

The three-neuron corneal reflex circuit and modulation of second-order corneal responsive neurons

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Abstract Neurons located in the border region between the interpolaris and caudalis subdivisions of the spinal trigeminal nucleus (Vi/Vc) are second order neurons of the corneal reflex, receiving corneal afferents and projecting to the lid closing, orbicularis oculi (OO) motoneurons. Recordings of Vi/Vc neurons identified by antidromic activation from stimulation of the facial nucleus and non-identified Vi/Vc neurons reveal two neuron types, phasic and tonic. Corneal stimulation elicits A δ latency action potentials that occur early enough to initiate OO contraction and C-fiber latency action potentials that can modulate the end of the blink in phasic Vi/Vc neurons. Tonic Vi/Vc neurons exhibit a constant irregular, low frequency discharge as well as the cornea-evoked activity exhibited by phasic neurons. For both phasic and tonic neurons, blink amplitude increases with the total number of spikes evoked by the corneal stimulus. Peak firing frequency predicts peak orbicularis oculi EMG activity. Paradigms that suppress cornea-evoked blinks differentially affect Vi/Vc neurons. Microstimulation of the border region between the spinal trigeminal caudalis subdivision and the C1 spinal cord (Vc/C1) significantly reduces the number of spikes evoked by corneal stimulation and suppresses blink amplitude. In the

paired stimulus paradigm, a blink evoked by a corneal stimulus 150 ms after an identical corneal stimulus is significantly smaller than the blink elicited by the first stimulus. Vi/Vc neuron discharge, however, is slightly larger for the second blink. Our data indicate that second-order Vi/Vc neurons do not determine the specific pattern of OO muscle activity; rather Vi/Vc neurons initiate OO motoneuron discharge and program the activity of another circuit that generates the late phase of the blink. The Vc/C1 suppression of Vi/Vc neurons suggests that the Vc/C1 region provides an “internal model” of the intended blink.

Keywords Blinking · Cornea · Corneal reflexes · Trigeminal nuclei · Blink adaptation

Introduction

In its simplest form, the corneal reflex circuit has three components: (1) primary cornea afferents; (2) second order trigeminal complex neurons; and (3) orbicularis oculi (OO) motoneurons. In rats, primary corneal afferents predominantly terminate in the ventral interpolaris-caudalis transition zone of the spinal trigeminal nucleus (Vi/Vc) and in the spinal trigeminal caudalis-C1 spinal cord border region (Vc/C1) (Marfurt and Del Toro 1987). It follows that the second order trigeminal neurons of the three-neuron corneal reflex circuit must be in one of these two regions. Anatomical and physiological studies show that the Vi/Vc region in the rat projects directly to OO motoneurons (Meng et al. 1997; Hirata et al. 1999, 2004). Consistent with the anatomy, damage to the Vi/Vc severely impairs the production of cornea-evoked blinks in humans (Ongerboer

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de Visser and Moffie 1979; Ongerboer de Visser 1983) and guinea pigs (Pellegrini et al. 1995), whereas Vc/C1 damage does not block cornea-evoked blinks in guinea pigs (Pellegrini et al. 1995). Although several studies provide careful and thorough descriptions of the discharge of Vi/Vc cornea-responsive neurons in paralyzed rodents (Meng et al. 1997, 1998; Hirata et al. 1999, 2000, 2004), the contribution of the Vi/Vc neurons to reflex blink generation is unknown. Our experiments investigate the role of these second order neurons in corneal reflex blinks.

One way to explore the role of Vi/Vc neurons in cornea-evoked blinks is to characterize their activity in paradigms that modify reflex blinking. In the paired stimulus paradigm, the blink evoked by the second of two identical blink-evoking stimuli is smaller than that evoked by the first (Cruccu et al. 1986; Pellegrini and Evinger 1995; Powers et al. 1997; Peshori et al. 2001). Another paradigm that suppresses corneal reflex blinks is microstimulation of the Vc/C1 region of the trigeminal complex (Henriquez and Evinger 2005). Although both paradigms produce similar levels of corneal-reflex blink suppression, they utilize different mechanisms (Henriquez and Evinger 2005). Our experiments investigate changes in the discharge pattern of blink-related Vi/Vc neurons during blink suppression produced by these two paradigms.

Methods

Single unit recordings of cornea responsive neurons in Vi/Vc were made in 46 male, 175–400 g Sprague–Dawley rats. Animals were maintained on a 12-h light/dark cycle and fed ad libitum. All procedures adhered to Federal, State, and University guidelines concerning the use of animals in research and received prior IACUC approval.

Preparation

Under urethane (1.2 g/kg i.p.) and xylazine (1 mg/kg i.m.) anesthesia, rats were placed in a stereotaxic and prepared for orbicularis oculi electromyogram (OOemg) and trigeminal nucleus recording and stimulation. To access the medulla, the dorsal portion of the C1 vertebral bone was removed. In some animals, the occipital bone was partially removed. The brain stem was kept moist with warm saline.

A pair of Teflon coated, silver wires (0.008" bare, 0.011" coated; AM Systems) were used to stimulate the cornea. To elicit blinks, a single, monophasic, electrical current (0.1 ms duration) was passed between the electrodes. In

all experiments, the stimulus current used to evoke blinks ranged from 0.3 to 3.0 mA. Excess tears were wicked off periodically to reduce current shorting between the corneal electrodes. If the lids moved the stimulating electrodes during a blink, the electrodes were repositioned to evoke a reliable blink.

The OOemg was recorded with pairs of Teflon coated, stainless steel wires (0.003" bare, 0.0055" coated; AM Systems) implanted in the OO at the medial and lateral margins of the lid. A silver wire (0.015" bare; AM Systems) attached to skull with a screw was the reference electrode. OOemg signals were amplified (AM systems, model 1700, 4-channel differential amplifier), filtered at 0.3–5 k Hz, collected at 10 k Hz (Data Translation DT 2821, 12-bit A/D resolution) and stored for off-line analysis.

For extracellular recordings, a glass microelectrode, (Corning #7740 AM Systems; capillary filament 2.0 mm O.D. × 1.16 mm I.D.) was filled with 2M NaCl saturated with fast green. The electrode tip was broken to produce an impedance between 5 and 30 MΩ. The electrode was positioned in the Vi/Vc transition region, between 1.0 mm anterior and 1.5 mm posterior to the obex and from 1.0 to 2.0 mm lateral from the midline of the brainstem using an electrode angle of 15° lateral and 20° caudal. Trigeminal unit activity was amplified (AM Systems, model 1800, 2-channel microelectrode amplifier), filtered 0.3–5 k Hz, collected at 10 k Hz (Data Translation DT 2821, 12-bit A/D resolution) and stored for off-line analysis. A neuron was defined as cornea-related if the neuron responded to electrical stimulation of the cornea and light brushing of cornea. In addition, the peri-orbital region was lightly brushed to determine whether the neuron's receptive field extended beyond the cornea. A neuron was defined as blink-related if it was cornea-related and if the unit discharge began before or coincident with the OOemg activity evoked by corneal stimulation. To localize recording sites, fast green was deposited at the site of the recording (Thomas and Wilson 1965).

Vc/C1 microstimulation was delivered through a glass microelectrode identical to that used for single unit recording. The electrode was positioned in the caudal Vc/C1 border region, from 2.0 to 4.0 mm posterior to the obex, between 1.0 and 1.5 mm lateral from the midline and from 0.5 to 1.5 mm below the brainstem surface using a posteriorly directed 40° angle. Final electrode placement was determined by testing whether a 70 ms train of 20 μA stimuli (0.1 ms duration, 200 Hz) visibly suppressed a corneal reflex blink evoked 5 ms after the termination of the stimulus train (Henriquez and Evinger 2005). At the end of each

experiment, fast green was deposited at the microstimulation site.

In twelve rats, a concentric bipolar stimulating electrode (concentric bipolar, SNE 100, 50 mm; Rhodes Medical Instruments) was placed in the facial nucleus (FN), between 10.3 and 10.8 mm caudal to bregma, 1.8 mm lateral to the midline and 8.0 mm dorsal from the dura surface using a 10° rostral angle. Antidromically driven Vi/Vc neurons were identified using a 1 Hz search stimulus (40 μ A, 0.1 ms duration). Antidromic field potentials were amplified (AM systems, model 1800, 2-channel microelectrode amplifier) and filtered 0.01–5 kHz. After isolation of a Vi/Vc unit, FN electrode stimulation strength was adjusted to a current intensity that evoked an antidromic action potential on approximately one half of the trials, the threshold for Vi/Vc neuron activation. To verify antidromic identification, neurons were tested for their ability to follow FN stimulus trains of 200 and 300 Hz at supra-threshold current (1.25–1.5 times threshold). Collision testing was not carried out. FN electrode location was marked at the end of experiments, by passing 10 μ A of anodal current for 10 s.

Protocols

Two blink modification paradigms were used to test for changes in the neuronal activity of Vi/Vc neurons and blink amplitude. In the primary paradigm, a Vc/C1 microstimulation train (70 ms, 200 Hz, 0.1 ms duration stimuli) was presented 75 ms prior to the onset of the corneal stimulus. Trials alternating between with and without Vc/C1 microstimulation were presented every 20 s (Henriquez and Evinger 2005). To test for suppression of cornea-evoked blinks and Vi/Vc neuron activity responses using the paired stimulus paradigm, two identical intensity stimuli were presented to the cornea with a 150 ms interstimulus interval. These paired stimulus trials were presented every 40 s.

Histology

At the end of the experiment, the deeply anesthetized rat was perfused transcardially with 6% dextran in 0.1 M phosphate buffer followed by 10% formalin in 0.1 M phosphate buffer. The brainstem, cerebellum and spinal cord to the C2 level were removed and placed in 30% sucrose in 0.1 M phosphate buffer for 24 h. The brain was frozen, cut into 100 μ m coronal sections, mounted on subbed slides and stained with cresyl violet. Recording and stimulation sites were identified and transferred to a standardized series of brain outlines (Paxinos and Watson 1998).

Data analysis

OOemg and neuronal activity were analyzed off-line using laboratory-developed software. Blink characteristics were established from the rectified OOemg signal (Fig. 1). Blink duration was determined by marking the start and end of the OOemg activity. Blink amplitude was calculated by integrating the OOemg activity between the marked beginning and end of the blink. Total OOemg activity following a stimulus was calculated by integrating over a 100 ms interval starting immediately after the stimulus artifact.

Several components of neuronal discharge were quantified. The total number of spikes in the 100 ms interval

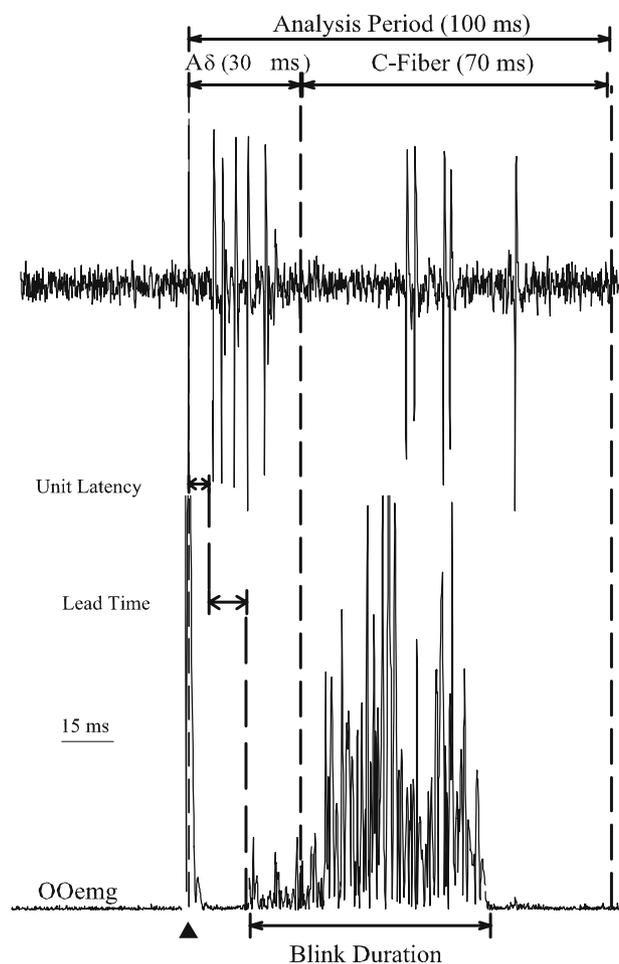


Fig. 1 Measurement of unit and OOemg activity. Vi/Vc unit activity in the first 100 ms following corneal stimulation (Analysis Period) was subdivided into an initial 30 ms (A δ) and subsequent 70 ms (C-Fiber) analysis intervals. The unit latency was the time between the corneal stimulus and the first spike. The lead time was the time between the first action potential and the onset of the reflex blink. Blink duration was the time between the start and end of the rectified OOemg activity and amplitude was the integrated OOemg activity during this period. The record is a single trial

following corneal stimulation provided a measure of the neuron's overall response to corneal stimulation (Fig. 1, Analysis Period). The first 100 ms following corneal stimulation was subdivided into a 30 (Fig. 1, A δ) and 70 ms analysis window (Fig. 1, C-Fiber). Previous investigators (Meng et al. 1997, 1998) showed that the activity of Vi/Vc neurons driven by A δ -fiber cornea afferents occurred within the first 30 ms after an electrical corneal stimulus, whereas C-fiber afferent input occurred at least 30 ms after the corneal stimulus. The latency of the first A δ elicited spike following a corneal stimulus (Fig. 1, Unit Latency), the time between the first A δ elicited spike following a corneal stimulus and the beginning of OOemg activity (Fig. 1, Lead Time), and the peak and average firing frequency in the 30 and 70 ms windows were determined. For tonically active neurons, activity in the period preceding corneal stimulation was used to calculate the tonic firing frequency. Vc/C1 microstimulation effects on Vi/Vc neuron activity were compared between pairs of sequential trials, one with (Test; *T*) and one without (Control; *C*) Vc/C1 microstimulation preceding the corneal stimulus. Both trials of the pair were discarded from the analysis if data from either the control or test trial was unusable because of problems with the corneal stimulus, e.g., movement of the electrode by the eyelid or tears shorting the stimulating electrodes.

To compare unit and OOemg activity among neurons, OOemg activity and the different measures of unit activity for each recording were normalized to their median control value. For all statistical analyses, an alpha level of <0.05 was deemed significant. Statistical analysis was performed using SPSS (SPSS Inc, Chicago, IL, USA). An analysis of variance (ANOVA) of normalized unit and OOemg data was used to determine the significance of decreases in blink amplitude and number of action potentials produced by Vc/C1 microstimulation. We performed post-hoc paired *t* tests to identify significant changes for individual neurons. A normalized difference score was also used to compare unit and OOemg activity. The normalized difference score was calculated by subtracting the control value from the test value and then dividing this difference by the sum of the test and control values ($(T - C)/(T + C)$). With this measure, suppression and facilitation have the same range from 0 to -1 or 0 to 1. In the paired stimulus experiments, comparisons were made between responses within a trial, rather than between trials.

Results

Forty-six cornea-responsive neurons in the Vi/Vc transition region were recorded from forty-two rats. Based

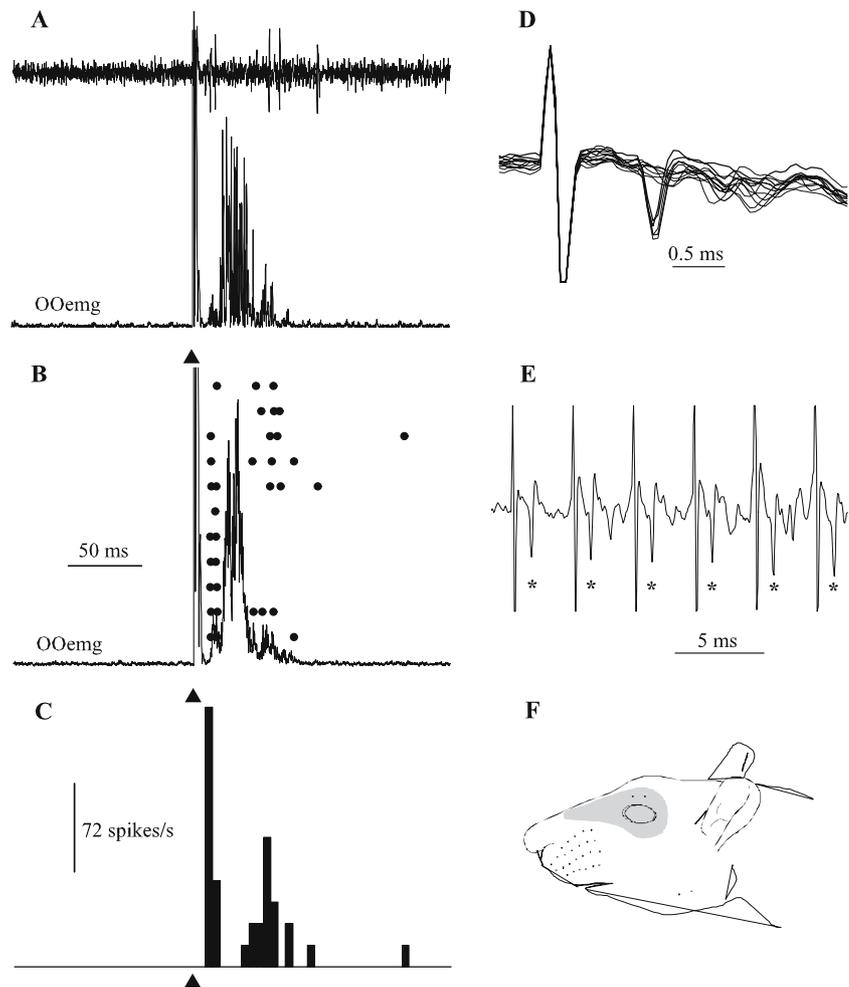
on their firing patterns, there were two groups of neurons, phasic (Fig. 2) and tonic (Fig. 3). Phasic neurons were quiescent until corneal stimulation, whereas tonic neurons exhibited a low level of discharge that increased following corneal stimulation. All neurons wide dynamic range receptive field properties, responding to electrical stimulation of the cornea, as well as to light brushing of the cornea and the fur around the eye (Figs. 2f; 3f). The latency for the 12 antidromically tested neurons ranged from 0.8 to 1.2 ms with a median latency of 1.1 ms. Based on a distance of 3.5 mm between stimulation and recording sites (Paxinos and Watson 1998), the conduction velocity of these neurons ranged from 2.9 to 4.4 m/s with a median conduction velocity of 3.2 m/s. Approximately 67% of the neurons were phasic. Nine of the twelve antidromically-identified and twenty-two of the thirty-two non-identified neurons were phasic. Both phasic and tonic neurons were recorded in the ventral portion of the spinal trigeminal nucleus near the caudal end of the Vi/Vc transition region (Fig. 4).

Phasic neurons

For both antidromically identified (Figs. 1, 2a–c) and non-identified phasic neurons (Fig. 6c), corneal stimulation typically evoked two components of neural activity that corresponded to A δ and C-fiber inputs. Nevertheless, a corneal stimulus did not always evoke both A δ and C-fiber elicited action potentials. For example, the phasic neuron illustrated in Fig. 2 generated A δ -elicited action potentials on ten of the eleven trials and C-fiber elicited action potentials on seven of the eleven trials (Fig. 2b). The appearance of C-fiber elicited action potentials did not depend on the occurrence of A δ activity. The phasic neuron illustrated in Fig. 2b had three C-fiber-elicited action potentials on the trial when it failed to generate A δ activity.

The discharge pattern of phasic neurons was consistent with a role in initiating cornea-evoked blinks. In seven of the eight antidromically identified phasic neurons in which corneal stimulation elicited a robust blink, the first spike following corneal stimulation began 1.6–10.6 ms before initiation of OOemg activity. The first spike occurred coincident with OOemg activity in the eighth neuron. Four of the seven antidromically identified phasic neurons had lead-times greater than 3.6 ms; discharging early enough to initiate OOemg activity. The average lead-times for non-identified phasic neurons was 4.9 ± 0.9 ms ($n = 22$). Nineteen of the twenty-two neurons began discharging before the start of OOemg activity and fifteen of the group began discharging early enough to initiate the

Fig. 2 Antidromically identified phasic Vi/Vc neuron. **a** Unit and rectified orbicularis oculi EMG (OOemg) for a single trial of corneal stimulation (filled triangle). **b** Raster showing occurrence of spikes for 11 trials superimposed on average rectified OOemg activity for those trials. **c** Histogram of spikes for the trials illustrated in (b) using 5 ms bins locked to the corneal stimulus. **d** Antidromic spikes evoked by facial nucleus stimulation at threshold. **e** Antidromic spikes (*) evoked by 300 Hz stimulation of the facial nucleus. **f** Receptive field of neuron determined by brushing



blink. Thus, the majority of phasic neurons began discharging early enough to initiate the blink and the other neurons would have contributed to the earliest OOemg activity.

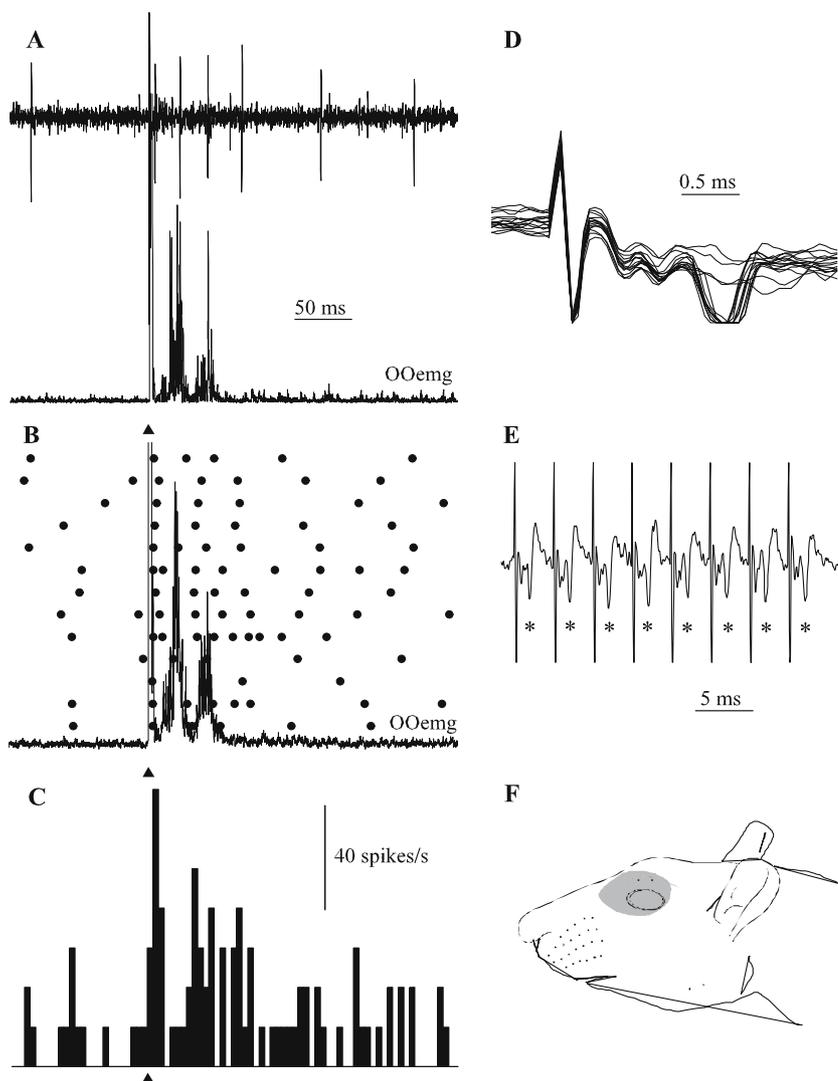
The total number of spikes in the phasic neuron discharge correlated with overall blink amplitude. Mean relative blink amplitude measured over the 100 ms interval after the corneal stimulus significantly increased with the mean relative number of spikes for the data from all non-identified phasic neurons (Fig. 5a, filled circle; $F = 37.117$, $P < 0.001$, $r^2 = 0.86$). The small number of points prevented the increase in blink amplitude with number of spikes for antidromically identified neurons from being significant (Fig. 5a, open circle; $F = 3.63$, $P > 0.05$, $r^2 = 0.64$). For all non-identified phasic neurons, the number of spikes for a median size blink ranged from 2 to 11 spikes with an average of 6.3 ± 2.6 spikes.

In contrast to the increase in blink amplitude with increasing numbers of action potentials, peak firing frequency in the initial A δ period did not show a consistent relationship with overall blink amplitude for either

antidromic ($F = 0.034$, $P > 0.05$, $r^2 = 0.01$) or non-identified phasic neurons ($F = 1.76$, $P > 0.05$, $r^2 = 0.25$). Peak OOemg amplitude, however, increased with the peak firing frequency in the A δ period (Fig. 5c). The increase in peak OOemg amplitude with peak firing frequency was significant for antidromically identified phasic neurons (Fig. 5c, open circle; $F = 20.63$, $P < 0.05$; $r^2 = 0.91$) and approached significance for the non-identified phasic neurons (Fig. 5c, filled circle; $F = 5.47$, $P = 0.06$, $r^2 = 0.47$). Based on the correlation between integrated OOemg amplitude and blink amplitude and peak OOemg amplitude and maximum down lid velocity (Evinger et al. 1991; Pellegrini et al. 1995), the total number of spikes and the peak firing frequency of phasic Vi/Vc neurons predicted blink amplitude and maximum down velocity, respectively.

The discharge of phasic neurons, however, did not accurately predict individual components of OOemg activity (Fig. 6). The mean relative number of spikes in the initial 30 ms failed to predict the initial 30 ms of blink amplitude for antidromic (Fig. 6a, open circle; $F = 3.44$, $P > 0.05$, $r^2 = 0.53$) and non-identified (Fig. 6a,

Fig. 3 Antidromically identified tonic Vi/Vc neuron. **a** Unit and rectified orbicularis oculi EMG (OOemg) for a single trial of corneal stimulation (filled triangle). **b** Raster showing occurrence of spikes for 13 trials superimposed on average-rectified OOemg activity for those trials. **c** Histogram of spikes for the trials illustrated in (b) using 5 ms bins locked to the corneal stimulus. **d** Antidromic spikes evoked by facial nucleus stimulation at threshold. **e** Antidromic spikes (*) evoked by 300 Hz stimulation of the facial nucleus. **f** Receptive field of neuron determined by brushing



filled circle; $F = 1.7_6$, $P > 0.05$, $r^2 = .25$) neurons. C-fiber spike activity also failed to predict the second component of the blink response because the C-fiber discharge rarely began before the second component of EMG activity (Figs. 2b, 6c, 8a, b).

Tonic neurons

Antidromically identified (Fig. 3a–c) and non-identified (Fig. 6d) tonic neurons exhibited a low frequency, irregular spontaneous activity (7.9–26.7 Hz). Similar to phasic neurons, corneal stimulation evoked an A δ and C-fiber discharge in tonic neurons (Figs. 3a–c, 6d) in which the first spike preceded the onset of OOemg activity by an average of 6.9 ± 2.8 ms. Sixty percent of these neurons began discharging at least 3.6 ms before the start of OOemg activity. For all tonic neurons, the total number of spikes in the 100 ms following the corneal stimulus was 6.1 ± 13.4 spikes.

The relationships between unit discharge and blink amplitude were similar for phasic and tonic neurons. Like phasic neurons, the mean relative blink amplitude increased with the mean relative number of spikes for non-identified (Fig. 5b, filled circle; $F = 84.5_4$, $P < 0.01$, $r^2 = 0.97$) tonic neurons. The small number of points prevented the increase in blink amplitude with number of spikes for antidromically identified neurons from being significant (Fig. 5b, open circle; $F = 12.2_2$, $P > 0.05$, $r^2 = 0.92$). Relative blink amplitude showed a significant increase with the mean relative peak A δ firing frequency of non-identified tonic neurons (Fig. 5d, filled circle; $F = 8.8_5$, $P < 0.05$, $r^2 = 0.61$). There were not enough values from the three antidromically identified tonic neurons to plot a peak A δ firing frequency—mean amplitude relationship. The increase in peak OOemg amplitude with peak A δ firing frequency, however was not significant ($F = 3.1_5$, $P > 0.05$, $r^2 = 0.44$). As with phasic neurons, neither the

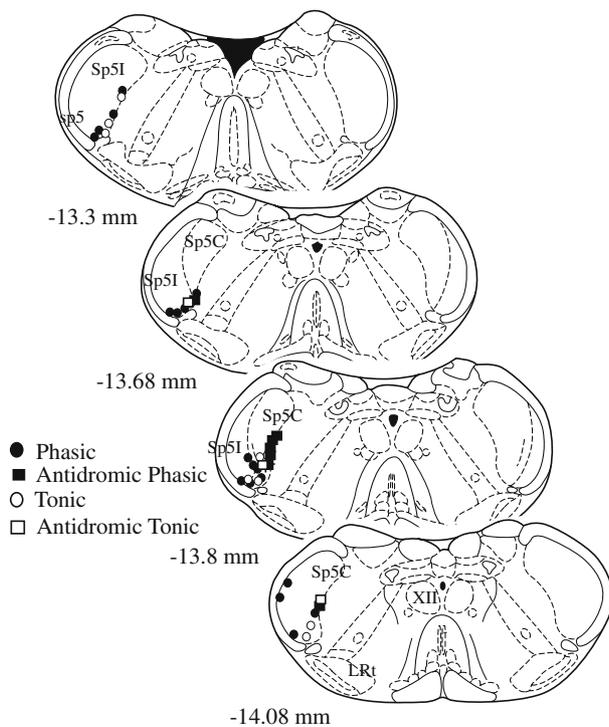


Fig. 4 Locations of Vi/Vc antidromic phasic (filled square), non-identified phasic (filled circle), antidromic tonic (open square), and non-identified tonic (open circle) neurons recorded at the Vi/Vc transition region of the spinal trigeminal nucleus plotted on schematics of the brainstem (Paxinos and Watson 1998). Numbers indicate distance from bregma. Abbreviations: *LRt* lateral reticular nucleus; *sp5* spinal trigeminal tract; *Sp5C* trigeminal caudalis subnucleus; *Sp5I* trigeminal interpolaris subnucleus; *XII* hypoglossal nucleus

mean relative number of spikes in the 30 ms after the corneal stimulation (Fig. 6b; $F = 3.9_3$, $P > 0.05$, $r^2 = 0.66$) nor the mean relative peak $A\delta$ firing frequency of tonic neurons ($F = 0.4_4$, $P > 0.05$, $r^2 = 0.12$) predicted the mean relative blink amplitude of the first component of OOemg activity.

Although the initial discharge of phasic and tonic neurons occurs early enough to initiate the OOemg response, Vi/Vc neurons are unlikely to determine the blink completely. The correlations between total number of spikes with overall blink amplitude (Fig. 5a, b) and peak $A\delta$ firing frequency with peak OOemg amplitude indicate that Vi/Vc neurons program the corneal reflex blink. Nevertheless, the overall neural discharge does not prefigure the pattern of OOemg activity (Figs. 2c, 3c, 6c, d). For example, the long latency C-fiber elicited discharge occurs too late to initiate or determine OOemg activity. Thus, Vi/Vc neurons may set the parameters of a cornea-evoked reflex blink but another brainstem circuit must detail the drive to OO motoneurons to generate the late components of the blink.

Vi/Vc discharge with blink oscillations

Corneal irritation causes blink oscillations in which a blink-evoking trigeminal stimulus produces a reflex blink plus one or more additional blinks (Peshori et al. 2001; Evinger et al. 2002). The source of these additional blinks is unknown. Two recordings of non-identified phasic Vi/Vc neurons in rats exhibiting blink oscillations suggest that Vi/Vc neurons may initiate and control these blinks (Fig. 7). Typical of blink oscillations, the additional blink (Fig. 7, Oscillation) was much larger than the reflex blink. For the oscillation, the discharge of the Vi/Vc neuron began 16 ms before the onset of the OOemg activity and the pattern of OOemg activity mirrored the instantaneous firing frequency. The discharge of both Vi/Vc neurons led the blink oscillations and their firing frequency predicted the pattern of OOemg activity.

Effect of Vc/C1 stimulation on Vi/Vc neuron discharge

As described previously (Henriquez and Evinger 2005), a train of Vc/C1 microstimulation significantly suppressed cornea-evoked blinks (Fig. 8b). If phasic Vi/Vc neurons initiated cornea-evoked blinks and programmed overall blink amplitude, then the blink suppression produced by Vc/C1 stimulation could have resulted from a reduction in the number of spikes evoked by corneal stimulation in Vi/Vc second order neurons. The majority of phasic neurons supported this prediction (Fig. 8b). An ANOVA revealed that Vc/C1 stimulation significantly suppressed the OOemg activity and number of spikes during recordings of antidromic phasic neurons ($F_{3,296} = 61.01952$; $P < 0.001$). Post-hoc paired t tests demonstrated that for all seven of the antidromically identified neurons tested with Vc/C1 stimulation, the stimulation significantly reduced the number of spikes and blink amplitude. An ANOVA showed that Vc/C1 stimulation also significantly suppressed the OOemg activity and number of spikes during recordings of non-identified phasic neurons ($F_{3,648} = 79.0548$; $P < 0.001$). Post-hoc paired t tests demonstrated a significant reduction in the total number of spikes for all neurons for which Vc/C1 stimulation significantly suppressed OOemg activity.

An ANOVA demonstrated that Vc/C1 stimulation significantly suppressed the OOemg activity and number of spikes generated by tonic neurons ($F_{3,360} = 34.874$; $P < 0.001$). Unlike phasic neurons, however, post-hoc paired t tests showed that only 50% of the tonic neurons for which Vc/C1 stimulation significantly suppressed OOemg activity exhibited a concomitant decrease in the total number of spikes.

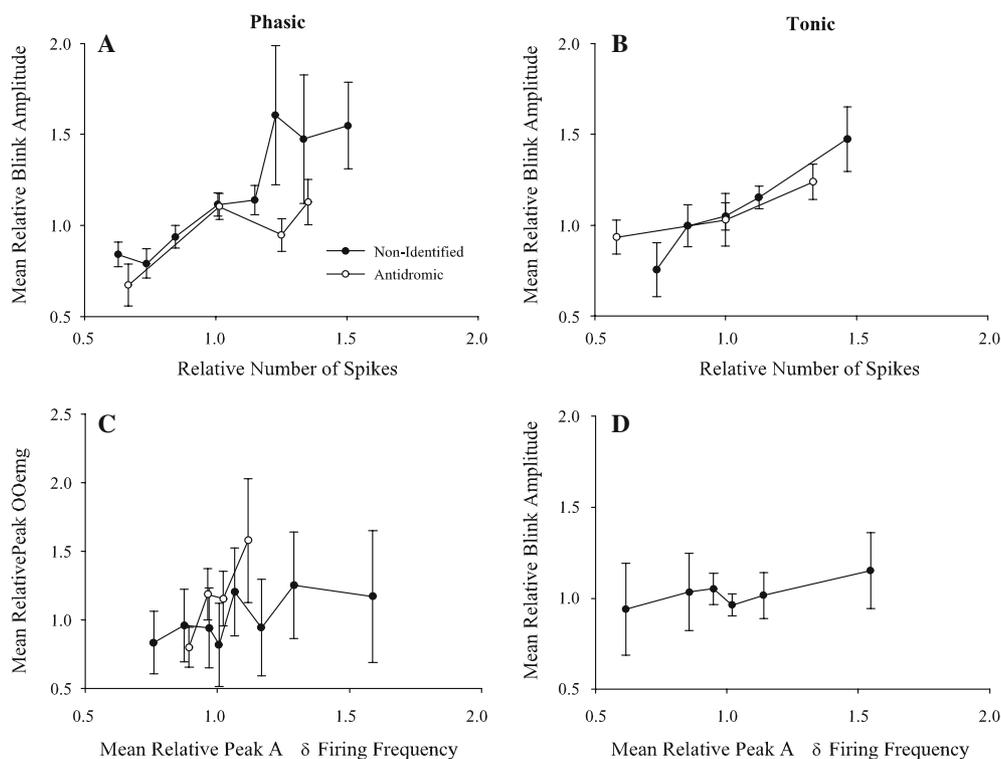


Fig. 5 Relationship between Vi/Vc neuron discharge and blink amplitude in the 100 ms interval following corneal stimulation. **a** Mean blink amplitude for the 100 ms following the corneal stimulus relative to the median blink amplitude as a function of the mean number of spikes relative to the median number of spikes for all of the non-identified (filled circle) and antidromically identified phasic neurons (open circle). **b** Mean blink amplitude for the 100 ms following the corneal stimulus relative to the median blink amplitude as a function of the mean number of spikes relative to the median number of spikes for all of the non-identified (filled circle) and antidromically identified (open circle) tonic neurons. **c** Mean Peak OOemg amplitude for the 100 ms following the corneal stimulus relative to the median Peak OOemg amplitude as a function of the mean peak A δ firing frequency relative to the median peak A δ firing frequency for all of the non-identified (filled circle) and antidromically identified (open circle) phasic neurons. **d** Mean blink amplitude for the 100 ms following the corneal stimulus relative to the median blink amplitude as a function of the mean peak A δ firing frequency relative to the median peak A δ firing frequency for all of the non-identified tonic neurons (filled circle). Error bars are SEM

Effect of paired corneal stimuli on Vi/Vc neuron discharge

Although our previous work demonstrated that blink suppression caused by Vc/C1 stimulation and pairing identical trigeminal stimuli used two different mechanisms (Henriquez and Evinger 2005), it was possible that they both created blink suppression by reducing the discharge of Vi/Vc neurons. We tested this proposal by comparing the normalized difference scores of changes in the total number of spikes and blink suppression produced by Vi/Vc stimulation and pairs of corneal stimuli with a 150 ms interstimulus interval for three tonic and three phasic neurons (Fig. 8c). Vc/C1 stimulation significantly reduced the total number of spikes in all three phasic neurons (Fig. 8b, c, filled circle; paired *t* tests, $P < 0.001$). In contrast, paired corneal stimuli caused a nonsignificant increase in the number of spikes evoked by the second of two corneal stimuli relative to the first stimulus for two of the three

neurons even though the paradigm significantly reduced blink amplitude (Fig. 8a, c, open circle; paired *t* tests, $P < 0.05$). For the tonic neurons, Vc/C1 stimulation significantly reduced the number of spikes for two of the three neurons (Fig. 8c, filled circle; paired *t* tests, $P < 0.05$) even though the reduction in blink amplitude did not achieve statistical significance. As with phasic neurons, paired corneal stimulation significantly increased the number of spikes for two of the three tonic neurons (Fig. 8c, open circle; paired *t* test, $P < 0.001$) even though the paradigm significantly reduced blink amplitude (Fig. 8c, open circle; paired *t* test, $P < 0.05$). Plotting the normalized difference scores for blink amplitude and the number of spikes in the Vc/C1 stimulation paradigm showed the expected concomitant reduction in number of spikes and blink amplitude (Fig. 8c, filled circle). As seen by the points with negative difference scores on the amplitude axis, the paired stimulus paradigm with a 150 ms interstimulus interval reduced the amplitude of the second blink

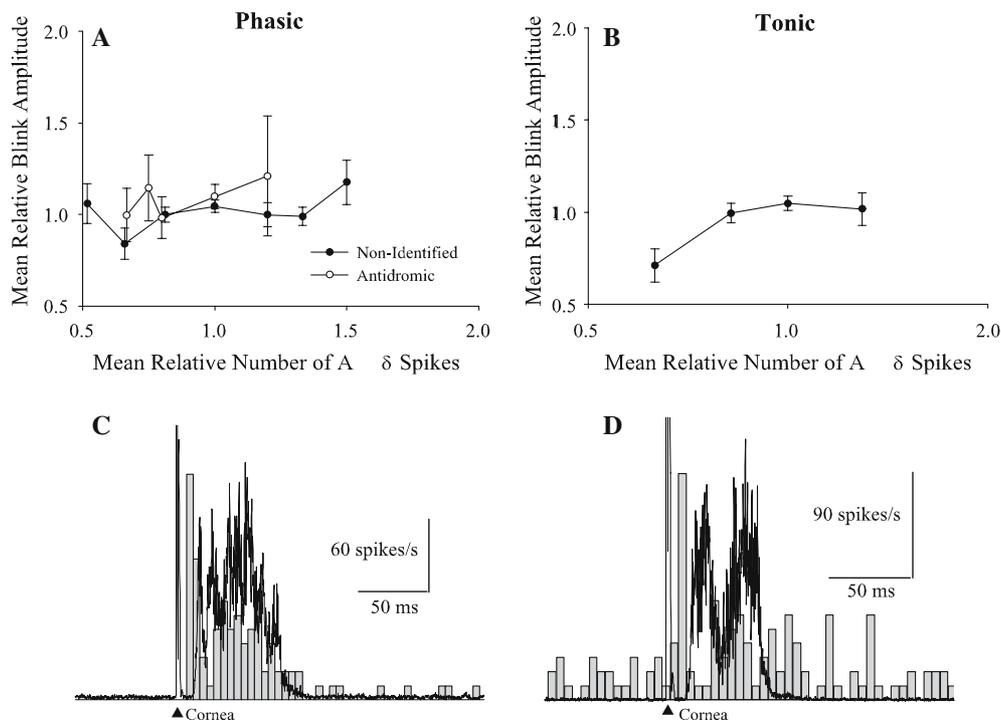


Fig. 6 Relationship between Vi/Vc neuron discharge and initial blink amplitude in the 30 ms interval following corneal stimulation. **a** Mean blink amplitude for the 30 ms following the corneal stimulus relative to the median blink amplitude as a function of the mean number of spikes relative to the median number of spikes for all of the non-identified (*filled circle*) and antidromically identified (*open circle*) phasic neurons. **b** Mean blink amplitude for the 30 ms following the corneal stimulus relative to median blink amplitude as a function of the mean number of

spikes relative to the median number of spikes for all of the non-identified (*filled circle*) tonic neurons. **c** Histogram of non-identified phasic neuron discharge using 5 ms bins locked to the corneal stimulus (*filled triangle* Cornea) superimposed on the average rectified OOemg activity from those trials. **d** Histogram of non-identified tonic neuron discharge using 5 ms bins locked to the corneal stimulus (*filled triangle* Cornea) superimposed on the average rectified OOemg activity from those trials. *Error bars* are SEM

relative to the first for five of the six neurons (Fig. 8c, open circle). Nevertheless, the difference score for the total number of spikes was positive, indicating that the second corneal stimulus evoked more spikes than the first. Thus, Vc/C1 stimulation suppressed the discharge of most Vi/Vc neurons, whereas the paired stimulus paradigm facilitated the discharge of those same neurons.

Discussion

Role of second order Vi/Vc neurons in the corneal reflex blink

The three-neuron component of the corneal reflex circuit appears to initiate corneal reflex blinks. In a previous study, Pellegrini et al. (1995) demonstrate that OO motoneuron activity begins 2 ms before OOemg activity in rodents. Based on our median antidromic latency, a Vi/Vc neuron action potential requires an average of 1.1 ms to reach the facial nucleus. Assuming

a 0.5 ms synaptic delay, Vi/Vc neurons must discharge at least 3.6 ms before OOemg activity to initiate OO muscle contraction. Sixty-six percent of phasic and sixty percent of tonic neurons begin discharging at least 3.6 ms before the start of OOemg activity. Eighty-seven percent of the phasic and all of the tonic neurons begin discharging before the start of OOemg activity. Thus, the discharge of the majority of Vi/Vc neurons is early enough to initiate and modulate the initial components of corneal reflex blinks.

The discharge pattern of Vi/Vc neurons suggests that they program corneal reflex blink amplitude and peak velocity in addition to initiating the blink. The size of the lid closure, blink amplitude, estimated from the integrated OOemg (Evinger et al. 1991; Pellegrini et al. 1995) increases with the total number of spikes for both phasic and tonic neurons (Fig. 5a, b). The peak firing frequency of the A δ discharge provides a weak prediction of the peak OOemg magnitude, a measure of maximum down velocity of the (Evinger et al. 1991) (Fig. 5). The late C-fiber driven discharge, however, occurred in the middle of OOemg activity

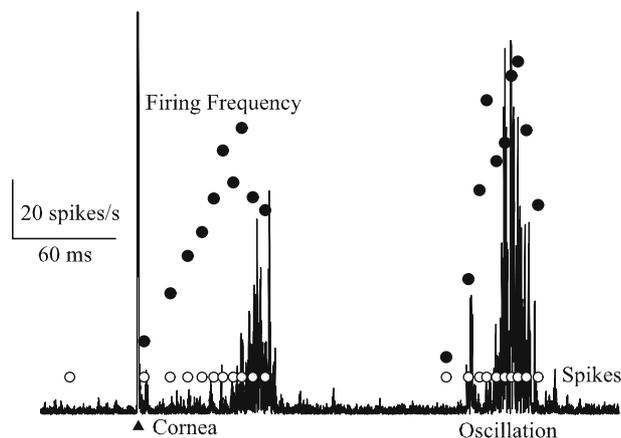


Fig. 7 Example of phasic Vi/Vc neuron activity with blink oscillations. Superimposed on the blink evoked by corneal stimulation (*filled triangle* Cornea) and blink oscillation (Oscillation) are the occurrence of action potentials (*open circle*) and the instantaneous firing frequency (*filled circle*) of those spikes for a single trial

rather than initiating the second component of OOemg blink activity (Figs. 2b, 6c, 8a, b). Thus, the simplest interpretation of the role of Vi/Vc neurons in generating corneal reflex blinks is that they initiate the blink by depolarizing OO motoneurons and program the reflex blink through activation of a reticular circuit (Holstege et al. 1986; Inagaki et al. 1989; Morcuende et al. 2002; Zerari-Mailly et al. 2003). The reticular circuit details the drive onto OO motoneurons to produce the later components of the cornea-evoked blink. Consistent with this interpretation, the instantaneous firing frequency of cat OO motoneurons correlates with the eyelid velocity of reflex blinks (Trigo et al. 1999). The lack of a correlation between the instantaneous firing frequency of Vi/Vc neurons and the pattern of OOemg activity with a reflex blink indicates that another circuit must create the correlation of OO activity and eyelid velocity (Fig. 8). The exception to this conclusion is that the instantaneous firing frequency of Vi/Vc neurons matches the pattern of OOemg activity with blink oscillations (Fig. 7).

It is unclear whether tonic and phasic Vi/Vc neurons are distinct groups or two ends of a continuum. In support of the continuum proposal, the total number of spikes evoked by a corneal stimulus of both tonic and phasic neurons predicts blink amplitude. Whether a Vi/Vc neuron is tonic or phasic may simply reflect the degree of chronic corneal activation. Thus, corneal irritation would convert a phasic neuron into a tonic neuron. Consistent with the two group proposal, tear production may involve tonic but not phasic neurons. Tonically active Vi/Vc neurons are known to project to the superior salivatory nucleus and to cause tearing (Hirata et al. 2004). In accordance for separate roles

for tonic and phasic neurons, the correlation between neural activity and blink parameters is weaker for tonic than phasic neurons. Microstimulation in the facial nucleus can antidromically activate afferents to the superior salivatory nucleus (Hirata et al. 2004). Human studies suggest a strong linkage between blinking and tear release (Doane 1980; Prause and Norn 1987; Collins et al. 1989; Buehren et al. 2001; Owens and Phillips 2001; Bron et al. 2004). Thus, it is reasonable that corneal afferent inputs that evoke a blink through the phasic neurons should also elicit comparable magnitude tearing through tonic neurons.

Role of Vi/Vc neurons in blink suppression

Both microstimulation in Vc/C1 region (Henriquez and Evinger 2005) and the first of a pair of blink-evoking trigeminal stimuli (Cruccu et al. 1986; Pellegrini and Evinger 1995; Powers et al. 1997; Peshori et al. 2001) suppress subsequent blinks (Fig. 8). Nevertheless, Vi/Vc neurons respond differently in these blink suppression paradigms. Vc/C1 stimulation reduces the number of spikes evoked by corneal stimulation, whereas the paired stimulus paradigm slightly increases the discharge of Vi/Vc neurons. This discontinuity between the response of second order trigeminal neurons projecting to the facial nucleus and the OOemg response in the paired stimulus paradigm complements the argument that a reticular circuit rather than Vi/Vc neurons establishes the pattern of OO motoneuron discharge and suggests that the paired stimulus paradigm suppression acts on this reticular circuit.

Although Vc/C1 suppression of the discharge of the majority of Vi/Vc neurons (Fig. 8) accounts for the reduction in blink amplitude with Vc/C1 microstimulation, the role of Vc/C1 in reflex blinking is unclear. The cornea and supraorbital primary afferents terminate preferentially in the Vi/Vc and Vc/C1 regions of the rodent trigeminal complex (Marfurt and Del Toro 1987; Marfurt and Echtenkamp 1988; Pellegrini et al. 1995; van Ham and Yeo 1996) and a blink evoking corneal stimulus elicits similar patterns of activity in Vi/Vc and Vc/C1 neurons (Meng et al. 1997; Hirata et al. 1999). Nevertheless, lesioning the Vc/C1 region does not block corneal reflex blinks (Ongerboer de Visser 1980; Pellegrini et al. 1995). Generating similar responses to blink-evoking inputs in two regions of which only the rostral region appears to play a role in generating reflex blinks indicates that the caudal region performs another role. One possibility is that the Vc/C1 activity evoked by a corneal stimulus represents an “internal model” of the intended blink initiated by the Vi/Vc region.

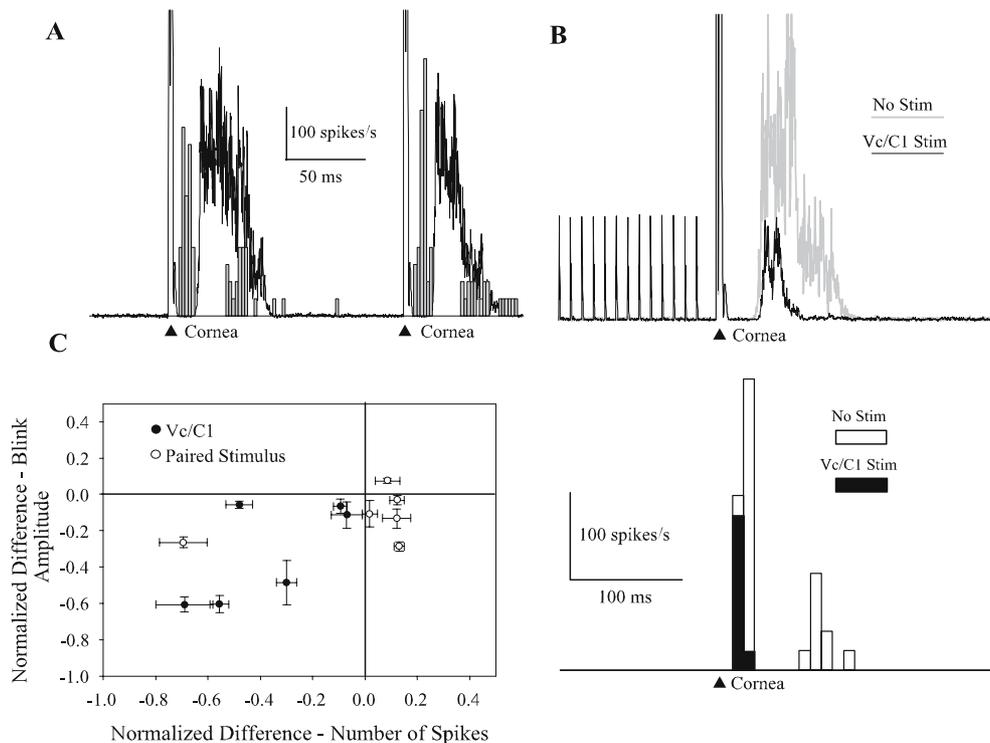


Fig. 8 Comparison of Vi/Vc activity with paired corneal stimuli and Vc/C1 microstimulation. **a** Histogram (2 ms bins) of phasic neuron discharge produced by identical corneal stimuli (filled triangle Cornea) superimposed on the average rectified OOemg activity from those trials. **b** The mean rectified OOemg activity with (solid line) and without (gray line) preceding Vc/C1 microstimulation and a histogram (2 ms bins) showing neural discharge from the same trials with (filled bars) and without (open bars) pre-

ceding Vc/C1 microstimulation. **a** and **b** show data from the same neuron. **c** The normalized difference for blink amplitude plotted as a function of normalized difference for the number of spikes in the 100 ms following a corneal stimulus for identical pairs of corneal stimuli (open circle) or Vc/C1 microstimulation (filled circle) for three phasic and three tonic Vi/Vc neurons. Each point is the mean of at least five trials \pm SD

In order to modify the blink reflex adaptively, the nervous system must compare the sensory inputs from the actual lid closure with the anticipated sensory signals from the movement (Evinger and Manning 1988; Evinger et al. 1989; Pellegrini and Evinger 1997; Schicatano et al. 2002). The delayed inhibition of the rostral Vi/Vc neurons by caudal Vc/C1 neurons (Fig. 8) (Meng et al. 1998; Henriquez and Evinger 2005) may suppress Vi/Vc neurons' response to the trigeminal feedback from the actual blink. Because Vc/C1 and Vi/Vc neurons respond similarly to the blink evoking corneal stimulus, this trigeminal interaction is a functional comparison of the "actual" and the "intended" blink. If Vc/C1 activity representing the "intended" blink matches the trigeminal feedback from the actual blink, then sensory signals from the blink would not be able to activate brainstem or cerebellar circuits. Repeated activation of brainstem or cerebellar circuits by sensory feedback from a blink, however, would indicate a difference between intended and expected blinks and initiate blink adaptation (Evinger and Manning 1988; Evinger et al. 1989; Schicatano et al. 2002).

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