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Increase of capsaicin-induced trigeminal Fos-like immunoreactivity by 5-HT₇ receptors

José Terrón¹, Esther Martínez-García¹, Marcello Leopoldo², and Enza Lacivita²

¹Cinvestav-IPN – Pharmacology, Mexico D.F., Mexico

²Università degli Studi di Bari - Dipartimento Farmaco-Chimico, Bari, Italy

Abstract

Objective—To explore whether pharmacological stimulation of the 5-HT₇ receptor modulates Fos-like immunoreactivity in the trigeminal nucleus caudalis of rats.

Background—The serotonin 5-HT₇ receptor was proposed to be involved in migraine pathogenesis and evidence suggests it plays a role in peripheral nociception and hyperalgesia through an action on sensory afferent neurons.

Methods—The potential activating or sensitizing role of 5-HT₇ receptors on trigeminal sensory neurons, as visualized by Fos-like immunoreactivity in the superficial layers of the trigeminal nucleus caudalis in rats, was investigated using the 5-HT₇ receptor agonist, LP-211, in the absence and the presence of intracisternal capsaicin, respectively. The agonist effect was characterized with the 5-HT₇ receptor antagonist, SB-656104A. Male Wistar rats received a subcutaneous injection of LP-211, SB-656104A, and SB-656104A + LP-211. They were then anesthetized and prepared to receive an intracisternal injection of capsaicin or its vehicle. Animals were perfused and brains removed; sections of the brain stem from the area postrema to the CI level were obtained and processed for Fos immunohistochemistry.

Results—Capsaicin but not its vehicle induced Fos-like immunoreactivity within laminae I and II of trigeminal nucleus caudalis. Pretreatment with LP-211 had no effect on Fos-like immunoreactivity but strongly increased the response produced by capsaicin; this effect was abolished by SB-656104A. Interestingly, capsaicin-induced Fos-like immunoreactivity was abolished by SB-656104A pretreatment thus suggesting involvement of endogenous 5-HT.

Conclusions—Data suggest that 5-HT₇ receptors increase activation of meningeal trigeminovascular afferents and/or transmission of nociceptive information in the brain stem. This mechanism could be relevant in migraine and its prophylactic treatment.

Keywords

5-HT₇ receptors; Fos-like immunoreactivity; trigeminal nucleus caudalis; capsaicin; cephalic pain; rat

Introduction

It is generally accepted that the trigeminovascular system is the anatomical substrate of neurovascular headaches, including migraine and cluster headaches.¹ This system consists of A and C type nerve fibers arising from the trigeminal ganglion that transmit, through their peripheral and central endings, respectively, nociceptive information from meningeal

blood vessels to the trigeminal nucleus caudalis (Sp5C),²⁻⁴ which contains the pain-receiving and transmitting zones of the brain stem.⁵ Immunoreactivity to the Fos protein, which is the product of the proto-oncogene c-fos, in the outer layers or laminae (LI-II) of Sp5C has been widely used as a marker of cephalic pain in experimental animals in response to chemical⁶⁻¹⁰ and mechanical¹¹ stimulation of intracranial nerves. Thus, Fos-like immunoreactivity (Fos-LI) in Sp5C (LI-II) in response to aversive stimuli may be employed to elucidate trigeminovascular mechanisms of neurovascular headaches.

Serotonin (5-hydroxytryptamine; 5-HT) has long been implicated in migraine and other vascular headaches but the nature of its involvement has not been defined. Regarding a potential ability of 5-HT to functionally modulate the trigeminovascular system, available studies have limited to the analysis of inhibitory mechanisms (5-HT_{1D}/5-HT_{1F} receptors) as potential targets of abortive treatments for migraine (e.g. sumatriptan),^{10,12,13} but no studies have focused on the search for excitatory mechanisms that might play a role in the activation of this system. In this regard, it is interesting that 5-HT has actually been demonstrated to play a role in pain and hyperalgesia in both humans¹⁴ and experimental animals^{15,16} thus implying that the monoamine may indeed play an excitatory role in nociceptive pathways. Among the numerous 5-HT receptor (sub)types classified to date, evidence has become available to suggest that the 5-HT₇ receptor is involved in pain and hyperalgesia¹⁷⁻²⁰ probably through a direct activating action on C type fiber neurons¹⁶ by mechanisms that may involve neuronal excitation.²¹ Whether the 5-HT₇ receptor might also be present in the trigeminovascular system and play a role in cephalic pain and hyperalgesia is not known. The reported expression of the 5-HT₇ mRNA in human trigeminal ganglia²² however seems consistent with this possibility.

On the basis of the above it was therefore hypothesized that pharmacological activation of the 5-HT₇ receptor could possibly lead to activation of trigeminovascular afferents supplying the meninges, as reflected by an increase of Fos-LI in Sp5C (LI-II). Furthermore, it has been shown that the cAMP/protein kinase A (PKA) second messenger cascade is implicated in hyperalgesia induced by inflammatory mediators and that this pathway mediates sensitization of dural nociceptive neurons.²³ Since this signaling pathway represents the major transduction mechanism of 5-HT₇ receptors,²⁴ it was also hypothesized that 5-HT₇ receptor stimulation could promote sensitization of trigeminovascular sensory fibers to the activating action of capsaicin. The aim of the present study in rats was therefore to evaluate the effect of a selective 5-HT₇ receptor agonist on Fos-LI in Sp5C (LI-II) in the absence and the presence of intracisternal (i.c.) capsaicin, and characterize this effect by using a 5-HT₇ receptor antagonist. The results suggest that 5-HT₇ receptors may play a modulatory role in the trigeminovascular system by increasing capsaicin-induced activation of trigeminovascular sensory fibers. A preliminary account of this investigation has been presented in abstract form.²⁵

Methods

Animals

Male Wistar rats (250-300 g; n=49) were purchased from Harlan Mexico (Mexico City). The animals were individually housed for at least 3 days before the experiments and kept at constant temperature and a 12 hour light:dark cycle with food and water provided *ad libitum*. Efforts were made to restrict the number of animals employed and to avoid any unnecessary suffering of them. All procedures and protocols complied with federal regulations on the care and use of laboratory animals and were approved by the Cinvestav-IPN ethics committee (CICUAL).

Intracisternal injection of capsaicin

After anesthesia with sodium pentobarbital (50 mg/kg i.p.), the animals were placed in a stereotaxic frame and prepared to receive an i.c. injection of capsaicin. For this purpose, a midline skin incision was made from the occipital protuberance to the cervical area under semi-sterile conditions and a 27-gauge needle coupled to a Hamilton syringe was inserted into the *cisterna magna*; the correct position of the needle in the *cisterna magna* was confirmed by extracting a small amount of cerebrospinal fluid. During this procedure, animals were mechanically ventilated through the trachea with a Harvard Apparatus rodent ventilator (58 cycles/min; 2 ml/100 g body weight). Capsaicin was administered with the aid of a micro-injection pump at a rate of 20 μ l/min during 5 min for a total volume of 100 μ l; this 100 μ l i.c. injection has been widely employed by other research groups with the vehicle shown to be devoid of remarkable effects on background levels of Fos-LI in Sp5C.^{26,27} To prevent capsaicin leakage, the needle was removed 10 min after the injection was completed. In order to facilitate appropriate distribution of capsaicin, the animals were kept in a reverse *trendelenberg* position for 30 min and then in a lateral position for 90 min. Control animals received an i.c. injection of vehicle. Evans Blue (0.2%) staining of the epidural space was used to demarcate the distribution of capsaicin in the skull; the dye was co-injected with capsaicin or its vehicle. The staining pattern, which was used to verify the effectiveness of the procedure, exhibited the typical pattern reported in other studies with a stronger coloration in the dura mater ventral from the cerebellum, around the brain stem and the upper spinal cord;²⁸ the animals which did not show this pattern were excluded from the study. The body temperature of the animals was maintained at 37 ± 1 °C with a thermostatically regulated homeothermic blanket and anesthesia was maintained by supplemental injections of pentobarbital (10 mg/kg, i.p.). At the end of the 2 h period animals were perfused via the ascending aorta with 0.1M PBS at pH 7.4 for 6 min, and 4% paraformaldehyde in 0.1M PBS for 30 min. The brain stem with attached cervical cord was stored overnight in the same fixative and then placed in a cryoprotectant (30% sucrose in 0.1M PBS) for 48 h. Serial coronal sections (40 μ m thick) were prepared at -20°C with a SM2000R Leica freezing microtome; sections were taken from the area postrema to the C1 level with every third section being saved and processed for immunohistochemistry.

Fos immunohistochemistry

Induction of the Fos protein was characterized immunohistochemically in free floating sections with the avidin-biotin procedure using commercially available kits. Sections were rinsed in 0.1M PBS, pretreated with 2% bovine serum albumin and 1% hydrogen peroxide in 0.1M PBS for 2 h at room temperature. They were then incubated with a primary rabbit polyclonal Fos antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1M PBS with 0.3% triton X-100 for 24 h; after this period sections were rinsed and placed in 0.1M PBS with 0.02% triton X-100 containing biotinylated goat anti-rabbit (1:100) secondary antibody (Vector Laboratories, Burlingame, CA) for 2 h. After several washes in 0.1M PBS with 0.02% triton X-100, the sections were incubated with an avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC kit, 1:200; Vector Laboratories, Burlingame, CA) in 0.1M PBS with 0.3% triton X-100 for 1-2 h at room temperature. After thorough washing with 0.1M PBS, the presence of peroxidase was revealed with 3,3'-diaminobenzidine substrate Kit (Vector Laboratories, Burlingame, CA) containing tris-buffer, 3,3'-diaminobenzidine, hydrogen peroxide and nickel in distilled water. Sections were then washed, mounted on gelatin-coated slides and cover-slipped. All procedures were performed under gentle tridimensional agitation.

Fos-immunoreactive cell counting

All Fos-immunoreactive cells were counted bilaterally in LI-II of Sp5C in 7 sections from each animal containing the trigeminal nucleus from the area postrema to the C1 level. Then,

the mean number of labeled cells per section was calculated for each animal and then an average number for each animal group was obtained.

Experimental protocol

Rats were divided into eight experimental groups which received the following treatments: 1) vehicle for capsaicin (n=6); 2) capsaicin (1 μ M) (n=7); 3) LP-211 (10 mg/kg) + vehicle for capsaicin (n=6); 4) LP-211 (10 mg/kg) + capsaicin (1 μ M) (n=6); 5) SB-656104A (3 mg/kg) + vehicle for capsaicin (n=6); 6) SB-656104A (3 mg/kg) + capsaicin (1 μ M) (n=6); 7) SB-656104A (3 mg/kg) + LP-211 (10 mg/kg) + vehicle for capsaicin (n=6); and 8) SB-656104A (3 mg/kg) + LP-211 (10 mg/kg) + capsaicin (1 μ M) (n=6). Exception made of capsaicin and its vehicle, which were injected into the *cisterna magna*, all the other treatments were given subcutaneously between 20 min (LP-211) and 40 min (SB-656104A) before the animals were anesthetized and prepared to receive the i.c. injection. The s.c. route was preferred over the i.p. route in order to maximally reduce stressful manipulation of the animals.

Drugs

Apart from the anesthetic, the drugs used in the present study (obtained from the sources indicated) were the following: capsaicin (8-methyl-N-vanillyl-6-nonenamide; Tocris); LP-211 (*N*-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide) (Dr. Marcello Leopoldo, Università degli Studi di Bari, Bari, Italy); and SB-656104 hydrochloride (SB-656104A; Gift from Glaxo-SmithKline, Harlow, U.K.). Capsaicin (3.05 mg) was diluted in 1 ml of saline:ethanol:tween 80 (8:1:1) and sonicated for 30 min. This stock solution (10 mM) was diluted to a final 1 μ M concentration (305 ng/ml) of capsaicin. Solutions of LP-211 and SB-656104A were prepared by dissolving the drugs in 20% dimethyl sulfoxide in distilled water; this vehicle did not alter Fos-LI in Sp5C (LI-II) (data not shown). Fresh solutions were prepared for each experiment.

Data presentation and statistical analysis

The number of Fos-immunoreactive cells per treatment group is expressed as the mean per section $\pm$ S.E.M. Comparison between the different group treatments was performed with one way ANOVA followed by a Newman-Keuls test to determine differences using the statistical tools of GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). In all cases, statistical significance was accepted at $P < 0.05$ (two-tailed).

Results

Consistent with previous findings in this model,²⁶⁻²⁹ i.c. injection of 1 μ M capsaicin -but not of its vehicle- (Fig. 1) significantly increased Fos-LI in Sp5C (LI-II) (Fig. 2). Capsaicin-induced Fos-LI was observed in clusters that were located predominantly in the dorsal and ventral aspects of Sp5C and they were present bilaterally. Subcutaneous administration of the selective 5-HT₇ receptor agonist, LP-211 (10 mg/kg), followed by i.c. injection of capsaicin's vehicle did not change Fos-LI in Sp5C (LI-II) as compared to the effect of the vehicle alone; a trend towards an increase however was noted (values for capsaicin's vehicle alone and LP-211 + capsaicin's vehicle were, respectively, 42 ± 18 and 77 ± 24 immunopositive cells per section) (Fig. 2). Interestingly, notwithstanding, pretreatment of the animals with LP-211 strongly and significantly increased Fos-LI induced by i.c. capsaicin (values for capsaicin alone and LP-211 + capsaicin were, respectively, 164 ± 29 and 368 ± 38 immunopositive cells) (Figs. 1 and 2); this response was also much higher than the one induced by LP-211 + capsaicin's vehicle (Fig. 2) thus suggesting that stimulation of the 5-HT₇ receptor may increase capsaicin-induced activation of meningeal trigeminal fibers perhaps through a sensitizing action.

Once the ability of capsaicin to induce Fos-LI in Sp5C (LI-II) was verified, and the effects of LP-211 on Fos-LI in vehicle- and capsaicin-treated rats were tested, the involvement of the 5-HT₇ receptor was characterized. Pretreatment with the selective 5-HT₇ receptor antagonist, SB-656104A (3 mg/kg, s.c.),³⁰ completely abolished the amplifying effect of LP-211 on capsaicin-induced Fos-LI in Sp5C (LI-II) (Figs. 1 and 2). Interestingly, the antagonist followed by capsaicin's vehicle had no effect on Fos-LI but it significantly inhibited the Fos response induced by i.c. capsaicin (values for capsaicin, and SB-656104A + capsaicin were, respectively, 164 ± 29 and 55 ± 11 immunopositive cells) thereby suggesting involvement of endogenous 5-HT in the effect of the irritant (Fig. 2).

Discussion

The major finding of the present study is that the 5-HT₇ receptor agonist, LP-211, given at a dose consistent with specific stimulation of 5-HT₇ receptors *in vivo*,^{31,32} strongly increased Fos-LI induced by i.c. capsaicin in LI-II of Sp5C. This result seems remarkable as it represents an evidence for an excitatory serotonergic mechanism in the trigeminovascular system which might play a role in the pathogenesis of migraine, as hypothesized previously.³³ The 10 mg/kg dose of LP-211 was selected since this was previously demonstrated to rapidly reach the systemic circulation in mice achieving a mean C_{max} of 0.76 ± 0.32 µg/ml at 30 min with quantifiable levels remaining evident for a period of 2 hours,³¹ and produce a significant hypothermic effect in wild type but not in 5-HT₇ receptor knockout mice, with the lower 3 mg/kg i.p. dose being inactive.³² Although the 30 mg/kg i.p. dose of LP-211 was reported to produce a higher hypothermic effect in wild type mice as compared to the 10 mg/kg dose, it also induced hypothermia in 5-HT₇ receptor knockout mice;³² furthermore, the hypothermic response to the 30 mg/kg dose of LP-211 in wild type mice, in addition to being blocked by the 5-HT₇ receptor antagonist, SB-269970, it also was partly inhibited by the 5-HT_{1A} receptor antagonist, WAY-100635, thus suggesting interaction of LP-211 with other receptor mechanisms at this higher dose.³² For these reasons, and due to the restricted number of animals to be employed in the study, it was decided to limit the use of LP-211 to the 10 mg/kg dose which, on the basis of the aforementioned studies, appears to selectively stimulate 5-HT₇ receptors. Regarding the 1-arylpiperazine metabolite of LP-211, which is more potent and selective than LP-211 for 5-HT₇ receptors,³² its plasma concentrations were shown to be below the detection limit of the analytical procedure when administered at the 3 mg/kg and 10 mg/kg doses in rats³² and mice;³⁰ it seems therefore unlikely that LP-211's metabolite may have played a significant role in the effects produced by the 10 mg/kg dose of the parent compound on Fos-LI. In spite of the fact that rats were employed in the present experiments and that no LP-211 disposition studies have been conducted in this species, it was assumed that this dose of the drug would most likely result in significant activation of 5-HT₇ receptors over a period of 2 hours after administration. Mediation by 5-HT₇ receptors in the above effect of LP-211 on capsaicin-induced Fos-LI was confirmed by the ability of the 5-HT₇ receptor antagonist, SB-656104A,³⁰ to completely inhibit this response. The 3 mg/kg s.c. dose of SB-656104A was chosen to ensure consistent blockade of 5-HT₇ receptors during the entire course of the experiment as an ED₅₀ oral dose of 2 mg/kg of the compound was previously determined to antagonize 5-CT-induced hypothermia in guinea pigs with a maximal inhibition being observed 2 hours after administration.³⁰ In agreement with this observation, a 3 mg/kg i.p. dose of SB-656104A given 1 hour before was recently reported to reverse the learning impairment induced by the *N*-methyl-D-aspartate receptor antagonist, MK-801, in the Morris water maze test in rats.³⁴ Since in the present experiments SB-656104A (3 mg/kg s.c.) was administered 40 min before i.c. treatments, the compound was clearly within a time frame consistent with its ability to block central and peripheral 5-HT₇ receptors.³⁴ This antagonist has been shown to exhibit reasonable 5-HT₇ receptor selectivity with over 12-, 31-, 45- and 91-fold higher affinity for the 5-HT₇ receptor (pK_i=8.7) with respect to 5-HT_{1D}

(pKi=7.6), 5-HT_{2A} (pKi=7.2), 5-HT_{2B} (pKi=7.04) and 5-HT_{5A} receptors (pKi=6.74), respectively.³⁰

Induction of the product of the immediate early gene c-fos is accepted to reflect functional activity in neurons,^{5,35} and immunoreactivity to the Fos protein in LI-II of Sp5C has been employed by a number of research groups to study the activity of the sensory part of the trigeminal system.^{8,9,12,13,29,36,37} Thus, it is likely that 5-HT₇ receptor activation might be involved in headache conditions that result from activation of the trigeminovascular system including migraine and cluster headache. This notion is consistent with a growing body of evidence supporting a role of 5-HT₇ receptors in nociception and hyperalgesia.¹⁷⁻²⁰ Indeed, the 5-HT₇ receptor mRNA was reported in sensory nerve structures such as the dorsal root ganglia of humans³⁸ and rats³⁹ whereas the protein was both detected in primary afferent nociceptors (which terminate in the superficial layers I and II of the spinal cord dorsal horn) and demonstrated to be involved in 5-HT-induced nociceptor activation.¹⁹ Relevant to the present findings and the potential involvement of the 5-HT₇ receptor in mechanisms of migraine headaches, evidence was provided suggesting that 5-HT₇ receptors might increase capsaicin-induced neurogenic inflammation in the rat knee joint.⁴⁰

Whether the 5-HT₇ receptor is expressed in the trigeminovascular system is not known at the present time, but its mRNA was found in human trigeminal ganglia.²² Since trigeminal nuclei in the medullary brain stem constitute an anatomical continuum of the spinal cord, it seems reasonable to expect that 5-HT₇ receptors may also be expressed and play a nociceptive role in this area which is highly relevant in the transmission of nociceptive information from meningeal tissues via trigeminal nociceptive fibers. The present data do not allow us to establish whether 5-HT₇ receptors mediating the amplifying effect of LP-211 on capsaicin-induced Fos-LI in Sp5C are expressed on peripheral trigeminovascular afferents and/or second-order neurons within the brain stem (see below). Further studies will be required to precisely locate 5-HT₇ receptors in the trigeminovascular system. It should be emphasized on the other hand that, since the single administration of LP-211 had no effect on Fos-LI with respect to controls, it is unlikely that additional direct/indirect peripheral or central effects of this brain penetrant agonist³² had played a significant role on capsaicin-induced Fos-LI in Sp5C.

It is finally interesting from a pathogenic standpoint, that capsaicin-induced Fos-LI was abolished by SB-656104A, which suggests that capsaicin-induced activation of trigeminovascular afferents was mediated by release of endogenous 5-HT (acting on 5-HT₇ receptors). It has been shown in this connection that a plausible source of 5-HT in the trigeminovascular system is the dural mast cell population, as electrical stimulation of the trigeminal ganglion, leading to activation of meningeal nociceptors, promotes degranulation of mast cells in the dura mater.^{41,42} Such activation of meningeal nociceptors has been hypothesized to take place during migraine with aura in response to local release of protons, potassium ions and glutamate.^{43,44} Activated meningeal nociceptors appear to release neuropeptides such as substance P and calcitonin-gene-related peptide (CGRP) that induce activation and degranulation of resident dural mast cells.^{45,46} The local release of inflammatory molecules from degranulated mast cells during neurogenic inflammation is believed to further stimulate meningeal nociceptors to promote a prolonged migraine headache. The ability of the 5-HT₇ receptor to amplify capsaicin-induced Fos-LI in Sp5C (LI-II) (present results) is actually consistent with this notion thus raising the possibility that the effect of 5-HT₇ receptor stimulation by 5-HT could involve CGRP release from trigeminovascular afferents with both substances acting synergistically to exacerbate activation of these fibers. In further support to this possibility, it was preliminary found that topical application of LP-211 on the dura mater encephali increases blood flow in the middle meningeal artery of anesthetized rats via a 5-HT₇ receptor-mediated mechanism that

involves CGRP release;⁴⁷ this functional evidence would certainly support the location of 5-HT₇ receptors in meningeal trigeminovascular afferents.

In conclusion the present study has provided evidence for an excitatory serotonergic mechanism mediating facilitation of capsaicin-induced Fos-LI in Sp5C (LI-II) via activation of 5-HT₇ receptors probably located in trigeminovascular afferents. In support of this possibility, during the preparation of this manuscript a report was published on the ability of the 5-HT₇ receptor antagonist, SB-269970, to inhibit CGRP release (as measured from the jugular vein) induced by electrical stimulation of the trigeminal ganglion in rats;⁴⁸ the putative 5-HT₇ receptor agonist, AS-19, failed to alter electrically-induced CGRP release upon single administration but it however reversed the inhibitory effect of SB-269970 on CGRP release.⁴⁸ Activation of 5-HT₇ receptors by 5-HT (probably released from activated dural mast cells) and their blockade by selective 5-HT₇ receptor antagonists could therefore be, respectively, relevant mechanisms in the pathogenesis and prophylactic treatment of migraine headaches.³³

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List of abbreviations

5-HT	5-Hydroxytryptamine
Fos-LI	Fos-like immunoreactivity
i.c.	intracisternal
Sp5C	trigeminal nucleus caudalis
LI-II	laminae I and II

Figure 1.

Distribution of Fos-immunoreactive cells in Sp5C (LI-II). Treatments were as follows: vehicle (i.c.) (A,B); capsaicin (1 μ M, i.c.) (C,D); LP-211 (10 mg/kg, s.c.) + capsaicin (1 μ M, i.c.) (E,F); and SB656104A (3 mg/kg, s.c.) + LP-211 (10 mg/kg, s.c.) + capsaicin (1 μ M, i.c.) (G,H). Upper and lower panels show, respectively, 10 $\times$ and 20 $\times$ microphotographs of the same preparation. Inserts in upper panels demarcate the areas shown in the lower panel microphotographs. Bars = 200 μ m (upper panels) and 100 μ m (lower panels).

Figure 2.

The Fos response in the trigeminal nucleus caudalis after intracisternal (i.c.) or subcutaneous (s.c.) administration of vehicle for capsaicin (VEH; i.c.), capsaicin (CAP; 1 μ M, i.c.), LP-211 (LP; 10 mg/kg, s.c.) and SB-656104A (SB; 3 mg/kg, s.c.) combined as indicated. CAP caused a robust induction of Fos-like immunoreactivity within laminae I and II as detected 2 h after injection. Each bar represents the mean number of Fos-immunopositive cells per 40- μ m section and vertical lines denote the standard error of the mean of 6-7 observations. * $P < 0.01$ vs VEH; ** $P < 0.001$ vs CAP or LP + VEH; + $P < 0.01$ vs CAP; # $P < 0.001$ vs LP + CAP