GABA<sub>A</sub> Receptor Activation Modulates Corneal Unit Activity in Rostral and Caudal Portions of Trigeminal Subnucleus Caudalis

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Hirata, Harumitsu, Keiichiro Okamoto, and David A. Bereiter. GABA<sub>A</sub> receptor activation modulates corneal unit activity in rostral and caudal portions of trigeminal subnucleus caudalis. J Neurophysiol 90: 2837–2849, 2003. First published July 30, 2003; 10.1152/jn.00544.2003. Corneal nociceptors terminate at the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition and subnucleus caudalis/upper cervical spinal cord (Vc/C1) junction regions of the lower brain stem. The aims of this study were to determine if local GABA<sub>A</sub> receptor activation modifies corneal input to second-order neurons at these regions and if GABA<sub>A</sub> receptor activation in one region affects corneal input to the other region. In barbiturate-anesthetized male rats, corneal nociceptors were excited by pulses of CO<sub>2</sub> gas, and GABA<sub>A</sub> receptors were activated by microinjections of the selective agonist muscimol. Local muscimol injection at the site of recording inhibited all Vi/Vc and Vc/C1 units tested and was reversed partially by bicuculline. To test for ascending intersubnuclear communication, muscimol injection into the caudal Vc/C1 junction, remote from the recording site at the Vi/Vc transition, inhibited the evoked response of most corneal units, although some neurons were enhanced. Injection of the nonselective synaptic blocking agent, CoCl<sub>2</sub>, remotely into the Vc/C1 region inhibited the evoked response of all Vi/Vc units tested. To test for descending intersubnuclear communication, muscimol was injected remotely into the rostral Vi/Vc transition and enhanced the evoked activity of all corneal units tested at the caudal Vc/C1 junction. These results suggest that GABA<sub>A</sub> receptor mechanisms play a significant role in corneal nociceptive processing by second-order trigeminal brain stem neurons. GABA<sub>A</sub> receptor mechanisms act locally at both the Vi/Vc transition and Vc/C1 junction regions to inhibit corneal input and act through polysynaptic pathways to modify corneal input at multiple levels of the trigeminal brain stem complex.

INTRODUCTION

A distinctive feature of the trigeminal sensory system is the representation of orofacial tissues at more than one rostrocaudal level of the trigeminal brain stem complex (Bereiter et al. 2000; Kruger and Young 1981). Multiple representation is particularly evident for structures supplied by the ophthalmic branch of the trigeminal nerve such as the cornea. Corneal afferents project to different levels of the trigeminal brain stem complex (Marfurt 1981; Marfurt and del Toro 1987; Panneton and Burton 1981), and correspondingly, noxious stimulation of the cornea (Lu et al. 1993; Meng and Bereiter 1996; Strassman and Vos 1993) produces a bimodal pattern of Fos-positive neurons with a high-density at the trigeminal subnucleus interpolaris/caudalis transition (Vi/Vc) and subnucleus caudalis/upper cervical spinal cord (Vc/C1) junction regions. The significance of multiple representation is not certain and two broad interpretations have been considered (Bereiter et al. 2000). Multiple representation of the cornea may provide functional redundancy to ensure that sensory information is faithfully processed to protect and maintain a vital sense organ, the retina. Another possible interpretation is that corneal afferents project to separate groups of second-order neurons that serve different aspects of ocular homeostasis.

To better understand the significance of multiple somatotopic representation, it is necessary to consider the possible role played by longitudinal fiber connections. The trigeminal brain stem complex is comprised of a principal nucleus and spinal nucleus (Vsp). The Vsp is further subdivided, from rostral to caudal, into subnucleus oralis (Vo), interpolaris (Vi), and caudalis (Vc) based on cytological features (Olszewski 1950). It is well established that the subnuclei of Vsp are interconnected longitudinally by fibers that course within the spinal trigeminal tract dorsal and lateral to Vsp as well as by a “deep bundle system” that runs within and ventral to substantia gelatinosa in the laminated portion of Vsp, i.e., Vc, and within the main body of Vsp at more rostral subnuclei (Gobel and Purvis 1972; Ikeda et al. 1984; Jacquin et al. 1990; Kruger et al. 1977; Nasution and Shigenaga 1987; Panneton and Burton 1982; Voisin et al. 2002). Although the anatomical features of intersubnuclear connections have been well described, the function of intersubnuclear communication in mediating various aspects of trigeminal nociception remains uncertain. Intersubnuclear connections may contribute to sensory processing since blockade of Vc or interruption of the spinal trigeminal tract at the level of Vc alters the response properties of nociceptive neurons in rostral portions of the complex (see Sessle 2000) and reduces the expression of central sensitization among neurons in rostral subnuclei following peripheral nerve injury (Chiang et al. 2002; Hu et al. 2002). Previously, we reported that microinjection of mu opioid agonists into the Vc/C1 junction region altered the responses of a high percentage of corneal units recorded at the Vi/Vc transition region (Hirata et al. 2000; Meng et al. 1998). Thus intersubnuclear communication between Vc and more rostral subnuclei may underlie some aspects of opioid analgesia in corneal nociception.

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The neurotransmitter systems that mediate intersubnuclear communication in Vsp are not well defined. In addition to opioid receptors (Hirata et al. 2000; Meng et al., 1998), glutamateergic (Meng et al. 1998; Woda et al. 2001) and purinergic receptors (Hu et al. 2002) may contribute to intersubnuclear communication in trigeminal nociception. However, despite a well-established role in regulation of central neural excitability (Mody et al. 1994) and a dominant role in local inhibitory neural circuits at the spinal level (see Hammond 1997; Todd and Spike 1993), little is known regarding GABAergic involvement in intersubnuclear communication in the trigeminal system. GABA-induced inhibition is mediated by two main receptor subtypes, ionotropic GABA_A receptors and G protein–coupled GABA_B receptors (Hammond 1997), and anatomical evidence indicates that both presynaptic and postsynaptic mechanisms are possible (see Todd and Spike 1993). GABAergic neurons are distributed throughout the Vsp with high densities in the superficial laminae of Vc (DiFiglia and Aronin 1990; Ginestal and Matute 1993; Iliaakis et al. 1996; Matthews et al. 1989; Polgar and Antal 1995; Wang et al. 2000) and at the Vi/Vc transition region (Matthews et al. 1988, 1989). Similarly, GABA_A receptors are found in superficial laminae of Vc (Kondo et al. 1995) and at the ventral Vi/Vc transition region (Fritschy and Mohler 1995) that receives corneal input. Most previous studies that have examined GABAergic effects on trigeminal nociceptive processing have emphasized the influence on cutaneous inputs (Chiang et al. 1999; Takeda et al. 2000). However, unlike cutaneous tissue, the cornea is not supplied by large diameter afferent fibers (MacIver and Tanelian 1993; Rozsa and Beuerman 1982). The primary aim of this study was to assess the effects of GABA_A receptor activation on corneal nociceptive input to trigeminal brain stem neurons. A second aim was to determine if intersubnuclear pathways for corneal nociceptive processing involve GABA_A receptors. Since most previous studies of intersubnuclear communication in the trigeminal system have examined only ascending effects of Vc activity on more rostral regions of Vsp, the present study also determined if GABAergic blockade of the rostral Vi/Vc transition region modified corneal unit activity in superficial laminae at the caudal Vc/C1 junction region. The corneal afferent system is well suited to examine intersubnuclear communication in trigeminal nociception. Most forms of corneal stimulation are perceived as painful by humans (Acosta et al. 2001; Belmonte et al. 1997) and the close proximity of nerve endings to the outer epithelial layer allows direct stimulation of corneal nociceptors without surgical preparation or injury to surrounding tissues. Also, the Vi/Vc transition and Vc/C1 junction regions that receive corneal input are separated by 3–4 mm in the rat (Marfurt and del Toro 1987), allowing the delivery of small volumes of drugs locally to one region without diffusion to the other region (Arikan et al. 2002; Hirata et al. 2000).

**Methods**

The protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital and conformed to the established guidelines set by The National Institute of Health guide for the care and use of laboratory animals.

**Animal preparation**

Male rats (289–487 g, Sprague-Dawley, Harlan) were anesthetized with pentobarbital sodium (70 mg/kg, ip) prior to surgery. The left femoral artery (blood pressure monitor) and jugular vein (anesthesia and drug infusions) were catheterized, and after tracheotomy, animals were respired artificially with oxygen-enriched room air. Adequate depth of anesthesia was confirmed by the absence of corneal and nictitans withdrawal reflexes. Animals were maintained by a continuous infusion of methohexitonal sodium (35–40 mg/kg/h) and switched to a mixture of methohexitonal sodium (26–40 mg/kg/h) and the paralytic agent, gallamine triethiodide (14–32 mg/kg/h), after completion of all surgical procedures and just prior to the recording session. The animal was placed in a stereotaxic frame and a portion of the occipital bone and C1 vertebrae was removed to expose the dorsal surface of the medulla. The brain stem surface was kept moist with warm mineral oil. A dental drill was used to remove a small portion of bone on the right side of the skull for placement of the array of anterograde stimulating electrodes. Expiratory end-tidal CO₂ was monitored continuously and kept at 4–5% by adjusting tidal volume. Mean arterial pressure (MAP) remained above 100 mmHg throughout each experiment. Body temperature was maintained at 38°C with a heating blanket and thermal probe.

**Electrophysiology recording**

Extracellular unit activity was recorded with tungsten electrodes (9–15 MΩ, FHC; Bowdoinham, ME) as described previously (Hirata et al. 1999, 2000). Neurons recorded at the rostral Vi/Vc transition were approached at an angle of 28° off vertical and 45° off midline, and 1.5–2.5 mm below the brain stem surface (see Fig. 3). Neurons recorded in superficial laminae (I-II) at the caudal Vc/C1 junction region were approached at an angle of 43° off vertical, 60° off midline and were found just before exiting the lateral dorsal horn, 300–500 μm after surface penetration. Mechanical (fine paint brush, von Frey filaments) and then electrical (bipolar electrode, 0.1–1 ms duration, <1.0 mA, 0.2 Hz) stimuli were used to search for responsive neurons. Unit activity was amplified, displayed on a digital oscilloscope to monitor spike shape and amplitude, and passed through a window discriminator. The window discriminator allowed single units to be isolated by amplitude as well as time (model DIS-1, BAK Electronics, Mt. Airy, MD). Discriminated corneal units, MAP, and a stimulus marker for CO₂ pulses were recorded on-line with an Apple computer (G3) through a DAQ interface board using LabVIEW software (National Instruments, Austin, TX). These data also were recorded on a four-channel DAT/SCSI based acquisition system (model CDAT4, Cygnus Technology, Delaware Water Gap, PA) for further off-line analyses.

The general response properties of each unit were determined prior to testing with CO₂ pulses. Units also were tested for A- and/or C-fiber type corneal input (electrical stimuli) and responses occurring at latencies of >30 ms were assumed to indicate C-fiber input (Hu 1990; Meng et al., 1997). The mechanical threshold for corneal input was determined with calibrated von Frey filaments. Each unit was tested for convergent cutaneous input from the ipsilateral face. Corneal units with a convergent cutaneous receptive field (RF) were classified as low threshold mechanoreceptive (LTM), wide dynamic range (WDR), or nociceptive specific (NS) units as described previously (Hirata et al. 1999; Hu 1990). LTM corneal units responded best to brushing the skin, while WDR units were excited by brush or indentation of the skin surface with low threshold von Frey filaments (1.2 g force) and showed a greater response to press or pinch. NS corneal units were activated by press or pinch of the facial skin but not by brushing. Many corneal units at the Vi/Vc transition region were classified as “complex” and displayed an inhibitory cutaneous RF that was contiguous with the corneal surface. Nearly all complex cells also were inhibited by intranasal mechanical stimulation. Neurons with no apparent cutaneous RF were classified as cornea only (CO) units.
Corneal stimulation by carbon dioxide

The details for delivery of CO2 pulses to the corneal surface were reported previously (Hirata et al. 2000). Briefly, variable concentrations (0–80%) of CO2 gas were obtained by mixing the outflow from tanks of 100% CO2 and air through a proportional gas mixer as monitored from the bleeder valve output by an infrared detector (CapStar 100, CWE, Ardmore, PA). Humidified CO2 gas mixtures were delivered at constant flow rate to the left cornea via polyethylene tubing positioned approximately 5 mm from the surface. The timing and duration of CO2 pulses (40-s duration, minimum of 15 min between pulses) were computer-controlled by LabVIEW software. In most experiments only the responses to 0% (air) and 80% CO2 pulses were compared. However, in initial experiments, the responses to a full range of CO2 concentrations (0, 30, 60, and 80%) were examined to confirm that the threshold and slope of the stimulus-response curves were similar to those reported previously (Hirata et al. 1999). Typically, 80% CO2 produced a near maximum neural response. Special care was taken to keep the cornea moist during surgery and throughout the recording period with normal saline or an artificial tear fluid.

Drugs

The following drugs were delivered by pressure microinjection (Neuro-Phore, Medical Systems, Greenvale, NY) in a volume of 300 nl via a single or dual barrel thick-walled micropipette (40–80 μm OD): selective GABA_A receptor agonist, muscimol hydrobromide (Sigma; 0.1–20 mM, pH = 6.7); selective GABA_A antagonist, bicuculline methiodide (BMI; Sigma; pH = 6.06, 0.1 mM); the nonsynaptic blocking agent, cobalt chloride (CoCl_2; Sigma; 10 and 100 mM, pH = 5.6); and the selective mu opioid receptor agonist, d-Ala,N-Me-Phe,Gly-ol-enkephalin (DAMGO; Sigma; 0.01 mM, pH = 7.3). All drugs were dissolved in artificial cerebrospinal fluid (150 mM NaCl, 2.6 mM KCl, 1.3 mM CaCl_2, 1.8 mM MgCl_2; pH = 5.7). Drugs were mixed with a 10% solution of Evan’s Blue dye to visualize the location of injection.

Experimental design

A single corneal unit was studied in each preparation. Three general experimental designs were used to deliver drugs while recording from corneal neurons. These approaches are represented schematically in Fig. 1: (a) local drug injection via a separate micropipette and recording at the caudal Vc/C1 junction region or (b) local drug injection via a separate micropipette and recording at the rostral Vi/Vc transition region (Fig. 1A), (c) drug injection into caudal Vc/C1 junction region while recording from the rostral Vi/Vc transition region (Fig. 1B), and (d) drug injection into rostral Vi/Vc transition region while recording from the caudal Vc/C1 junction region (Fig. 1C). For the experimental design shown in Fig. 1A, the tips of the glass microinjection pipette (thick-walled glass, 40–80 μm, OD) and the recording electrode were angled stereotaxically to target a position 0.5–0.8 mm apart. This proximity was confirmed histologically following each experiment from the deposition of Evan’s Blue dye and a lesion at the recording site. After characterizing the mechanical properties for each unit, pulses of 0% CO2 (room air, 40 s duration) were applied to the corneal surface to establish the threshold air flow rate necessary to evoke neural activity. A flow rate just below this threshold value was used to deliver repeated pulses of 80% CO2 (40 s duration, ≥15 min between successive pulses). After a second 80% CO2 pulse, drugs were injected slowly over 5 min from a micropipette connected to the pneumatic pressure device followed at 5 min by additional 80% CO2 test pulses. In experiments in which muscimol was injected, at 60 min postmuscimol, the GABA_A antagonist, BMI, often was injected from the second barrel of a dual micropipette assembly followed by additional 80% CO2 test pulses. In some cases, muscimol-evoked changes in corneal activity were not tested for reversibility with BMI to determine if spontaneous recovery occurred. In each experiment only one single concentration of drug (muscimol, BMI, or CoCl_2) was tested. The target position of the microinjection pipette was based on the coordinates used to record corneal units in previous studies (Hirata et al. 1999, 2000; Meng et al. 1997, 1998). Microscopic observation of the meniscus movement monitored the rate (60 nl/min) and volume (300 nl) of injection. Previously, a volume of 300 nl was shown to spread spherically over approximately 1 mm to completely bathe laminae I-V at the caudal Vc/C1 junction without diffusing to the Vi/Vc transition region, 3–4 mm rostral to Vc/C1 (see example in Hirata et al. 2000; Fig. 2).

Data analysis

Neural recording data were acquired and displayed by LabVIEW as peristimulus time histograms (PSTHs) of spikes per 1-s bins, exported to a spreadsheet and analyzed off-line. Since most units displayed a variable background activity during the interstimulus period between CO2 pulses, evoked activity was assessed statistically by first calculating a response magnitude (R_mag). R_mag was determined by subtracting the mean plus 2 SD of the background activity from the total spike count for each bin. The total R_mag for a given stimulus was defined as the cumulative sum of spikes for those contiguous bins in which the spike count exceeded the mean + 2 SD of the background activity. The total R_mag was calculated for each stimulus period and should be thought of as equivalent to the “area under the curve.” The response duration was defined as the time interval after stimulus onset until three consecutive bins with a positive spike count occurred above background (initial latency) and until the value of three consecutive bins no longer exceeded the mean + 2 SD above background activity. The neural response to CO2 was seen as an early (latency = <5 s) and late (latency = 7–22 s) component in which only the late component was proportional to CO2 concentration (Hirata et al. 1999). Corneal units activated by CO2 were further classified as type I in which only a late excitatory phase was seen or as type II units in which an early inhibitory phase preceded the late excitatory component (see examples in Figs. 2A, 4A, and 5A). Total R_mag values reported in the text refer to the late excitatory component. Average total R_mag values for...
each CO₂ test series (i.e., 0 and 80% CO₂) under different drug conditions were assessed statistically by ANOVA, corrected for repeated measures (Winer 1971), and individual comparisons by Newman-Keuls. χ² analysis determined if different classes of CO₂-responsive units (e.g., type I vs. type II) were associated with different cutaneous RF properties (i.e., LTM, WDR, NS, complex, cornea only). Corneal units responsive to mechanical stimulation of the corneal surface were isolated and tested subsequently for chemical sensitivity by CO₂ pulses. As shown in Table 1, 60% of 71 Vi/Vc units (85%) and 23 of 26 Vc/C1 units (88%) responded to CO₂ pulses. The remaining units were classified as cold units and were not analyzed further. Also, several cells recorded at the Vi/Vc transition (n = 2) and Vc/C1 junction region (n = 5) were classified as cold units and were not included in further analyses.

Histology

At the end of the experiment, the animal was given a bolus of methohexital sodium (60 mg/kg, iv) and was perfused through the heart with saline followed by 10% formalin containing potassium ferrocyanide. Blocks of medulla were frozen, sectioned at 40 μm, and stained with cresyl violet. Recording sites at the Vi/Vc transition and Vc/C1 junction regions were reconstructed and drawn on a standardized series of brain outlines adapted from the atlas of Paxinos and Watson (1997).

RESULTS

General properties of corneal units

Seventy-one units at the Vi/Vc transition and 26 units at the Vc/C1 junction region responsive to mechanical stimulation of the corneal surface were isolated and tested subsequently for chemical sensitivity by CO₂ pulses. As shown in Table 1, 60 of 71 Vi/Vc units (85%) and 23 of 26 Vc/C1 units (88%) responded to CO₂ pulses. The remaining units were classified as mechanical only and not analyzed further. Also, several cells recorded at the Vi/Vc transition (n = 2) and Vc/C1 junction region (n = 5) were classified as cold units and were not included in further analyses.

FIG. 2. Muscimol injected locally near the site of recording inhibited the spontaneous activity and evoked responses of corneal units at the Vi/Vc transition and Vc/C1 junction regions. A: peristimulus time histogram showing an example of muscimol (0.1 mM) injected into the rostral Vi/Vc transition on a complex unit. Asterisks indicate the initial inhibitory phase to CO₂ pulses characterizing this cell as a type II unit. The bar graph to the right of the histogram summarizes the total Rmag response for each stimulus. Stimulus periods: 1 = 0% CO₂; 2 = 80% pre-muscimol; 3 = 80% post-muscimol; 4 = 80% post-musotin; 5 = 80% post-bicuculline methiodide (BMI). Each stimulus was separated by a 15-min interval. B: example of muscimol (0.1 mM) injected into the caudal Vc/C1 junction on a WDR unit. Stimulus periods as in A. Note that local injection of BMI (0.1 mM) caused a partial reversal of the muscimol-induced inhibition. C: summary of the effects of muscimol (at 20 min postinjection) and BMI on corneal units. *P < 0.05, **P < 0.01 vs. premuscimol value.
inhibited by local muscimol. The example shown in Fig. 2A was a complex unit with a type II pattern to CO₂ pulses.

Local effects of muscimol injection near the recording site

To determine if local GABA_A receptor activation modified the response to corneal stimulation, muscimol (0.1 mM, 300 nl) was injected by micropipette near the recording site. In nearly all cases (7 of 8), spontaneous activity of units at the Vi/Vc transition or Vc/C1 junction was inhibited (>50% decrease vs. background) by muscimol and displayed a partial reversal after the GABA_A antagonist, BMI (0.1 mM, 300 nl, see examples in Figs 2, A and B). The CO₂-evoked response of each Vi/Vc corneal unit tested (n = 4; 1 type I and 3 type II units) was inhibited by local muscimol. The example shown in Fig. 2A was a complex unit with a type II pattern to CO₂.

### Table 1. Classification of cornea units recorded at the Vi/Vc transition and Vc/C1 junction regions

<table>
<thead>
<tr>
<th>RF Class</th>
<th>CO₂ Response</th>
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<tbody>
<tr>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>Vi/Vc units</td>
<td></td>
</tr>
<tr>
<td>Cornea only</td>
<td>13</td>
</tr>
<tr>
<td>WDR</td>
<td>12</td>
</tr>
<tr>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>LTM</td>
<td>2</td>
</tr>
<tr>
<td>Complex</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
</tr>
<tr>
<td>Vc/C1 units</td>
<td></td>
</tr>
<tr>
<td>WDR</td>
<td>9</td>
</tr>
<tr>
<td>NS</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
</tr>
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Receptive field classes: complex, inhibitory, and excitatory cutaneous RFs; cornea only, no cutaneous RF; LTM, low threshold mechanoreceptive; NS, nociceptive specific; WDR, wide dynamic range. CO₂ response classes: Type I, excitatory response only; Type II, inhibitory-excitatory biphasic response to CO₂; M only, mechanoreceptive only.

A convergent inhibitory cutaneous RF (complex units) were found exclusively at the Vi/Vc transition region (Table 1). Although corneal units at the Vi/Vc transition with a type I-like pattern to CO₂ were represented among all cell classes, most units with a type II-like pattern to CO₂ pulses were classified as complex cells. The inhibitory RF of complex units was contiguous with the corneal surface and often included the ipsilateral nostril (see example in Fig. 4B). The relationship between CO₂ response pattern (type I vs. II) and cutaneous RF property (CO, excitatory cutaneous RF, complex inhibitory RF) among Vi/Vc transition units was highly significant ($\chi^2 = 17.98$, $df = 2$, $P < 0.001$). By contrast, all CO₂-responsive units at the caudal Vc/C1 junction region had an excitatory convergent cutaneous nociceptive RF (WDR and NS) and displayed a type I-like response to CO₂ pulses.

Electrical stimulation of the corneal surface evoked a short latency (<30 ms) response in 10 of 28 type I Vi/Vc units consistent with A-fiber only input, while 18 type I units displayed latencies consistent with A+C fiber input. Eleven of 16 type II Vi/Vc units tested displayed A-fiber-only input and five units (all with a complex inhibitory RF) had A+C fiber input. Seven of 13 Vc/C1 units tested had A fiber-only input, and six units displayed activity consistent with A+C fiber inputs. These results indicated that small diameter myelinated and unmyelinated corneal afferent fibers do not project selectively to the Vi/Vc transition or Vc/C1 junction regions.

FIG. 3. Experimental design for recording corneal units at the Vi/Vc transition region while injecting drugs into the caudal Vc/C1 junction region. A: location of Vi/Vc corneal units classified according to cutaneous RF properties. Note that units are not segregated within the Vi/Vc transition by cell class. B: sagittal view of brain stem with general set-up for unit recording (LabVIEW) and pressure microinjection (PSI). C: extent of dye spread determined in 5 cases by injection of Evan’s Blue dye (10%, 300 nl) at the end of the experiment. Note that dye spread included the entire lateral portion of the dorsal horn, the site of termination for a majority of corneal afferent fibers.
Muscimol inhibition of CO₂-evoked activity of Vi/Vc units was independent of cutaneous RF class since this sample was comprised of one CO, one NS, and two complex units. Similarly, local injection of muscimol into the caudal Vc/C1 junction inhibited the CO₂-evoked response of each Vc/C1 unit clearly, local injection of muscimol into the caudal Vc/C1 junction did not alter the spontaneous or CO₂-evoked activity of Vi/Vc units when followed over 60 min after injection (n = 4; 2 type I and 2 type II units, data not shown).

**INJECTION OF CoCl₂.** Microinjection of the nonselective synaptic blocking agent, CoCl₂, into the Vc/C1 junction region caused a dose-related reduction in CO₂-evoked activity and an increase in spontaneous activity of Vi/Vc units (Fig. 4C). Spontaneous activity increased during the injection of high dose CoCl₂ and continued to increase over 60 min as shown by the example in Fig. 4A. This unit was classified as a complex cell (note the inhibitory RF shown in Fig. 4A) with a type II-like response pattern to CO₂. By 5 min after injection of high dose CoCl₂ (100 mM), the CO₂-evoked total $R_{mag}$ was reduced by at least a 50% in each cell tested. The CO₂-evoked response duration was reduced by 5 min after injection of low-dose (35 ± 4 vs. 20 ± 5 s, pre- vs. post-CoCl₂, $P < 0.01$) or high-dose CoCl₂ (25 ± 4 s vs. 5 ± 3 s, $P < 0.01$, ANOVA) with a gradual recovery by 60 min (data not shown). Since spontaneous activity was increased while evoked responses were reduced after CoCl₂ blockade of the Vc/C1 junction region, this suggested that spontaneous and evoked activity of corneal units at the Vi/Vc transition were mediated, at least in part, by different neuron pools (see Fig. 8).

**INJECTION OF MUSCIMOL.** Microinjection of the selective GABA_A receptor agonist, muscimol, into Vc/C1 junction region had no consistent immediate effect on spontaneous activity, in contrast to the increase seen after CoCl₂. Neither low (0.1 mM) or high doses (≥0.2 mM) of muscimol altered spontaneous activity during first 20 min after injection; however, spontaneous activity was reduced significantly (46.7 ± 18.6%, $P < 0.05$, Fig. 5A) by 60 min after high-dose muscimol. In contrast to the rapid effect of CoCl₂ on CO₂-evoked responses, muscimol-induced reduction in total $R_{mag}$ developed slowly. Although the total $R_{mag}$ was reduced significantly by 5 min postinjection, a maximum effect was seen only by 20 min (Fig. 5B). A partial reversal of the inhibition after high

**Tests for ascending intersubnuclear communication: cobalt chloride or muscimol injection into the Vc/C1 junction while recording at the Vi/Vc transition region**

The general set-up for the experimental design is shown in Figs. 1B and 3B. Corneal units were recorded at the Vi/Vc transition region mainly along the ventral borders of the nucleus, independent of convergent cutaneous RF properties, and were not segregated by cutaneous RF class (Fig. 3A). Drug injections were made at the Vc/C1 junction region, 3–4 mm caudal to the recording site (Fig. 3C). The spread of drug (300 nl total volume) included the superficial and deep laminae of caudal Vc, but did not infringe on the recording site (see example in Hirata et al. 2000; Fig. 2). In control experiments, artificial cerebrospinal fluid injection (300 nl) into the Vc/C1 junction did not alter the spontaneous or CO₂-evoked activity of Vi/Vc units when followed over 60 min after injection (n = 4; 2 type I and 2 type II units, data not shown).

**FIG. 4.** Effects of CoCl₂ injection into the Vc/C1 junction on corneal units recorded at the Vi/Vc transition region. A: peri-stimulus time histogram displaying the effect of CoCl₂ on a Vi/Vc unit with a type II-like response pattern to CO₂. Asterisks indicate the initial inhibitory phase to each CO₂ pulse characterizing this cell as a type II unit. B: shaded region indicates the inhibitory cutaneous receptive field (RF) of the unit shown in A classified as a complex cell. C: summary of the effect of CoCl₂ injection into the caudal Vc/C1 junction on the total $R_{mag}$ response to CO₂ pulses (left) and spontaneous activity (right); *$P < 0.05$; **$P < 0.01$ vs. predrug value; ‡$P < 0.01$ low- vs. high-dose CoCl₂.
dose muscimol was seen by 5 min after injection of the GABA_A antagonist, BMI, through the second barrel of a dual pipette. This slow onset pattern following high dose muscimol was a consistent finding since 8 of 10 cells tested (6 type I and 2 type II) displayed greater inhibition of total R
mag at 20 than 5 min. Although the predominant effect of muscimol injection into the caudal Vc/C1 junction on rostral Vi/Vc units was inhibitory, three additional units (1 WDR and 2 complex units) displayed an enhanced CO_2-evoked total R
mag after high-dose muscimol (>50% increase vs. background, Fig. 6A). To determine if muscimol and mu opioid agonists acted at similar sites in the Vc/C1 junction to modulate Vi/Vc units, 4 additional Vi/Vc units (all type I units) were tested after muscimol (0.1 mM) and then DAMGO (0.01 mM). As shown by the example in Fig. 6B, DAMGO caused a prompt increase in CO_2-evoked total R
mag and response duration in two of four units, while two units were not affected.

**Tests for descending intersubnuclear communication:** muscimol injection into the Vi/Vc transition while recording at the Vc/C1 junction region

Muscimol was injected into the rostral Vi/Vc transition region to determine if rostral blockade modifies the activity of corneal units at the caudal Vc/C1 junction region (Figs. 1C and 7A). As shown by the example of Fig. 7C, injection of 0.5 mM muscimol into the Vi/Vc transition enhanced the CO_2-evoked total R
mag and response duration. By 20 min after injection, the average total R
mag was increased by 47% and response duration by 33% compared with the preinjection value (Fig. 7D). This was a consistent finding since CO_2-evoked responses were enhanced in four of four Vc/C1 units tested after high-dose muscimol. Injection of low-dose muscimol (0.1 mM) had no significant effect; however, in one case (WDR unit), the CO_2-evoked total R
mag was increased by 48% above the pre-muscimol value at 60 min. Muscimol had no consistent effect on spontaneous activity (Fig. 7C) as only two of five units displayed an increase that exceeded 50% of the pre-muscimol value. Injection of low-dose CoCl_2 (10 mM) into the Vi/Vc transition did not modify the responses of any Vc/C1 units tested (n = 5); however, higher doses were not tested. 

**Effects of CoCl_2 and muscimol on MAP**

Injection of CoCl_2 (10 mM) into the Vc/C1 junction region caused a prompt increase in resting MAP within 1 min (+8 ± 1 mmHg, P < 0.01) and blocked completely the CO_2-evoked pressor response (+10.5 ± 2.5 and 0.3 ± 1.7 mmHg, pre- vs.
FIG. 6. Examples of enhanced CO₂-evoked responses of corneal units at the Vi/Vc transition. A: example of enhanced response of a Vi/Vc unit after muscimol (0.5 mM) injection into the caudal Vc/C1 junction region. Stimulus periods: 1 = 80% CO₂ premuscimol; 2 = 80% premuscimol; 3 = 80%, 5 min postmuscimol; 4 = 80%, 20 min postmuscimol; 5 = 80%, 35 min postmuscimol; 6 = 80%, 50 min postmuscimol. Note that the effect of muscimol, although apparent by 20 min, continues to increase for about 35 min. B: example of enhanced response of a Vi/Vc unit after injection of the selective mu opioid agonist, DAMGO (0.01 mM), into the Vc/C1 junction region. Note that this unit was not affected by prior injection of low dose muscimol (0.1 mM) into the caudal Vc/C1 junction region. Naloxone (1 mg/kg, intravenous) reversed the effect of DAMGO. Stimulus periods: 1 = 80% CO₂ premuscimol; 2 = 80% premuscimol; 3 = 80%, 5 min postmuscimol; 4 = 80%, 20 min postmuscimol; 5 = 80%, 5 min post-DAMGO; 6 = 80%, 20 min post-DAMGO; 7 = 80% postnaloxone. Other CO₂ test pulses in A and B were separated by 15-min intervals.

FIG. 7. Experimental design for recording corneal units at the caudal Vc/C1 junction region while injecting drugs into the Vi/Vc transition region. A: sagittal view of brain stem with general set-up for recording and microinjection (see also Fig. 1C). B: recording location of the Vc/C1 unit shown in C. C: peristimulus time histogram of a corneal unit classified as wide dynamic range (WDR) displaying an enhanced response to muscimol injection into the Vi/Vc transition region. D: summary of the effect of muscimol injection into the Vi/Vc transition region on the total Rmag (top) and response duration (bottom) to CO₂ pulses. *P < 0.05, **P < 0.01 vs. predrug value; aP < 0.05, bP < 0.01 low- vs. high-dose muscimol.
These results indicated a prominent role for GABA_\text{A} receptor antagonists in modulating nociceptive input to corneal units in lower trigeminal brain stem regions. Local GABA_\text{A} receptor activation at the site of recording caused a rapid inhibition of spontaneous and evoked activity of corneal units at both the Vi/Vc transition and Vc/C1 junction regions. GABA_\text{A} receptor activation also contributed to intersubnuclear communication since corneal stimulation-evoked activity recorded in the Vi/Vc transition or Vc/C1 junction region was modified significantly by microinjection of muscimol into the other region. However, in contrast to the rapid effects observed after local injection of muscimol at the site of recording, GABA_\text{A} receptor involvement in intersubnuclear processing of corneal input developed more slowly and reached a maximum only by 20 min after injection. Since injection of CoCl_2 or DAMGO into the caudal Vc/C1 junction region caused rapid changes in corneal unit activity at Vi/Vc transition region, these results suggested that intersubnuclear communication relevant to corneal nociceptive processing involves multiple mechanisms.

**Effects of local GABA_\text{A} receptor activation**

Local microinjection of muscimol near the site of recording caused a rapid and sustained decrease in spontaneous and CO_2-evoked corneal unit activity at both the Vi/Vc transition and Vc/C1 junction regions. Rapid inhibition of corneal unit activity after local muscimol was consistent with previous results in which units driven by cutaneous stimuli recorded in deep laminae of Vc (Takeda et al. 2000) or superficial or deep laminae in spinal dorsal horn (Willcockson et al. 1984) were inhibited by iontophoretic application GABA_\text{A} receptor agonists or enhanced by local application of GABA_\text{A} receptor antagonists (Chiang et al. 1999; Nam et al. 2002; Takeda et al. 2000). The pronounced decrease in spontaneous activity after local muscimol suggested that corneal units at the Vi/Vc transition and in superficial laminae at the Vc/C1 junction regions normally are not under high tonic levels of GABA_\text{A} receptor-mediated control, a conclusion supported by behavioral studies (Kaneko and Hammond 1997). Takeda et al. (2000) also reported that local iontophoretic application of muscimol reduced the spontaneous activity and RF size of all cells tested in deep laminae of Vc. Although RF areas were not determined routinely in this study, in two caudal Vc/C1 units the convergent cutaneous RF area was noticeably reduced by 5 min after local muscimol and in one case the corneal RF area was reduced in a Vi/Vc unit classified as a cornea only cell. The dose of muscimol (0.1 mM) injected locally was comparable to that applied topically to the spinal cord surface to block electrical-evoked input to deep dorsal horn neurons from myelinated and unmyelinated afferents (Sokal and Chapman 2003) or the muscle pressor reflex (Wilson 2001). Corneal stimulation-evoked pressor responses also were reduced by muscimol injection into the Vc/C1 junction region in the present experiments.

**GABA_\text{A} receptor activation and intersubnuclear communication**

Possible GABA_\text{A} receptor contributions to ascending intersubnuclear communication were tested by muscimol injection into the Vc/C1 junction while recording corneal unit activity at the Vi/Vc transition region. This approach revealed a dose-dependent modulation of corneal unit activity in which high dose muscimol caused either inhibition or enhancement of the responses of Vi/Vc units (defined as >50% change from pre-injection R_\text{mag} value). Several lines of evidence suggest that these results cannot be explained on the basis of diffusion from the site of injection. First, some corneal units were enhanced while others were inhibited after remote injections of muscimol into the Vc/C1 junction, whereas local injection and recording at the Vi/Vc transition revealed only inhibition of corneal units. Second, the distance between the sites of injection and recording (approximately 3 mm) exceeded the distance at which a small volume (300 nl) injection would be expected to diffuse in significant concentration to influence unit activity (Arikant et al. 2002). Third, injection of CoCl_2 or DAMGO into the Vc/C1 junction caused prompt (by 5 min) dose-related increases in spontaneous (CoCl_2) or evoked activity (DAMGO) of some corneal units at the Vi/Vc transition region. The differences in time-course and directional change after injection of muscimol, CoCl_2 and DAMGO suggested that ascending intersubnuclear communication pathways involve GABAergic as well as non-GABAergic mechanisms. Similarly, GABA_\text{A} receptor involvement in descending intersubnuclear communication, as tested by muscimol injection into the Vi/Vc transition while recording corneal units at the Vc/C1 junction region also developed slowly over 20 min. Since muscimol injection into the Vi/Vc transition enhanced the activity of four of five units at the Vc/C1 junction, while all Vc/C1 units were inhibited after local muscimol injection at the Vc/C1 junction (Fig. 2B), these findings further supported the conclusion that these effects were not due to drug diffusion.

The delayed onset of changes in corneal unit activity after muscimol injection into a remote region compared with the rapid inhibitory effect of local application at the site of recording suggested that GABA_\text{A} receptor contributions to intersubnuclear communication did not involve direct connections between the Vi/Vc transition and Vc/C1 junction regions. Several previous studies have noted delayed effects following muscimol injections. For example, in the primate muscimol injection into forebrain regions remote from the recording site evoked changes in neural activity after a variable delay of up to several minutes (Hikosaka and Wurtz 1985). Muscimol injections into motor or somatosensory cortex caused pronounced effects on grip force in monkeys; however, no changes occurred before 15–30 min (Brochier et al. 1999). Intrathecal administration of muscimol caused tactile allodynia that developed progressively over 15–60 min in a rat neuropathic pain model (Hwang and Yaksh 1997). Corneal units in superficial laminae at the Vc/C1 junction can be driven antidromically from sites in the ventrolateral Vi/Vc transition region (Hirata et al. 2000); however, it is not known if GABAergic fibers connect directly the Vi/Vc and Vc/C1 regions that process corneal input. Although GABAergic neurons in Vc project some distance to regions such the trigeminal motor nucleus (Li et al. 1996), they apparently do not project to either the principal trigeminal nucleus or the pontine parabrachial region (Haring et al. 1990). GABAergic involvement in corneal nociceptive processing by intersubnuclear communication also could have occurred through activation of supraspinal brain regions. GABAergic neurons in the pontine parabrachial region (Haring et al. 1990).
the rostral ventromedial medulla (RVM) project to the superficial laminae of the spinal dorsal horn (Antal et al. 1996) and direct stimulation of the RVM inhibits corneal units at the Vi/Vc transition and Vc/C1 junction regions (Meng et al. 2000). However, even if muscimol action at the caudal Vc/C1 junction region affected Vi/Vc unit activity by acting through supraspinal pathways, it seems unlikely that a delay of several minutes could be explained simply by a polysynaptic circuit. An alternative explanation for the delayed effects of muscimol was that these actions were mediated through second messenger systems and subsequent alteration in genomic activity. For example, a 3 h exposure to muscimol was sufficient to reduce brain-derived nerve growth factor (BDNF) and nerve growth factor (NGF) in cultured hippocampal neurons (Zafra et al. 1991). BDNF and NGF are well-established endogenous modulators of nociceptive circuits in the spinal dorsal horn (McMahon 1996; Thompson et al. 1999). The Vi/Vc transition and the superficial laminae at the Vc/C1 junction regions that receive corneal input display significant levels of BDNF protein (Conner et al. 1997).

Previously, we reported that injection of mu opioid agonists into the Vc/C1 junction region modulated corneal unit activity at the Vi/Vc transition region (Hirata et al. 2000; Meng et al. 1998). Since disinhibition of GABAergic outflow is established in opioid-mediated analgesia (Vaughn et al. 1997), one prediction from the present experiments was that GABA _A_ receptor activation would mimic the effects of opioid agonists. Although muscimol and DAMGO injection into the Vc/C1 junction region modified corneal unit activity at the Vi/Vc transition region, the time-course for these effects were quite different (see Fig. 6), since the effects of DAMGO occurred immediately after injection, while those of muscimol developed more slowly. Thus although opioid and GABAergic mechanisms in corneal nociceptive processing may share common elements, they are not coexistent. Thus one possible circuit for ascending intersubnuclear communication between the caudal Vc/C1 junction and the rostral Vi/Vc transition regions includes at least two populations of cells (Fig. 8): 1) GABA_A-positive neurons at the Vc/C1 junction that have little tonic influence on resting activity of Vi/Vc units and act only indirectly to alter the evoked activity of Vi/Vc cells, and 2) GABA_A-negative neurons that are tonically active (T, in Fig. 8), greatly influence resting activity as revealed after blockade by CoCl_2 and may act directly on corneal primary afferents that project to Vi/Vc units to inhibit evoked responses. Furthermore, these findings underscore concerns regarding the interpretation of results following focal pharmacological blockade of complex brain circuits (see Malpeli 1999). Although CoCl_2 and muscimol block synaptic activity without disrupting fibers of passage, the present results indicate clearly that these two agents act, at least in part, on different elements.

It is not possible to exclude a contribution from GABA_B receptors in corneal nociceptive processing, since this was not tested systematically in this study. In preliminary experiments, a single low dose (0.1 mM) of the selective GABA_B agonist, baclofen, was injected into the Vc/C1 junction region and had no effect on CO_2-evoked activity of six Vi/Vc units tested. Others have examined the relative contribution of GABA_A and GABA_B receptor subtypes in acute sensory processing and reported that GABA_A-related drugs generally were more effective. For example, spontaneous activity and cutaneous RF areas of units in deep laminae of Vc were increased after bicuculline, but not after the GABA_B antagonist, saclofen (Chiang et al. 1999). The C-fiber evoked nociceptive flexion withdrawal reflex was enhanced by GABA_A but not by GABA_B antagonists in the spinal rat (Sivilotti and Woolf 1994), while spinal administration of GABA_A, but not GABA_B antagonists prevented the release of excitatory amino acids into dorsal horn after inflammation (Sluka et al. 1994) and blocked the inhibition of nociceptor-evoked dorsal horn activity that normally occurs during periaqueductal gray stimulation (Lin et al. 1996). These experiments were performed under barbiturate anesthesia, a class of drugs known to act in part through GABA_A receptor activation (see Fragen and Avram 2000). Although it was possible that the choice of anesthetic may have influenced GABA_A receptor-mediated events, the use of continuous infusion with constant monitoring of blood pressure and expiratory CO_2 suggested that depth of anesthesia was similar for all animals.
Intersubnuclear circuitry and trigeminal nociception

Intersubnuclear communication in trigeminal sensory processing has been examined for a variety of forms of input including facial skin (Dallel et al. 1998; Greenwood and Sessle 1976; Woda et al. 2001), tooth pulp (Chiang et al. 2002; Hu et al. 2002), and dural membranes (Davis and Dostrovsky 1988). The majority of these studies have emphasized ascending modulation and the relationship between Vc and the more rostral subnucleus oralis (Vo). However, anatomical studies indicate extensive descending as well as ascending projections between different nuclear regions of the trigeminal brain stem complex (Ikeda et al. 1984; Jacquin et al. 1990; Kruger et al. 1977; Nasution and Shigenaga 1987). The present study differs from previous reports in that both ascending and descending interations were assessed, and interactions between the rostral and caudal portions of Vc were examined and did not include the Vo. In previous studies, we have argued that the Vi/Vc transition region that processes corneal input may serve specialized functions in nociception such as the recruitment of descending controls (Hirata et al. 1999, 2000; Meng et al. 1998). This conclusion was based largely on the findings that a significant percentage of Vi/Vc corneal units were enhanced following systemic morphine or direct microinjection of mu opioid agonists into the caudal Vc/C1 junction region. Although muscimol injection into the caudal Vc/C1 junction also enhanced the evoked activity of some rostral Vi/Vc units, it is not likely that the GABAergic properties of some corneal units at the Vi/Vc transition compared with segmental controls in spinal dorsal horn, the unique properties of some corneal units at the rostral portion of Vc not seen at the caudal portion, plus the unequal effects of GABAergic involvement in ascending and descending controls suggests specialization within the trigeminal system not seen at the spinal level.

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