Neocortical Spreading Depression Provokes the Expression of C-fos Protein-like Immunoreactivity within Trigeminal Nucleus Caudalis via Trigeminovascular Mechanisms

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The effects of neocortical spreading depression (SD) on the expression of immunoreactive c-fos protein were examined within the superficial laminae of trigeminal nucleus caudalis (TNC), a brainstem region processing nociceptive information. KCl was microinjected into the left parietal cortex at 9 min intervals over 1 hr, and SD was detected by a shift in interstitial DC potential within adjacent frontal cortex. The stained cells in lower brainstem and upper cervical spinal cord were counted on both sides after tissues were sectioned (50 μm) and processed for c-fos protein-like immunoreactivity (LI) using a rabbit polyclonal antisemur. C-fos protein-LI was visualized in the ventrolateral TNC, chiefly in laminae I and IIo and predominantly within spinal segment C, (e.g., −1.5 to −4.5 mm from obex) ipsilaterally. SD significantly increased cell staining within ipsilateral TNC. The ratio of cells in laminae I and IIo on the left:right sides was 1.32 ± 0.13 after 1 M KCl, as compared to 1.06 ± 0.05 in control animals receiving 1 M NaCl instead of KCl microinjections (p < 0.01). The ratio was reduced to an insignificant difference after chronic surgical transection of meningeal afferents and recurrent SD (1.09 ± 0.11). Pretreatment with intravenous sumatriptan, a 5-HT1-like receptor agonist that selectively blocks meningeal C-fibers and attenuates c-fos protein-LI within TNC after noxious meningeal stimulation, also reduced the ratio to an insignificant difference (1.10 ± 0.09). Sumatriptan or chronic surgical transection of meningeal afferents, however, did not reduce the ability of KCl microinjections to induce SD. On the other hand, combined hyperoxia and hypercapnia not only reduced the number of evoked SDs from 6.3 ± 1.0 to 2.5 ± 1.2 after 0.15 M KCl microinjection, but also significantly (p < 0.01) reduced associated c-fos protein-LI in TNC.

These data indicate that multiple neocortical SDs activate cells within TNC. The increase in c-fos protein-LI, observed predominantly ipsilaterally, was probably mediated by SD-induced stimulation of ipsilaterally projecting unmyelinated C-fibers innervating the meninges. If true, this is the first report demonstrating that neurophysiological events within cerebral cortex can activate brainstem regions involved in the processing of nociceptive information via trigeminovascular mechanisms.

[Key words: c-fos immunoreactivity, spreading depression (SD), trigeminovascular mechanisms, trigeminal nucleus caudalis (TNC), sumatriptan, migraine, meninges]

Spreading depression (SD) is a propagating phenomenon associated with a transient loss of membrane potential and electrophysiological activity in brain cells including neocortex (for review, see Bures et al., 1974). SD develops within cerebral cortex of animals and possibly humans (Avoli et al., 1991) in response to noxious stimuli. Single episodes are associated with dramatic changes in interstitial [K+], [H+], [Na+], [Ca2+], [Cl−] (Nicholson and Kraig, 1981), and [HCO3−] (Kraig and Cooper, 1987), as well as in levels of glutamate, lactate (Krivanek, 1961; Mutsch and Hansen, 1984), and arachidonic acid and metabolites such as the prostaglandins (Lauritzen et al., 1990). In otherwise normal brain, ionic concentrations return to baseline by 3–4 min whereas elevated lactate and arachidonate levels persist for up to 10 min. The consequences of repeated SDs appear to be more long lasting but have been less well studied. In one report, repeated SDs caused reactive astrocytosis as evidenced by dramatic glial fibrillary acidic protein staining and hypertrophy that persisted for several weeks (Kraig et al., 1991). However, recurrent SDs were not sufficient to cause irreversible neuronal injury, at least as assessed by hematoxylin and eosin staining (Nedergaard and Hansen, 1987). Kraig et al., 1991).

The trigeminovascular system innervates blood vessels within pia mater and arachnoid surrounding neocortex (Mayberg et al., 1981, 1984; Liu-Chen et al., 1983a; Suzuki et al., 1989). Axons, predominantly unmyelinated (Liu-Chen et al., 1986), are neuromuscle-containing (Liu-Chen et al., 1983b, 1984; Moskowitz et al., 1983; Norregaard and Moskowitz, 1983; McCulloch et al., 1986; Saito et al., 1987a,b) and originate from the ipsilateral ophthalmic trigeminal division (Mayberg et al., 1984). Trigeminovascular fibers also detect and transmit nociceptive information. Hence, noxious chemicals placed into the subarachnoid space (e.g., blood, carrageenan) activate cells within the superficial laminae I and...
and closed with stainless steel surgical clips. Animals were returned to
the home cage. The skull incision was coated with 5% lidocaine cream
and closed with cloth tapes.

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tivity (LI) provided a measure of neuronal activation, and this
was examined within laminae I and II of TNC, a region con-
trigeminovascular system. Accordingly, SDS were evoked in
the trigeminal nuclei caudalis (TNC), but much less so after
meningeal deafferentation (Nozaki et al., 1992a,b). An important
gain of the experiments reported herein is to determine
whether trigeminovascular activation develops after more subtle
forms of stimulation (e.g., in response to neurophysiological
events such as SD).

We tested the possibility that SD could activate the ipsilateral
trigeminovascular system. Accordingly, SDs were evoked in the
cerebral cortex of the laboratory rat by KCl microinjections.
The number of cells expressing c-fos protein–like immunoreac-
tivity (LI) provided a measure of neuronal activation, and this
was examined within laminae I and II of TNC, a region con-
taining both the synapses of primary afferent fibers concerned
with transmission of nociceptive information and second and
higher order neurons that transmit nociceptive information to
rostral centers. Recurrent SDs caused a significant increase in
c-fos protein–LI within ipsilateral TNC that was reduced by (1)
decreasing nociceptive neural transmission through denervation
of meningeal afferents or sumatriptan pretreatment, or by (2)
decreasing the number of evoked SDs through exposure to com-
bined hyperxia and hypercapnia (hypercarbia).

**Materials and Methods**

**Animal preparation and recording.** Wistar, male rats (250-450 gm; Har-
lan Sprague–Dawley, Indianapolis, IN) were placed in individual cages
in a 12 hr night/12 hr day, thermoregulated (22°C) room and were
allowed free access to water but were fasted 12-16 hr before surgery. Animals (n = 7) were anesthetized with halothane (3% for induction,
3% during surgical procedures, and 1.5-2.5% during physiological re-
cordings) via a 20% oxygen-balance nitrogen mixture. Animals were
warmed (37 ± 0.5°C) during surgical procedures by a heating lamp
directed at their dorsal surface and controlled via a rectal temperature
probe. A tail artery was cannulated for sampling blood and monitoring
arterial blood pressure. The incision site for the arterial catheter
was coated with 5% lidocaine cream and closed with cloth tapes.

A left parasagittal skin incision (approximately 1 cm long) was made
2-3 mm from the midline. The wound edges were spread and the skull
bone scraped. Two small (1.2 mm diameter) craniotomies were then
drilled. The first was made 2 mm lateral and 3 mm anterior to bregma.
The second was made 6 mm posterior and 5 mm lateral to bregma. Both
 craniotomies were carefully drilled so that the underlying dura
was not torn. The skull incision was coated with 5% lidocaine cream
and closed with stainless steel surgical clips. Animals were returned to
individual cages and maintained at 37 ± 0.5°C via a heat lamp regulated
by a rectal temperature probe until they were awake. For the next 6-7
hr, animals were allowed free access to water in individual cages before
being reanesthetized as described above.

When anesthetized, surgical clips were quickly removed and 5% li-
docaine cream reapplied to the wound edges. Animals were then placed
on a thermoregulated bed within a stereotaxic frame and maintained
at 37 ± 0.5°C. The animal’s nose was held in place by a standard
stereotaxic rodent nose bar clamp that was gently secured around the
animal’s nose and upper incisors. To minimize nociceptive stimulation,
sterile ear bars were not used. The skull area was warmed by Ringer's
superfusion fluid (35-37°C) that contained (in mM) Na+: 143.5, K+:
3.0; Ca²+: 1.5; Mg²+: 1.5; Cl−: 115; HCO₃−: 26.4; glucone: 9.6,
and glucose, 5.0, which, when aerated with 95% oxygen and 5% carbon
dioxide, gave a pH of 7.3-7.4 (modified from Bretag, 1969). Arterial
pH, carbon dioxide tension (PaCO₂), and oxygen tension (PaO₂) were
periodically monitored with a Corning 168 blood gas analyzer (Corning
Diagnostics Corp., Medfield, MA). Blood glucose was measured
with a Glucometer (Miles Laboratories, Naperville, IL). Experiments
were performed at a level of anesthesia in which there was no withdrawal
or blood pressure response to ear or paw pinch. This level of anesthesia,
achieved using halothane, was associated with mild respiratory acidosis
(55-75 torr), mild reduction in systolic arterial blood pressure, but nor-
mal blood glucose (Table 1).

SD was induced by microinjection of KCl via a fused silica needle
(#2-1361, Supelco, Inc., Bellefonte, PA) that was placed 1000 μm below
the pial surface of the posterior craniotomy (Kraig et al., 1987). Injections
(0.5 μl/min for 1 min) of KCl (1 M or 0.15 M KCl) were made
every 9 min for a period of 1 hr (e.g., a total of six injections). Sham
controls were injected with 1 M NaCl at an analogous rate, duration,
and frequency. A glass micropipette (filled with 150 mM NaCl; tip di-
diameter, 2-4 μm) was positioned 1000 μm below the pial surface through
the anterior craniotomy to monitor DC neocortical potentials and thus
confirm that SD had occurred. Microelectrodes were connected to an
Axograph A-1 amplifier system (Axon Instruments, Burlingame, CA),
and DC signals were displayed on a chart recorder. An indifferent elec-
trode filled with 3% agar and 150 mM NaCl was positioned on the left
temporalis muscle.

Seven experimental animal groups were studied. Group I (n = 6)
received 1 M NaCl injections to the left parietal cortex (1 M NaCl).
Group II (n = 7) consisted of only 1 M KCl injections to the left parietal
cortex (1 M KCl). Group III (n = 8) received an intravenous dose of sumatriptan [3-(2-diethylamino)ethyl-N-methyl-1H-indole-5-methane
sulfonamide; Glaxo, Ware, England; 300 μg/kg] 30 min before the ini-
tiation of KCl injections as described for group II above (1 M KCl/
sumatriptan). Group IV (n = 5) was subjected to unilateral meningeal
dorsal rhizotomy (left side) by transection of trigeminal afferents at
the ethmoid foramen 2 weeks prior to 1 M KCl microinjection (left
side) as described previously (Kano et al., 1991) (1 M KCl/sensory

| Group | pH | PaCO₂ (torr) | PaO₂ (torr) | BP (mm Hg) | Glucose (% mmol) | Hct (%) | Temp (°C) |
|-------|----|-------------|-------------|------------|-----------------|--------|----------|
| NaCl 1 M | 7.23 | 62 | 106 | 101 | 6.4 | 42 | 37.0 |
| (n = 6) | ±0.02 | ±6 | ±10 | ±8 | ±0.9 | ±2 | ±0.4 |
| KCl 1 M | 7.26 | 58 | 109 | 101 | 6.8 | 43 | 37.2 |
| (n = 7) | ±0.02 | ±2 | ±8 | ±9 | ±0.7 | ±4 | ±0.3 |
| KCl 1 M/Suma | 7.23 | 63 | 93 | 109 | 6.1 | 42 | 37.5 |
| (n = 5) | ±0.02 | ±5 | ±3 | ±4 | ±0.2 | ±1 | ±0.6 |
| KCl 1 M/Sx | 7.26 | 59 | 107 | 103 | 5.1 | 45 | 37.3 |
| (n = 5) | ±0.03 | ±3 | ±15 | ±7 | ±0.2 | ±2 | ±0.5 |
| KCl 1 M/CO₂ | 7.20 | 64 | 104 | 107 | 6.7 | 45 | 37.3 |
| (n = 5) | ±0.03 | ±5 | ±17 | ±2 | ±0.6 | ±2 | ±0.4 |
| KCl 0.15 M | 7.73 | 56 | 102 | 113 | 6.1 | 48 | 37.3 |
| (n = 4) | ±0.02 | ±4 | ±14 | ±9 | ±2.0 | ±1 | ±0.2 |
| KCl 0.15 M/CO₂ | 7.23 | 62 | 100 | 105 | 8.0 | 44 | 37.3 |
| (n = 6) | ±0.04 | ±4 | ±12 | ±4 | ±2.0 | ±2 | ±0.4 |

Data are means ± SD. Suma, sumatriptan; Sx, chronic sensory denervation; CO₂, hyperxia–hypercapnia. BP, blood
pressure; Hct, hematocrit; Temp, rectal temperature.
These lesions are associated with significant reductions (40-80%) in the density of calcitonin gene-related peptide-containing fibers on the ipsilateral circle of Willis (Suzuki et al., 1989). To suppress SD after KCl microinjection, animals (n = 5) in group V were first exposed to hyperoxia (e.g., 100% oxygen) for 10-15 min before raising inhaled carbon dioxide tension so that PaCO₂ exceeded 100 torr (1 atm KC1 at hyperoxia-hypercapnia). The animals in group V also received KCl microinjections as done in group II.

SD was not suppressed by combined hyperoxia and hypercapnia in group V animals as well as it was when 1 M KCl was administered by superfusion (Kraig et al., 1991). This reduced ability of raised O₂ and CO₂ to suppress SD may have occurred because 1 M KCl by microinjection is a stronger stimulus for SD than is 1 atm KCl by superfusion. 

Infusion of hyperoxia and hypercapnia may be more effective in suppressing SD when KCl is microinjected at a lower molarity. To test this possibility and to further clarify the relative importance of SD versus potassium on trigeminal nucleus c-fos staining, two additional groups were studied. Animals in each group received microinjections of 0.15 M KCl. However, the rate of injection needed to be raised to 1.0 μl/min to induce SD reliably. Thus, SD in animals of group VI (n = 4) and group VII (n = 6) was induced by injection of 0.15 M KCl at 1.0 μl/min for 1 min every 9 min for a period of 1 hr. Group VI animals were similar to group II in that they received KCl injections (0.15 M KCl). Group VII animals were similar to group V animals in that, in addition to these injections, animals were treated with combined hyperoxia and hypercapnia (0.15 M KCl/hyperoxia-hypercapnia).

**Immunohistochemical procedures.** After 1 hr recording periods, animals were processed for immunohistochemical staining of c-fos protein within brain. Inhaled halothane was raised to 5%. When systolic blood pressure fell below 60 mm Hg, animals were killed by intracardiac perfusion fixation. Perfusion was injected at 110 mm Hg and first consisted of 150 ml NaCl (200 ml) that was followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Brains with attached upper cervical cords were removed and stored in the same fixative for 2 hr before being placed in 0.15 M phosphate buffer (pH 7.2) and shipped to Boston from Chicago via overnight mail. The specimens were placed in 20% sucrose and 30% ethylene glycol in 0.1 M phosphate buffer (pH 7.2) for 48 hr at 4°C prior to sectioning. They were sectioned coronally (50 μm) on a freezing microtome.

Free-floating sections were processed immunohistochemically by the avidin–biotin procedure using commercially available kits (Vectastain ABC, Vector Labs, Burlingame, CA) as described previously (Nozaki et al., 1992a). Briefly, sections were incubated with 10% normal goat serum and 0.03% hydrogen peroxide in 0.1 M phosphate-buffered saline (PBS; pH 7.3) for 30 min at room temperature. Subsequently, sections were incubated with primary antisera (1:5000 dilution) in PBS with 0.3% Triton X-100 (Sigma Labs, St. Louis, MO) overnight at room temperature. Gentile agitation was used for all incubations unless otherwise noted. The antisera was kindly provided by Dr. Dennis Sla- moff, Department of Neurology and Neurosurgery, University of California at Los Angeles. The polyclonal antisera was prepared in rabbits and directed against an in vitro translated product of the c-fos gene. Antisera was preabsorbed against acetone-dried rat liver powder overnight to reduce nonspecific background staining. The staining pattern produced by this antisera is comparable to a commercially available monoclonal antibody, but we are not against a synthetic peptide that consists of residues 4-17 of the c-fos protein (Presley et al., 1990).

After washes in PBS (15 min × 3), sections were placed in biotinylated anti-rabbit IgG antisera (1:200 dilution in PBS; Vector Labs, Burlingame, CA) at room temperature for 7 hr. After PBS washes (15 min × 3), sections were placed in avidin–biotin–peroxidase complex (Vector Labs) for 2 hr at room temperature. After PBS washes (15 min × 3), sections were placed in a solution of 3,3’-diaminobenzidine tetrahydrochloride (40 mg/ml; Sigma) and 0.003% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.6) for 30 min. After the diaminobenzidine reaction, sections were mounted on gelatin-coated slides, air dried, and coverslipped.

Forebrains were sectioned coronally between 1.0 mm rostral and 5.0 mm caudal to bregma (seven sections). The brainstem and upper spinal cord were sectioned serially (50 μm) from midbrain to the second cervical segment, and every third section (total 100 sections) was processed for immunohistochemistry and examined in each animal. Anatomical boundaries were determined by coordinates established by the Paxinos and Watson rat brain atlas (Paxinos and Watson, 1986). Tissue sections were evaluated using a Zeiss microscope and 25 x objective by an investigator unaware of the treatment group or results of the c-fos protein staining within cerebral cortex. Labeled cells were counted in brain regions in which c-fos protein staining was either dense, asymmetric, or different between groups. Hence, positive cells were counted selectively within TNC, parabrachial nucleus, dorsal raphe nucleus, and nucleus of the solitary tract.

Immunostained cells within TNC (laminar I and II) were counted on both sides. The area postrema and second cervical segment were chosen as its rostral and caudal extent, respectively. TNC was divided into spinal and brainstem segments at the cervicomedullary junction (inferior border of pyramidal d cussation, 1.5 mm caudal to obex). Approximately 45 sections (13 sections for rostral TNC and 32 sections for caudal TNC) were examined in each animal. Trigeminal nucleus interstitialis and oralis exhibited few positive cells, and cell counts were not obtained. The number of positive cells within nucleus of the solitary tract was counted 0.3 mm caudal to 0.9 mm rostral from obex, wherein the majority of positive cells were observed; approximately nine sections were evaluated in each animal. Cells within parabrachial and dorsal raphe nuclei were counted each in a single section. 9.1 mm and 7.8 mm caudal to bregma, respectively. A qualitative scale (0, no positive cell; +, sparse positive cells; ++, moderate positive cells; ++++, numerous positive cells) was used to evaluate cell staining within neocortex, hippocampus, thalamus, hypothalamus, periaqueductal gray, inferior olive and all other brainstem nuclei.

By counting and comparing positive cells on the two sides within each nucleus, variabilities usually attributed to staining, cell counting, "stress" and related hormones, drugs and anesthetics, and physiological parameters such as PaCO₂ and PaO₂, are minimized.

**Statistical procedures.** The data are expressed as the number of stained cells ± standard deviation per section on the two sides. For statistical analysis, the cell counts were transformed by the square root to stabilize the variance. The difference between the two sides was then computed for rostral and caudal segments of TNC. A two-way analysis of variance was used to test for differences between the experimental groups, correcting for experiment (the study was done in a sequence of four experiments). The Ryan–Einot–Gabriel–Walsch multiple F test was used to correct for multiple comparisons. p value less than 0.05 was considered significant.

**Results**

**Spreading depression**

Several pilot experiments (35 animals) were conducted before the experimental protocols described above were finalized. Initially, SD was induced as previously described by Kraig et al. (1991). Briefly, 1 M KCl was applied to the exposed parietal cortex of one cerebral hemisphere while 1 M NaCl was applied to the contralateral side for a period of 30 min, 1 hr, or 2 hr. Furthermore, animals were killed by perfusion–fixation as described above immediately after the recording periods. Others were allowed to awaken for 24 hr before being reanesthetized and killed by perfusion–fixation. In still other experiments, the anesthetic was changed from halothane to pentobarbital (given as a 50 mg/kg intraperitoneal injection). None of these experimental paradigms were sufficiently sensitive to detect a change in left/right labeling caused by SD. We speculate that the effects of SD within TNC were obscured by the trauma associated with drilling of craniotomies, opening of the dura, and application of KCl to the skull and dura. Accordingly, the dura was left intact in our final preparation. In addition, KCl was not applied to the dura, but instead was microinjected 1000 μm below the pial surface via a silica needle that pierced the intact dura. Furthermore, animals were allowed to recover for 6–7 hr after surgery before being reanesthetized for the induction of recurrent SD.

Together, these technical innovations allowed us to show that recurrent SD significantly increases c-fos protein staining within the ipsilateral TNC. Based on our present knowledge of the employed antisera, we cannot say with certainty whether the observed immunoreactivity represents c-fos protein itself or a related antigen(s).
Table 2. Number of spreading depressions and c-fos expression evoked in the cortex by microinjection of NaCl or KCl

|                        | Number of SD  | c-fos       |
|------------------------|---------------|-------------|
| NaCl 1 m (n = 6)       | 0–1 (0.2 ± 0.4) | 0–+        |
| KCl 1 m (n = 7)        | 6–8 (6.6 ± 1.0) | + + +       |
| KCl 1 m/sumatriptan (n = 5) | 6–7 (6.2 ± 0.4) | + + +       |
| KCl 1 m/sensory denervation (n = 5) | 6–9 (7.2 ± 1.3) | + + +       |
| KCl 1 m/hypercapnia-hyperoxia (n = 5) | 4–5 (4.4 ± 0.5) | + + + +     |
| KCl 0.15 m (n = 4)     | 5–7 (6.3 ± 1.0) | + + +       |
| KCl 0.15 m/hypercapnia-hyperoxia (n = 6) | 1–4 (2.5 ± 1.2) | 0–+        |

Qualitative scale: 0, no positive cells; +, sparse positive cells; + +, moderate positive cells; + + +, numerous positive cells. Data in parentheses are means ± SD.

Physiologic variables were similar in all experimental groups (Table 1). The mean number of SDS for each experimental group are shown in Table 2. Continuous records representative of the treatment groups that evoked SDS are shown in Figure 1. SD always occurred after microinjection of 1 m KCl [group II, 6.6 ± 1.0 SD (range, 6–8)] or 0.15 m KCl [group VI, 6.3 ± 1.0 SD (range, 5–7)]. Treatment with intravenous sumatriptan or chronic sensory denervation did not affect the number of SDS [group III, 6.2 ± 0.4 SD (range, 6–7); group IV, 7.0 ± 1.4 SD (range, 6–9)]. Sumatriptan pretreatment did, however, slightly alter the DC waveform of SD recorded in frontal cortex (Fig. 1). The waveform was sharper with less plateau. The significance of this alteration is unknown. Similarly, in the example shown, spontaneous electrical activity stopped at the onset of the fourth SD (evidenced by the narrowing of the baseline DC signal). Such a diminution in baseline activity was not regularly seen in the sumatriptan group. Combined hyperoxia and hypercapnia reduced the number of evoked SDS after KCl microinjection [group V, 4.4 ± 0.5 SD (range, 4–5); group VII, 2.5 ± 1.2 SD (range, 1–4)]. Microinjection of 1 m NaCl (group I) never evoked SD, although insertion of the injection cannula did evoke a single SD in one animal [0.2 ± 0.4 SD (range, 0–1)].

C-fos protein response in forebrain

C-fos protein–L1 was expressed intensely within cerebral cortex on the side ipsilateral to SD (Table 2, Fig. 2b). Sumatriptan or chronic sensory denervation did not reduce the degree of neocortical staining induced by SD (Table 2, Fig. 2c,d). Positive cells were particularly evident in laminae II, III, and VI. Staining was reduced after combined hyperoxia and hypercapnia and was only faintly observed after NaCl microinjection (Fig. 2a,e).

Positive cells were also noted within ipsilateral hippocampus in at least half the animals after KCl microinjections, all animals after KCl/sumatriptan treatment, and four out of five animals after KCl/sensory denervation. After NaCl microinjections, one of six animals did show prominent ipsilateral hippocampal staining; this animal exhibited a single SD during the recording period. Only faint c-fos protein staining was present in the KCl/hyperoxia–hypercapnia group.

Many labeled cells were observed bilaterally in the thalamus such as habenular, paraventricular thalamic, mediodorsal, intermediodorsal, and centromedian nuclei, in the hypothalamus such as supraoptic, paraventricular, and dorsomedial nuclei, in the medial, lateral, and triangular septal nuclei, and in the medial and lateral preoptic area. There were no apparent differences between groups, however. A few c-fos protein–labeled cells were observed in the medial and mediodorsal parts of caudate-pu-
Figure 2. C-fos LI in the cingulate cortex taken from the frontal region after animals received microinjections into left posterior parietal cortex of 1 μM NaCl (a), 1 μM KCl (b), 1 μM KCl/sumatriptan (c), 1 μM KCl/sensory denervation (d), or 1 μM KCl/hyperoxia-hypercapnia (e). C-fos LI is visualized in cells within ipsilateral cerebral cortex after KCl (b) but much less so after NaCl (a) microinjections. Hyperoxia–hypercapnia prior to KCl microinjection (e) but not sumatriptan (c) or sensory meningeal denervation (d) reduces c-fos expression in the frontal cortex. L, Left side; R, right side. Scale bar, 300 μm.
tamen bilaterally after 1 M KCl injection, and to a lesser degree after 1 M NaCl injection, but c-fos protein expression was not reduced after KCl injection that accompanied combined hypoxia and hypercapnia, or sumatriptan treatment or after chronic meningeal deafferentation. Rare c-fos protein-labeled cells were detected in globus pallidus.

C-fos protein response in brainstem

C-fos protein-labeled cells were observed bilaterally within multiple brainstem nuclei (Table 3). The numbers were greatest within TNC, parabrachial nucleus, nucleus of the solitary tract, dorsal raphe nucleus, periaqueductal gray, and inferior olive. The numbers of positive cells within nucleus of the solitary tract and dorsal raphe nucleus did not differ between groups (data not shown). The numbers of positive cells within parabrachial nucleus, periaqueductal gray, and inferior olive were greater in the KCl groups. However, only within parabrachial nucleus were fewer positive cells counted after 1 M KCl/sumatriptan, 1 M KCl/sensory denervation, or 1 M KCl/hyperoxia–hypercapnia treatment [146 ± 25 (1 M KCl), 100 ± 13 (1 M NaCl, p < 0.05), 69 ± 15 (1 M KCl/sumatriptan, p < 0.01), 74 ± 19 (1 M KCl/sensory denervation, p < 0.01), 73 ± 19 (1 M KCl/hyperoxia–hypercapnia, p < 0.01)].

C-fos protein response in TNC

Labeled cells were found throughout TNC bilaterally in all groups (Figs. 3, 4). Positively stained cells were located chiefly in laminae I and II, and were particularly evident within the ventrolateral segment corresponding to the region receiving trigeminal afferent inputs from the ophthalmic division (Fig. 4). Sparse labeling was present in laminae III–V. Most positive cells were located between −1.5 and −4.5 mm from obex. Consequently, labeled cells were counted above and below −1.5 mm to define a rostral and caudal TNC part, respectively. Approximately 80% of total positive cells were found in the caudal segment [74 ± 15 (caudal) as compared to 45 ± 14 (rostral) cells per section after 1 M KCl]. Differences between the experimental groups were more significant in the caudal segment as well. For the above reasons, and because the spinal portion of the TNC corresponds to the termination sites for the opthalmic division (Darian-Smith, 1966), the results obtained for rostral and caudal TNC will be described separately.

Caudal TNC. The greatest number of c-fos protein–positive cells was found after 1 M KCl microinjections alone (right plus left; 74 ± 15, p < 0.01). Labeled cells ipsilateral to the KCl

Table 3. Expression of c-fos protein in brainstem nuclei

| Brainstem Nuclei               | 1 M NaCl | 1 M KCl | 1 M KCl/Suma | 1 M KCl/Sx | 1 M KCl/CO2 |
|--------------------------------|----------|---------|--------------|-----------|-------------|
| Periaqueductal gray            |          | ++      | ++           | ++        | ++          |
| Dorsal raphe nucleus           |          | ++      | ++           | ++        | ++          |
| Parabrachial nucleus           |          |         |              |           |             |
| Medial                         | 0-+      | 0-+     | 0-+          | 0-+       | 0-+         |
| Lateral                        | ++       | ++      | +            | +         | +           |
| Substantia nigra               |          | +       | +            | +         | +           |
| Compacta                       |          | +       | +            | +         | +           |
| Reticularis                    | 0        | 0       | 0            | 0         | 0           |
| Locus coeruleus                | 0-+      | 0-+     | 0-+          | 0-+       | 0-+         |
| Lateral reticular nucleus      | ++       | ++      | ++           | ++        | ++          |
| Inferior olive                 | ++       | ++      | ++           | ++        | ++          |
| | Nucleus of the solitary tract |          |         |              |           |             |
| Rostral                        | 0-+      | 0-+     | 0-+          | 0-+       | 0-+         |
| Caudal                         | ++       | ++      | ++           | ++        | ++          |
| Area postrema                  | +        | +       | +            | +         | +           |
| Trigeminal nucleus             |          |         |              |           |             |
| Oralis                         | 0        | 0       | 0            | 0         | 0           |
| Interpolaris                   | +        | +       | +            | +         | +           |
| Caudalis                       | ++       | ++      | ++           | ++        | ++          |

The same qualitative scale is used as in Table 2. Suma, sumatriptan; Sx, chronic sensory denervation; CO2, hyperoxia–hypercapnia.
microinjection accounted for this increase. The numbers for the other treatment groups are as follows: 61 ± 11 (1 M NaCl), 35 ± 11 (1 M KCl/sumatriptan), 47 ± 13 (1 M KCl/sensory denervation), and 21 ± 6 (1 M KCl/hyperoxia-hypercapnia). Fewer cells were counted after 0.15 M KCl [74 ± 15 (1 M KCl) vs. 25 ± 6 (0.15 M KCl)]. There were no significant differences between groups treated with 0.15 M KCl and 0.15 M KCl/hyperoxia-hypercapnia (25 ± 6 vs. 21 ± 9, respectively).

When the data are expressed as the ratio of positive cells on the two sides (left:right), variability between individual experiments (e.g., due to differences in chromogen development, etc.) is taken into consideration. When expressed in this way, the differences between groups were highly significant (p < 0.01).

The ratios (as well as number of cells per section on the left vs. right sides, respectively) were 1.37 ± 0.19 (43 ± 10 vs. 31 ± 6, 1 M KCl) and 1.35 ± 0.08 (15 ± 4 vs. 11 ± 3, 0.15 M KCl), respectively. The groups treated with sumatriptan or sensory denervation did not show significant differences between the two sides [1.13 ± 0.11, p < 0.05 (18 ± 6 vs. 16 ± 5), 1 M KCl/sumatriptan; 1.11 ± 0.12, p < 0.05 (24 ± 6 vs. 23 ± 7), 1 M KCl/sensory denervation], nor did the animals microinjected with NaCl [1.06 ± 0.06, p < 0.01 (32 ± 6 vs. 30 ± 5)]. Similarly, combined hyperoxia and hypercapnia administered prior to 1 M KCl or 0.15 M KCl significantly reduced the ratio and differences in cell numbers between the two sides [1.09 ± 0.10, p < 0.01 (10 ± 2 vs. 10 ± 3), 1 M KCl; 1.04 ± 0.05, p < 0.01 (11 ± 5 vs. 10 ± 4), 0.15 M KCl].

**Rostral TNC.** Group differences based on total cell numbers were not found in this segment of TNC.

Statistically significant differences were noted between the two sides, however, after 1 M KCl microinjection versus 1 M NaCl microinjection (1.17 ± 0.06, p < 0.05). The number of positive cells per section on the left and right sides was 25 ± 8 and 22 ± 7, respectively. No significant asymmetries occurred in those animals treated with 0.15 M KCl, 1 M NaCl, 1 M KCl/sumatriptan, 1 M KCl/sensory denervation, or KCl/hyperoxia–hypercapnia, however.

**Discussion**

**C-fos response**

The nuclear phosphoprotein c-fos may play a role in long-term alterations of cellular function (Sheng and Greenberg, 1990; Morgan and Curran, 1991). Trauma, ischemia, seizures, and more subtle forms of neuronal stimulation induce c-fos antigen and the related family of antigens (FRAs) in neural tissues (Curran and Morgan, 1985; Dragunow and Robertson, 1987, 1988; Sagar et al., 1988; Onodera et al., 1989; Sheng and Greenberg, 1990). C-fos protein–LI can be used to identify areas of neuronal activity and to map functionally related neuronal pathways (Sagar et al., 1988; Menetrey et al., 1989; Bullitt, 1990).

The induction of c-fos protein–LI occurs in the trigeminal nucleus caudalis and dorsal horn of the spinal cord following peripheral noxious and non-noxious stimulation (Hunt et al., 1987, Presley et al., 1990, Antou et al., 1991, Gugas et al., 1991). Continuous or prolonged stimuli evoke the most robust response, and the number of labeled cells correlates with stimulus intensity. Presley et al. (1990) reported the induction of c-fos protein–LI within subpopulations of spinal cord neurons after formalin injection into the hind paw of a rat; the number of positively labeled cells correlated with a behavioral response indicative of pain. Similarly, Nozaki et al. (1992a,b) noted the appearance of immunoreactive cells within TNC (laminae I and II) in response to autologous blood injected into the subarachnoid space of a rat. The number of positive cells corresponded to the amount of administered blood (Nozaki et al., 1992a). Morphine significantly attenuated c-fos expression in both models (Presley et al., 1990; Nozaki et al., 1992b).

Positive cells were also present within ipsilateral hippocampus. Bures et al. (1974) reported that SD does not propagate between noncontiguous gray matter regions. Hence, the response in hippocampus probably developed as a consequence of transynaptic activation via connections between neocortex and entorhinal/piriform cortex or perhaps via unilateral activation of the septohippocampal pathway.

Within TNC, immunoreactivity was prominent within ipsilateral laminae I and II in ventrolateral segments of the spinal region corresponding to inputs from the ophthalmic division (Shigenaga et al., 1986; Anton et al., 1991). More than likely, trigeminal meningeal afferents stimulated this response inasmuch as meningeal deafferentation blocked the response, and contralateral TNC (dorsomedial as well as ventrolateral segments) would have featured prominently following stimulation by descending somatosensory connections from neocortex. The data suggest that c-fos expression may be mediated by lamina-specific inputs from trigeminovascular fibers.

SD-induced brainstem labeling was not restricted to the TNC. Staining, observed within parabrachial nucleus, is consistent with assigned visceral afferent functions for this nucleus (Cechetto et al., 1985; Hyden et al., 1989). Nozaki et al. (1992a) reported the presence of c-fos protein–LI within parabrachial nucleus following the instillation of blood into the subarachnoid space. The response was diminished by meningeal deafferentation and by pretreatment with a 5 HT1 agonist sumatriptan (Nozaki et al., 1992b). Similarly, the c-fos response to recurrent SDs was less after sumatriptan treatment and meningeal deafferentation, and after KCl microinjections combined with hyperoxia and hypercapnia.

Recurrent SDs most likely stimulated c-fos protein expression within TNC as suggested by the staining pattern after KCl/hypercapnia and hyperoxia. Inadvertent spread of KCl from the injection site to the meninges, a second possibility, seems much less likely. We cannot completely exclude possible effects of elevated PaCO2 and PaO2 on c-fos protein expression per se, although we know of no precedent for this in the literature. However, hypercapnia–hyperoxia did not suppress c-fos protein–LI in all locations, as immunostaining within brainstem nuclei other than TNC and the parabrachium was unaffected (Table 3). It follows that spreading depression is probably not the stimulus for c-fos protein LI activity within these other brainstem nuclei.

As reported previously, the extent and magnitude of the c-fos response within brain and spinal cord may be influenced by the depth and duration of anesthesia and choice of anesthetics, as well as by the presence of stimuli extraneous to the particular experiment (Bullitt, 1990). These factors, plus possible differences in scalp incisions, skull trauma, and drilling, plus the delicate nature of the experimental preparation, may have accounted for the relatively large number of labeled cells within TNC on both sides in all treatment groups and for the differences in cell numbers between groups. Primary afferent inputs into TNC bilaterally may have contributed as well (Jacquín et al., 1982). C-fos protein–LI was also observed to an equivalent extent in all treatment groups within brainstem nuclei such as dorsal raphe nucleus, periaqueductal gray, nucleus of the solitary tract,
area postrema, inferior olive, and in some hypothalamic and thalamic nuclei, although additional experiments are needed to provide a more detailed examination in each of these brain regions. C-fos protein-L1 was not observed within cells in any of the above noted brainstem or forebrain regions when normal, unoperated, unstressed, and untouched subjects were subjected to brain immunohistochemical examination using the employed antisera (M. A. Moskowitz, K. Nozaki, and R. P. Kraig, unpublished observations).

Sumatriptan

The importance of the trigeminovascular system to SD-induced labeling is further underscored by the actions of sumatriptan (Humphrey et al., 1988; Moskowitz, 1992). Sumatriptan, a polar indolamine that does not penetrate readily into brain parenchyma, inhibits trigeminovascular activation via prejunctional mechanisms when tested in a model system of neurogenic inflammation within the meninges (Buzzi and Moskowitz, 1990; Buzzi et al., 1991, 1992). Pharmacological studies indicate that sumatriptan inhibits neuropeptide release from perivascular fibers (Buzzi et al., 1991; Moskowitz, 1992). Intracranial, but not extracranial, trigeminal afferents appear to possess this receptor. A second drug action relates to sumatriptan’s ability to inhibit neural transmission specifically within the trigeminovascular system. Sumatriptan blocks c-fos protein-L1 within TNC following noxious irritation of meningeal afferents induced by blood. The drug response is specific, inasmuch as the 5-HT,-like agonists sumatriptan or dihydroergotamine did not inhibit c-fos protein-L1 within TNC induced by formalin application to the nasal mucosa (Nozaki et al., 1992b). An action on primary to the nasal mucosa (Nozaki et al., 1992b). An action on primary afferent fibers was proposed. Of course, the studies reported herein do not exclude the unlikely possibility that this hydrophilic 5-HT analog blocks c-fos protein-L1 within TNC directly.

Possible mechanisms of trigeminovascular activation

What then constitutes an effective stimulus for trigeminovascular activation after recurrent SDs? To our knowledge, there are no detailed reports describing the electrophysiological properties of primary nociceptors within the meninges. In other tissues, the vast majority of nociceptors are polymodal and respond to noxious, thermal, mechanical, and chemical stimuli (Bessou and Perl, 1969; Kumazawa and Mizumura, 1980; Lang et al., 1990). Local release or new synthesis of chemical constituents such as bradykinin, potassium, hydrogen ion, and certain neurotransmitters such as 5-HT (Lang et al., 1990; Steen et al., 1992) activates or sensitizes primary afferent fibers. Neugebauer et al. (1989) provided evidence for nociceptor sensitization to passive joint motion following intratracheal bradykinin or prostaglandin injections. Some of the same agents are released into neocortex during SD. For example, hydrogen ions and potassium concentrations increase within the interstitial space 5- to 20-fold during a single SD; levels of prostaglandins increase as well. Oxygen free radicals, mediators of tissue injury, are generated during the formation of prostaglandins (Kontos, 1989). Cumulative or tonic stimulation by recurrent SDs was required inasmuch as signals generated by single SDs did not raise the number of stained cells (this assumes that elevated PaCO₂ + O₂ had no other effects on c-fos protein-L1 in TNC). Alternatively, detection of positive cells after single SDs may have been obscured by the relatively large population of labeled cells present under baseline conditions.

Glutamate-mediated mechanisms and the NMDA receptor may initiate and facilitate SD (Van Harreveld, 1984; Hansen et al., 1988; Marrannes et al., 1988). Under certain conditions, glutamatergic neurotransmission promotes neural injury (Choi, 1988). Unmyelinated C-fibers are stimulated by the chemical constituents released during and after tissue injury (see above). NMDA antagonists such as MK-801 block cortical SD and the attendant c-fos response in neocortex (Marrannes et al., 1988; Herrera and Robertson, 1990). We speculate that nitric oxide, formed within brain cells as a consequence of NMDA receptor activation, may reach the vasculature after local synthesis (Dawson et al., 1991; Bredt and Snyder, 1992). Nitric oxide itself or a free radical derivative (Hogg et al., 1992) stimulates trigeminovascular activation as well. We have shown that NO stimulates perivascular trigeminovascular fibers as a unique vasodilating mechanism within the pial circulation (H. A. Kontos, E. P. Wei,
and M. A. Moskowitz, unpublished observations). As demonstrated for nitroglycerin and sodium nitroprusside (Wei et al., 1992), the effects of nitric oxide were significantly attenuated by the addition of calcitonin gene-related peptide (8-37), a competitive receptor antagonist.

SD may be one of several neurophysiological events capable of activating nociceptive mechanisms in cerebral cortex. Generalized seizures activate trigeminovascular fibers, and by so doing, increase blood flow in neocortex (Sakas et al., 1989).

Injurious stimuli to brain (such as ischemia; Moskowitz et al., 1989) and to blood vessels (e.g., acute, severe hypertension; Sakas et al., 1989) also can activate this system. Pain and headache develop in humans during seizures, ischemia, and severe hypertension. In fact, headache of migraine with aura develops following a focal neurological event (most often referable to neocortex) that some have likened to the spreading depression of Leao (Milner, 1958; Olesen et al., 1981).

The findings are consistent with the following formulation: neurophysiologically driven ionic and metabolic mechanisms (e.g., SD) promote the release of nociceptive substances from neocortex into the interstitial space. Within the perivascular space, released substances activate and sensitize trigeminovascular fibers that surround pial vessels supplying and draining neocortex within Virchow-Robin spaces. As a result, impulses are conveyed to TNC and then to more rostral centers. Pain may ensue. Less likely, products released from cortex may stimulate meningeal vessels, which in turn release nociceptive molecules. We speculate that our findings may be relevant to headache pathophysiology and to the pathogenesis of photophobia and phonophobia accompanying stimulation of meningeal afferents.

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