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Activation of Oral Trigeminal Neurons by Fatty Acids is Dependent upon Intracellular Calcium

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Abstract

The chemoreception of dietary fat in the oral cavity has largely been attributed to activation of the somatosensory system that conveys the textural properties of fat. However, the ability of fatty acids, which are believed to represent the proximate stimulus for fat taste, to stimulate rat trigeminal neurons has remained unexplored. Here, we found that several free fatty acids are capable of activating trigeminal neurons with different kinetics. Further, a polyunsaturated fatty acid, linoleic acid (LA), activates trigeminal neurons by increasing intracellular calcium concentration and generating depolarizing receptor potentials. Ion substitution and pharmacological approaches reveal that intracellular calcium store depletion is crucial for LA-induced signaling in a subset of trigeminal neurons. Using pseudorabies virus (PrV) as a live cell tracer, we identified a subset of lingual nerve-innervated trigeminal neurons that respond to different subsets of fatty acids. Quantitative real-time PCR of several transient receptor potential (TRP) channel markers in individual neurons validated that PrV labeled a subset but not the entire population of lingual-innervated trigeminal neurons. We further confirmed that the LA-induced intracellular calcium rise is exclusively coming from the release of calcium stores from the endoplasmic reticulum in this subset of lingual nerve-innervated trigeminal neurons.

Keywords

Somatosensory system; calcium imaging; Pseudorabies virus; linoleic acid

Introduction

Dietary fat contributes significantly to food palatability. In general, higher fat content is associated with greater hedonic value in food [18]. Also, fat is the most energy dense of all dietary macronutrients. The excessive energy intake associated with high dietary fat intake is considered one of the contributing factors for the obesity epidemic in developed countries in recent decades [7,8]. Although the link between fat intake and obesity is obvious, few studies have focused on the mechanisms underlying how the somatosensory system recognizes dietary fat. Understanding these detailed mechanisms may help combat the incidence of dietary-induced obesity in modern society.

Perhaps the most salient sensory cue for dietary fat lies in its texture or “mouth feel”, a property generally attributed to the somatosensory system [46,51]. However, in recent years there has been a focus on the ability of fat, in the form of free fatty acids, to stimulate the gustatory system. To this end there has been mounting evidence that the gustatory system can respond to fatty acids, consistent with their being a taste of fat [1,11,24,38,42]. Over a decade ago, it was shown that free fatty acids could activate mammalian taste cells by

blocking delayed rectifying potassium (DRK) channels [21-23]. More recently, several fatty acid-responsive proteins have been identified that may play a role in fatty acid transduction, including the fatty acid-binding protein, CD36 [2,20,32,48] and several fatty acid activated G-protein-coupled receptors (GPCRs) [14,25,37,41,45]. Very recently, Liu et al. [36] provided evidence that a polyunsaturated fatty acid, linoleic acid (LA), could directly activate taste receptor (Type II) cells by eliciting an intracellular Ca^{2+} rise and subsequent depolarization through activation of transient receptor potential channel type M5 (TRPM5) [36], the same pathway that appears involved in sweet, bitter and umami tastes [16,31,49]. While it is clear that fatty acids are capable of activating the peripheral taste system, little is known about their ability to stimulate the sensory neurons that underlie the textural properties attributed to dietary fats in the oral cavity. In fact, despite the intensive studies in somatosensation in the central nervous system, there is virtually nothing known about the ability of dietary fat to activate oral trigeminal neurons.

In the present study, we show for the first time that several free fatty acids, including short chain saturated, medium and long chain monounsaturated and long chain polyunsaturated fatty acids could stimulate trigeminal neurons by increasing intracellular Ca^{2+} levels. To specifically identify the subpopulation of oral trigeminal neurons we developed techniques using pseudorabies virus (PrV) to trace retrogradely the lingual nerve-innervated subpopulation of trigeminal neurons. Moreover, by focusing on the prototypical taste-involved fatty acid, LA, we were able to show that intracellular Ca^{2+} stores play an important role in fatty acid transduction in a significant proportion of identified trigeminal neurons. Taken together, these data show for the first time that fatty acids can stimulate somatosensory neurons and begin to unravel the mechanisms of fat somatosensation in lingualinnervated rat trigeminal neurons, where there is a critical role of intracellular Ca^{2+} stores.

Methods

Primary culture of trigeminal neurons

Trigeminal neurons were isolated from male adult and neonate (P0-P7) Sprague-Dawley rats according to methods modified from Simon and colleagues [34,35]. Anesthesia procedures were approved by Utah State University IACUC. Briefly, sodium pentobarbital (50 mg/kg) was injected i.p. to achieve anesthesia in adult rats. For neonate rats, anesthesia was achieved by placing the pups in a water-tight container (latex-glove) and immersing them in ice-cold water for 2-3 min to induce hypothermia. For each series of experiments, rats were decapitated and the paired trigeminal ganglia were dissected aseptically and washed in Hank's balanced salt solution (HBSS). Ganglia were cut into pieces and incubated (37 °C, 5% CO_2) for 30 min in 2.5 mg/ml collagenase (Type XI, Sigma). Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by 10-min incubation at 37 °C in 100 $\mu\text{g}/\text{ml}$ DNase I (sigma). Cells were centrifuged, resuspended and plated onto glass coverslips in culture medium (1:1 DMEM/F12 with 10% FBS and 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin). For the PrV infected neurons, 400 $\mu\text{g}/\text{ml}$ foscarnet was supplemented in the culture media.

Calcium imaging and electrophysiology

Trigeminal neurons were loaded with Fura-2/AM (5 μM ; Invitrogen) for 1 hour in Tyrode's (physiological saline) solution with 0.5% pluronic acid at 37 °C. A monochromator (Bentham FSM150, Intracellular Imaging Inc.) was used as the excitation light source. The cell fluorescence was collected by a CCD camera (PixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2™, Intracellular Imaging). The ratio of fluorescence (340 nm/380 nm) was directly converted to Ca^{2+} concentrations using

a standard curve generated by the imaging system using Fura-2 Ca^{2+} imaging calibration kit (Invitrogen). Stimuli were applied extracellularly with a bath perfusion system at a flow rate of at least 4 ml/min permitting complete exchange of the extracellular solution in less than 20 seconds. All the free fatty acids were dissolved in Tyrode's solution (in mM: 140 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose and 10 sodium pyruvate, pH 7.4) unless stated otherwise. Ca^{2+} and Na^+ free solutions were made by substitution with the relevant amount of EGTA or mannitol, respectively. For generation of concentration-responsive curves, some trigeminal neurons were treated with LA in an ascending concentration series, while others were tested in random order. No differences were observed using these two stimulus paradigms. In all experiments, solution presentation order was randomized for different fatty acids. To quantify the adaptation associated with responses to C18:1 and C14:0 administration, a first order exponential fit was used to estimate the time constant (τ). To deplete intracellular Ca^{2+} stores, thapsigargin (1 μM) was added during Fura-2 loading for a period lasting as long as 1 hour.

Current clamp recording was performed to measure LA-induced membrane potential change with an Axopatch-200B amplifier (Molecular Devices) controlled by pClamp software (v. 10; Molecular Devices). Pipette that had resistances of $\sim 2 \text{ M}\Omega$ were used to perform patch clamp recording on trigeminal neurons that had a diameter between 20-30 μm corresponding to medium size nociceptors [5]. The membrane potential of trigeminal neurons was recorded continuously before, during and after bath application of LA using the current clamp mode of the amplifier while holding the cell at its zero current level. Intracellular solution for current clamp recording contains: 140 K gluconate, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH 7.2 adjusted with KOH; 310 mosM.

PrV labeling of lingual-innervated trigeminal neurons

DNA of the PrV Kaplan strain that contained GFP as the reporter gene (PrV- Ka Δ gGfp) was generously provided by Dr. Mettenleiter (Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Germany). Detailed characteristics of the PrV Kaplan strain has been previously described [47]. The DNA was transfected into PK15 cells for virus recovery. It was further propagated in PK15 cells and harvested after the complete cytopathic effect was shown. Viral stocks containing 3.2×10^8 PFU/ml were aliquoted in 100 μl volumes and stored at -80°C .

For injection of PrV into the tongue, P0-P7 neonate rats were anaesthetized by ~ 3 min hypothermia and placed on their backs. The tongue was gently extended with a blunt forceps, and 10 μl of high titer PrV was injected with a sterile Hamilton syringe. The injection site was several mm anterior from the frenulum of the tongue and inserted in a posterior fashion into the tongue muscle along the tract of the lingual nerve. The PrV solution was applied while withdrawing the needle from back toward the tip of the tongue. The neonate rats were placed back with their mothers for 12-24 hours before the trigeminal ganglia were collected.

qRT-PCR

Procedures for quantitative and regular qRT-PCR have been described previously [22]. Primers for CD36 (sense/antisense/probe; 5'-GGTGTGCTGGACATTGG-3'/5'-CTATGCTCATCTTCGTTAGG-3'/5'-TCAAGCCTTCGATAGGTTCTGAGACATCA-3'; 1000-1016/1108-1127/1077-1105), GPR40 (sense/antisense/probe; 5'-TTCTTTCTGCCCTTGTTAT-3'/5'-GCCCTGAGCTTCCGTTTG-3'/5'-ACTGCTTTCTGCTATGTGGGCTGCCTC-3'; 575-594/652-669/596-622), GPR43 (sense/antisense/probe; 5'-GCGGGCATCAGCATAGAA-3'/5'-CCCACCTGCTCGGTTGAGTT-3'/5'-

TGGCTTTCCCGGTGCAGTACAAGCTATC-3'; 301-318/451-470/332-359), and GPR120 (sense/antisense/probe; 5'-CTGCACATTGGATTGGC-3'/5'-TCTGGTGGCTCTCGGAGTAT-3'/5'-CTCTCCGCGAGGCTTTCGTGATCTGT-3'; 624-640/783-802/738-764) were designed using Oligo 6.0 Primer Analysis Software (Molecular Biology Insights). For GPR84 detection, a commercially available TaqMan Gene Expression Assay (ABI, Foster City CA) was used.

Statistical analysis

The significant effects of all the treatments were determined by unpaired Student's t-test ($\alpha=0.005$) compared with their controls as described in the text. Data are presented as mean $\pm$ SEM, unless otherwise indicated.

Results

Fatty acids elicit an intracellular Ca^{2+} rise and depolarize trigeminal neurons

To test whether somatosensory neurons are capable of responding to fatty acids, we used cell-based approaches including functional Ca^{2+} imaging and patch-clamp recording. First, ratiometric Ca^{2+} imaging was used to explore the ability of a series of fatty acids to elicit an intracellular Ca^{2+} rise in rat trigeminal neurons. As shown in Fig. 1a and 1b, trigeminal neurons were treated with several fatty acids including polyunsaturated (C18:2, C20:4 and C22:6), monounsaturated (C18:1) and saturated (C6:0 and C14:0) fatty acids. Ca^{2+} transients could be induced in trigeminal neurons by some of these fatty acids in a robust and reversible fashion. Interestingly, long chain polyunsaturated fatty acids activated trigeminal neurons with a comparatively slow onset and the responses showed little adaptation. However, some monounsaturated and saturated fatty acids we tested (C18:1 and C14:0, respectively) activated trigeminal neurons in a decidedly different manner. Responses to these fatty acids were characterized by an initial delay followed by rapid onset (delay time: C18:1, 141.5 ± 37.8 s, $n=11$; C14:0, 167 ± 18.9 s, $n=17$) and subsequent adaptation (τ approximately 68 sec). Different G protein-coupled receptors (GPCRs) have been implicated in the transduction of different classes of fatty acids. GPR40 and GPR120 are GPCRs which respond to medium and long chain fatty acids *in vitro* and *in vivo* [14,9,27,26,33], GPR84 is activated by medium chain fatty acids [52] while GPR41 and GPR43's ligands are short chain fatty acids [10,55]. These GPCRs are each expressed in trigeminal ganglia by qRT-PCR (Fig. 1e) and, our responses are consistent with multiple functional fatty acid activated GPCRs in the somatosensory system.

To characterize the fatty acid transduction pathway in greater detail, we chose to focus our efforts on linoleic acid (LA), a well-characterized long chain polyunsaturated fatty acid that has been the focus of the majority of research on fatty acid taste. Initially, we performed a series of experiments to characterize the concentration-response function for LA-induced responses in isolated trigeminal neurons. LA (1, 3, 10, 30 and 100 μM) elicited concentration-dependent changes in intracellular Ca^{2+} with an EC_{50} of 19.5 μM (Fig. 1c), a value near the one found in taste cells (13.7 μM) [36]. Accordingly, we chose to use 30 μM LA as our prototypical concentration for fatty acid stimulation, since it produced significant but non-saturating responses. Following these data, we also checked whether LA (30 μM) had the ability to depolarize trigeminal neurons using the current clamp mode in patch clamp recording. In 9 cells tested with an average resting potential of -62.7 ± 3.3 mV, 7 showed large depolarizing responses (61.5 ± 6.4 mV) to LA (Fig. 1d). Noticeably, the responses also exhibited a slow onset after stimulation, which was comparable with the time course of responses recorded in Ca^{2+} imaging assays.

LA-induced Ca^{2+} responses depend on release from intracellular stores in a subpopulation of trigeminal neurons

To begin to explore the mechanisms underlying the ability of fatty acids to stimulate the somatosensory system, we investigated the ionic dependence of the LA-induced intracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{in}}$) change. As shown in Fig. 2a and 2b, replacement of extracellular Na^+ with mannitol caused a modest decrease in the magnitude of LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ rise. Replacement of Ca^{2+} with EGTA, on the other hand, had highly variable effects, leading to either a complete inhibition (Fig. 2a, trace 1), moderate inhibition (Fig. 2a, trace 2) or a lack of inhibition of LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ increase (Fig. 2a, trace 3), which indicated part of the intracellular Ca^{2+} increase could be attributed to influx from the extracellular environment. When comparing the dependence of the response on extracellular and intracellular Ca^{2+} , there was no obvious evidence of distinct subgroups (Fig. 2c).

Since the ionic substitution experiments indicated that the source of $[\text{Ca}^{2+}]_{\text{in}}$ increase could be variable among cells, we investigated the dependence of the LA response on endoplasmic reticulum Ca^{2+} store. To do so, we treated trigeminal neurons with thapsigargin (TG; $1 \mu\text{M}$), a sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor. Interestingly, the effects of TG treatment revealed that there were two apparent types of responses in trigeminal neurons based upon their relative sensitivity to TG (Fig. 2d and 2e). After 1h incubation of $1 \mu\text{M}$ thapsigargin, one subgroup of trigeminal neurons (TG-insensitive) was still responding robustly to LA even in Ca^{2+} free Tyrode's buffer (Fig. 2d), whereas the TG-sensitive subgroup of trigeminal neurons (Fig. 2e) showed attenuated abilities to stimulate neurons, and also a complete inhibition of responses in Ca^{2+} free buffer. When comparing the mean $[\text{Ca}^{2+}]_{\text{i}}$ increase in physiological saline (Tyrode's) between TG-insensitive and TG-sensitive neurons, it was clear that in TG-sensitive neurons the LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ rise was dependent upon a significant contributions from intracellular sources and from the extracellular environment (Fig. 2f). In contrast, the mechanisms and physiological roles of the TG-insensitive trigeminal neurons remain unclear.

Lingual trigeminal neurons show variable fatty acid specificity

We were interested in determining if the variability in the ion dependence of LA responses was attributable to different populations of trigeminal neurons (i.e. of oral, nasal, or facial origin). To test this idea, we explored the use of viral tracers to identify the subpopulation of trigeminal neurons that innervates the tongue. Pseudorabies virus (PrV) is a porcine herpes virus that has been successfully utilized as a 'live-cell' tracing tool in neuroscience research [13]. Its neurotropic feature allows nerve endings to pick up the virions and spread the infectious progeny across synapses within a circuit [12]. Specifically, PrV-Bartha and PrV-Kaplan strains which carried the DNA of the fluorescent protein GFP have been made and used as tracers of nasal and cutaneous trigeminal neurons [17,47]. In the present study, we injected into the tongues of rats an engineered PrV-Kaplan strain, PrV- $\text{Ka}\Delta\text{gGfp}$, with GFP as the reporter gene. In order to obtain the best infection efficiency, we injected PrV into neonate rats instead of adult rats, which were used in the earlier studies. A 12-24 hour post inoculation (hpi) time-window was chosen to allow the broadest possible PrV infection, but limit the spread of non-specific infection [47]. Previous reports have indicated that the PrV needs ~12 hours to reach the cell bodies of trigeminal neurons [47]. Further, by selecting a time from 0-12 hours after cell body infection (12-24 hpi) we were within a time frame where there would be no expected electrophysiological changes in the cells due to infection [39]. Foscarnet ($400 \mu\text{g}$) was supplemented to regular culture medium for trigeminal neurons to inhibit the non-specific viral spread [17]. In order to obtain the largest labeled neuron population in the trigeminal ganglia, PrV was injected into the tongue muscle in the vicinity of the lingual nerve without any lateral restrictions. Though no specific cell counts were made in this study, as shown in Fig. 3a, about 10% of the trigeminal neurons

isolated from PrV-injected rats expressed GFP, which indicated their lingual origin. It was also clear that GFP was observed in isolated trigeminal neurons with different soma diameters, which is indicative of different functional classes of somatosensory neurons (i.e. trigeminal neurons and dorsal root neurons). However, in the present study, cells with soma diameter less than $\sim 20 \mu\text{m}$ were much more commonly labeled and it was this population of cells that was used in subsequent studies.

To validate that PrV+ trigeminal neurons maintained their physiological function, we performed Ca^{2+} imaging experiments to test the responsiveness of PrV+ trigeminal neurons to a series of prototypical trigeminal irritants. The labeled neurons were tested with menthol, mustard oil and capsaicin, which have been shown to activate somatosensory neurons by triggering the TRPM8, TRPA1 and TRPV1 channels activities alone or together [3,28,19,30]. These chemicals reflect the cooling, tingling or spicy features of foods that are mediated by the somatosensory system [3,4]. All three stimulants could activate PrV-labeled neurons (PrV+) although they might not stimulate the same cell (Fig. 3b and 3c). We also collected PrV+ trigeminal neurons for single cell qRT-PCR assay on TRPM8 and TRPA1. Consistent with the calcium imaging data, some neurons apparently expressed both TRPM8 and TRPA1, while some other neurons were only positive for TRPM8 (data not shown). Taken together, the 'live-cell' tracing tool PrV- $\text{Ka}\Delta\text{gGgfp}$ was successfully adopted and refined for identifying a functional population of lingual trigeminal neurons.

Next, we re-examined fatty acids' ability to stimulate PrV+ trigeminal neurons. Since we were initially interested in whether these single neurons respond to multiple classes of fatty acids, we applied three fatty acids from different categories sequentially on the same PrV+ neurons: C18:2 (LA), C18:1(oleic) and C14:0 (myristic). Fig. 4a and 4b show examples of different fatty acid classes activating individual trigeminal neurons with their characteristic response profile (i.e. slow vs. rapid initiation of $[\text{Ca}^{2+}]_{\text{in}}$ increase). For 165 PrV+ trigeminal neurons tested with these three fatty acids, we found that LA activated the largest population (67%), followed by oleic (28%) and myristic acids (22%) (Fig.4c). There was a significant amount of overlap in the response profile to different fatty acids. For example, 9% of the entire PrV+ population responded to all three fatty acids (Fig. 4c). However, some neurons only responded to individual fatty acid (i.e. 47%, 3% and 3% of trigeminal neurons only responded to linoleic, oleic and myristic acids, respectively). Interestingly, as shown in Fig. 4d, trigeminal neurons from neonatal rats exhibit less robust responses to LA than those from adults. Further, we found no significant difference in magnitude of the LA-induced responses in PrV- and PrV+ neurons. Clearly, the PrV tracing technique was not able to isolate the entire population of LA-sensitive lingual trigeminal neurons. Further, it is likely that trigeminal neurons that innervate other areas may also have the capability of being activated by LA.

LA-induced intracellular Ca^{2+} rises in PrV+ trigeminal neurons are dependent on intracellular Ca^{2+} release

In order to study specifically how fatty acids stimulate trigeminal neurons from lingual origin, we investigated the ionic dependence of LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ increases on PrV+ and PrV- neurons, which in neurons from adult rats showed a variable dependence on $[\text{Ca}^{2+}]_{\text{in}}$. As seen in trigeminal neurons from adults, replacement of extracellular Na^+ had no obvious effect on the LA-induced $[\text{Ca}^{2+}]_{\text{in}}$ increase in either PrV+ and PrV- neurons (Fig. 4a). For the trigeminal neurons from PrV injected rats, we compared the LA responses in PrV+ and PrV- neurons for their relative dependence upon extracellular and intracellular Ca^{2+} (Fig. 4b). In general, there were distinct differences between PrV+ and PrV- neurons in the source of the LA-induced Ca^{2+} increase (Fig. 4b). Taken together as a group, PrV+ neurons were more highly dependent upon release of Ca^{2+} from intracellular stores than were the PrV- neurons (Fig. 4c). Therefore, we concluded that in lingual trigeminal neurons (i.e. PrV

), the LA-induced $[Ca^{2+}]_{in}$ increase was independent of extracellular Na^+ and significantly dependent upon release from intracellular stores.

To further confirm the LA-induced $[Ca^{2+}]_{in}$ increase is coming from the intracellular Ca^{2+} store, again, we pretreated trigeminal neurons from PrV injected animals with thapsigargin (1 μM) to deplete the ER Ca^{2+} stores, and asked whether LA could still activate the neurons. As shown in Fig. 4d, after a 1-h thapsigargin treatment, LA-induced $[Ca^{2+}]_{in}$ increases in Tyrode's buffer were significantly reduced in PrV+ neurons compared to PrV- ones (Fig. 4e), which was consistent with previous data from TG-sensitive trigeminal neurons pooled from adult rats (Fig. 2d & 2e). To further confirm the role of intracellular Ca^{2+} store, we removed extracellular Ca^{2+} again in TG treated neurons. The $[Ca^{2+}]_{in}$ increase was abolished in both PrV+ and PrV- trigeminal neurons. Noticeably, the TG-insensitive population still existed in both populations, but was reduced (PrV+, 10% and PrV-, 13%, compared with 32% from pooled data). Therefore, PrV tracing failed to isolate an entirely uniform population of trigeminal neurons that were sensitive to intracellular store depletion. Nonetheless, we concluded that the intracellular Ca^{2+} increase initiated by LA stimulation of lingual trigeminal neurons was largely dependent upon release from the intracellular Ca^{2+} stores.

Discussion

In addition to taste and smell, oral somatosensation contributes significantly to the sensory cues provided by food. For example, mustard oil and carbon dioxide could incorporate a pungent feel to food and drink through somatosensory neurons by targeting TRPA1 and TRPV1, or TRPA1 alone, respectively [53,3,28,19,30]. Capsaicin generates hot and spicy sensations by activation of TRPV1 [15,29]. Cooling mouth feel caused by menthol and icilin is contributed by activation of TRPM8 and TRPA1 [40,43,50,30], making both TRPM8 and TRPA1 cold sensors. Another pungent agent 'sanshool' from Szechuan pepper activates sensory neurons through inhibiting background potassium channels to give a 'tingling and numbing' feel to the mouth [4]. Thus, the contributions of the somatosensory system to chemosensory perception of food are complex.

Until quite recently, fat was thought to be tasteless and that its only salient cues were provided by its texture via activation of the somatosensory system [46,51]. High fat content diet generally gives a mouth feel of creaminess, slipperiness or oiliness. Given the recent data showing that free fatty acids are able to activate the taste system in a similar mechanism to those activated by more classical tastants like sweet, umami and bitter [36], we sought to determine if trigeminal neurons could respond in a similar fashion to the chemical cues (fatty acids) that lead to taste cell activation. In this report, we showed that several free fatty acids were capable of stimulating trigeminal neurons by depolarizing and increasing intracellular Ca^{2+} , in a concentration range ($EC_{50}=19.5 \mu M$) similar to that of the taste system. This is the first evidence showing that physiologically relevant concentrations of free fatty acids are capable of activating the somatosensory system as well.

LA is a polyunsaturated fatty acid that is rich in diets from different cultures and has been used as the prototypical fat stimulus in studies of the taste. Using ion substitution, we showed that in trigeminal neurons from adult rats, both intracellular Ca^{2+} stores and extracellular Ca^{2+} contributed to the fatty acid-induced intracellular Ca^{2+} increase (Fig. 2). This is consistent with the heterogeneous nature of trigeminal neurons. Nonetheless, since extracellular Na^+ does not contribute significantly to the LA-induced intracellular Ca^{2+} increase (Fig. 2), we could conclude that, unlike taste cells [36], downstream sodium-permeable channels like TRPM5 are not involved in this pathway in trigeminal neurons. Although depletion of intracellular Ca^{2+} stores with thapsigargin (ER Ca^{2+} ATPase

inhibitor) was not effective in all trigeminal neurons, our data clearly indicate that the involvement of intracellular Ca^{2+} stores in the fatty acid transduction pathway in the somatosensory system. Also, consistent with data from the taste system [36], our results showed evidence that ER depletion triggered store-operated Ca^{2+} entry (SOCE) existed in trigeminal neurons. After incubation with thapsigargin, the initial resting Ca^{2+} level increased significantly (Fig. 2d & 2e, arrows). The application of LA first decreased basal Ca^{2+} levels, then increased Ca^{2+} concentration gradually. This is probably due to the fact that fatty acids like LA inhibit SOCE, followed by the LA-induced activation of the trigeminal neurons [54]. However, more studies have to be done to further address the role of SOCE, if any, in fatty acid transduction.

In order to more specifically target the ionic dependence of fatty acid induced changes in intracellular Ca^{2+} to the lingual population of trigeminal neurons, we utilized pseudorabies virus (PrV) as a tool for tracing trigeminal neurons of lingual origin. Several groups have reported tracing nasal or oral trigeminal neurons or feeding neural circuits successfully [13,17,44], but little functional or physiological roles of PrV labeled neurons have been reported. We attempted to verify the lingual origin of PrV+ cells by checking their responsiveness to menthol, mustard oil and capsaicin. Some PrV+ trigeminal neurons were able to be activated by all three stimulants, while most other neurons were only responsive to menthol. This is consistent with the previous findings that menthol activates a distinct subset of about 10% of sensory neurons [6], which might indicate that small TRPM8 positive neurons are mainly coming from lingual nerve innervation. Therefore, we can conclude that although PrV did not label the entire population of lingual trigeminal neurons, the PrV+ subset of cells exclusively comes from lingual innervation. Indeed, in experiments using PrV+ neurons, LA-induced Ca^{2+} mobilization was largely dependent on release from ER Ca^{2+} stores and did not show the high degree of variability seen in the pooled trigeminal neurons from adult rats.

In the present study, we have shown that fatty acids can depolarize and elicit a rise in intracellular Ca^{2+} in trigeminal neurons. As a first approach to understanding this pathway, we have attempted to identify the source of the LA-induced rise in intracellular Ca^{2+} . Clearly, as stated above, the rise in intracellular Ca^{2+} is dependent to a significant degree on release from ER stores for the trigeminal neurons of lingual origin. In the case of taste cells [36], the LA-induced depolarization is downstream of this Ca^{2+} increase. We suspect that a similar membrane channel is involved in trigeminal neurons also. However, we have not at the present time identified the presumed channel responsible for the LA-induced depolarization. Given the fact that the LA-induced responses are completely independent of extracellular sodium, it is unlikely a monovalent cation-permeable TRPM5-like channel is involved. Moreover, TRPM5 is not expressed in trigeminal neurons (data not shown). Obviously, while we have shown as a first step that fatty acids at physiological concentrations are effective in activating lingual trigeminal neurons, specifics of the signaling elements of this pathway require further exploration.

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Fig. 1.

Fatty acids elicit a rise in intracellular Ca^{2+} in adult rat trigeminal neurons. (a) Specificity of fatty acid responsiveness in isolated trigeminal neurons. Various fatty acids (C14:0, C18:1, C20:4 and C22:6) were able to induce intracellular Ca^{2+} increases in subsets of trigeminal neurons, with the exception of the short chain saturated fatty acid, caproic acid (C6:0). All fatty acids were applied at a final concentration of 30 μM . (b) LA (C18:2) initiates a robust intracellular Ca^{2+} increase in single trigeminal neurons. (c) LA dose responsive curve, with EC_{50} at 19.5 μM ($n=77$). LA was applied at 1-100 μM final concentration. (d) LA (30 μM)-induced depolarization of trigeminal neurons using current clamp recording holding the cell at the zero current level (i.e. rest). (e) Expression of putative fatty acids receptive proteins CD36, GPR40, GPR43, GPR84 and GPR120 relative to a calibrator (GAPDH; [22]).

Fig. 2.

LA-induced $[Ca^{2+}]_{in}$ increase in trigeminal neurons from adult rats was dependent on both extracellular and intracellular Ca^{2+} , but not extracellular Na^{+} ions. LA was applied at a final concentration of 30 μ M. (a) Three subtypes of responses (traces 1, 2, 3) were recorded when extracellular Na^{+} and extracellular Ca^{2+} were subsequently removed after regular Tyrode's buffer. LA could stimulate all three types of trigeminal neurons without the presence of extracellular Na^{+} , but the responses could be dependent (trace 1), moderately dependent (trace 2), or independent (trace 3) of extracellular Ca^{2+} . (b) Normalized mean responses ($[Ca^{2+}]_{in}$ increase in Na⁺ free Tyrode's / Tyrode's $\pm$ SEM) to LA in Tyrode's and Na⁺ free Tyrode's (n = 153; p=0.006) (c) Histogram of the normalized $[Ca^{2+}]_{in}$ increase ($[Ca^{2+}]_{in}$ increase in Ca²⁺-free Tyrode's / Tyrode's). (d-e) Trigeminal neurons could be both thapsigargin (TG, 1 μ M) insensitive and sensitive. After 1 h TG incubation, trigeminal neurons were treated with LA in Tyrode's buffer, followed by Ca²⁺ free Tyrode's. Arrows are pointing to the initial resting Ca²⁺ levels before application of LA. TG-insensitive trigeminal neurons were still able to be activated by LA in regular and Ca²⁺ free Tyrode's (d), while TG-sensitive trigeminal neurons were showing reduced and diminished responses in Tyrode's and Ca²⁺ free Tyrode's (e). (f) Mean responses (rise in $[Ca^{2+}]_{in}$) $\pm$ SEM to LA in Tyrode's and Ca²⁺ free Tyrode's in TG-insensitive (n=38) and TG-sensitive (n=81) trigeminal neurons. *, p<<0.005 compared with Tyrode's responses in TG-insensitive trigeminal neurons. **, p<<0.005 compared with Tyrode's responses in TG-sensitive trigeminal neurons.

Fig. 3.

Pseudorabies virus (PrV) injected to the tongue successfully labeled a subpopulation of trigeminal neurons, which could be activated by menthol (1000 μM), mustard oil (100 μM) and capsaicin (3 μM). (a) Fluorescence image showing PrV labeled trigeminal neurons ~24 hours after injection. Arrows point to trigeminal neurons that were successfully infected by PrV. Scale bar, 100 μm . (b-c) Bath application of menthol, mustard oil and capsaicin successfully stimulated PrV-labeled (PrV+) trigeminal neurons with varying degrees of efficacy.

Fig. 4. PrV-labeled (PrV+) trigeminal neurons were able to be activated by different fatty acids (30 μ M). (a-b), bath application of LA (C18:2), oleic (C18:1) and myristic (C14:0) acids stimulate PrV-labeled (PrV+) trigeminal neurons. (c) The Venn diagram of the proportion of fatty acid responsiveness seen in PrV+ neurons. In PrV+ neurons, 67% (n=186) of trigeminal neurons respond to LA, while 28% (n=186) and 22% (n=186) of neurons respond to oleic acid (gray circle) and myristic acid (dashed circle), respectively. The overlap of three circles is the proportion of trigeminal neurons that responded to all three fatty acids (9%). (d) Mean responses (rise in $[Ca^{2+}]_{in}$) $\pm$ SEM induced by LA in trigeminal neurons from adult rats, neonatal rats (total number), PrV- neurons and PrV+ neurons. *, $p < 0.005$.

Fig. 5.

LA-induced $[Ca^{2+}]_i$ increase in PrV+ trigeminal neurons was dependent on extracellular Ca^{2+} but not extracellular Na^+ . LA was applied at a final concentration of 30 μM . (a) Two representative graphs of LA-induced $[Ca^{2+}]_i$ increase in regular and Na^+ free Tyrode's in PrV+ neuron (black trace) and PrV- neuron (gray trace). (b) Histograms of mean normalized percentages (LA-induced $[Ca^{2+}]_i$ increase in Ca^{2+} free Tyrode's/Tyrode's, %) in PrV+ (n=117, black squares) and PrV- trigeminal neurons (n=101, gray squares). (c) Ratio of the >91% population to 0-10% population in (b). (d) One hour thapsigargin (1 μM) treatment reduced the LA-induced $[Ca^{2+}]_i$ increases in Tyrode's in PrV+ (black trace) neurons and inhibited responses in both PrV+ (black trace) and PrV- (gray trace). (e) Mean responses (rise in $[Ca^{2+}]_i$) $\pm$ SEM induced by LA in Tyrode's and Ca^{2+} free Tyrode's in both PrV+ (n=93) and PrV- TGNs (n=96) when pretreated with TG. *, $p < 0.005$.