Reflex responses of human masseter motor units to mechanical stimulation of the teeth

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1Institute of Physiology and Pharmacology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania; 2Centre for Brain Research, Ege University, Bornova, Izmir, Turkey; and 3Koç University School of Medicine, Sariyer, Istanbul, Turkey

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Uginčius P, Atiš ES, Türker KS. Reflex responses of human masseter motor units to mechanical stimulation of the teeth. J Neurophysiol 111: 51–61, 2014. First published October 16, 2013; doi:10.1152/jn.00478.2013.—Our aim was to investigate the jaw reflexes using both the probability- and the discharge rate-based analysis methods. Twelve consenting volunteer subjects participated in this study. Subjects bit gently on bite bars that carried the impression of their teeth. Surface and intramuscular electrical activity of the masseter was recorded. With the help of audio feedback from one motor unit, each subject bit to discharge the unit at a fixed rate. While the subject continuously activated the selected motor unit, 4-N stimuli were delivered to the upper right central incisor either at a rapid or a slow rate. For each trial, ≥ 300 stimuli were delivered, and, once a trial was completed, local anesthetic block was applied around the stimulated tooth, and the experiment was repeated. While preceding local anesthesia, the rapid-rate stimuli (“tap”) induced substantial inhibitory reflex responses; during local anesthetic block, the same stimulus induced excitatory and inhibitory reflex responses. Slow-rate stimuli (“push”), on the other hand, usually generated a combination of inhibitory and excitatory responses that disappeared completely during the local anesthetic block. This study discovered that the strength of the inhibitory reflex response to a tooth-tap stimulus was much larger than previously reported. This study also found that whereas the probability-based analyses were better for illustrating the existence and latency of small earlier responses, the discharge rate-based method was better for indicating the duration of earlier responses and the existence, sign, and duration of later responses.

masseter reflex; periodontal mechanoreceptor; muscle spindle

ALTHOUGH CENTRAL PATTERN GENERATOR sets the basic rhythm of chewing (Dellow and Lund 1971), ongoing modulation of masticatory forces to overcome varying resistances encountered during chewing are provided by the peripheral sensory feedback (Morimoto et al. 1989). It has been claimed that two of the most important peripheral sensory inputs that help control mastication come from periodontal mechanoreceptors (PMRs) around the teeth and muscle spindles in the jaw muscles (Lavigne et al. 1987).

Previous studies to indicate the importance of these receptors in feedback control of mastication can be criticized since they have been performed either on anesthetized animals or on human subjects using only the probability-based analyses. There are several reasons for repeating these experiments. First, the use of general anesthetics is likely to give us erroneous results as general anesthetics work by means of directly affecting the cell membranes and synaptic transmission between neurons (Nicoll 1972; Nicolson et al. 1976). Such preparations also lack supraspinal inputs that normally help activate the interneurons that are essential for the completion of reflex circuitries (Matthews 1972). Therefore, functional information on neuronal pathways obtained from reduced animal experiments may be misleading.

Second, all previous work on human jaw reflexes has used probability-based analyses such as the spike-triggered averaging of the surface electromyogram (SEMG), peristimulus time histogram (PSTH), and raster dots of single-motor-unit (SMU) spikes (reviewed in Türker 2002). Recent experiments on brain slices have shown that the probability-based analyses are subject to significant errors (reviewed in Türker and Powers 2005). On the other hand, frequency-based methods are free from such errors (Türker and Cheng 1994; Türker and Powers 2003).

Third, most researchers have used masseter SEMG to study reflex connections of receptors in the trigeminal area. The use of SEMG of the masseter muscle is open to cross talk from mimic muscles that cover the masseter (Warwick and Williams 1973).

Other than these recording and analysis problems in the trigeminal reflex studies, the stimulus used to generate reflex responses could also be criticized. The reflex connections of the PMRs are normally studied using mechanical stimuli. However, the profile of the stimuli has been subject of criticism as the exact stimulus profile that was delivered to the tooth was not precisely controlled in most experiments (for review, Türker 2002). Therefore, resulting reflex response could not be compared with the experiments where stimulus profile was recorded and regulated using a proportional-integral-derivative controller system to deliver exact stimuli each time.

To study the connection of the trigeminal receptors to the masseter motoneurons, therefore, we have decided to use normal human subjects and SMU techniques to avoid the general anesthetic- and cross talk-related issues. We have also used precisely controlled stimuli to activate PMRs and used local anesthetic (LA) blocks to make sure that the reflex originated from around the stimulated tooth. Furthermore, we have used both the classic and the frequency-based analysis methods to compare the two methods and to reduce count and synchronization type errors of the classic methods.

Our principal hypothesis in this study was that the previously established neuronal pathways, that are activated when a tooth is mechanically stimulated, are likely to be incorrect.
METHODS

Ethical approval. The protocol was approved by the Human Ethics Committee of Ege University, and all of the procedures used conformed to the Declaration of Helsinki. The subjects were informed about the procedures and signed the informed consent forms.

Subjects and methods. Twelve neurologically normal volunteer subjects with healthy dentitions and no history of masticatory dysfunction or orthodontic treatment (3 men and 9 women) aged between 19 and 40 yr (mean ± SD = 27.1 ± 7.6) participated in this study. Consumption of analgesics <24 h before the study was not allowed.

General protocol. Subjects were instructed to bite gently into a semirigid dental impression material mounted on two bite bars. The impression material was cut away from around the upper right central incisor to allow stimulation and leave space for this tooth to “move” in response to the stimulus (more details in Türker et al. 2004; Yang and Türker 1999). Tooth stimulation was achieved by small, rubber-tipped, stimulating probe. Subjects were instructed to bite gently into impression material so that a selected single masseter motor unit fired at a fixed rate (10–25 Hz) using audio feedback. While the subject bit and discharged the motor unit of the masseter regularly at the instructed rate, stimuli were delivered to the upper right central incisor. Usually >300 stimuli were applied to the tooth in each trial to obtain reliable peristimulus frequencygram (PSF) and PSTH records. At least 2 trials were obtained to test the effects of the tap or the push stimuli. The duration of the rest period between the trials was ~15 min. Once the stimulation procedures have been performed, ~4 ml of LA (Xylocaine, lignocaine hydrochloride) was infiltrated buccally and palatally (in the vicinity of the incisive foramen) around the stimulated tooth to block periodontal input from canine to canine. At least 2 more trials with similar number of stimuli were performed while the subject could not feel the tap stimulus on the locally anesthetized tooth.

Stimuli. Tooth stimulation was performed via a probe aligned orthogonally to the labial surface of the tooth. A computer-generated force profile was used as the input signal to a small mechanical vibrator connected to the tooth via this probe. A preload of ~0.5 N was maintained on the tooth to minimize high-frequency stimulation components (Türker et al. 1997). The tap stimulus was a 4-N sinusoidal waveform with a rise time of 5 ms (800 N/s). The push stimulus was also sinusoidal in profile and 4 N in size. It had a rise time of ~100 ms (40 N/s). The interval between the stimuli was randomly altered between 0.8 and 1.2 s to avoid predictive stimulus application. Profile of the stimulus was maintained using a proportional-integral-derivative controller system based on LabVIEW software.

Recording SEMG. The skin over the right masseter muscle belly was prepared to reduce the interelectrode resistance to <10 kΩ, and adhesive bipolar EMG electrodes (Duotrode; Myotronics) were placed. The SEMG was amplified (3,000×), band-pass filtered (20–500 Hz), and sampled at 2,000 Hz. Grounding of the subject was achieved by the use of a lip-clip electrode (Türker et al. 1988).

SMU potentials. The activity of a SMU was recorded from the right masseter muscle using custom-made, intramuscular, fine-wire, bipolar electrodes made of two Teflon-insulated silver wires. These wires were inserted to a depth of ~1 cm into the belly of the muscle using 25-gauge needles. The needle was withdrawn immediately, leaving the two “fish-hooked” wires in the muscle. The wires were insulated except their tips to record single-unit activity. Subjects were asked to contract the muscle to fire a clearly identifiable motor unit at a fixed rate (10–25 Hz) with help of audio feedback. SMU potentials were discriminated online using a microprocessor-based waveform analysis method, which matched the shape of the action potentials to preestablished templates [Spike2; Cambridge Electronic Design (CED)].

Analysis. For each trial, the SEMG of the masseter was band-pass filtered (20–500 Hz), full-wave rectified, extracted around the time of the stimuli (time interval: ~160 to 250 ms), and averaged. Cumulative sum (CUSUM; Ellaway 1978) was then constructed. Each CUSUM record was expressed in k units, with k being the prestimulus average bin value (time interval: ~160 to 0 ms, where 0 is the time of the stimulus delivery). For each trial, an error box was built that uses the largest prestimulus deflection to form a symmetrical box (Brinkworth and Türker 2003; Türker et al. 1997; Yang and Türker 1999). A significant reflex response was determined when poststimulus SEMG-CUSUM deflection was larger than error box limits (Türker and Powers 2003). Poststimulus time interval for detecting significant reflex responses was 0 to 90 ms for the tap stimulus and 0 to 160 ms for the push stimulus, matching to the minimum reaction time of the subjects to these stimuli (see also Brodin et al. 1993a).

When analyzing SMU, offline discrimination of the shape of action potentials was performed using preestablished templates (Spike2 system). Data were then used to construct PSFs, PSF-CUSUMs, PSTHs, and PSTH-CUSUMs. For all CUSUM calculations, the same analyses as to the one described for the SEMG (above) were used. The reflex was considered to be a significant event only when it was larger than the error box before the reaction time set for that stimulus. Further calculations were performed only when these conditions were met. Initial inhibitory reflex latency was taken as the time between 0 ms (initiation of the stimulus) and first turning point of significant CUSUM deflection in PSTH and SEMG (Fig. 1, bottom graph). Reflex latency for the PSF was taken from the PSTH-CUSUM as it represents the latency of the very first reflex better (Todd et al. 2012). Similarly, the endpoint of the reflex was determined as the second significant turning point of the CUSUM. The duration of the reflex was the interval between the first and the second turning points of the CUSUM. Magnitude of the reflex was determined as the size of the CUSUM between the first and second turning points divided by the number of stimuli used for that experiment. This value was then normalized to the 100% reflex response using the formula below (Brinkworth and Türker 2003):

\[ \text{100\% reflex amplitude} = \frac{k \times \text{reflex duration in bins}}{\text{number of stimuli}} \]

Here, k represents the average prestimulus bin value. Therefore, 100% reflex amplitude represents maximum possible inhibition, i.e., no spikes in any of the bins throughout the duration of the reflex. In each experiment and for each of the conditions (before LA, during LA, and for the subtraction), distinctive 100% reflex size was calculated and then compared with the actual size of the reflex obtained from the CUSUM in that trial. This approach gave us percentage strength of the reflex responses independent to the number of stimuli used and the duration of the reflex.

Statistical analysis. After determining significant reflex responses using the error box approach, further statistical analysis was performed only on the significant responses. Cross-tabulations 2 × 2 and \( \chi^2 \) test with Fisher exact test (2 ways) were performed to compare reflex incidences, latencies, durations, and strengths obtained from PSF-CUSUM, PSTH-CUSUM, and SEMG-CUSUM before and during LA conditions. Significances between the same reflex parameters using tap and push stimuli before LA and during LA conditions (and between during LA and subtraction during LA from before LA conditions using tap stimulus) were detected using nonparametric Mann-Whitney U test [Exact Sig. (2-tailed)]. Then, post hoc Bonferroni corrections were made for the reflex parameters, which were used for comparison more than once. The level of significance was set at \( P < 0.05 \).

RESULTS

Reflex responses of single masseter motor units to mechanical tooth stimulation were studied in 75 units in 12 subjects. Because of unavoidable movement of the electrode within the muscle during the application of LA, it was often not possible to keep the same motor unit throughout an experiment, i.e.,
before and during LA. However, we managed to keep the same unit throughout the experiment in some cases (n = 8, 5 for tap and 3 for push experiments), and the reflex responses of the same unit were obtained both before and during LA block. Confirmation of the identity of the unit was obtained using the Macro EMG method used by several investigators that indicates the extent of the surface contribution of a SMU via most or all of its participating muscle fibers (O’Connor and Türker 2001; Schmied and Türker 2001; Stålb erg 2011). The rest of the units (n = 67) were recorded either before or during the LA. The subtraction process was always performed in the same experiment only in the CUSUM responses of the same unit before and during LA. On average, 448 ± 143 (mean ± SD) stimuli were delivered in each of the successful trials. The average number of stimuli for tap experiments was 483 ± 142 and for push experiments was 373 ± 116. The results are presented for the tap and the push stimuli using the 3 different conditions (before LA, during LA, and after subtraction).

**Tap stimulus.** It was possible to examine the responses of 24 motor units to tap stimuli before LA and 25 motor units during LA. Figure 1 illustrates the analysis procedure of a typical tooth-tap experiment. As can be seen, tooth tap induced a period of absolute silence in the discharge of a SMU. The lack of spikes is clearly shown in the PSTH and the PSF records as empty spaces. As can be seen in the SEMG and PSTH records, the silent period disappeared, and an early excitatory response (although small in size compared with the inhibitory response) appeared during the LA block (middle column). When the CUSUM obtained during LA block is subtracted from the CUSUM obtained before LA (right column), the extent and the size of the PMR reflex response without the overlapping effect from other vibration-sensitive receptors have been indicated. Initiation of the silent period (I1) has been taken to indicate the latency of the inhibitory reflex response. In the expanded section of the figure (bottom graphs), the latency of the inhibitory reflex (downgoing CUSUM deflection that is larger than the size of the error box; indicated by horizontal, dashed lines) is shown by a solid, vertical line (I1). End of the inhibitory period is also indicated by a solid, vertical line at the turning point of the CUSUM record. The latencies and the endpoints of the excitatory reflex responses (E1, E2, and E4; where upgoing CUSUM deflections are larger than the size of the error box) are also shown using vertical, dashed lines.

When all units were considered, PSF-CUSUMs demonstrated early inhibition (I1) in 17 motor units before and 11 motor units during LA [not significant (N.S.); Table 1]. Early excitation (E1) before LA did not manifest in any of the motor units tested, whereas during LA 11 motor units displayed E1 with a latency of 19.9 ms and duration of 5.4 ms, which was not preceded by I1.

The incidences of significant reflex responses are summarized in Table 1. PSTH-CUSUMs indicated I1 in 23 of the 24 motor units tested before LA and only in 2 motor units during LA (P < 0.001). E2 following inhibition manifested in 14 motor units before LA, and E1 was detected in 11 motor units during LA. In addition, I3 was observed in 4 motor units before LA, and I2 was detected in 9 motor units during LA. Eight motor units before LA displayed E4, and three units during LA displayed E3 response.

In SEMG-CUSUM analysis, 22 of 24 masseter records (which were simultaneously recorded with SMUs), showed I1 before LA, whereas only 1 I1 was manifested during LA (P < 0.001). Excitation following inhibition (E2) was seen in 17 trials before LA, and E1 was detected in 22 of the 25 during LA. I3 was seen in 6 trials before LA, and I2 was detected in 18 trials during LA. E4 manifested in 9 SEMG-CUSUMs before LA, and E3 was detected in 6 SEMG-CUSUMs during LA (N.S.).

To obtain the latency, duration, and the strength of the reflex responses that are mainly PMR of origin, we used the same SMU recordings before and during LA, and we have subtracted point-by-point CUSUM values obtained during LA from the CUSUM values obtained preceding the LA. The results of this calculation are shown in Tables 1–3 under Subtraction.

In general, the following points can be made regarding the two different forms of analyses as detailed in Tables 1–3.

**Tap stimulus before the LA block:** 1) it generates an early inhibition (I1), 2) I1 latency was significantly shorter (P < 0.01) in both PSF- and PSTH-CUSUM compared with the SEMG-CUSUM. 3) I1 size was significantly larger (P < 0.01) in PSTH-CUSUM compared with the SEMG-CUSUM. 4) For the E1, the latency and duration were shorter (P < 0.05 and P < 0.01, respectively), and size was stronger (P < 0.01) in the PSTH-CUSUM compared with the SEMG-CUSUM. For the PSF-CUSUM, only the strength of the I1 differed significantly between before and during LA conditions (P < 0.01; Table 3). 6) This early inhibition was followed by a period of excitation (E2) in SEMG and PSTH at around 40 and 34 ms, respectively (N.S.). 7) There was no E2 response in the PSF; instead, the discharge rate of units actually declined significantly during the period that was labeled as E2 in SEMG and PSTH. 8) Therefore, the E2 is a synchronization artifact and not a genuine reflex. 9) Hence, the duration of the I1 response was much longer when it was determined using the PSF method compared with the PSTH and SEMG methods (Table 2; P < 0.01).

During the LA block with the tap stimulus: 1) I1 was replaced by an early excitation (E1; latency: 20 ms for the PSTH and 22 ms for the SEMG). 2) E1 latency and duration during LA block were significantly different from the values of E2 preceding the LA block for both SEMG and PSTH (P < 0.01 for all comparisons). 3) E1 duration was shorter, but its size was stronger (both P < 0.01) in PSTH-CUSUM compared with the SEMG-CUSUM. 4) This new E1 was a genuine excitation as it was the very first spike gathering after the stimulus (Türker and Powers 1999, 2003). 5) E1 may originate from fast conducting fibers likely to be spindles in the jaw muscles (Carels and van Steenberghhe 1985).

Subtraction of response during LA from before LA: although the latency and the duration of reflexes did not change after the subtraction, the strength of the I1 increased dramatically, including in the PSF records (P < 0.05). This indicates that the tap stimulus activates excitatory and inhibitory receptors simultaneously and that the strength of the inhibitory receptors is reduced during the LA block. LA block also exposed the response of the receptors that are not affected by the LA block (vibration-sensitive spindles).

**Push stimulus.** In 5 subjects, 13 motor units before LA and 13 motor units during LA were analyzed. Figure 2 illustrates the responses of a SMU that continue to be active before and during the LA. In total, only 3 units were active before and during LA in the push experiments. The push stimuli induced a small inhibitory followed by a large excitatory reflex re-
response. Push-induced excitatory response was a genuine reflex as indicated by the increase in the discharge rate of the motor units tested. PSF-CUSUMs demonstrated (Tables 1–3) I1 in 8 motor units before and none during LA ($P < 0.01$). E1 before LA manifest in 8 motor units, whereas during LA only 2 motor units showed excitation ($P < 0.05$), which were not preceded by I1. One motor unit before LA demonstrated I2 following by I1, and one more motor unit showed E2 followed by E1.
Table 1. Incidences and mean reflex latencies for tap and push stimuli using PSF-CUSUM, PSTH-CUSUM, and SEMG-CUSUM methodology

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Response</th>
<th>Tap Stimulus</th>
<th>Push Stimulus</th>
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<tbody>
<tr>
<td></td>
<td>Before LA (PMR and spindle)</td>
<td>During LA (spindle)</td>
<td>Subtraction (PMR alone)</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>17.5 ± 1.3 [22]</td>
<td>22.4 ± 1.9 [22]</td>
</tr>
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</table>

Values are shown for the significant reflex responses only. n Shows the total number of single units studied for each section of the experiment. Incidences of significant reflex responses are indicated in square brackets for each of the experimental conditions. Nonsignificant results were still used for subtraction purposes. Latencies were not placed in the table if the number of observations was 1 or 0. Note that statistical analysis was only performed on the values of significant reflex responses (results). Subtractions were only performed if the data were available before and during local anestheisa (LA) in the same unit (5 units in tap experiments). Also note that the table does not include the pre-NA and subtraction values for the push experiments since LA block completely obliterated the reflex responses for the push stimulus. PSF, peristimulus frequencygram; CUSUM, cumulative sum; PSTH, peristimulus time histogram; SEMG, surface electromyogram; PMR, periodontal mechanoreceptor; E1, E2, E3, and E4, 1st, 2nd, 3rd, and 4th excitation; I1, I2, and I3, 1st, 2nd, and 3rd inhibition.

PSTH-CUSUMs indicated inhibition in 9 motor units before LA, and only 1 motor unit demonstrated inhibition during LA (P < 0.01). E1 following inhibition manifested in 6 motor units before LA and in 2 motor units during LA (P > 0.05). In addition, the late inhibition (I2) was observed in 1 motor unit before LA.

In SEMG-CUSUM analysis, 11 of 13 records analyzed showed a significant I1 before LA, whereas only 2 manifested II during LA (P < 0.01). E1 following II was seen in 12 SEMG-CUSUMs before LA and 2 SEMG-CUSUMs during LA (P < 0.001). I2 was seen in 4 SEMG-CUSUMs before LA and in 1 SEMG-CUSUM during LA (P > 0.05).

Using PSF-CUSUM, two SMUs showed I1 with a latency of 25 ms each (calculated using the PSTH-CUSUM), duration 60 and 41 ms, and reflex strength –6.9 and –10.3%, respectively. Using PSTH-CUSUM for these two SMUs, I1 latency was 25 ms, duration was 20 ms for both units, and the reflex strengths were –108.3 and –55%, respectively. PSF-determined duration for I1 was significantly longer than the same reflex duration determined using the PSTH (P < 0.01).

In PMR in push stimulus in PSF and PSTH-CUSUM, one SMU demonstrated E2, which followed E1, and different SMU showed E2 after II. Three SMUs showed manifestations of E2 followed by I1 and E1 in SEMG-CUSUM during push stimulus application were detected.

Before and during the LA block with the push stimulus: 1) the size of both the I1 and E1 were stronger with the PSTH-CUSUM compared with the SEMG-CUSUM (both P < 0.01). 2) Push stimulus induced small inhibition followed by a large excitation during the LA block. 3) No significant reflex response was observed during the LA block. 4) Therefore, push stimulus only activates receptors that are disabled during the LA block. 5) These receptors may have different characteristics to the ones activated by the tap stimuli as they mainly induce excitation rather than inhibition. 6) This study showed that the PMR response can be modified depending on the rate of force delivery to a tooth. Also, unlike the tap stimulus where an excitatory reflex response was observed during the LA block, no such reflex occurred with the push stimulus during the LA block. 7) Therefore, when the response during LA block is subtracted from the response without LA (right column), there was little change from the reflex response before the LA block.

Figure 3 illustrates the force profiles used in these experiments as well as the CUSUMs of the measured variables. The profiles of the stimuli were fixed throughout the experiments.
importance of trigeminal receptors in feedback control of muscle spindles. Push stimulus, on the other hand, activates the inhibitory reflex pathway originating from the muscle spindles, and jaw-muscle motoneurons. This suggestion is supported by several layers of mimic muscles such as the zygomaticus major, platysma, and risorius (Warwick and Williams 1973), which are activated without conscious intention of the subject. These mimic muscles are activated especially when their investigation of the feedback properties and neuronal connections of these afferents. This muscle is, however, covered by several layers of mimic muscles such as the zygomaticus major, platysma, and risorius (Warwick and Williams 1973), which are activated without conscious intention of the subject. These mimic muscles are activated especially when mastication have been put forward either in experiments where anesthetized animals chewed while various receptor inputs were excluded from feedback mechanism or in human experiments where SEMG and probability-based analyses (spike-triggered averaging of SEMG or PSTH of SMU potentials) were used. We felt that previous approaches to study jaw reflexes should be reexamined for the following reasons. First, the general anesthesiology work by means of directly affecting the neuron membrane, excitability of interneurons, and the normal functions of synapses (Nicoll 1972; Richards 1995; Scholfield 1980). Therefore, the synaptic activity must be already altered in these animals. Furthermore, such preparations also lack supraspinal inputs that normally help activate the interneurons that are essential for the completion of reflex circuitries (Matthews 1972). Therefore, the synaptic organization and neuronal pathways may not be correctly indicated in experiments on anesthetized animals. We could not directly confirm this suggestion since the experimental procedures are different in animals. Whereas in animals nerve fibers can be stimulated and recordings obtained from the motoneurons (Lund 1991), this is not possible in conscious human subjects. However, since the stimuli are more natural, and the recordings occurred by conscious participation of subjects, the results can be more realistic in human experiments.

Second, although the contribution of the PMRs and muscle spindles to jaw muscles during the static and dynamic conditions has been widely studied (Brinkworth et al. 2003; Brodin et al. 1993b; Ottenhoff et al. 1992a,b; Svensson et al. 2000; Türker et al. 1994; reviewed in Sowman et al. 2010; Türker 2002), most of these studies used SEMG of the masseter in their investigation of the feedback properties and neuronal connections of these afferents. This muscle is, however, covered by several layers of mimic muscles such as the zygomaticus major, platysma, and risorius (Warwick and Williams 1973), which are activated without conscious intention of the subject. These mimic muscles are activated especially when

Table 2. *Mean reflex durations for tap and push stimuli are shown for the 3 different analysis methods*

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Response</th>
<th>Tap Stimulus Before LA (PMR and spindle) [n = 24]</th>
<th>Tap Stimulus During LA (spindle) [n = 25]</th>
<th>Push Stimulus Subtraction (PMR alone)</th>
<th>Push Stimulus Before LA (PMR alone) [n = 13]</th>
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<tr>
<td></td>
<td>E3</td>
<td>14.5 ± 4.8 [4]</td>
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<td></td>
<td>E4</td>
<td>14.8 ± 4.1 [8]</td>
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<td></td>
<td>I2</td>
<td></td>
<td></td>
<td></td>
<td>31.3 ± 4.6 [4]</td>
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<tr>
<td></td>
<td>E2</td>
<td>19.0 ± 5.9 [17]</td>
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<td></td>
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<tr>
<td></td>
<td>I3</td>
<td>58.2 ± 14.2 [6]</td>
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<td></td>
<td>E3</td>
<td></td>
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<tr>
<td></td>
<td>E4</td>
<td>17.7 ± 4.3 [9]</td>
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</table>

Similar to the statement in Table 1, we could only perform statistical comparisons when there were significant values for both before and during LA conditions (METHODS). Also, similar to the situation in Table 1, this table does not include the pre-LA and subtraction values for the push stimulus since LA block completely obliterated the reflex responses for the push stimulus.

**DISCUSSION**

Supporting our hypotheses, the current study found that the previously established pathways that connect PMRs to masseter motoneurons are likely to be erroneous. Although the classic methods used in the current study confirmed the previous findings on the pathways of these receptors, the PSF analysis indicated that they might be wrong. Basically, the experimental protocol used in the current study showed three unique findings that should change the neuronal map of the trigeminal system. These are: 1) tap-induced vibration activates excitatory reflexes that are not affected by the LA block of the stimulated tooth. 2) The aid of the LA block and subtracting the reflex during the LA from the reflex preceding the LA indicated that the duration and the strength of the inhibitory reflex pathway connecting PMRs to masseter motoneurons were grossly underestimated in previous studies. The inhibitory reflex originating from the PMRs lasts twice as long (as indicated in PSF records; Table 2) and twice as strong (Table 3) compared with the values reported in previous studies. 3) The classically described excitatory response that followed the inhibitory response in SEMG or PSTH has been found to be a synchronization error. PSF indicated that it is a continuation of the inhibitory reflex as the discharge rate of units declined significantly during that period. 4) Current findings suggest a new wiring diagram among the PMRs, spindles, and jaw-muscle motoneurons. This suggestion is depicted in Fig. 4 and summarized as follows: tap stimulus activates the inhibitory reflex pathway originating from the PMR and the excitatory pathway originating from the jaw-muscle spindles. Push stimulus, on the other hand, activates both the inhibitory and the excitatory PMR pathways (with the cell bodies in the trigeminal ganglion and trigeminal mesencephalic nucleus, respectively; refer to Türker 2002 for details) to the jaw closers.

**Comparison of the current findings with the literature.** The importance of trigeminal receptors in feedback control of
the subject is anxious about the experimental procedure, fatigued, in pain, uncomfortable, or simply because he/she unintentionally moves the face or blinks. Therefore, the EMG activity recorded using surface electrodes over the masseter can come from any of these mimic muscles as well as from the masseter itself. It is hence suggested that researchers consider these limitations when evaluating SEMG from the masseter. Current results justify this criticism as it indicates a genuine difference between results obtained using the SEMG and the SMUs (RESULTS).

Third, recent experiments on brain slices have shown that the classic probability-based analyses are subject to major errors (Türker and Powers 1999, 2003). These classic analysis techniques rely on the number of occurrences of action potentials at a particular time after the stimulus. It generates significant errors, as the action potentials are synchronized by the stimulus-induced synaptic potential. These synchronized spikes will then induce autocorrelation-related peaks and troughs in the probability-based analyses that can be identified as excitatory or inhibitory reflex responses. On the other hand, the frequency-based method is free from such errors, as it only considers the changes in the instantaneous discharge rate of the spikes, which are not affected by the gathering of spikes at any particular time after the stimulus.

![Fig. 2. Reflex response of a unit to push stimuli before and during the LA block. The number of triggers used to obtain this figure were 423 before LA and 341 during LA block.](image-url)
Current results indicate that, despite the unavoidable synchronization-based errors, the SEMG and PSTH have been shown to be useful in identifying existence and latencies of early reflexes. PSF, on the other hand, has been found to be most useful for indicating the durations of reflexes. PSF has also been useful for confirming whether the long-latency reflexes (as identified in SEMG and PSTH) are genuine events.

Reflex strengths were only calculated for the significant reflex responses. Similar to the statement in Table 1, we could only perform statistical comparisons when there were significant values for both before and during LA conditions (METHODS). Also, similar to the situation in Table 1, this table does not include the pre-LA and subtraction values for the push stimulus since LA block completely obliterated the reflex responses for the push stimulus.

Reflex responses of 2 units to tap and push stimuli before LA block. The 1st and 2nd columns show the reflex responses of the motor units to tap and push stimuli, respectively, in the form of PSF-CUSUM, PSTH-CUSUM, and SEMG-CUSUM. Tap-stimulus profile used is shown in the left bottom panel, and push-stimulus profile is showed in the right bottom panel.

Table 3. Mean reflex strengths for tap and push stimuli calculated from the CUSUMs of PSF, PSTH, and SEMG

<table>
<thead>
<tr>
<th>Methodology Response</th>
<th>Tap Stimulus</th>
<th>Push Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before LA (PMR and spindle)</td>
<td>During LA (spindle)</td>
</tr>
<tr>
<td>E1</td>
<td>-18.5 ± 11.5 [18]</td>
<td>43.6 ± 9.0 [5]</td>
</tr>
<tr>
<td>I2</td>
<td>36.7 ± 19.1 [17]</td>
<td>15.5 ± 4.1 [9]</td>
</tr>
<tr>
<td>E2</td>
<td>-11.7 ± 5.4 [6]</td>
<td></td>
</tr>
<tr>
<td>I3</td>
<td>29.6 ± 17.0 [6]</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>29.6 ± 17.0 [6]</td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>29.6 ± 17.0 [6]</td>
<td></td>
</tr>
</tbody>
</table>

Reflex strengths were only calculated for the significant reflex responses. Similar to the statement in Table 1, we could only perform statistical comparisons when there were significant values for both before and during LA conditions (METHODS). Also, similar to the situation in Table 1, this table does not include the pre-LA and subtraction values for the push stimulus since LA block completely obliterated the reflex responses for the push stimulus.
responses can also be criticized. 1) Delivering exact stimulus profile: the connection of the PMRs is normally studied using mechanical stimuli. However, the profile of the stimuli has been subject of criticism, as most previous experiments did not illustrate the exact profile that was delivered to a tooth (reviewed in Türker 2002). Therefore, resulting reflex response could not be compared with the experiments where stimulus profile was recorded in series with the stimulus probe and regulated using feedback systems to deliver exact stimulus profile each time. 2) Spread of the vibration from the stimulus: even if the mechanical stimulation is well-controlled and delivered consistently to activate PMRs, it induces vibration, which activates vibration-sensitive receptors such as the muscle spindles (Türker and Jenkins 2000). Therefore, a jaw reflex that is induced via a tap to a tooth does not necessarily belong to the PMRs alone.

**PMR connection to masseter motoneurons: tap stimulus.** It has been well-established that tooth tap evokes an inhibitory reflex response in the masseter muscle when the electrical activity is detected using either intramuscular or surface EMG (reviewed in Türker 2002). This inhibitory period has been reported to be followed by an excitatory phase in most previous studies (e.g., Bonte and van Steenberghe 1989). However, although the existence of the early inhibitory period has been widely held, the excitatory phase that immediately follows this inhibition has been criticized. For example, it has been claimed that the excitatory period was caused by synchronously occurring spikes that were delayed by an earlier inhibitory postsynaptic potential (IPSP; Brinkworth et al. 2003).

The current study differs from all previous work as it has not only used LA to bring out and then exclude the spindle contribution, but also used the discharge rate-based analysis method to overcome errors imbedded into the classic analysis methods (see also Kahya et al. 2010; Todd et al. 2012). As the LA block overwhelmed much of the PMR contribution to the reflex, remaining reflex response has been thought to belong to the muscle-spindle circuitry. Therefore, subtracting the reflex response during LA from the reflex response before LA indicated, for the first time, the extent of the PMR reflex response.

Results from units before and during LA have shown clearly that the PMR reflex is much stronger and much longer-lasting than previously thought. Furthermore, it has become clear that the excitatory response that immediately followed the inhibition was due to synchronous occurrence of delayed spikes.

**PMR connection to masseter motoneurons: push stimulus.** Unlike the excitatory response that followed the initial inhibitory response in the tap stimulus, push-induced excitatory response was a genuine reflex as indicated by the increase in the discharge rate of the motor units tested. This experiment shows that the PMR response can modify depending on the rate of the force delivered on a tooth (Brodin et al. 1993b). Whereas a tap stimulus represents a dangerous force on the tooth, and hence activation of the inhibitory pathway to jaw-closer motoneurons has advantages, push stimulus represents a slowly rising force similar to the one that is encountered during normal chewing, and hence inhibition is not warranted (Fig. 4).

**Muscle-spindle connection to masseter motoneurons.** Responses of the muscle spindles to tap and push stimuli are illustrated in these experiments during the LA block of the PMRs. Whereas the tap stimuli activated the spindles and induced short-latency excitatory reflex responses, the push stimuli hardly activated them. This further confirms the differential stimulation of these receptors. Whereas rapidly rising tap stimuli generate vibration, and spread of this vibration activates the vibration-sensitive spindles, slowly rising push stimuli hardly cause vibration and hence no spindle activation (Fig. 4).
4A for speculative pathways for the reflex connection of PMRs and spindles to the masseter motor neuron pool).

**Analysis methods.** In this study, we used three methods to determine the reflex responses: PSF, PSTH, and rectified and averaged SEMG. As has been previously shown in regularly discharging motoneurons in brain slices, the PSTH and SEMG techniques have count and synchronization errors that give wrong impressions regarding the neuronal networks (Türker and Powers 1999, 2003, 2005). A number of recent studies have compared these methods of spike analysis to illustrate the differences between them (Binboğaz et al. 2011; Deriu et al. 2005; Kahya et al. 2010; Norton et al. 2008; Prasartwuth et al. 2008; Rogasch et al. 2012; Todd et al. 2012). In general, the following findings are noted from these recent studies. 1) CUSUMs of averaged SEMG records are better for illustrating small but persistent changes than the raw averaged records. 2) Latency of an early inhibition is better represented by the CUSUMs of SEMG or PSTH. This is due to the fact that the reduction of spike density can be seen with ease in the CUSUMs of SEMG or PSTH records but not in the PSF. PSF, on the other hand, cannot display any discharge rates during this period of gap (refer to Fig. 1, 1st column) and hence cannot be used for pinpointing the latency of an inhibitory period. 3) As it indicates the area of change in the record, CUSUM should be preferred for estimating the size of the reflex response to the rectified averaged records. 4) Existence of small early excitation or inhibition is better presented using the CUSUMs of SEMG or PSTH than the CUSUM of PSF. 5) Duration of an inhibition or excitation, on the other hand, should be determined using the PSF method as the other methods underestimate durations. 6) CUSUM of PSF should be used to indicate the existence, duration, and size of later events.

Many of these suggestions are established in the current study. For example, whereas PSTH and SEMG illustrated the timing of early small excitatory postsynaptic potentials (EPSPs) better (see, for example, Figs. 1 and 2, middle columns), PSF was superior for displaying later events as it was less affected by the count and synchronization type errors (Prasartwuth et al. 2008; Türker and Powers 2005). This property of the PSF analysis is a major advantage as it can recognize synchronous gathering of spikes following an IPSP. Such dense spike occurrences due to synchronous recurrence of delayed spikes are by default labeled as excitatory events and low spike densities as inhibition in the PSTH and SEMG records as these methods rely on recognition of spike densities. Since PSF examines the instantaneous discharge rate of spikes, it can indicate whether these increased spike densities represent excitatory events (see Türker and Powers 2003).

**Criticism of the current methodology.** Muscle-spindle activation during vibration: we put forward that the vibration induced by the tap stimulus activates the muscle spindles in the trigeminal region and hence causes an excitatory reflex response in the masseter muscle. This is due to the fact that the muscle spindles are found almost exclusively in the jaw closers (Kubota and Masegi 1977). Although muscle spindles are exquisitely sensitive to vibration, there are other receptors with similar sensitivity to vibration. Although vibration-sensitive Pacinian receptors were not observed in the trigeminal region in the microneurography studies (Johansson et al. 1988), inner-ear receptors and vestibular receptors exist in the region and could be activated by the tap-induced vibration. The sound generated by the tap stimulus was hardly audible and hence is not expected to cause a significant reflex response. However, even if it did, the reflex response would have been an inhibitory (Sato et al. 1994; van der Glas et al. 1988) and not, as observed, a short-latency excitatory reflex response. Vestibular receptors are also inhibitory to the jaw closers (Deriu et al. 2005) and hence could not be responsible for the excitatory reflex response.

Although the reflex response of the PMRs was blocked during the LA application, some inhibitory responses were still observed in a number of cases in the current study. The auditory and vestibular receptors may be responsible for the continuing inhibition during the LA block where PMR contribution is minimized. However, the remaining inhibition can also come from the stimulation of the PMRs in the unblocked region in the dental arch. Tap-induced vibration can be transmitted through bone or via interproximal teeth contacts to activate other PMRs. It is also possible that the stimulated tooth and/or some of the neighboring teeth are not fully anesthetized during the LA block and continue to contribute to the reflex. This possibility was minimized by making sure that the subject did not feel the tooth tap during the LA block. The extent of these effects will be the subject of a future study in this laboratory.

**PSF method.** Although PSF method is found to be less prone to count and synchronization type errors, it is not efficient for indicating the latency of inhibitory responses as well as small but genuine responses (Piotrkiewicz and Kudina 2012). This is due to the fact that in the beginning of an inhibitory response the spike occurrences are delayed, and hence no spikes are available to determine discharge rate. Therefore, it is suggested that the latencies of inhibitory periods need to be obtained from the PSTH or SEMG. However, these probability-based analyses wrongly indicate the end of the inhibitory periods as the timing of synchronous occurrence of the delayed spikes (Türker and Powers 1999, 2003). PSF is used to determine the endpoint of these periods where the reduction of the discharge rate ends. Also, small inhibitory or excitatory responses do not alter the discharge rate of units significantly and can be missed by the PSF records. It is therefore suggested that both types of analyses should be used for correct identification of neuronal circuitries (also refer to the review Türker and Powers 2005).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

K.S.T. conception and design of research; P.U., E.S.A., and K.S.T. performed experiments; P.U. and E.S.A. analyzed data; P.U. and K.S.T. interpreted results of experiments; P.U. and E.S.A. prepared figures; P.U. and K.S.T. drafted manuscript; K.S.T. edited and revised manuscript; P.U., E.S.A., and K.S.T. approved final version of manuscript.

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