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Non-NMDA glutamate receptors modulate capsaicin induced c-*fos* expression within trigeminal nucleus caudalis

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1 We examined the effects of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzol[f]quinoxaline-7-sulphonamide (NBQX), the kainate receptor antagonists γ-(R-)-glutamylaminomethanesulphonic acid (GAMS) and 6,7,8,9-tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione-3-oxime (NS-102), and the group III metabotropic glutamate receptor (mGluR) agonist 2-amino-4-phosphono-S-butanoic acid (L-AP4) on c-*fos*-like immunoreactivity (c-*fos* LI) in trigeminal caudalis (Sp5C), lateral reticular (LRt), medullary reticular (Md) and solitary tract (Sol) nuclei, after intracisternal injection of capsaicin in urethane anaesthetized Sprague-Dawley rats.

2 Few c-*fos* labelled cells were observed within Sp5C in capsaicin-vehicle treated animals. The number of positive c-*fos* cells increased by 17 fold after intracisternal capsaicin (5 nmol) administration.

3 Pretreatment with CNQX (0.02, 0.1, 0.6, 3 and 15 mg kg^{-1}) or NBQX (0.01, 0.1 and 1 mg kg^{-1}), administered intraperitoneally 15 min before capsaicin, significantly reduced labelled cells within Sp5C by a maximum of 45 and 34%, respectively. The number of c-*fos* LI cells within LRt, Md and Sol was not affected. Pretreatment with L-AP4 (1, 3 and 10 mg kg^{-1}) decreased the number of Sp5C c-*fos* LI cells by a maximum of 30%, whereas GAMS (1 and 10 mg kg^{-1}) and NS-102 (1 and 5 mg kg^{-1}) did not show any significant effect.

4 These results suggest that blockade of AMPA receptors, but not kainate receptors, or the activation of group III mGluRs, decrease the response of Sp5C neurons to trigeminovascular activation. Thus, in addition to NMDA receptors, mGluRs and AMPA receptors may modulate cephalic pain and may provide a potential therapeutic target for antimigraine drugs.

Keywords: AMPA/kainate receptors; metabotropic glutamate receptors; meninges; trigeminal system; pain; migraine; headache

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; c-*fos* LI, c-*fos*-like immunoreactivity; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; GAMS, γ-(R-)-glutamylaminomethanesulphonic acid; Glu, glutamate; HR, heart rate; L-AP4, 2-amino-4-phosphonono-S-butanoic acid; LRt, lateral reticular nucleus; MABP, mean arterial blood pressure; Md, medullary reticular nucleus; mGluR, metabotropic glutamate receptor; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide disodium; NMDA, N-methyl-D-aspartate; NS-102, 6,7,8,9-tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione-3-oxime; Sol, solitary tract nucleus; Sp5C, trigeminal nucleus caudalis; TG, trigeminal ganglion

Introduction

The trigeminal nerve transmits nociceptive information from the meninges to the brain stem trigeminal nucleus caudalis (Sp5C) in part *via* capsaicin sensitive fibres (Strassman *et al*., 1986; Bove & Moskowitz, 1997). These fibres contain neuropeptides such as substance P (Liu-Chen *et al*., 1986), calcitonin gene related peptide (CGRP; Uddman *et al*., 1985) and neurokinin A (Saito *et al*., 1987). As shown in several species, activated trigeminal fibres release neuropeptides from peripheral and central axons and transmit impulses to synaptic endings within Sp5C (Buzzi *et al*., 1991; Nozaki *et al*., 1992a; Strassmann *et al*., 1986; Goadsby & Hoskin 1997).

Glutamate (Glu) is the major excitatory amino acid in the mammalian central nervous system acting both at ligand-gated ion channels (ionotropic) and G-protein coupled metabotropic receptors. Several studies have implicated glutamate in nociception. It has been identified in peripheral and central terminals of primary afferent neurons, co-existing with substance P in some central nerve endings (Battaglia & Rustioni, 1988; Wanaka *et al*., 1988; Smullin *et al*., 1990; Baranauskas & Histri, 1998). Presynaptic Glu-immunoreactive terminals are found within lamina II of Sp5C (Iliakis *et al*., 1996). High densities of N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and metabotropic Glu receptor (mGluR) binding sites are present within lamina I, II of Sp5C (Tallaksen-Greene *et al*., 1992) and mRNA expression for NMDA receptors was found within trigeminal ganglion (TG) cells (Watanabe *et al*., 1994). At the spinal cord level, NMDA, kainate and AMPA receptors are expressed within both the dorsal horn (Furuyama *et al*., 1993) and the dorsal root ganglion (Sato *et al*., 1993; Huettner, 1990). Using microdialysis techniques Bereiter & Benetti (1996) showed that noxious stimulation to the face excite small trigeminal C-fibres and release acutely Glu and aspartate within Sp5C. Administration of Glu, or its receptor agonists NMDA, AMPA, and kainate results in mechanical or thermal allodynia and hyperalgesia (Zhou *et al*., 1996; Jackson *et al*., 1995; Lawand

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et al., 1997), while blockade of Glu receptors antagonizes the nociceptive effects (Raigorodsky & Urca, 1990; Birder & Groat, 1992; Eisenberg *et al*., 1993; Bereiter *et al*., 1996).

Expression of the immediate early gene protein product c-*fos* has been used as a marker of neuronal activity (Abbadie *et al*., 1997; Carrive & Meyer-Carrive, 1997). Treatment with Glu, NMDA, and AMPA induce c-*fos* expression within neurons both *in vitro* (Figiel & Kaczmarek, 1997; Lauritzen *et al*., 1997; Griffiths *et al*., 1997) and *in vivo* (Sharp *et al*., 1990; Berretta *et al*., 1992). Neurons within Sp5C express c-*fos* in response to noxious meningeal stimulation by the irritant capsaicin or autologous blood (Nozaki *et al*., 1992a; Cutrer *et al*., 1995b). This expression is attenuated in animals pretreated with drugs effective in the treatment of migraine headache, such as sumatriptan, dihydroergotamine or valproate (Nozaki *et al*., 1992b; Hoskin *et al*., 1996a, b; Cutrer *et al*., 1995a).

We recently showed that the potent and selective NMDA receptor antagonist MK-801 – (5R, 10S)-(+)-5-methyl-10,11-dihydro - 5H - dibenzo[a,d]cyclo - hepten - 5,10-imine hydrogen maleate – blocks capsaicin induced c-*fos* LI within Sp5C in rats (Mitsikostas *et al*., 1998). In addition, NMDA as well as non-NMDA receptor antagonists reduces c-*fos* LI within Sp5C after corneal or facial stimulation (Eisenberg *et al*., 1993; Bereiter *et al*., 1996; Bereiter & Bereiter, 1996). In this report we investigate the effects of non-NMDA receptor agonists and antagonists on capsaicin induced c-*fos* response within brain stem nuclei.

Methods

*Animal preparation and c-*fos *immunohistochemistry*

Male Sprague-Dawley rats (250–300 g, Charles River Laboratories, Wilmington, MA, U.S.A.) were anaesthetized with intraperitoneal (i.p.) urethane (1.2 g kg^{-1}) and maintained with 0.2 g kg^{-1} urethane i.p., every 2 h as needed to suppress the withdrawal response to hindpaw stimulation. A soft catheter (PE-10, 0.28 mm internal diameter; Intramedic, Clay Adams, Parsippany, NJ, U.S.A.) was introduced into the cisterna magna and after 45 min either the vehicle or drug was administered i.p. Fifteen minutes later, a capsaicin solution (0.1 ml; 50 μM) was injected into the cisterna magna *via* the catheter. Capsaicin was diluted in artificial CSF (see drugs). Animals were euthanized by an overdose of pentobarbitone (80 mg kg^{-1}, i.p.) 2 h after capsaicin administration and perfused immediately *via* the ascending aorta with 0.9% saline (200 ml), followed by 4% formaldehyde (500 ml) in 0.1 M phosphate buffer (PB). Brain stems with attached cervical cords were stored overnight in the same fixative and then placed in a cryoprotectant (20% sucrose, 30% ethylene glycol in 0.1 M PB) until sectioning (50 μm thick; from 3 mm rostral to obex to the C2 level) with a freezing microtome (Reichert-Jung, 2000 Leica, Deerfield, IL, U.S.A.). Every third tissue section was saved for immunohistochemistry. We used the free floating, avidin-biotin procedure, as has been previously described (Mitsikostas *et al*., 1998). The primary c-*fos* antibody (Oncogene Research Products, Cambridge, MA, U.S.A.) was diluted in 0.1 M PB (1 : 8000). Biotinylated goat antirabbit serum (Vector, Burlingame, CA, U.S.A.) was used as a secondary antibody (1 : 600).

Cell counting

C-*fos* positive nuclei were counted by an observer naive to the treatment groups (D.D. Mitsikostas) and confirmed (in randomly selected sections) by another investigator (M. Sanchez del Rio) under similar conditions. C-*fos* LI cells were counted in laminae I, II of Sp5C using the weighted average method, previously described and validated in guinea-pigs (Cutrer *et al*., 1995b) and rats (Mitsikostas *et al*., 1998). Briefly, based on the observation that c-*fos* LI was maximal at the level −2.00 to −2.30 mm and decreased linearly both rostrally and caudally, six 50 μm sections (every third section) were counted at each of three levels from 0 (obex) to −0.90 mm (mid-point −0.45 mm), −1.80 to −2.30 (mid-point −2.05 mm) and −6.00 to −6.50 (mid-point −6.25 mm). The mean number of labelled cells at these three levels was determined (x_1, x_2 and x_3, respectively). The trapezoid area under the curve was $8.5 \cdot x_1 + 22.5 \cdot x_2 + 15 \cdot x_3$. The weighted average was calculated by dividing this area by 45 (i.e. the number of 50 μm sections counted every 150 μm from obex −0.45 to obex −6.25). This value reflects the total c-*fos* expression within the entire Sp5C. An assessment of the extent of c-*fos* LI in solitary tract nucleus (Sol; visible in six serial sections), lateral reticular nucleus (LRt; six sections) and medullary reticular nucleus (Md; six sections) was also performed. In these nuclei the average number of labelled cells per section was calculated.

*Effect of catheter placement on c-*fos *expression*

Since mechanical and chemical (blood within the subarachnoid space) stimulation of C-fibres can occur as a result of surgery and induce c-*fos* expression within Sp5C, preliminary experiments investigated the effect of catheter placement into the cisterna magna on c-*fos* LI within Sp5C. A total number of 28 urethane anaesthetized animals were studied in three groups. Three intact animals were anaesthetized and euthanized 3 h later. A second group of 22 animals were euthanized 2 ($n = 3$), 3 ($n = 4$), 4 ($n = 3$), 5 ($n = 3$), 6 ($n = 3$), 7 ($n = 3$) and 8 h ($n = 3$) after i.c. catheter placement (no capsaicin treatment). And a third group of animals ($n = 3$) was treated with i.c. capsaicin (5 nmol) 1 h after catheter placement and sacrificed 2 h later. Brain stems from all animals were sectioned as described above and c-*fos* stained cells were counted in Sp5C, at the obex level (six sections for each animal).

Drug treatment

Dose ranges of drugs tested in the present study were chosen based on the previously observed ratios between potency in the c-*fos* paradigm and *in vitro* affinity of drugs at the presumed target. Previously tested agents include the 5-HT_1 receptor agonist CP-93,129 (Nozaki *et al*., 1992b), the NK_1 receptor antagonist RPR-100893 (Cutrer *et al*., 1995b), the $GABA_A$ receptor antagonist bicuculline (Cutrer *et al*., 1995a) and the NMDA receptor antagonist MK-801 (Mitsikostas *et al*., 1998) and the 5-hydroxytryptamine$_{1B/1D/1F}$ receptor agonist sumatriptan (Mitsiko *et al*., 1999 in press). Each drug was first tested at a relatively high dose. If a significant activity was observed, the dose was gradually decreased until the drug lost significant efficacy. Because day to day variations were observed in the c-*fos* response to intracisternal capsaicin, a separate control group was used for each drug-treatment. Drug vehicle (normal saline, i.p., $n = 10$) and the selective AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) 15 mg kg^{-1} ($n = 8$), 3 mg kg^{-1} ($n = 8$), 0.6 mg kg^{-1} ($n = 8$), 0.1 mg kg^{-1} ($n = 6$), or 0.02 mg kg^{-1} ($n = 6$) were injected i.p. in urethane anaesthetized rats. The selective AMPA receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide disodium (NBQX)

1 mg kg^{-1} ($n=7$), 0.1 mg kg^{-1} ($n=7$), and 0.01 mg kg^{-1} ($n=7$), or drug vehicle (normal saline, $n=9$) were administered in another set of animals. The kainate receptor antagonists γ-(R-)-glutamylaminomethane-sulphonic acid (GAMS) 1 mg kg^{-1} ($n=7$) and 10 mg kg^{-1} ($n=8$) and the selective agonist of group III mGluRs 2-amino-4-phosphono-S-butanoic acid (L-AP4) 3 mg kg^{-1} ($n=5$) and 10 mg kg^{-1} i.p., ($n=12$) were injected i.p. in a separate group of animals, a control group for GAMS and L-AP4 treatments received drug-vehicle (normal saline; $n=11$). The competitive Glu receptor antagonist with high selectivity for the low-affinity [^{3}H]-kainate binding site 6,7,8,9-tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione-3-oxime (NS-102) 1 mg kg^{-1} ($n=5$) and 5 mg kg^{-1} ($n=6$), or drug-vehicle (dimethyl sulphoxide (DMSO), $n=6$), were also administered in another set of animals. Because CNQX and NBQX are short acting drugs (Chizh *et al*., 1994; Birder & de Groat, 1992; Shinozaki *et al*., 1990), they were administered 15 min before i.c. capsaicin treatment; GAMS, NS-102 and L-AP4 were injected 30 min before capsaicin.

Systemic physiological parameters

Physiological monitoring was carried out in 16 animals. After anaesthesia with i.p. urethane and placement of the intracisternal catheter, a catheter (PE-50, internal diameter 0.58 mm; Becton Dickinson Co., Sparks, MD, U.S.A.) was placed in the left femoral artery. The effects of i.c. capsaicin injection on arterial pH, $PaCO_2$ and PaO_2, mean arterial blood pressure (MABP) and heart rate (HR) were measured before drug administration, 15 min after drug treatment, 10 and 30 min after capsaicin administration. HR and MABP were monitored continuously for 60 min in animals pretreated with i.p. CNQX 1 mg kg^{-1} ($n=4$), NBQX 1 mg kg^{-1} ($n=4$), L-AP4 10 mg kg^{-1} ($n=4$) and drug vehicle ($n=4$). Blood gases and pH measurements were performed four times in each animal (15 min after the placement of the catheter, i.e. baseline; 10 min after i.p. drug treatment; 10 and 30 min after i.c. capsaicin injection) using a blood gas/pH analyzer (Corning 178; Ciba-Corning Diagnostics, Medford, MA, U.S.A.). MABP and HR were monitored using Mac/Lab8 data acquisition system (AD Instruments, Medford, MA, U.S.A.) equipped with ETH 400 transducer amplifier. Core temperature was maintained at 36–37°C by a homeothermic blanket (Harvard Apparatus No. 551, South Natick, MA, U.S.A.).

Drugs

A fresh capsaicin solution (8-methyl-N-vanillyl-6-nonenamide, Sigma, St. Louis, MO, U.S.A) was made 2 h before use. Capsaicin (1.5 mg) was diluted in 1 ml of saline : ethanol : Tween 80 (8 : 1 : 1) and sonicated for 30 min. The solution was further diluted into artificial CSF (in mM): NaCl 132, KCl 3, $MgCl_2$ 0.6, $CaCl_2$ 1.5, $NaHCO_3$ 49, urea 6.6, D(+)-glucose 7.4, HEPES 5, pH adjusted to 7.4) to a final concentration of 50 μM. CNQX disodium, NBQX disodium, GAMS and L-AP4 (Research Biochemical International, Natick, MA, U.S.A.) were dissolved in normal saline. NS-102 (Research Biochemical International) was dissolved in DMSO (Sigma). Urethane (ethyl carbamate, Sigma) was diluted in water (3.4 M). Sodium pentobarbitone was obtained from Abbott Laboratories (Chicago, IL, U.S.A.).

Statistics

Data are expressed as a weighted average ± standard error of cells per 50 μm section. The weighted averages derived from

Table 1 Systemic physiological parameters after drug- and capsaicin-treatment

Group	*Baseline*	*Drug treatment*	*Capsaicin 10 min*	*Capsaicin 30 min*	*Capsaicin 60 min*
			pH		
Drug-vechicle	7.41 (0.01)	7.42 (0.01)	7.41 (0.01)	7.45 (0.02)	
CNQX	7.26 (0.1)	7.40 (0.01)	7.41 (0.01)	7.29 (0.1)	
NBQX	7.39 (0.01)	7.39 (0.1)	7.40 (0.01)	7.39 (0.01)	
L-AP4	7.40 (0.01)	7.41 (0.02)	7.42 (0.01)	7.45 (0.02)	
			PaO_2		
Drug-vechicle	105.6 (4.2)	105.8 (5.6)	100.1 (10.0)	97.0 (6.3)	
CNQX	105.5 (2.8)	108.4 (3.4)	119.5 (3.7)	100.9 (5.2)	
NBQX	97.5 (3.1)	108.4 (7.3)	103.7 (6.3)	93.9 (6.2)	
L-AP4	111.1 (6.9)	104.9 (7.2)	108.3 (4.9)	97.0 (6.3)	
			$PaCO_2$		
Drug-vechicle	33.5 (3.9)	32.5 (4.7)	36.1 (4.6)	32.0 (4.1)	
CNQX	24.4 (5.5)	35.6 (3.6)	24.8 (4.7)	33.1 (1.2)	
NBQX	32.8 (4.6)	34.7 (2.5)	34.3 (1.1)	29.4 (1.9)	
L-AP4	24.0 (2.5)	27.5 (3.7)	22.4 (3.8)	29.2 (1.8)	
			HR		
Drug-vechicle	313.0 (26.7)	325.9 (34.6)	372.9 (56.6)*	392.2 (55.8)*	406 (45)*†
CNQX	351.8 (26.2)	352.5 (28.4)	379.4 (14.8)	397.8 (36.1)	392 (36)†
NBQX	321.8 (24.1)	356.4 (37.5)	357.4 (23.9)	379.9 (24.7)*	370 (24)†
L-AP4	334.8 (18.6)	335.3 (17.1)	418.9 (16.5)*	400.1 (8.8)*	418 (21)*†
			MABP		
Drug-vechicle	75.7 (4.5)	78.2 (6.5)	101.7 (19.8)*	91.7 (15.5)	95 (12)†
CNQX	86.7 (7.8)	88.7 (3.8)	129.2 (4.7)*	112.2 (11.4)*	98 (9)*†
NBQX	85.2 (5.3)	87.0 (5.4)	92.7 (6.8)	91.2 (7.3)	92 (6)#
L-AP4	86.5 (5.8)	86.7 (5.6)	118.7 (9.9)*	101.5 (5.2)	98 (9)†

Intracisternal capsaicin (5 nmol) caused an increase in heart rate (HR) and mean arterial blood pressure (MABP) that lasted at least 60 min. Data are presented as mean (standard error). A two-way ANOVA (factor 1 capsaicin treatment and factor 2 drug treatment) followed by paired sample *t*-test was performed. †Significant effect ($P<0.005$) of capsaicin-treatment; #significant effect ($P<0.05$) of drug-treatment. *Significant difference compared with baseline- and after treatment-values.

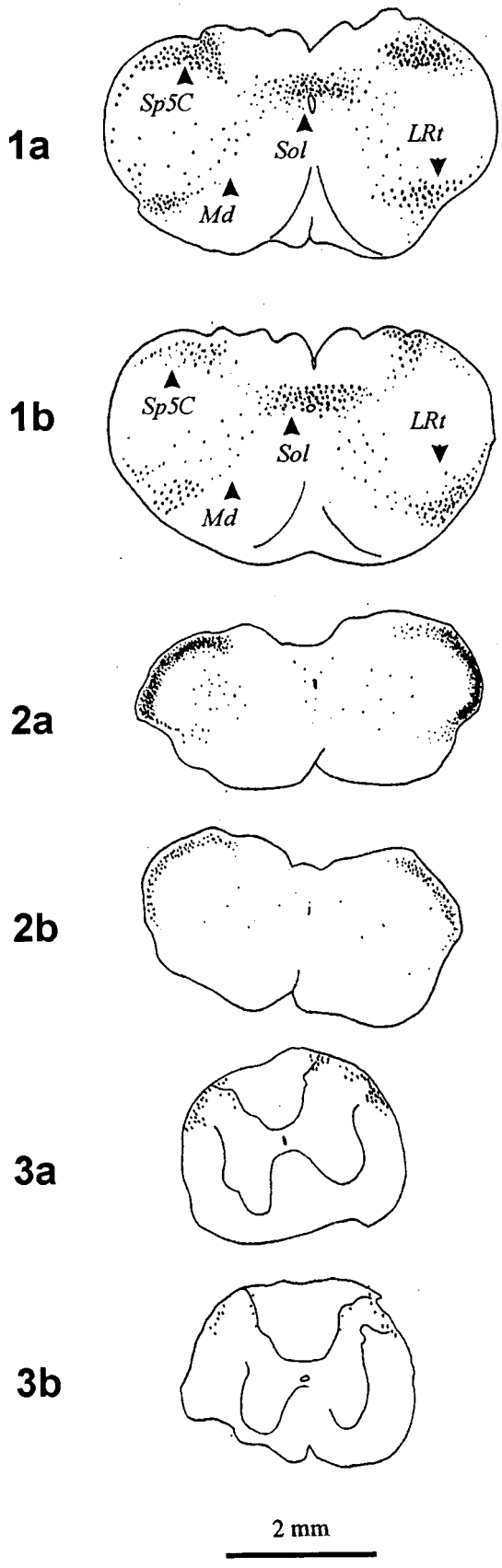

Figure 1 Camera lucida drawings showing the location of c-*fos* antigen immunostained cells (dots) within superficial laminae of trigeminal nucleus caudalis (Sp5C) and the deeper laminae of solitary tract nucleus (Sol), lateral reticular nucleus (LRt) and medullary reticular nucleus (Md), in coronal brain stem sections taken from two representative animals treated with intracisternal capsaicin (5 nmol). C-*fos* expression was detected bilaterally and most intensely within dorsal than ventral aspects of laminae I and II, being greatest at −2.05 mm (2a), as previously has been reported (a images, drug-vehicle treated animal). Pretreatment with CNQX (15 mg kg^{-1}; b images) reduced the c-*fos* positive cells per 50 μm section at each level of Sp5C, from dorsal (obex, 1b) to caudal (obex − 6.45 mm, 3b), but not in Sol, LRt and Md. These nuclei are also labelled in capsaicin-vehicle treated animals, or intact anaesthetized animals, suggesting that intraperitoneal injection, or urethane, or both, could be the primary trigger. Sections 1, obex; sections 2, −2.05 mm; and sections 3, spinal cord (−6.25 mm). All the drawings have the same magnification.

the cell counting method (see Methods) were compared by one-way ANOVA followed by Tukey's *post hoc* procedure. Student's two-tailed *t*-test was used when appropriate for simple comparisons between means. Data from physiological parameters were compared by two-way ANOVA (factor 1, capsaicin-treatment and factor 2, drug-treatment), followed by paired sample *t*-test. *P* values of 0.05 or less were considered significant.

Results

Physiological parameters

Capsaicin and drug treatment had no significant effect on pH, PaO_2 and $PaCO_2$ (Table 1). Capsaicin caused a significant increase in HR and MABP, that lasted at least 30 min. An effect of capsaicin on HR was also observed in animals pretreated with CNQX, NBQX, or L-AP4. In the presence of NBQX the effect of capsaicin on MABP was not observed. No significant interaction of two factors (capsaicin and drug treatment) was revealed (Table 1).

*Distribution of c-*fos*-LI positive brain stem neurons after capsaicin*

Capsaicin increased c-*fos* LI bilaterally in lamina I, II of Sp5C (Figure 1) and was more intensely expressed within dorsal aspects at each sampled level. C-*fos* expression was greatest at −2.05 mm. Cells in Sol, AP, LRt and Md were also labelled, as were in the leptomeninges (Figure 1). Staining was also found in association with arachnoid and pial blood vessels and was most prominent in that portion of meninges overlying the Sp5C or the dorsal horn of the spinal cord; the identity of this immunostaining was not determined. No evidence of sub-arachnoid haemorrhage was present.

There was no statistically significant change in the number of c-*fos* positive neurons between 2 and 8 h post surgery (data not shown). In capsaicin treated animals the average number of c-*fos* LI neurons within Sp5C was at least 17 times higher than in capsaicin-vehicle treated rats (3 h after surgery) (Table 2). Thus, surgical catheter placement does not significantly contribute to the estimated number of capsaicin induced c-*fos* LI neurons within Sp5C. AP, Sol, LRt and Md were also labelled even in intact (only anaesthetized) animals, and showed no changes with time (data not shown). Based on these findings, c-*fos* expression was studied 3 h after surgery in all subsequent experiments.

Drug treatment

Both AMPA receptor antagonists CNQX and NBQX reduced c-*fos* LI within Sp5C, with ID_{50} values of 0.07 ± 0.03 and 0.03 ± 0.01 mg kg^{-1}, respectively (0.25 ± 0.1 μmol and

Table 2 Capsaicin-induced c-*fos* immunoreactive cells within brain stem nuclei after drug treatment

			Sp5C						
Drug	*Dose* (mg kg^{-1})	n	*weighted*	*obex*	*obex−2.05 mm*	*bex−6.25 mm*	*Sol*	*Md*	*LRt*
CNQX									
	vehicle	10	313 (19)	206 (27)	512 (27)	60 (13)	75 (7)	43 (7)	54 (8)
	0.02	6	282 (16)	203 (14)	480 (33)	40 (13)			
	0.1	6	236 (19)*	151 (16)*	383 (24)*	31 (7)*			
	0.6	8	205 (14)*	108 (15)*	341 (29)*	40 (11)			
	3	8	192 (22)*	117 (18)*	334 (41)*	37 (16)			
	15	8	175 (19)*	115 (23)*	290 (33)*	33 (4)*	80 (8)	41 (8)	41 (5)
NBQX									
	vehicle	9	270 (15)	195 (18)	438 (18)	58 (5)	97 (12)	73 (13)	71 (7)
	0.01	7	242 (24)	198 (42)	394 (42)	33 (6)			
	0.1	7	196 (21)*	136 (23)*	309 (40)*	58 (14)			
	1	7	179 (20)*	120 (14)*	280 (37)*	57 (13)	68 (15)	68 (13)	60 (13)
GAMS									
	vehicle	11	260 (17)	206 (19)	400 (33)	50 (10)	62 (6)	45 (8)	50 (10)
	1	7	232 (22)	162 (27)	371 (27)	58 (11)			
	10	8	204 (26)	137 (14)	332 (49)	45 (10)	65 (10)	35 (8)	35 (4)
NS-102									
	vehicle	6	270 (38)	178 (19)	445 (56)	48 (17)	68 (7)	30 (4)	54 (6)
	1	5	208 (24)	112 (14)	353 (34)	40 (4)			
	5	6	207 (13)	117 (18)	340 (27)	51 (14)	62 (4)	27 (5)	43 (3)
L-AP4									
	vehicle	11	260 (17)	206 (19)	400 (33)	50 (10)	62 (6)	45 (8)	50 (10)
	3	5	204 (43)	160 (26)	345 (79)	13 (4)*			
	10	12	190 (18)*	154 (23)	295 (32)*	48 (30)	85 (10)	50 (4)	42 (2)
Baseline		4	12 (3)	17 (3)	16 (4)	9 (2)			

Data are presented as mean of c-*fos* LI cells per section (standard error). Baseline represents the mean of c-*fos* LI cells per section in capsaicin-vehicle and drug-vehicle treated animals (note that these animals also underwent surgical catheter placement). Sp5C, trigeminal nucleus caudalis; Sol, solitary tract nucleus; Md, medullary reticular nucleus; LRt, lateral reticular nucleus; $*P<0.05$ compared with drug-vehicle treated group of animals.

0.08 ± 0.02 μmol kg^{-1}, Figures 1 and 2). The effect of both CNQX and NBQX was dose-dependent, with a threshold at 0.1 mg kg^{-1}. At obex, −2.05 mm and −6.45 mm, CNQX (15 mg kg^{-1}) reduced c-*fos* LI by 44, 43 and 45%, respectively. The maximum reduction caused by NBQX at these levels was 38, 36 and 2% respectively. CNQX, or NBQX did not change the average number of c-*fos* positive neurons per section in Sol, LRt and Md (Table 2).

The metabotropic receptor agonist L-AP4 (10 mg kg^{-1}) reduced the weighted average of c-*fos* LI within the entire Sp5C by 30% (Table 2). The reduction was significant only at obex −2.05 mm level. At lower dose of L-AP4 (3 mg kg^{-1}) did not change the c-*fos* LI (only at −6.25 mm level a significant decrease was seen). Pretreatment with the putative kainate receptor antagonist GAMS (1 and 10 mg kg^{-1}) did not significantly affect expression within laminae I, II nor did treatment with the selective antagonist of low affinity kainate receptors NS-102 (1 and 5 mg kg^{-1}). Pretreatment with L-AP4, GAMS or NS-102 did not change the c-*fos* expression within Md, LRt and Sol (Table 2).

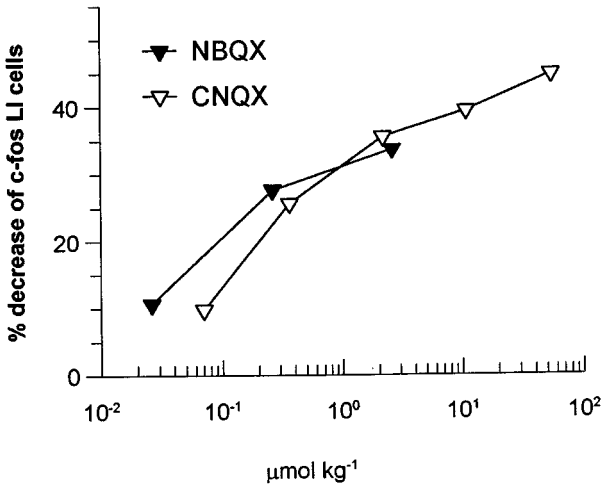

Figure 2 Pretreatment with CNQX and NBQX dose-dependently decreased capsaicin-induced c-*fos* immunoreactivity (c-*fos* LI) within trigeminal nucleus caudalis (laminae I, II). Drugs were given intraperitoneally followed by intracisternal injection of capsaicin (5 nmol). Animals were euthanized 2 h after menigeal irritation with capsaicin. Data are presented as percentage of decrease of c-*fos* LI cells per section (weighted average, see methods).

Discussion

AMPA/kainate receptor antagonists

We used a panel of Glu receptor antagonists to determine the importance of two Glu receptor subtypes to induce c-*fos* expression in Sp5C after administering the irritant capsaicin into the subarachnoid space. Treatment with the AMPA/kainate receptor antagonists CNQX, NBQX significantly and dose-dependently decreased the number of capsaicin induced c-*fos* LI neurons within Sp5C. GAMS and NS-102, two antagonists at low-affinity kainate receptor did not reduce the number of c-*fos* labelled neurons within Sp5C. None of the above treatments blocked capsaicin induced c-*fos* expression within Md, LRt and Sol nuclei. Drug-treatment did not change the animals' respiratory function, at variance with previous reports (Engberg *et al*., 1993; Foutz *et al*., 1994; Pierrefiche *et al*., 1994). It is possible that the doses used in our study were

too low or that simultaneous blockade of multiple glutamate receptor subtype is required to affect respiratory function. It is unlikely that the effect on c-*fos* are due to cardiovascular changes since none of the tested drugs had any effect on c-*fos* LI within Sol, which is the most important nucleus involved in baroreceptor afferent integration (Zhang & Mifflin, 1998). Taken together these data suggest that AMPA receptors can modulate c-*fos* expression and possibly neurotransmission within the trigeminovascular pain system after noxious meningeal stimulation by capsaicin. Kainate receptors do not seem to participate in this modulation. Interestingly, none of the compounds tested was able to decrease c-*fos* expression to background levels (i.e. as observed in animals receiving an intracisternal injection of capsaicin vehicle alone). Similar findings have been reported previously for other drugs attenuating c-*fos* expression in Sp5C, e.g., valproate (Cutrer, 1995a), the NK-1 receptor antagonist RPR 100893 (Cutrer *et al*., 1995b), the NMDA receptor antagonist MK-801 (Mitsikostas *et al*., 1998) and the 5-hydroxytryptamine$_{1B/1D/1F}$ receptor agonist sumatriptan (Mitsikostas *et al*., 1999 in press), suggesting that multiple neurotransmitter systems activate second order Sp5C neurons.

Both CNQX and NBQX are potent and selective AMPA receptor antagonist (K_i values 0.27 and 0.06 μM, respectively) and show low affinity for NMDA (K_i values 25 and >100 μM) and kainate receptors (K_i values 1.8 and 4.1 μM, respectively) (Shimizu-Sasamata *et al*., 1996; Dev *et al*., 1996). Thus, while CNQX shows only limited selectivity for AMPA versus kainate receptors (affinity ratio ≈ 7), NBQX is a more potent AMPA receptor antagonist (affinity ratio ≈ 70). NBQX is estimated to be three times more effective than CNQX in our model. Thus, it seems that AMPA more than kainate receptors can modulate capsaicin-induced c-*fos* expression within Sp5C.

The importance of kainate receptors was examined by administering GAMS. In most studies GAMS showed a preferential antagonistic action at kainate-type receptors in spinal cord neurons (Davies & Watkins, 1983; 1985), or in seizures induced by excitatory amino acids (1 μmol; Turski *et al*., 1985). Additionally, GAMS discriminates between kainate and AMPA receptors *in vitro* (Zhou *et al*., 1993; Zhou & Parks, 1992). However, GAMS showed only limited selectivity for the kainate-preferring receptor subtype expressed by rat dorsal root ganglion neurons (K_B value 360 μM) and the AMPA preferring subtype expressed by neurons from rat cerebral cortex (K_B value 750 μM) (Wilding & Huettner, 1996). Because GAMS also blocks NMDA receptors at >1 mM (Raigorodsky & Urca, 1990), we did not use doses higher than 10 mg kg^{-1} (40 μmol kg^{-1}).

To further investigate the importance of kainate receptors in capsaicin-induced c-*fos* LI within Sp5C, we administered NS-102, a non-NMDA receptor antagonist that selectively displaces low-affinity [^{3}H]-kainate binding (Johansen *et al*., 1993; Verdoorn *et al*., 1994). Wilding & Huettner (1996) found that the K_B value of NS-102 was 115 μM and 6 μM for neurons within the rat cerebral cortex (AMPA receptor) and the dorsal root ganglion (kainate receptor), respectively, suggesting that NS-102 is about ten times more potent than GAMS at kainate receptors. Similar to GAMS, NS-102 did not significantly change c-*fos* LI within Sp5C. The maximum dose of NS-102 used in the present study is 50 times higher than the threshold dose for CNQX. However, the affinity of NS-102 at kainate receptors is only five times smaller than that of CNQX for AMPA receptors (Wilding & Heuttner, 1996), suggesting than kainate receptors are unlikely to be involved in the c-*fos* response.

It is worth mentioning that urethane weakly blocks kainate and NMDA receptors (Dalo & Larson, 1990). However, we previously reported reduction of c-*fos* response when an NMDA receptor antagonist was given in the presence of urethane anaesthesia (Mitsikostas *et al*., 1998). It is thus unlikely that a partial attenuation of the c-*fos* response by the anaesthetic accounts for the lack of effect of kainate receptor antagonists in our study.

We cannot determine the exact location of the relevant AMPA receptors mediating the c-*fos* response. Current evidence favours an action on brain stem neurons because expression of AMPA receptors within primary trigeminal afferents has not been documented (Iida 1997; Sahara *et al*., 1997).

Metabotropic glutamate receptors

L-AP4 was administered to examine the potential importance of the group III mGluRs to capsaicin-induced c-*fos* response. L-AP4 inhibits glutamate release (Roberts 1995; Pin & Duvoisin, 1995; Pisani *et al*., 1997). It binds with high affinity to mGluR4 (0.4 μM), mGluR6 (0.9 μM) and mGluR8 (0.4 μM), whereas the affinities for the other mGluR subunits and ionotropic Glu receptors are very low (Conn & Pin, 1997; Eriksen & Thomsen, 1995). These receptors are probably pre-synaptic auto-receptors that fine-tune synaptic transmission by reducing calcium flux (Herrero *et al*., 1992; Thomsen, 1997). The mGluR4 and mGluR7 subtypes are present within rat TG and Sp5C neurons (Ohishi *et al*., 1995; Li *et al*., 1996). L-AP4 also modulates GABAergic activity within neo-cortex in other pain models (Thomsen, 1997). Thus, L-AP4 may modulate nociceptive pathways at many levels from the primary afferent neurons to the somatosensory cortex.

In conclusion the presented data suggest that AMPA and group III mGlu receptors modulate noxious responses within second order trigeminal neurons after meningeal irritation by capsaicin. Based on the lack of effect of GAMS and NS-102, it seems that kainate receptors are not involved. Since this model may be predictive for treatment of vascular headaches (Moskowitz & MacFarlane, 1993), AMPA and MGluR4 receptors may also serve as potential targets for the development of new anti-migraine drugs.

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