nerve of rats, even at saturating concentrations (Silver and Moulton, 1982). The second odorant tested was lavender oil (Sigma-Aldrich, Munich, Germany, #61718), diluted to 1:10.000 with Milli-Q water. The chemical stimuli were pipetted onto a piece of filterpaper that was placed in a small (30 mm × 25 mm) perforated plastic box (paraffin embedding box Histosette 1, M 491–2, Simport, Beloel, Canada). The box was positioned either in the center of an IVC cage that was closed...
subsequently with a plexiglas lid, or in the center of the open-field arena, also closed subsequently. In open-field experiments, 2 μl AITC was applied either alone or together with 15 μl odorant to the filterpaper; for c-Fos experiments, 15 μl AITC was applied either alone or together with 15 μl odorant. For stimulation a separate IVc cage was cleaned and filled with approximately 3 cm of fresh bedding material (Altromin International, Germany). The box containing odorant-soaked filter paper was placed in the cage 10 min prior to introducing the mouse. The mouse remained in the cage for a 10-minute exposure period. Each mouse was then returned to its home cage and, one hour after stimulation, a time point when c-Fos expression was found to reach a peak, mice were sacrificed and prepared for c-Fos immunohistochemistry (see below). Five mice were tested for each stimulus condition. From each mouse, 12 brainstem sections were obtained, corresponding to a total of 50–60 sections per stimulus condition. In each of these coronal sections, 8 areas were imaged (areas 1–4 on both sides of SpVc), corresponding to a total of 400–480 images per stimulation for the entire experiment.

Open field test
Open field tests were used to quantify general locomotor activity of mice during trigeminal stimulations. Eight mice were tested per day, always between 8 am and 3 pm. Each animal was tested only once and was then sacrificed. No cross-over design was used, and the single observer was not blinded to the experimental condition. On the experimental day, mice were habituated to the laboratory for 1 hour before exposure to chemical stimuli. Experiments were conducted in an open-field test arena (42 cm × 42 cm × 42 cm). Chemical stimuli were placed into the arena 10 min prior to the mouse. Trigeminal and olfactory stimuli (see above) were applied in a plastic box and placed in the middle of the test arena, which was then closed with a Plexiglas lid. Each mouse was tested once and, during this time, activity was monitored using a video-taped tracking system (SygnisTracker, Sygnis, Heidelberg, Germany). All animals were placed at the same starting position, and their explorative running was recorded for 10 min. After stimulation, mice were returned to their home cages and the test arena was cleaned and prepared for the next mouse. The distance traveled under each stimulus condition was averaged over 7–11 mice and is presented with SEM in the graphs.

Mouse grimace scale
Before and after each period of chemical stimulation in the open field test, the mouse grimace scale (MGS) (Langford et al., 2010) was applied to assess the subjective pain level experienced by each animal. To quantify this level, the observer assigned a value between 0 and 2 to each “facial action unit” (FAU; Langford et al. 2010). A score of 0 indicated the absence of pain for each FAU, a score of 1 indicated moderate pain, and a score of 2 indicated severe pain. MGS scores obtained before stimulation were close to 0 in all test animals and were used to determine the baseline. Averaged MGS scores obtained immediately after stimulation are presented in the graphs.

Free-floating immunohistochemistry
Sacrificed mice were immediately perfused intracardially, first with ice-cold phosphate-buffered saline (PBS, 150 mM NaCl, 8.1 mM Na2HPO4 × 2H2O, 1.9 mM NaH2PO4 × H2O, pH adjusted to 7.4) and then with 4% ice-cold paraformaldehyde (PFA, in PBS). The dissected tissue was postfixed in 4% PFA for a further 30 min. Brainstems were cut in ice-cold PBS with a vibratome (Leica, VT1000S) to obtain 150 μm thick sections. Sections were fixed again for 30 min in 4% PFA and washed 4x10 min with PBS. After incubating the sections in blocking solution (5% goat serum (Sigma-Aldrich), 0.5% Triton X-100 and 0.05% Na3) for 2 h, a primary antibody against c-Fos (Synaptic Systems, #226003, 1:1000) diluted in blocking solution was applied overnight. The c-Fos antibody was produced in rabbit by immunization with a synthetic peptide corresponding to amino-acid residues 2 to 17 from rat c-Fos (UniProt Id: P12841). It stained a cell pattern in the mouse brain similar to previous reports (Wu et al., 2020; Liu et al., 2021; Roth et al., 2021). Sections were washed 4 × 10 min with PBS containing 0.5% Triton X-100 (PBST), followed by a 2 h incubation with a secondary antibody conjugated to a fluorescent tag (goat α rabbit Alexa Fluor 488) and diluted in blocking solution. After washing 4 × 10 min in PBST, sections were mounted on gelatin-coated glass slides using non-fluorescent Aqua-Poly/Mount (Polysciences). All sections were co-stained with the nuclear dye 4’,6-diamidin-2-phe nylindol (DAPI, Sigma-Aldrich; 0.3 μM) during incubation with the secondary antibody.

Confocal microscopy and automated counting of c-Fos expressing cells
Sections were examined using a confocal laser scanning system (D-Eclipse 90i/C1; Nikon) using a 20x objective for brainstem microscopy. Images were taken as z-stacks and saved as maximum intensity projections. Confocal optical sections consisted of 7 stacks, each approximately 2 μm thick, resulting in images that represent 11 μm of each brainstem slice. For counting c-Fos-positive cells, we applied an automated analysis routine that avoided any bias associated with manual counting. Images were pre-processed using the interactive learning and segmentation toolkit iLastik 1.3.2 (Kreshuk lab, EMBL, Heidelberg, Germany). To this end, a subset of 30 images showing c-Fos immunosignal in SpVc were used to train this machine-learning-based image analysis tool. After the software learned to distinguish signal from background, iLastik workflows could be applied to the remaining data. The resulting probability map displayed, which pixels belonged to a c-Fos expressing nucleus and was used directly for counting activated cells by ImageJ (NIH, USA) using the Analyze Particles Tool. Finally, the counts were normalized to the area of the brainstem.
slice evaluated, to obtain the number of c-Fos positive cells per mm². No normalization to the total cell number or to the number of neurons within each section was attempted, as such normalization would introduce additional sources of technical bias. Moreover, no attempts were made to distinguish neurons from glial cells or different types of neurons from each other. The criteria that justified averaging of data (c-Fos positive cells per mm²) obtained from different animals were: (1) same anatomical location of the section; (2) same experimental treatment in all stages; (3) same set of machine-learned parameters to identify c-Fos-positive cells. The results were mean values indicating general cell activity in SpVc under different experimental conditions. Editing of images was performed with CorelPHOTO-PAINT 2017 (Corel Corporation).

Statistics
Statistics were calculated using OriginLab 9.0 (OriginLab Corporation, Northampton). Before starting with statistical analysis, normality was tested using the Shapiro–Wilk test. Normally distributed c-Fos data were analyzed using two-way ANOVA, followed by a Bonferroni post hoc test with correction for multiple comparison. For the analysis of behavioral tests, which were not normally distributed, a Mann–Whitney’s U test was applied. Data are presented as box-and-whisker diagrams with boxes representing the 25 and 75 percentiles, whiskers representing 5 and 95 percentiles, and red bars illustrating that the aversive strength of AITC stimulation was significantly weakened by PEA. We next tested whether this cross-modal effect was specific for PEA or whether it could also be triggered by another odorant. We chose lavender oil for this test, because lavender scent and its predominant odorant linalool were previously suggested to exert analgesic effects (Nascimento et al., 2014; Tashiro et al., 2016; Donatello et al., 2020; Higa et al., 2021; Kashiwadani et al., 2021). Since undiluted lavender oil acts both as an olfactory and a trigeminal stimulus, we used strongly diluted lavender oil (1:10,000) that still produces an intense lavender scent.

RESULTS
Effects of odor co-stimulation on nocifensive behavior in mice
To investigate possible olfactory-trigeminal interactions in mice, we first tested whether odorant-stimulation could change pain-related nocifensive behavior. We used the irritant substance allyl isothiocyanat (AITC), a known TRPA1 agonist, for trigeminal stimulation. AITC is a volatile, pungent stimulus that evokes nocifensive behavior and c-Fos expression in rodent SpVc (Anton et al., 1991; Ebersberger et al., 1995; Bereiter, 1997; Takeda et al., 1999; Nomura et al., 2002; Ro et al., 2003). For olfactory co-stimulation, we applied phenylethyl alcohol (PEA), a major component of rose oil, one of only a few known odorants that don’t stimulate nasal trigeminal fibers, even at high concentrations (Doty et al., 1978; Silver and Moulton, 1982). Since PEA appears to be inert to the trigeminal system, it was employed as a bona fide pure olfactory stimulus. The stimuli were presented to awake, freely moving mice in the center of an open-field arena. PEA alone did not change patterns of explorative running in the arena, confirming that PEA had no detectable aversive quality (Fig. 1A, B). In contrast, running activity was severely reduced in the presence of AITC, as the animals tended to stay away from the source of the irritant (Fig. 1C). When PEA was co-applied with AITC (Fig. 1D), the suppression of motor activity was partly mitigated. Quantification of 10 minute motor activity is shown in Fig. 1E with a mean reduction of 59.4% in the presence of AITC alone (red bar) and 34.9% upon co-stimulation (hatched bar), illustrating that the aversive strength of AITC stimulation was significantly weakened by PEA. We next tested whether this cross-modal effect was specific for PEA or whether it could also be triggered by another odorant. We chose lavender oil for this test, because lavender scent and its predominant odorant linalool were previously suggested to exert analgesic effects (Nascimento et al., 2014; Tashiro et al., 2016; Donatello et al., 2020; Higa et al., 2021; Kashiwadani et al., 2021). Since undiluted lavender oil acts both as an olfactory and a trigeminal stimulus, we used strongly diluted lavender oil (1:10,000) that still produces an intense lavender scent.

In our open-field experiments, the diluted lavender oil alone did not alter running activity (Fig. 1F, G), but alleviated AITC-induced nocifensive behavior (Fig. 1H) with an efficiency comparable to PEA (Fig. 1I). Quantification of motor activity (Fig. 1J) yielded a similar result as observed with PEA: Explorative running was reduced by 64.4% with AITC alone (red bar) and by 45.5% upon co-stimulation with lavender (hatched bar). Thus, the aversive response to AITC (set to 100% for AITC alone) decreased by 41.3% (p < 0.05) with PEA and by 29.4% (p < 0.05) with diluted lavender, demonstrating effective mitigation of AITC avoidance by these odorants in mice.

To gain some degree of insight into the quality of sensory perception that AITC stimulation elicited in the animals without and with odor co-stimulation, we analyzed their facial expression during each test according to the standardized mouse grimace scale criteria (Langford et al., 2010). For this analysis, five facial features related to pain perception were assessed: orbital tightening, nose bulging, cheek bulging, ear positioning and whisker change. Each feature was quantified on a three-point scale with scores of 0 indicating no pain, 1 moderate pain, and 2 severe pain. While neither PEA nor lavender alone caused any discernible change of facial expression (Fig. 2A, B, red bars), the presence of AITC in the open field arena produced signs of severe pain (Fig. 2A, B, red bars), probably the stinging perception we know from mustard oil, horseradish or wasabi, all products that contain AITC as their main pungent component (Terada et al., 2015). Importantly, co-stimulation with AITC and a test odorant (hatched bars) reduced the grimace score by 32.7% (PEA, p < 0.01) or 29.7% (lavender, p < 0.01) to levels that indicated only moderate pain. These results demonstrate a measurable analgesic effect that odorants can exert on the perception of trigeminal pain in mice.

Odor-induced modulation of neural activity in mouse SpVc
Does the analgesic effect of odorants result from signal integration in SpVc? To examine this question, we evaluated c-Fos expression in the afferent trigeminal
pathway upon stimulation with AITC. In our experiments, airborne AITC molecules had access to trigeminal sensory terminals in the facial mucosae of the nose, eyes and mouth. To monitor stimulus-induced neural activity in the medullary brainstem, we analyzed c-Fos expression in four distinct areas of SpVc known to process afferent signals that originate from different parts of the trigeminal system. Fig. 3A depicts the SpVc in a brainstem section from an SNS\textsuperscript{tdTomato} mouse that expressed the red fluorescent protein tdTomato under the promoter of Na\textsubscript{1.8} voltage-gated sodium channels, a marker for sensory neurons in the pain system (Agarwal et al., 2004). A template of four areas, for which the density of c-Fos expressing cells was determined in each individual section, is shown in Fig. 3B. In rodents, the ventral area 1 roughly represents the ophthalmic V1 input from nose, eyes and meninges, the medial area 2 represents the maxillary V2 input from infraorbital and zygomatic regions, and the dorsal area 3 represents the mandibular V3 input from the buccal and lingual regions. While areas 1–3 cover superficial layers directly targeted by afferent fibers (red fibers in Fig. 3A, B), area 4 represents the deeper magnocellular layer of SpVc containing downstream levels of the SpVc network. Exposure of test mice to AITC elicited neural activity in SpVc, visualized by c-Fos immunofluorescence emanating from the nuclei of brainstem cells (Fig. 3C, D). This stimulus-dependent signal provided a measure for the total neural activity (of neuronal and glial origin) within each SpVc section analyzed; it did not yield specific information on the types of cells responding (Mitsikostas et al., 1999; Mitsikostas and Sanchez del Rio, 2001). Responding cells were
Expression of pain perception according to the standardized mouse grimace scale (MGS) with scores of 0 indicating no pain, 1 indicating moderate pain and 2 indicating severe pain. Scores for test animals suggested that animals experienced severe pain upon exposure to AITC (red bar; mean score 1.857 ± 0.037; Wilcoxon-Mann-Whitney test, p < 0.001; n = 7), but not to PEA (white bar; mean score 0.018 ± 0.018; p = 1; n = 11). Co-stimulation with PEA reduced the AITC score by 32.7% to 1.25 ± 0.073 (p < 0.001; n = 8) (hatched bar). (B) Diluted lavender oil (1:10.000) alleviated pain perception when co-applied with AITC, while not changing basal score by itself (white bar; 0 ± 0; p = 1; n = 8). Mean scores with AITC alone were 1.850 ± 0.033 (p < 0.001; n = 8) (red bar), but 1.3 ± 0.076 (Wilcoxon-Mann-Whitney test, p < 0.001; n = 8) upon co-stimulation with lavender (hatched bar), indicating an odor-induced reduction of MGS score by 29.7%.

Fig. 2. The aversive behavior elicited by AITC has the sensory quality of pain perception. (A) Expression of pain perception according to the standardized mouse grimace scale (MGS) with scores of 0 indicating no pain, 1 indicating moderate pain and 2 indicating severe pain. Scores for test animals suggested that animals experienced severe pain upon exposure to AITC (red bar; mean score 1.857 ± 0.037; Wilcoxon-Mann-Whitney test, p < 0.001; n = 7), but not to PEA (white bar; mean score 0.018 ± 0.018; p = 1; n = 11). Co-stimulation with PEA reduced the AITC score by 32.7% to 1.25 ± 0.073 (p < 0.001; n = 8) (hatched bar). (B) Diluted lavender oil (1:10.000) alleviated pain perception when co-applied with AITC, while not changing basal score by itself (white bar; 0 ± 0; p = 1; n = 8). Mean scores with AITC alone were 1.850 ± 0.033 (p < 0.001; n = 8) (red bar), but 1.3 ± 0.076 (Wilcoxon-Mann-Whitney test, p < 0.001; n = 8) upon co-stimulation with lavender (hatched bar), indicating an odor-induced reduction of MGS score by 29.7%.

Quantification of SpVc activity and of possible modulatory effects required a standardized counting procedure for c-Fos immunosignals in coronal sections. We approached this by first training an algorithm under manual guidance to detect c-Fos-positive nuclei and, based on that training, to establish a probability map for each section, which could then be evaluated in terms of the number of c-Fos-positive cells per mm² (see Experimental procedures). Importantly, the same trained algorithm and the same area template was applied to each individual section to ensure comparability between data sets from different animals. Judging from the low variance of results that were derived from similarly processed sections, this routine proved to be fairly reliable. The density of c-Fos-expressing cells was calculated separately for each of the four areas and the averaged density of the four regions is provided in the following graphs to assess overall SpVc activity. Moreover, a graphical representation of c-Fos immunofluorescence provides an overview of where AITC-induced neural activity is concentrated within SpVc. The shape and size of SpVc changes profoundly along its rostrocaudal axis and spatial aspects of activity can yield essential information on initial signal processing in the SpVc network. Fig. 4 provides a qualitative graphical representation of how AITC-induced c-Fos immunosignals were distributed between five planes (P1–P5) of coronal sections along the rostrocaudal axis. The density of dots in these drawings represents the proportionate numbers of activated neurons within the different layers of SpVc. It can be seen that a region with high activity occurs at the ventral end of the nucleus within the middle segment of SpVc, 600–1050 μm caudal to the obex. Apart from this region, the majority of c-Fos immunosignal was detected in lamina II, and lamina III. An additional small hotspot can be localized to lamina II, of the most caudal plane (P5) of the nucleus. These data illustrate that the AITC-induced neuronal activity occurred within the main input layers of the SpVc network, laminae II and III, that a large proportion of signals reached the ventral V1 region, and that almost no activity could be detected in the superficial lamina I and in the deep magnocellular layer of SpVc.

Determining the density of c-Fos expressing cells allowed comparison of the overall SpVc activity under different experimental conditions. As representative images obtained from the most responsive area 1 illustrate (Fig. 5A, B), exposure to PEA alone did not increase c-Fos immunosignal over the basal value, confirming earlier reports that this odor has no trigeminal potency (Doty et al., 1978; Silver and Moulton, 1982). In contrast, exposure to AITC alone strongly increased the overall density of c-Fos expressing cells in SpVc (Fig. 5C, image from area 1), a result consistent with previous findings in rat (Anton et al., 1991; Ebersberger et al., 1995; Takeda et al., 1999). Finally, co-application of AITC and PEA resulted in less extensive c-Fos expression (Fig. 5D, image from area 1), indicating reduced network activity in the presence of the odorant. Quantitative evaluation of X, the mean of areas 1 to 4 (A1–A4 in Fig. 3E) as a measure of the overall SpVc activity. The value of X increased significantly (p < 0.001) upon AITC stimulation (Fig. 3E).

To repeat these experiments with lavender as an odorant, we diluted lavender oil 1:10.000 so as to avoid trigeminal stimulation. Indeed, the diluted compound did not induce any discernible rise in c-Fos signal intensity (Fig. 6A, B), consistent with the lack of irritant quality of the diluted odor. Exposure to the AITC stimulus induced
strong c-Fos expression (Fig. 6C) that was efficiently counteracted by co-stimulation with lavender (Fig. 6D). Evaluation of these data yielded a result similar to the PEA experiment. The strong c-Fos response to stimulation by AITC (Fig. 6E, red bar) was reduced by 55.2% in the presence of lavender scent (hatched bar; \( p < 0.001 \)), in line with the analgesic effect caused by that odor in the behavioral tests.

Information as to the origin of afferent trigeminal signals can be gleaned from comparing AITC-induced c-Fos expression within the four areas depicted in Fig. 3B. While all four areas displayed similar levels of c-Fos expression in the absence of AITC (Fig. 7, black bars), area 1 produced by far the highest c-Fos density upon stimulation (Fig. 7, red bars), indicating that the largest fraction of AITC-induced signals reached SpVc through the ophthalmic nerve from the nasal cavities, the eyes and possibly the meninges. Smaller but significant contributions came from the mandibular (area 2) and the maxillary (area 3) nerves, suggesting some nociceptive input from lingual, buccal and labial origin. Importantly, while the analgesic effect of odorants reduced the AITC response by 50% in area 1 (Fig. 7, hatched bars), a clear effect could also be detected in areas 2 and 3. Upon co-stimulation by AITC and PEA (Fig. 7A) or AITC and lavender (Fig. 7B) the c-Fos count did not rise significantly over control in these areas. It thus appears that the inhibitory impact of odorant stimulation is not restricted to the area of highest activity but is, instead, effective throughout the entire SpVc.

Taken together, these data demonstrate that the neuronal response of the irritant AITC is effectively suppressed by odorants at the first level of the central trigeminal pathway, a network effect consistent with the reduced nocifensive behavior in the presence of odors.

**DISCUSSION**

**Trigeminal pain modulation in mice**

The present study relied on experimental methods that provide a reproducible and quantifiable read-out for perceived trigeminal pain intensity in mice. Such methods are required to assess the extent of pain modulation and to identify modulating agents like, in this study, odorants. To assess trigeminal pain-related behavior in mice, we combined a distinctive, quantifiable, aversive pattern of behavior, the inhibition of explorative running, with the analysis of facial features that indicate the severity of pain experienced by the animals. Together, these parameters fulfilled criteria required to identify nocifensive behavior in response to trigeminal stimuli (Huang et al., 2016) and could be used to assess how effectively pain was modulated by the test odorants. The noxious stimulus AITC was presented in such a way that the animals, upon feeling the sting of the irritant during initial exploration, were able to avoid the immediate vicinity of the AITC source and restrict their movements to the edges of the open-field arena. This inhibition of free exploration was significant and it was partly mitigated by co-stimulation with either PEA or lavender. Does this result indicate a reduced level of pain perception in the presence of odor-
Fig. 4. Spatial distribution of c-Fos immunosignals in the mouse SpVc. Schematic representation illustrating the relative frequency of c-Fos signals in five coronal planes (P1–P5) following stimulation with AITC. The approximate position of each plane is indicated by red lines in the inset (sagittal aspect of the mouse brainstem), and the distance from obex is indicated under each plane. Dots represent local densities of c-Fos expressing cells. Immunosignals were observed in laminae II and III of all sections with prominent clustering in the ventral areas of P2 and P3 as well as a smaller dorsal cluster in P5. CC, central canal; MR, magnocellular region; SpVi, spinal trigeminal nucleus interpolaris; SpVc, spinal trigeminal nucleus caudalis; TT, trigeminal tract; VZ, ventral zone. The obex is the site where the fourth ventricle connects to the central canal of the medulla oblongata.

Fig. 5. Olfactory co-stimulation inhibits neural activity in SpVc. (A–D) Examples of c-Fos immunosignals in the most responsive area 1 of SpVc without stimulation (control) and following application of the indicated stimuli. (E) While exposure to PEA had no detectable effect on c-Fos density (white bar), exposure to AITC strongly increased the density of c-Fos positive cells (red bar; 102.3 ± 4.4c-Fos positive cells/mm² (two-way ANOVA, p < 0.001; n = 5 mice; mean value obtained from 475 images of 60 slices). Upon co-stimulation with PEA, the rise of c-Fos density was less severe (68.7 ± 3.1c-Fos positive cells/mm²; two-way ANOVA, p < 0.001; n = 5 mice; mean value obtained from 482 images of 61 brainstem slices) (hatched bar), indicating a 50.9% suppression of AITC-induced network activity in the SpVc as a consequence of olfactory co-stimulation.
ants? The facial features of the test animals would suggest so, as the score of the mouse grimace scale decreased from values indicating severe pain to values indicating moderate pain. Our data thus suggest a pronounced analgesic effect of odorants in the trigeminal system, an effect of suppressive pain modulation elicited by a non-nociceptive sensory modality.

This observation is consistent with the ability of PEA to mitigate avoidance of AITC in a water-drinking paradigm (Maurer et al., 2019). Importantly, this study also demonstrated that trigeminal fibers residing in the nose are neither directly affected by the presence of odorants nor by the activity of the olfactory system. Thus, PEA and lavender do not display any inhibitory effect on the nasal trigeminal terminals and its receptors; interaction does not occur in the periphery. This finding is in accordance with the recent demonstration of an analgesic effect that the main lavender-odorant linalool exerts on somatosensory pain in mice, as brought about by the animals’ response to noxious stimuli applied to the tail (Tashiro et al., 2016). Importantly, this modulatory effect did not depend on direct linalool effects on nociceptive fibers but, instead, depended on the integrity of the olfactory system, as the analgesic effect was absent in anosmic mouse models (Kashiwadani et al., 2021). The link between olfactory and nociceptive systems underlying this cross-modal effect appears to be the hypothalamus, mediated by an inhibitory effect of the hypothalamic neuropeptide orexin on neurons in the spinal cord dorsal horn (Higa et al., 2021). Thus, a concept for odor-dependent descending inhibition of pain processing is already established for the spinal cord. Our results indicate that a similar mode of pain modulation may limit trigeminal pain and, thus, may help to explain beneficial odor effects in patients suffering from headache (Martin, 2006; Prescott and Wilkie, 2007; Bartolo et al., 2013; Riello et al., 2019). It still remains to be explored whether the analgesic effect is specific for the odorants PEA and lavender oil, or whether a larger variety of odors have the capacity to antagonize trigeminal pain. In this context, pleasant odorants may exert different effects compared to aversive smells, and effective stimulus intensities still have to be determined. The odor-induced changes in AITC avoidance behavior reported here suggest that this mouse model is suited to examine such questions.

Fig. 6. Lavender oil inhibits nociceptive processing in the SpVc. (A–D) c-Fos immunosignals obtained from the most responsive area 1 of SpVc without (control) and with the indicated stimuli. (E) The diluted lavender oil (1:10,000) did not raise c-Fos density significantly (white bar), indicating its lack of trigeminal potency. Exposure to AITC, however, induced intense c-Fos expression (red bar; 91.8 ± 5.2 c-Fos positive cells/mm² (two-way ANOVA, p < 0.001; n = 5 mice; mean value obtained from 464 images of 58 slices), which was reduced to 56.3 ± 3.3 c-Fos positive cells/mm² (two-way ANOVA, p < 0.001; n = 5 mice; mean value obtained from 466 images of 59 slices) by co-stimulation with lavender (hatched bar). Thus, the lavender odor inhibited AITC-induced neural activity in SpVc by 55.2%.

Fig. 7. The analgesic effect of odorants operates throughout SpVc. (A) Density of c-Fos expressing cells in areas 1 to 4 (A1–A4) of the SpVc without stimulation (black bars), upon stimulation with AITC (red bars), and during co-stimulation with AITC and PEA (hatched bars). Localization of areas according to Fig. 3B, representing dominant afferent supply by the ophthalmic (A1), mandibular (A2) and maxillary (A3) nerves. In addition, area 4 (A4) marks the magnocellular layer of SpVc. Data evaluated from 406–482 images of 55–61 slices from 5 mice each. (B) c-Fos densities in areas 1–4 during co-stimulation with AITC and lavender (1:10,000) (hatched bars) illustrate odor-induced suppression of SpVc activity in the entire SpVc. Each mean value was obtained from 5 mice obtained from 444–466 images of 58–60 slices, respectively; significance values result from two-way ANOVA with *p < 0.05, **p < 0.01 and ***p < 0.001; n. s.: not significant.
SpVc neural activity as measure of nociceptive signal intensity

In a number of studies, stimulus-induced changes of c-Fos expression in the rodent SpVc were used to monitor afferent nociceptive signals at the initial stage of the central trigeminal system. The focus was often on the somatotopic representation of incoming signals, the question of spatial dispersion of afferent fibers originating from different parts of the head (Anton and Peppel, 1991; Sugimoto et al., 1994; Takeshita et al., 2001; Nomura et al., 2002; Ro et al., 2003). However, c-Fos immunosignals were also used successfully to evaluate subtle changes in SpVc activity, changes caused by trigeminal pain modulators such as morphine (Ebersberger et al., 1995; Bereiter, 1997), GABA (Cutrer et al., 2001; Nomura et al., 2002; Ro et al., 2003). The analysis of c-Fos expression in SpVc is, therefore, an established method to visualize activity modulation in SpVc, providing complementary data to electrophysiological recordings. Key to the reliability of this method is the way c-Fos immunopositive cells are being counted. This is a challenging task because the nuclear immunosignals in each section occur in various levels of brightness, so that a detection threshold has to be imposed for the identification of positive signals. Here we have employed a machine-learning system that we first trained to separate positive signals from background and then applied these learned constraints to the analysis of all subsequent images by using the same set of acquired parameters for each section. This procedure avoided bias that can occur upon manual counting, and it yielded robust results, indicated by SEM values < 11% of the calculated means for pooled data from up to 48 mice. We are, therefore, confident that the AITC-induced increase of c-Fos density in SpVc, as well as the odorant-dependent reduction of this c-Fos density, mirror real differences in neural activity upon stimulation and co-stimulation.

To appreciate the efficacy of PEA and lavender on mitigating AITC-evoked SpVc activity, a comparison with other pain modulators is interesting. Upon intraperitoneal injection of morphine hydrochloride into rats before the experiment, nasal stimulation with AITC caused 55% less c-Fos immunosignals in SpVc than without morphine (Ebersberger et al., 1995). Similarly, treating guinea pigs with the anti-migraine drug valproic acid before intracisternal capsaicin injection reduced c-Fos expression in SpVc by 52% (Cutrer et al., 1995). Thus, the reduction of AITC-induced c-Fos expression by 50.9–55.2% in our experiments illustrates the substantial analgesic power of odorant co-stimulation. In fact, on the level of the medullary brainstem, odor co-stimulation is as effective in suppressing network activity as pharmacological compounds for headache relief. Although too early to suggest clinical significance, our results corroborate the interest in a possible beneficial role of PEA and lavender in treating various forms of headache conditions including trigeminal neuralgia and migraine.

Olfactory gate or descending inhibition?

The neural pathway that allows odor-induced signals to impinge on nociceptive processing in the SpVc still needs to be identified. A mechanism comparable to the gate-control theory appears unlikely, as this concept requires non-nociceptive afferents to interfere with the pain system through collateral connections in the spinal cord dorsal horn. Thus, collaterals of touch receptor afferents exert presynaptic inhibition on nociceptors through inhibitory interneurons (Comitato and Bardon, 2021). In contrast, the axons of olfactory sensory neurons do not form collaterals before entering the olfactory bulb; a direct connection between those axons and neurons in the SpVc is, therefore, not possible. Furthermore, within the nasal epithelia, the excitability of trigeminal fibers was found to be unaffected by activity in the olfactory system (Maurer et al., 2019), although a modulation in the reverse direction, an inhibition of olfactory activity by trigeminal neuropeptides, can be demonstrated (Daiber et al., 2013; Genovese et al., 2017). Thus, it appears that odor-induced signals originating in the olfactory epithelium have neither direct access to trigeminal afferents within the nose, nor to the trigeminal ganglia or to the SpVc. Consequently, olfactory signals are unlikely to operate directly in modulating gating control for nociceptive afferents.

An established pathway for signal flow from the main olfactory bulb to the brainstem leads through the posterolateral amygdala and the hypothalamus (Krettek and Price, 1978; Mucignat-Caretta, 2021), pointing to the possibility that hypothalamic neuromodulators may be involved in mediating the odorant effect on SpVc. Indeed, the hypothalamic neuropeptides oxytocin and orexin are well documented pain modulators in the spinal cord (Boll et al., 2018; Kumamoto, 2019) and oxytocin was also shown to inhibit neural activity in the SpVc (Garcia-Boll et al., 2018). Moreover, tracing studies have revealed that no less than five hypothalamic nuclei are directly and bilaterally connected to SpVc, in particular to the V1 region of ophthalmic input (Abdallah et al., 2013), which represents the most responsive area in our experiments (cf. Fig. 3B, area 1). This tight connectivity suggests that olfactory signals may stimulate hypothalamic networks, which then impinge on network activity in SpVc. Exposure to PEA or lavender may thus induce hypothalamic activity that inhibits AITC-induced processing in SpVc. Such a mechanism of pain modulation would be a mode of descending inhibition, comparable to the recently discovered orexin-mediated analgesic effect of linalool on nociceptive processing in the spinal cord dorsal horn (Higa et al., 2021; Kashiwadani et al., 2021). According to this concept, the hypothalamus mediates cross-modal interaction that limits pain intensity. For further exploration of this concept it is necessary to advance from recording stimulus-induced neural activity in SpVc, as analyzed in the present study, to monitoring activity-dependent expression of markers within distinct neuronal subsets expressing receptors for hypothalamic neuropep-
tides (Boll et al., 2018; Garcia-Boll et al., 2018; Althammer et al., 2021;). Such studies offer the potential to reveal distinct signaling pathways that originate in the olfactory system and target SpVc networks via the hypothalamus.

Taken together, this study has demonstrated that behavioral and neural responses to the irritant AITC are effectively mitigated by olfactory co-stimulation with PEA or lavender. The neural effect occurs at the first level of the central trigeminal pathway, the spinal trigeminal nucleus caudalis, and inhibits trigeminal pain processing.

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DECLARATION OF INTEREST

None.

REFERENCES

Abdallah K, Artola A, Monconduit L, Dallei R, Luccarini P (2013) Bilateral descending hypothalamic projections to the spinal trigeminal nucleus caudalis in rats. PLoS One 8 e73022.

Abdallah K, Monconduit L, Artola A, Luccarini P, Dallei R (2015) GABAergic inhibition or dopamine denervation of the A11 hypothalamic nucleus induces trigeminal analgesia. Pain 156:644–655.

Agarwal N, Offermanns S, Kuner R (2004) Conditional gene deletion in primary nociceptive neurons of trigeminal ganglia and dorsal root ganglia. Genesis 36:122–129.

Albrecht J, Kopietz R, Frasnelli J, Wiesmann M, Hummel T, Lundström JN (2010) The neuronal correlates of intranasal chemical stimulation of the nasal mucosa. Brain Res 62:183–196.

Althammer F, Eliava M, Grinevich V (2021) Central and peripheral release of oxytocin: relevance of neuroendocrine and neurotransmitter actions for physiology and behavior. Handb Clin Neurol 180:25–44.

Anton F, Herdgen T, Peppel P, Leah JD (1991) c-FOS-like immunoreactivity in rat brainstem neurons following noxious chemical stimulation of the nasal mucosa. Neuroscience 41:629–641.

Anton F, Peppel P (1991) Central projections of trigeminal primary afferents innervating the nasal mucosa: a horseradish peroxidase study in the rat. Neuroscience 41:617–628.

Bartolo M, Serrao M, Gagnebeili Z, Alparidze M, Perrotta A, Padua L, Pirelli F, Nappi G, et al. (2013). Modulation of the human nociceptive flexion reflex by pleasant and unpleasant odors. Pain 154:2054-2059.

Bascouër-Sandoval C, Salgado-Salgado S, Gómez-Milán E, Fernández-Gómez J, Michael GA, Gálvez-Garcia G (2019) Pain and distraction and action to sensory modalities: current findings and future directions. Pain Pract 19:686–702.

Bensafi M, Iannilli E, Gerber J, Hummel T (2008) Neural coding of stimulus concentration in the human olfactory and intranasal trigeminal systems. Neuroscience 154:832–838.

Bereiter DA (1997) Morphine and somatostatin analogue reduce c-fos expression in trigeminal subnucleus caudalis produced by corneal stimulation in the rat. Neuroscience 77:863–874.

Bereiter DA, Hirata H, Hu JW (2000) Trigeminal subnucleus caudalis: beyond homologies with the spinal dorsal horn. Pain 88:221–224.

Boll S, Almeida de Minas AC, Raftogianni A, Herpertz SC, Grinevich V (2018) Oxytocin and pain perception: from animal models to human research. Neuroscience 387:149–161.

Boyle JA, Frasnelli J, Gerber J, Heinke M, Hummel T (2007) Cross-modal integration of intranasal stimuli: a functional magnetic resonance imaging study. Neuroscience 149:223–231.

Brand G (2006) Olfactory/trigeminal interactions in nasal chemoreception. Neurosci Biobehav Rev 30:908–917.

Castro A, Raver C, Li Y, Uddin O, Rubin D, Ji Y, Masri R, Keller A (2017) Cortical regulation of nociception of the trigeminal nucleus caudalis. J Neurosci 37:11431–11440.

Chattoraj KG, Porreca F, Sessle B (2017) Mechanisms of craniofacial pain. Cephalalgia 37:613–626.

Comitato A, Bardoní R (2021) Presynaptic inhibition of pain and touch in the spinal cord: from receptors to circuits. Int J Mol Sci 22.

Cutrer FM, Limmroth V, Ayata G, Moskowitz MA (1995), Attenuation by valproate of c-fos immunoreactivity in trigeminal nucleus caudalis induced by intracisternal capsaicin. Br J Pharmacol 116:3199-3204.

Cutrer FM, Mitsikostas DD, Ayata G, Sanchez del Rio M (1999) Attenuation by butalbital of capsicain-induced c-fos-like immunoreactivity in trigeminal nucleus caudalis. Headache 39:697–704.

Daiber P, Genovese F, Schriefer VA, Hummel T, Möhrten F, Frings S (2013) Neuropeptide receptors provide a signalling pathway for trigeminal modulation of olfactory transduction. Eur J Neurosci 37:572–582.

Donatello NN, Emer AA, Salm DC, Ludtke DD, Bordignon SASR, Ferreira JK, Salgado ASI, Venzke D, Bretchta LC, Micke GA, Martins DF (2020) Lavandula angustifolia essential oil inhalation reduces mechanical hyperalgesia in a model of inflammatory and neuropathic pain: The involvement of opioid and cannabinoïd receptors. J Neuroimmunol 340:577145.

Doty RL, Brugger WE, Jurs PC, Orndorff MA, Snyder PJ, Lowy LD (1978) Intranasal trigeminal stimulation from odorous volatiles: psychometric responses from anosmic and normal humans. Physiol Behav 20:175–185.

Dusser G, Cao Y-Q (2016) TRPM8 and migraine. Headache 56:1406–1417.

Ebersberger A, Anton F, Töllf TR, Ziegglansberger W (1995) Morphine, 5-HT2 and 5-HT3 receptor antagonists reduce c-fos expression in the trigeminal nuclear complex following noxious chemical stimulation of the rat nasal mucosa. Brain Res 676:336–342.

Frasnelli J, Schuster B, Hummel T (2007) Interactions between olfaction and the trigeminal system: what can be learned from olfactory loss. Cereb Cortex 17:2268–2275.

García-Boll E, Martinez-Lorencana G, Condés-Lara M, González-Hernández A (2018) Oxytocin inhibits the rat medial dorsal horn Sp5/C1 nociceptive transmission through OT but not V1A receptors. Neuropharmacology 129:109–117.

Genovese F, Bauersachs HG, Gräßer I, Kupke J, Maglin L, Daiber P, Möhrten S-NF, Majewska A (2017) Possible role of calcitonin gene-related peptide in trigeminal modulation of glomerular microcircuits of the rodent olfactory bulb. Eur J Neurosci 45:587–600.

Gossrau G, Baum D, Koch T, Sabatowski R, Hummel T, Haehner A (2020), Exposure to odors increases pain threshold in chronic low back pain patients. Pain Med 21:2546-2551.

Han S-M, Ahn D-K, Youn D-H (2008) Pharmacological analysis of excitatory and inhibitory synaptic transmission in horizontal brainstem slices preserving three subnuclear of spinal trigeminal nucleus. J Neurosci Methods 167:221–228.

Higa Y, Kashivadani H, Sugimura M, Kuwaki T (2021) Orexergic descending inhibitory pathway mediates linalool odor-induced analgesia in mice. Sci Rep 11:9224.
Huang D, Ren L, Qiu CS, Liu P, Peterson J, Yanagawa Y, Cao YQ (2016) Characterization of a mouse model of headache. Pain 157:1744–1760.

Hummel T, Frasenelli J (2019) The intranasal trigeminal system. Handb Clin Neurol 164:119–134.

Kakeda T, Ogino Y, Moriya F, Saito S (2010) Sweet taste-induced analgesia: an fMRI study. Neuroreport 21:427–431.

Kashiwadani H, Higa Y, Sugimura M, Kuwaki T (2021) Linalool odor-induced analgesia. Korean Physiol Pharmacol 23:271–279.

Nomura H, Ogawa A, Tashiro A, Morimoto T, Hu JW, Iwata K (2002) Induction of Fos protein-like immunoreactivity in the trigeminal spinal nucleus caudalis and upper cervical cord following noxious and non-noxious mechanical stimulation of the whisker pad of the rat with an inferior alveolar nerve transaction. Pain 95:225–238.

Oyamaguchi A, Abe T, Sugiyos N, Niwa H, Takemura M (2016) Selective elimination of isolectin B4-binding trigeminal neurons enhanced formalin-induced nocifensive behavior in the upper lip of rats and c-Fos expression in the trigeminal subnucleus caudalis. Neurosci Res 103:40–47.

Pellegrino R, Drehser E, Hummel C, Warr J, Hummel T (2017) Bimodal odor processing with a trigeminal component at sub- and suprathreshold levels. Neuroscience 363:43–49.

Prescott J, Wilkie J (2007) Pain tolerance selectively increased by a sweet-smelling odor. Psychol Sci 18:308–311.

Proudfoot CJ, Garry EM, Cottrell DF, Rosie R, Anderson H, Robertson DC, Fleetwood-Walker SM, Mitchell R (2006) Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. Curr Biol 16:1591–1605.

Riello M, Cecchini MP, Zanini A, Di Chiappani M, Tinazzi M, Fiorio M (2019) Perception of phasic pain is modulated by smell and taste. Eur J Pain 23:1790–1800.

Ro JY, Harriott A, Crouse U, Capra NF (2003) Insensitive jaw movements increase c-fos expression in trigeminal sensory nuclei produced by masseter muscle inflammation. Pain 104:539–548.

Roth E, Benuot S, Quentin B, Lam B, Will S, Ma M, Heely N, Danwsh T, Shrestha Y, Gribble F, Reimann F, Pshenichnaya I, Yeo G, Baker DJ, Trevaskis JL, Blouet C (2021) Behavioural and neurochemical mechanisms underpinning the feeding-suppressive effect of GLP-1/CCK combinatorial therapy. Mol Metabol 43:101118.

Sandri A, Cecchini MP, Riello M, Zanini A, Nocini R, Fiorio M, Tinazzi M (2021) Pain, smell, and taste in adults: a narrative review of multisensory perception and interaction. Pain Ther 10:245–268.

Sessle BJ (2000) Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit Rev Oral Biol Med 11:57–91.

Silver WL, Moulton DG (1982) Chemosensitivity of rat nasal trigeminal receptors. Physiol Behav 28:927–931.

Sjöstrand C, Savic I, Laidon-Meyer E, Hillert L, Lodin K, Waldenlind C (2016) Characterization of a mouse model of headache. Pain 157:10:3546–3561.

Takeshita S, Hiraoka T, Morimoto T, Kuwaki T, Kashiwadani H (2016) Odour-induced analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. Curr Biol 16:1591–1605.

Terada Y, Hirata H, Bereiter DA (2001) Intensity coding by TMJ-responsive neurons in superficial laminae of caudal medullary dorsal horn of the rat. J Neurophysiol 86:2393–2404.

Tashiro S, Yamauchi R, Ishikawa S, Sakurai T, Kajita K, Kanamura Y, Kuwaki T, Kashiwadani H (2016) Odour-induced analgesia mediated by hypothalamic orexin neurons in mice. Sci Rep 6:37129.

Teraoka Y, Masuda H, Watanabe T (2015) Structure-activity relationship study on isothiocyanates: comparison of TRPA1-activating ability among allyl isothiocyanate and specific flavor components of wasabi, horseradish, and white mustard. J Nat Prod 78:1937–1941.

Tremblay C, Frasenelli J (2021) Olfactory-trigeminal interactions in patients with Parkinson’s disease. Chem Senses 46:1–8.

Tu C-Y, Fan C-H, Chiu N-H, Ho Y-J, Lin Y-C, Yeh C-K (2020) Targetted delivery of engineered auditory sensing protein for ultrasounr neustnoculation in the brain. Theranostics 10:3546–3561.

Yin H, Lee KE, Park SA, Bhatiar JP, Suh BJ, Jeon JG, Kim BG, Park SJ, Han SK (2009) Inhibitory effects of somatostatin on the substantia gelatinosa neurons of trigeminal subnucleus caudalis via somatostatin type 2 receptors in juvenile mice. Brain Res 1304:49–56.
Yin H, Park SA, Han SK, Park SJ (2011) Effects of 5-hydroxytryptamine on substantia gelatinosa neurons of the trigeminal subnucleus caudalis in immature mice. Brain Res 1368:91–101.

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