Published in final edited form as:

J Neurochem. 2009 August ; 110(3): 811–821. doi:10.1111/j.1471-4159.2009.06154.x.

CGRP Stimulation of iNOS and NO Release from Trigeminal Ganglion Glial Cells Involves MAP Kinase Pathways

CV Vause and PL Durham

Department of Biology, Missouri State University, Springfield, MO

Abstract

Clinical and basic science data support an integral role of calcitonin gene-related peptide (CGRP) in the pathophysiology of temporomandibular joint disorders. Recently, we have shown that CGRP can stimulate the synthesis and release of nitric oxide (NO) from trigeminal ganglion glial cells. The goal of this study was to determine the role of MAP kinase signaling pathways in CGRP regulation of iNOS expression and NO release from cultured trigeminal ganglion glial cells from Sprague-Dawley rats. CGRP treatment for 2 hrs significantly increased activity of the MAP kinase reporter genes, Elk, ATF-2, and CHOP. In addition, CGRP increased nuclear staining for the active forms of the MAP kinases ERK, JNK, and p38. This stimulatory event was not observed in cultures pretreated with the CGRP receptor antagonist peptide CGRP₈₋₃₇. Similarly, pretreatment with selective MAP kinase inhibitors repressed increases in reporter gene activity as well as CGRP-induced increases in iNOS expression and NO release mediated by MAP kinases. In addition, over-expression of MEK1, MEK3, MEK6, and MEKK significantly increased iNOS expression and NO production in glial cells. Results from our study provide evidence that CGRP binding to its receptor can stimulate iNOS gene expression via activation of MAP kinase pathways in trigeminal ganglion glial cells.

Keywords

glia; CGRP; MAP kinases; nitric oxide; iNOS; trigeminal

Calcitonin gene-related peptide (CGRP) is a multifunctional neuropeptide that plays an important role in the pathology of and other debilitating orofacial diseases such as temporomandibular joint disorders (TMD) (Appelgren *et al.* 1995). CGRP is a 37 amino acid product of alternative splicing of the calcitonin-CGRP gene (Amara *et al.* 1982, Rosenfeld *et al.* 1983) and is widely distributed in the central and peripheral nervous systems (Van Rossum *et al.* 1997, Wimalawansa 1996). Excitation of trigeminal nerves, which provide sensory innervation to most of the head and face, is thought to lead to peripheral release of CGRP that promotes an inflammatory response and central release that can cause activation of second order neurons resulting in pain (Kopp 2001, Hargreaves 2007, Pietrobon 2005). In addition, results from recent studies support an autocrine and paracrine function for CGRP within the trigeminal ganglion (Thalakoti *et al.* 2007, Zhang *et al.* 2007). These cellular effects of CGRP are mediated via activation of the CGRP receptor.

Although historically CGRP receptors have been divided into two classes referred to as CGRP1 and CGRP2, recent data have clarified that the CGRP1 receptor is the only CGRP receptor (Hay *et al.* 2008). Functional CGRP receptors are composed of a G protein-coupled

receptor known as the calcitonin-like receptor (CLR), a single transmembrane domain protein called receptor activity modifying protein type 1 (RAMP1), and a receptor component protein that defines the G-protein to which the receptor couples (Poyner *et al.* 2002). RAMP1 functions to traffic mature CRL proteins to the surface of the cell membrane and plays a critical role for receptor function since it defines the relative potency of ligands for the receptor (Mallee *et al.* 2002). CGRP receptors are expressed by vascular smooth muscle cells (Moreno *et al.* 1999, Oliver *et al.* 2002), as well as neurons and glia in the peripheral and central nervous systems, such as second order neurons and astrocytes (Morara *et al.* 2008, Levy *et al.* 2004), trigeminal ganglion neurons (Lennerz *et al.* 2008, Zhang *et al.* 2007) and satellite glial cells (Thalakoti *et al.* 2007). It is well established that activation of CGRP receptors couples to increases in cAMP and cGMP levels in a number of different cell types (Cheng *et al.* 1995, Fiscus *et al.* 1991, Poyner *et al.* 2002, Wimalawansa 1996). However, CGRP receptors have also been reported to couple to activation of mitogen-activated protein kinases (MAP kinases) (Parameswaran *et al.* 2000, Schaeffer *et al.* 2003).

MAP kinases are important signal transducing enzymes that connect activation of cell surface receptors to key regulatory events within the cell via a series of reversible phosphorylation events (Chang & Karin 2001, Seger & Krebs 1995). It is now known that at least four distinctly regulated groups of MAP kinases are present in mammalian cells, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 $\alpha/\beta/\gamma/\delta$), and ERK5, that are activated by specific MAP kinases (MAPK) (Chang & Karin 2001, Schaeffer & Weber 1999, Widmann *et al.* 1999). Importantly, many of the agents implicated in the initiation or maintenance of inflammation and pain have been shown to directly activate MAP kinase cellular signaling cascades in neurons and glial cells (Ji 2004a, Ji 2004b, Obata & Noguchi 2004).

The inducible form of nitric oxide synthase (iNOS), which is responsible for the production of large quantities of nitric oxide (NO), is known to be regulated by MAP kinases. While iNOS is not expressed at high levels in normal human temporomandibular joints (TMJs), iNOS expression in the synovial lining of diseased TMJs is greatly increased (Homma *et al.* 2001, Nagai *et al.* 2003, Takahashi *et al.* 2003). Furthermore, NO levels in synovial fluid obtained from patients with internal derangement and osteoarthritis of their TMJ were significantly increased when compared to control levels and correlated with disease stage and pain in the patients' joint area (Suenaga *et al.* 2001, Takahashi *et al.* 1999). In addition, elevated levels of NO are also implicated in the underlying pathology of migraine and infusion of NO can cause migraine attacks (Iversen & Olesen 1996, Olesen & Jansen-Olesen 2000). NO is produced by the nitric oxide synthase family of enzymes that includes neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS) (Liu *et al.* 2002). iNOS is expressed by a diverse array of cell types including both neuronal and glial cells found in the central and peripheral nervous systems. Despite the importance of iNOS and elevated NO levels in the development and maintenance of inflammation and pain, the cellular events involved in the expression of iNOS in glial cells is poorly understood.

In previous studies, we have shown that CGRP activation of CGRP receptors can increase the expression of iNOS and stimulate NO release from trigeminal ganglion glial cells (Li *et al.* 2008). In this study, we used glia-enriched cultures to determine the role of MAP kinase signaling pathways in CGRP regulation of iNOS expression and NO release from cultured trigeminal ganglion glial cells. Data from this study provide the first evidence, to our knowledge, that CGRP can stimulate iNOS gene expression via activation of MAP kinase pathways in trigeminal ganglion glial cells. Based on our results, we propose that CGRP released from cell bodies of trigeminal neurons could function to promote and maintain an

inflammatory cycle within the ganglion that mediates peripheral sensitization, and thus, play an important role in TMD pathology.

Materials and methods

Animals

All animal experimental procedures were conducted in accordance with institutional and National Institutes of Health guidelines. Every effort was made to minimize animal suffering and reduce the number of animals used. Pregnant female Sprague-Dawley rats (Charles River, Wilmington, MA) were housed in clean plastic cages on a 12-hour light/dark cycle with unrestricted access to food and water. Trigeminal ganglia from three to four day old neonatal rat pups were isolated and used to establish enriched glial cultures.

Primary trigeminal ganglia cultures

Primary cultures of trigeminal ganglia enriched in glial cells were established based on our previously published protocol (Bowen *et al.* 2006, Durham & Russo 1999, Durham & Russo 2003). Briefly, trigeminal ganglia were isolated from 20 to 24 pups and incubated in 10 mL L15 media (Leibovitz; Sigma, St. Louis, MO) containing 10 mg/mL Dispase II (Invitrogen Corporation., Carlsbad, CA), and 1 unit/ μ L RQ1 DNase (Promega, Madison WI) for 30 minutes at 37°C. Following centrifugation at $250 \times g$ for 1 minute, pellets were resuspended and dissociated in plating medium by vigorous trituration and then spun at $250 \times g$ for 3 minutes to pellet neuronal cells, and the resultant supernatant respun at $500 \times g$ for 5 minutes to concentrate glial cells. The resulting glial cell pellet was resuspended in L-15 medium containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 50 mM glucose, 250 μ M ascorbic acid, 8 μ M glutathione, 2 mM glutamine, and 10 ng/mL mouse 2.5 S nerve growth factor (Alomone Laboratories, Jerusalem, Israel). Penicillin (100 units/mL), streptomycin (100 μ g/mL), and amphotericin B (2.5 μ g/mL, Sigma) were also added to the supplemented L15 media. For transfection and NO release studies, dissociated cells were plated on 24-well poly-D-lysine coated tissue culture plates (Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ) at a density equivalent of two ganglia per well. For the immunocytochemistry studies, glial cells were plated at a density of half a ganglion on 11 mm glass coverslips coated with poly-D-lysine (relative MW 30,000-70, 000; Sigma). Cultures were incubated at 37°C at ambient CO₂. The culture medium was changed after 24 hours and every other day thereafter.

Transient transfection of trigeminal cultures

Glia-enriched trigeminal ganglia cultures were transiently transfected 3 days after plating with Lipofectamine 2000 (Invitrogen) essentially as described previously (Durham & Russo 1998, Durham *et al.* 1997). Cultured cells were co-transfected with 0.25 μ g Gal4-luciferase reporter, 0.25 μ g of the transactivator plasmids containing ELK1, c-JUN, ATF-2, and CHOP activation domains fused to the Gal4 DNA binding domain, as well as plasmid DNA for expression of MEK1, MEKK, MEK3, or, MEK6 under control of a CMV promoter (Stratagene, La Jolla, CA). In some experiments, CMV5 plasmid DNA was used as plasmid control. The DNA and Lipofectamine 2000 reagent (1: 2 ratio) were incubated together for 20 minutes in L15 medium (without supplements). The DNA-Lipofectamine complex was then added to the trigeminal glial-enriched cultures maintained in the fully supplemented L15 medium (including serum, 10 ng/ml NGF, antibiotics, and an antifungal agent) and incubated for ~24 hours before assaying for reporter activity. CGRP (1 μ M in sterile water; American Peptide, Inc., Sunnyvale, CA) was added 2 hours before harvesting. In some experiments, the cells were preincubated with selective MAP kinase inhibitors for p38 (SB239063; 10 μ M in dimethyl sulfoxide (Sigma; DMSO)); Tocris Biosciences, Ellisville, MO), JNK (SP600125; 1 μ M in DMSO, Tocris), ERK (U0126; 1 μ M in DMSO, Cell

Signaling Technology, Danvers, MA), or DMSO (1%) for 15 minutes prior to treatment with CGRP. Luciferase activity was measured using the Luciferase Assay System (Promega) while protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). In initial experiments, cell viability was determined using the trypan blue exclusion method following transfection with the different plasmids or treatments. Luciferase activities are reported as mean relative light units (RLU) per 50 μ g total protein with standard errors. Each experimental condition was repeated in at least three independent experiments performed in duplicate.

Immunocytochemistry

Immunocytochemical studies were conducted using untreated 2-day old primary glial cultures, cultures treated for 2 hours with 1 μ M CGRP or pretreated for 15 minutes with the CGRP₈₋₃₇ peptide (10 μ M in sterile water; Sigma), p38 inhibitor SB239063 (10 μ M), JNK inhibitor SP600125 (1 μ M), or ERK inhibitor U0126 (1 μ M) prior to CGRP stimulation, or 24 hours after transient transfection to overexpress MEK1, MEKK, MEK3, or MEK6. Coverslips were rinsed briefly with PBS and treated with 4% paraformaldehyde for 30 minutes at room temperature and with 0.1% triton X-100 in PBS for an additional 15 minutes to fix and permeabilize the cells. Fixed cells were incubated for 30 minutes in PBS containing 5% donkey serum and were stained with a mouse monoclonal antibody directed against glia fibrillary acidic protein (GFAP, 1:1000 in PBS; Chemicon International Inc., Temecula, CA), rabbit polyclonal antibodies directed against the phosphorylated, active forms of the MAP kinase proteins p38 (1:200; Cell Signaling), JNK (1:200; Cell Signaling), ERK (1:200; Cell Signaling), or a mouse monoclonal antibody directed against iNOS (1:100; Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ). Immunoreactive proteins were detected following incubation with Rhodamine Red-X-conjugated donkey anti-rabbit IgG antibodies (diluted 1:100 in PBS, Jackson ImmunoResearch Laboratories, West Grove, PA; for ERK, JNK, and p38) or Rhodamine Red-X-conjugated donkey anti-mouse IgG antibodies (for GFAP and iNOS). Prior to viewing, cells were mounted with Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to identify nuclei. As a control, some cultures were incubated with only the anti-mouse or anti-rabbit secondary antibodies. The number of neuronal cells that responded to CGRP stimulation was determined by analysis of immunopositive cells from ten random fields of cells from three coverslips. The reported numbers are the average of the counts obtained by two laboratory staff that were blinded to the experimental design. All images were taken at 1000X magnification on an Olympus microscope with a D41 fluorescent camera and analyzed using Microsuite Advanced Suite Five software.

Measurement of nitric oxide levels

NO levels were determined by the Griess reaction using a sodium nitrate standard (Promega). Glial-enriched cultures maintained for 1 day were left untreated or treated for 48 hours with lipopolysaccharide (LPS) (1 μ g/mL), CGRP (1 μ M; Sigma), or pretreated with CGRP₈₋₃₇ peptide (10 μ M), p38 inhibitor SB239063 (10 μ M), JNK inhibitor SP600125 (1 μ M), or ERK inhibitor U0126 (1 μ M) 15 minutes prior to addition of CGRP, or 48 hours after transient transfection to overexpress MEKK, MEK1, MEK3, or MEK6. The NO concentration for each well was normalized to the total protein level as determined by the Bradford method. Each condition was performed in triplicate in a minimum of three independent experiments in which laboratory personnel were blinded to the experimental design.

Statistics

Statistical analyses were performed using the non-parametric Kruskal Wallance test with Minitab 15 statistical software. Statistical significance was considered at $p < 0.05$.

Results

CGRP increases MAP kinase activity in trigeminal glial cells

Glial enriched cultures from trigeminal ganglia that were established as described in a previous study (Li et al. 2008) were transiently transfected with the MAP-kinase responsive reporter genes for Elk-1, ATF-2, and CHOP. Cells co-transfected with plasmids containing a gene encoding the transactivation domains of Elk-1, ATF-2, or CHOP linked to the yeast Gal4 DNA binding domain and a luciferase reporter plasmid containing Gal4 DNA binding sites were used to determine the effects of CGRP (1 μ M) on MAP kinase gene expression. CGRP treatment for 2 hours resulted in a 15-fold increase in Elk-1 and ATF-2 activities (basal levels were 6760 ± 197 RLU/50 μ g total protein and 9520 ± 812 RLU/50 μ g total protein, respectively) as well as a 20-fold increase in CHOP activity (basal level 28050 ± 562 RLU/50 μ g total protein) when compared to basal levels (Fig. 1). Cell viability, as monitored by trypan blue exclusion, was unaffected following transfection with the different plasmids or CGRP treatment when compared to control values (>90% viability for all conditions). These results demonstrate that CGRP can increase activity of MAP kinase-responsive genes in trigeminal ganglion glial cells.

CGRP increases MAP kinases in trigeminal glial cells

We estimate that >98% of the cells in the trigeminal ganglion cultures are glial cells based on images obtained using phase contrast microscopy as well as staining with the nuclear dye DAPI and antibodies directed against glia fibrillary acidic protein (GFAP) (Fig. 2A). To determine which specific MAP kinase proteins were regulated by CGRP, cultures were treated for 2 hours with CGRP (1 μ M) or pretreated for 30 minutes with CGRP₈₋₃₇ (10 μ M), a truncated peptide that blocks the CGRP receptor (Chiba et al. 1989). Untreated control cell cultures and treated cells were immunostained for the active (phosphorylated) forms of ERK (P-ERK), JNK (P-JNK), and p38 (P-p38). A summary of the immunostaining results for the active MAP kinase proteins are shown in Table 1. CGRP treatment resulted in a significant increase in the number of P-ERK immunoreactive cells (71% of total number of cells) when compared to control cultures (13%) (Fig. 2B). The greatest intensity of P-ERK staining was observed in the nucleus of the glial cells. Pretreatment with the CGRP receptor antagonist CGRP₈₋₃₇ essentially blocked the stimulatory effect of CGRP on P-ERK (5%; Fig. 2C). No P-ERK staining was observed when cultures were incubated with only the secondary antibodies (Fig. 2D).

Similarly, cultures stained for active-JNK and active-p38 showed significant increases in the number of immunopositive cells in response to CGRP treatment (72 and 92%, respectively) when compared to control untreated cultures (18% for both P-JNK and P-p38) or cultures pretreated with CGRP₈₋₃₇ (4 and 3%, respectively) (Fig. 3 and Table 1). The staining intensity for both P-JNK and P-p38 was greatest in the nucleus of the glial cells. These data provide evidence that activation of CGRP receptors on trigeminal glial cells leads to increased expression of the active phosphorylated forms of JNK and p38 in trigeminal glial cells.

Effects of MAP kinase inhibitors on CGRP regulation of iNOS

CGRP has previously been shown to upregulate iNOS expression in trigeminal glial cultures (Li et al., 2008). To determine whether CGRP regulation of iNOS involves activation of MAP kinase pathways, cultures were pretreated with MAP kinase inhibitors prior to addition of CGRP (1 μ M) and then stained with an antibody directed against iNOS. While iNOS staining was detected in less than 20% of the untreated cells, treatment of cultures with 1 μ M CGRP significantly increased the number of iNOS immunoreactive glial cells (85%) (Fig. 4 and Table 2). Preincubation with selective inhibitors for the MAP kinases p38 (SB239063;

10 μ M) and ERK (U0126; 1 μ M) reduced the number of cells with detectable levels of iNOS similar to the level of control unstimulated cultures. However, pretreatment of cultures with SP600125 (1 μ M), a JNK inhibitor, did not reduce the number of iNOS immunoreactive cells as much as seen with ERK and p38 inhibitors, but still was less than seen in response to CGRP stimulation. No significant change in the number of iNOS positive cells was seen in response to treatment to 1% DMSO, which served as a vehicle control. These immunostaining data suggest that CGRP regulation of iNOS involves activation of the MAP kinases ERK, JNK, and p38 since the selective inhibitors of these pathways inhibited the stimulatory effect of CGRP on iNOS.

Effects of MAP kinase inhibitors on CGRP induced NO production

We have recently shown that CGRP can also increase NO production in trigeminal ganglion glial cells (Li et al. 2008). To determine which MAP kinases are involved in CGRP regulation of NO production, the Greiss reaction was performed on media from glial-enriched cultures treated with CGRP as well as control cultures and cultures pretreated with MAP kinase inhibitors. CGRP (1 μ M) treatment increased NO levels almost 3-fold, while cultures pretreated with MAP kinase inhibitors for p38 (SB239063; 10 μ M) and ERK (U0126; 1 μ M) showed levels similar to control unstimulated cultures (Fig. 5). Similar to effects observed on iNOS in our cultures (Fig. 4), pretreatment with the JNK inhibitor SP600125 (1 μ M) did not repress the stimulatory effect of CGRP on NO as much as seen with the ERK and p38 inhibitors. However, the amount of NO was less than those observed with CGRP treatment alone and not statistically different than control levels. These results demonstrate that the MAP kinases p-38 and ERK, and to a lesser extent JNK, are involved in CGRP production of NO in trigeminal glial cells.

Effects of MAP kinase over-expression on iNOS and NO production in trigeminal glial cells

As a complementary approach to demonstrate that MAP kinases are involved in the regulation of iNOS and NO production, glial-enriched trigeminal cultures were transiently transfected with MEK CMV-expression vectors known to constitutively express the specific MAP kinases as previously described (Durham & Russo 1998, Durham & Russo 2003). Over-expression of the MEK1, MEKK, MEK3, or MEK6 proteins caused a significant increase in the number of iNOS expressing cells 24 hours after transfection when compared to control levels (Fig. 6 and Table 2). However, as a control, transfection with an equivalent amount of CMV5 plasmid did not cause a significant increase in iNOS (data not shown). These data provide further evidence that MAP kinase pathways are involved in the upregulation of iNOS in trigeminal glial cells.

Next, the effect of over-expressing MEK1, MEKK, MEK3, and MEK6 in trigeminal glial cells on NO release was determined. When compared to control levels, the amount of NO released from cultures over-expressing MEK1 or MEK3 was greatly increased and was comparable to that observed in response to treatment with 1 μ g/mL LPS (Fig. 7). While statistically different than the control, stimulation with MEKK was only 3-fold higher than the control level of NO secretion. In contrast, over-expression of MEK6 did not result in increased NO release. Similarly, transfection with an equivalent amount of CMV5 plasmid, which was used as control DNA, did not cause a significant increase in NO release (data not shown). These results further suggest that MAP kinases are involved in CGRP regulation of NO production in trigeminal glial cells.

Discussion

Previously we had shown that CGRP, a neuropeptide implicated in TMD, could increase expression of iNOS and the amount of NO released from cultured trigeminal ganglion glial

cells (Li et al. 2008). In this study, we extend our findings to provide evidence that CGRP stimulation of iNOS and NO release involves activation of several MAP kinases. In addition, our findings demonstrate that the effect of CGRP is mediated via activation of the CGRP receptor since pretreatment with the N-terminal truncated form of CGRP, CGRP₈₋₃₇, was able to block the cellular effects of CGRP. This shorter peptide has been reported to bind with high affinity to the CGRP receptor and thus prevent the binding of the endogenous full-length CGRP (Chiba et al. 1989). We had previously shown that trigeminal ganglion glial cells express the membrane protein RAMP1 (Li et al. 2008), which is required for functional CGRP receptors (Mallee et al. 2002, Poyner et al. 2002). While it is well established that activation of CGRP leads to increases in cAMP via stimulation of adenylyl cyclase, there have been only a few reports of CGRP receptor coupling to MAP kinase pathways. For example, CGRP was shown in smooth muscle cells to upregulate activity of ERK1/2 and p38 (Schaeffer et al. 2003). In addition, the cellular effects of CGRP on dorsal root ganglion neurons (Anderson & Seybold 2004) and in keratinocytes (Yu *et al.* 2006) were shown to involve the ERK1/2 MAP kinase signaling pathway. However, data from our study provide the first evidence that CGRP receptor activation leads to stimulation of MAP kinase activity and increased expression of the active, phosphorylated forms of ERK, JNK, and p38 via activation of the CGRP receptor in trigeminal ganglion glial cells.

We also found that CGRP could stimulate increased activity of the MAP-kinase responsive genes Elk1, ATF-2, and CHOP, which function as transcription factors to regulate inflammatory gene expression, in trigeminal ganglion glial cells. Interestingly, while trigeminal ganglia are comprised of both neuronal cells and glial cells, the glial cells greatly outnumber the neurons (Pannese *et al.* 2003), as is reported within the central nervous system (Watkins & Maier 2002). Within the ganglion, the cell bodies of sensory neurons are completely surrounded by satellite glial cells (Pannese 2002) and together are thought to form a functional unit (Hanani 2005) in which satellite glial cells play an important role in regulating the excitability state of the neurons (Pannese 1981, Pannese et al. 2003). For example, it was recently demonstrated that satellite glial cells mediate enhanced excitability of nociceptive trigeminal ganglion neurons following peripheral inflammation (Takeda *et al.* 2007). This increased excitability is likely to involve activation of MAP kinase pathways since these signaling pathways are known to lead to phosphorylation and activation of transcription factors that are responsible for regulating expression of inflammatory genes (Obata & Noguchi 2004). Thus, CGRP activation of its receptor on satellite glial cells would be expected to lead to increased expression of not only iNOS but also induction of other pro-inflammatory genes such as cytokines and interleukins that are known to be regulated by MAP kinases and are expressed by satellite glial cells (Kaminska 2005, Schindler *et al.* 2007). Although CGRP release from the cell body has not been directly demonstrated, it is likely that CGRP is released within the ganglia under basal as well as stimulated conditions since the vesicle docking protein SNAP-25 is present in the cell body of trigeminal neurons (Thalakoti et al. 2007). Furthermore, results from other studies on trigeminal ganglion have shown that other neuropeptides can be released from the cell body of stimulated neurons and can cause excitation of other neuronal cells as well as satellite glial cells (Neubert *et al.* 2000, Ulrich-Lai *et al.* 2001). Thus, CGRP released from the cell body would increase release of NO and other inflammatory agents that would mediate peripheral sensitization of trigeminal sensory neurons (McMahon *et al.* 2005). Peripheral sensitization, which is characterized by increased sensitivity and excitability of nociceptive neurons and a lower threshold of activation, involves changes in ion channel function as well as changes in gene expression (Dai *et al.* 2002). Importantly in animal models of tissue injury and inflammation, the active levels of the MAP kinases ERK and p38, which were found in our study to be increased in response to CGRP, are known to be involved in the development and maintenance of peripheral sensitization (Ji 2004a, Ji 2004b).

While iNOS is not usually expressed at high levels in normal human tissues, iNOS expression is greatly increased during pathological conditions where prolonged levels can cause tissue damage and cell death (Homma et al. 2001, Nagai et al. 2003, Takahashi et al. 2003). It is known that iNOS gene expression, similarly to other inducible genes, is regulated primarily at the level of transcription initiation by multiple intracellular signaling cascades (Saha & Pahan 2006). In our study, we have demonstrated that increased expression of iNOS in satellite glial cells in response to CGRP is mediated by activation of the MAP kinases ERK, JNK, and p38. However, based on our MAP kinase inhibitor results, it appears that the stimulatory effects of CGRP on NO production primarily involve activation of p-38 and ERK and to a lesser extent JNK activation. Our findings are in agreement with data from studies on astrocytes and microglia, glial cells found in the CNS, in that all three MAP kinase pathways are involved in iNOS gene regulation in response to inflammatory stimuli. In rat astrocytes, stimulation with LPS and IFN- γ was shown to cause activation of p38 and ERK1/2 (Bhat *et al.* 1998). Likewise, in another study, TNF- α and IL-1 β were reported to mediate their stimulatory effects via the ERK and p38 pathways (Marcus *et al.* 2003). A role of JNK in regulating iNOS expression in astrocytes has also been demonstrated in several studies (Bhat *et al.* 2003, Cvetkovic *et al.* 2004). Similarly, the involvement of one or more MAP kinases has been reported for iNOS regulation in microglia in response to inflammatory stimuli (Wang *et al.* 2004, Bhat *et al.* 1998, Bodles & Barger 2005). Studies of the molecular mechanisms involved in the regulation of iNOS transcription have identified multiple NF- κ B binding sites and NF- κ B is known to promote transcription of iNOS mRNA (Xie *et al.* 1994, Yamaza *et al.* 2003). Interestingly, p38 stimulation of iNOS transcription has been shown to involve regulation of NF- κ B in rat astroglia cells (Bhat *et al.* 2002). However, it appears that the involvement of MAP kinase regulation of NF- κ B is dependent on the particular stimulatory agent (Saha & Pahan 2006). Based on our findings, CGRP regulation of iNOS gene expression in glial cells likely involves a complex interplay of multiple signaling pathways in which MAP kinases and NF- κ B play a central role.

In summary, we have provided evidence that the stimulatory effects of CGRP on iNOS expression and NO production is mediated through CGRP receptor coupling to MAP kinase pathways in trigeminal ganglion glial cells. NO is an unstable free radical gas that functions as an important signaling molecule involved in the development and maintenance of inflammation and pain (Guzik *et al.* 2003, Liu *et al.* 2002, Naik *et al.* 2006, Yun *et al.* 1996). Thus, a large increase in the amount of NO within the trigeminal ganglion is likely to lead to activation of other satellite glial cells as well as trigeminal neurons resulting in increased synthesis and release of other inflammatory molecules. In support of this notion, we have previously shown that NO can stimulate the synthesis and release of CGRP from trigeminal ganglion neurons (Bowen *et al.* 2006). Thus, we propose that excitation of trigeminal neurons and subsequent release of CGRP from neuronal cell bodies would result in satellite glial cell activation and increased NO production, which would in turn cause increased expression of other inflammatory molecules in both neurons and glia. These events would lead to an inflammatory cycle within the ganglion that contributes to peripheral sensitization and hence, increased excitability of trigeminal neurons that likely play an important role in the underlying pathology of TMD and possibly migraine. The importance of blocking the physiological effects of CGRP has been demonstrated in clinical trials in which CGRP receptor antagonists were shown to abort migraine attacks (Ho *et al.* 2008, Olesen *et al.* 2004). It will be of clinical relevance to determine if CGRP receptor antagonists are equally effective in treating TMD since elevated levels of NO correlate with joint pathology and pain (Suenaga *et al.* 2001, Takahashi *et al.* 1999). Based on data from our study, it is likely that the effectiveness of CGRP antagonists will involve inhibition of MAP kinase pathways and hence, reduced iNOS expression and NO production in trigeminal ganglion glial cells.

Acknowledgments

This work was supported by NIH grant DE017805.

Abbreviations

CGRP	calcitonin gene-related peptide
NO	nitric oxide
TMJ	temporomandibular joint
TMD	temporomandibular joint disorder
CLR	calcitonin-like receptor
RAMP1	receptor activity modifying protein type 1
MAPKK	MAP kinase kinase
nNOS	neuronal nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
ERK	extracellular signal-regulated kinase
JNK	c-Jun amino-terminal kinase
RLU	relative light unit
GFAP	glia fibrillary acidic protein
LPS	lipopolysaccharide

REFERENCES

- Amara S, Jonas V, Rosenfeld M, Ong E, Evans R. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature*. 1982; 298:240–244. [PubMed: 6283379]
- Anderson LE, Seybold VS. Calcitonin gene-related peptide regulates gene transcription in primary afferent neurons. *J Neurochem*. 2004; 91:1417–1429. [PubMed: 15584918]
- Appelgren A, Appelgren B, Kopp S, Lundberg T, Theodorsson E. Neuropeptides in the arthritic TMJ and symptoms and signs from the stomatognathic system with special considerations to rheumatoid arthritis. *J Orofac Pain*. 1995; 9:215–225. [PubMed: 8995921]
- Bhat N, Zhang P, Lee J, Hogan E. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor- α gene expression in endotoxin-stimulated primary glial cultures. *J Neurosci*. 1998; 18:1633–1641. [PubMed: 9464988]
- Bhat NR, Feinstein DL, Shen Q, Bhat AN. p38 MAPK-mediated transcriptional activation of inducible nitric-oxide synthase in glial cells. Roles of nuclear factors, nuclear factor kappa B, cAMP response element-binding protein, CCAAT/enhancer-binding protein-beta, and activating transcription factor-2. *J Biol Chem*. 2002; 277:29584–29592. [PubMed: 12048217]
- Bhat NR, Shen Q, Fan F. TAK1-mediated induction of nitric oxide synthase gene expression in glial cells. *J Neurochem*. 2003; 87:238–247. [PubMed: 12969270]
- Bodles AM, Barger SW. Secreted beta-amyloid precursor protein activates microglia via JNK and p38-MAPK. *Neurobiol Aging*. 2005; 26:9–16. [PubMed: 15585341]
- Bowen E, Schmidt T, Firm C, Russo A, Durham P. Tumor necrosis factor- α stimulation of calcitonin gene-related peptide expression and secretion from rat trigeminal ganglion neurons. *J Neurochem*. 2006; 96:65–77. [PubMed: 16277606]

- Chang L, Karin M. Mammalian MAP kinase signaling cascades. *Nature*. 2001; 410:37–40. [PubMed: 11242034]
- Cheng L, Khan M, Mudge A. Calcitonin gene-related peptide promotes Schwann cell proliferation. *J Cell Biol*. 1995; 129:789–796. [PubMed: 7730412]
- Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T, Fukase M, Noda T, Fujita T. Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am J Physiol*. 1989; 256:E331–335. [PubMed: 2537579]
- Cvetkovic I, Popadic D, Vuckovic O, Harhaji L, Miljkovic D, Trajkovic V. 5-Aza-2'-deoxycytidine stimulates inducible nitric oxide synthase induction in C6 astrocytoma cells. *Brain Res*. 2004; 998:83–90. [PubMed: 14725971]
- Dai Y, Iwata K, Fukuoka T, Kondo E, Tokunaga A, Yamanaka H, Tachibana T, Liu Y, Noguchi K. Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. *J Neurosci*. 2002; 22:7737–7745. [PubMed: 12196597]
- Durham P, Russo A. Serotonergic repression of mitogen-activated protein kinase control of the calcitonin gene-related peptide enhancer. *Mol Endocrinol*. 1998; 12:1002–1009. [PubMed: 9658404]
- Durham P, Russo A. Regulation of calcitonin gene-related peptide secretion by a serotonergic antimigraine drug. *J Neurosci*. 1999; 19:3423–3429. [PubMed: 10212302]
- Durham P, Russo A. Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. *J Neurosci*. 2003; 23:807–815. [PubMed: 12574409]
- Durham P, Sharma R, Russo A. Repression of the calcitonin gene-related peptide promoter by 5-HT1 receptor activation. *J Neurosci*. 1997; 17:9545–9553. [PubMed: 9391009]
- Fiscus R, Zhou H, Wang X, Han C, Ali S, Joyce C, Murad F. Calcitonin gene-related peptide (CGRP)-induced cyclic AMP, cyclic GMP and vasorelaxant responses in rat thoracic aorta are antagonized by blockers of endothelium-derived relaxant factor (EDRF). *Neuropeptides*. 1991; 20:133–143. [PubMed: 1665896]
- Guzik T, Korb R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. *J Physiol Pharmacol*. 2003; 54:469–487. [PubMed: 14726604]
- Hanani M. Satellite glial cells in sensory ganglia: from form to function. *Brain Res Rev*. 2005; 48:457–476. [PubMed: 15914252]
- Hargreaves R. New migraine and pain research. *Headache*. 2007; 47:S26–43. [PubMed: 17425708]
- Hay DL, Poyner DR, Quirion R. International Union of Pharmacology. LXIX. Status of the calcitonin gene-related peptide subtype 2 receptor. *Pharmacol Rev*. 2008; 60:143–145. [PubMed: 18552275]
- Ho TW, Mannix LK, Fan X, Assaid C, Furtek C, Jones CJ, Lines CR, Rapoport AM. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. *Neurology*. 2008; 70:1304–1312. [PubMed: 17914062]
- Homma H, Takahashi T, Seki H, Ohtani M, Kondoh T, Fukuda M. Immunohistochemical localization of inducible nitric oxide synthase in synovial tissue of human temporomandibular joints with internal derangement. *Arch Oral Biol*. 2001; 46:93–97. [PubMed: 11163600]
- Iversen H, Olesen J. Headache induced by a nitric oxide donor (nitroglycerin) responds to sumatriptan. A human model for development of migraine drugs. *Cephalalgia*. 1996; 16:412–418. [PubMed: 8902249]
- Ji R. Mitogen-activated protein kinases as potential targets for pain killers. *Curr Opin Investig Drugs*. 2004a; 5:71–75.
- Ji RR. Peripheral and central mechanisms of inflammatory pain, with emphasis on MAP kinases. *Curr Drug Targets Inflamm Allergy*. 2004b; 3:299–303. [PubMed: 15379598]
- Kaminska B. MAPK signaling pathways as molecular targets for anti inflammatory therapy-from molecular mechanisms to therapeutic benefits. *Biochem Biophys Acta*. 2005; 1754:253–262.
- Kopp S. Neuroendocrine, immune, and local responses related to temporomandibular disorders. *J Orofac Pain*. 2001; 15:9–28. [PubMed: 11889652]
- Lennerz JK, Ruhle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin

- gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. *J Comp Neurol.* 2008; 507:1277–1299. [PubMed: 18186028]
- Levy D, Jakubowski M, Burstein R. Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT1B/1D receptor agonists. *Proc Natl Acad Sci USA.* 2004; 101:4274–4279. [PubMed: 15016917]
- Li J, Vause CV, Durham PL. Calcitonin gene-related peptide stimulation of nitric oxide synthesis and release from trigeminal ganglion glial cells. *Brain Res.* 2008; 1196:22–32. [PubMed: 18221935]
- Liu B, Gao H, Wang J, Jeohn G, Cooper C, Hong J. Role of nitric oxide in inflammation-mediated neurodegeneration. *Ann N Y Acad Sci.* 2002; 962:318–331. [PubMed: 12076984]
- Mallee J, Salvatore C, LeBourdelle B, Oliver K, Longmore J, Koblan K, Kane S. Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. *J Biol Chem.* 2002; 277:14294–14298. [PubMed: 11847213]
- Marcus JS, Karackattu SL, Fleegal MA, Sumners C. Cytokine-stimulated inducible nitric oxide synthase expression in astroglia: role of Erk mitogen-activated protein kinase and NF-kappaB. *Glia.* 2003; 41:152–160. [PubMed: 12509805]
- McMahon SB, Cafferty WB, Marchand F. Immune and glial cell factors as pain mediators and modulators. *Exp Neurol.* 2005; 192:444–462. [PubMed: 15755561]
- Morara S, Wang LP, Filippov V, Dickerson IM, Grohovaz F, Provini L, Kettenmann H. Calcitonin gene-related peptide (CGRP) triggers Ca²⁺ responses in cultured astrocytes and in Bergmann glial cells from cerebellar slices. *Eur J Neurosci.* 2008; 28:2213–2220. [PubMed: 19046367]
- Moreno M, Cohen Z, Stanimirovic D, Hamel E. Functional calcitonin gene-related peptide type 1 and adrenomedullin receptors in human trigeminal ganglia, brain vessels, and cerebromicrovascular or astroglial cells in culture. *J Cereb Blood Flow Metab.* 1999; 19:1270–1278. [PubMed: 10566974]
- Nagai H, Kumamoto H, Fukuda M, Takahashi T. Inducible nitric oxide synthase and apoptosis-related factors in the synovial tissues of temporomandibular joints with internal derangement and osteoarthritis. *J Oral Maxillofac Surg.* 2003; 61:801–807. [PubMed: 12856254]
- Naik A, Tandan S, Kumar D, Dudhgaonkar S. Nitric oxide and its modulators in chronic constriction injury-induced neuropathic pain in rats. *Eur J Pharmacol.* 2006; 530:59–69. [PubMed: 16364289]
- Neubert JK, Maidment NT, Matsuka Y, Adelson DW, Kruger L, Spigelman I. Inflammation-induced changes in primary afferent-evoked release of substance P within trigeminal ganglia in vivo. *Brain Res.* 2000; 871:181–191. [PubMed: 10899285]
- Obata K, Noguchi K. MAPK activation in nociceptive neurons and pain hypersensitivity. *Life Sci.* 2004; 74:2643–2653. [PubMed: 15041446]
- Olesen J, Diener H, Husstedt I, Goadsby P, Hall D, Meier U, Pollentier S, Lesko L. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N Engl J Med.* 2004; 350:1104–1110.
- Olesen J, Jansen-Olesen I. Nitric oxide mechanisms in migraine. *Pathol Biol (Paris).* 2000; 48:648–657. [PubMed: 11072643]
- Oliver K, Wainwright A, Edvinsson L, Pickard J, Hill R. Immunohistochemical localization of calcitonin receptor-like receptor and receptor activity-modifying proteins in the human cerebral vasculature. *J Cereb Blood Flow Metab.* 2002; 22:620–629. [PubMed: 11973435]
- Pannese E. The satellite cells of the sensory ganglia. *Adv Anat Embryol Cell Biol.* 1981; 65:1–111. [PubMed: 7013430]
- Pannese E. Perikaryal surface specializations of neurons in sensory ganglia. *Int Rev Cytol.* 2002; 220:1–34. [PubMed: 12224547]
- Pannese E, Ledda M, Cherkas P, Huang T, Hanani M. Satellite cell reactions to axon injury of sensory ganglion neurons: increase in number of gap junctions and formation of bridges connecting previously separate perineuronal sheaths. *Anat Embryol.* 2003; 206:337–347. [PubMed: 12698360]
- Parameswaran N, Disa J, Spielman W, Brooks D, Nambi P, Aiyar N. Activation of multiple mitogen-activated protein kinases by recombinant calcitonin gene-related peptide receptor. *Eur J Pharmacol.* 2000; 389:125–130. [PubMed: 10688975]

- Pietrobon D. Migraine: new molecular mechanisms. *Neuroscientist*. 2005; 11:373–386. [PubMed: 16061523]
- Poyner D, Sexton P, Marshall I, Smith D, Quirion R, Born W, Muff R, Fischer J, Foord S. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev*. 2002; 54:233–246. [PubMed: 12037140]
- Rosenfeld M, Mermod J-J, Amara S, Swanson L, Sawchenko P, Rivier J, Vale W, Evans R. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature*. 1983; 304:129–135. [PubMed: 6346105]
- Saha R, Pahan K. Regulation of inducible nitric oxide synthase gene in glial cells. *Antioxid Redox Signal*. 2006; 8:929–947. [PubMed: 16771683]
- Schaeffer C, Vandroux D, Thomassin L, Athias P, Rochette L, Connat J. Calcitonin gene-related peptide partly protects cultured smooth muscle cells from apoptosis induced by an oxidative stress via activation of ERK1/2 MAPK. *Biochim Biophys Acta*. 2003; 1643:65–73. [PubMed: 14654229]
- Schaeffer H, Weber M. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol*. 1999; 19:2435–2444. [PubMed: 10082509]
- Schindler J, Monahan J, Smith W. p38 pathway kinases as anti-inflammatory drug targets. *J Dent Res*. 2007; 86:800–811. [PubMed: 17720847]
- Seger R, Krebs E. The MAPK signaling cascade. *FASEB J*. 1995; 9:726–735. [PubMed: 7601337]
- Suenaga S, Abeyama K, Hamasaki A, Mimura T, Noikura T. Temporomandibular disorders: relationship between joint pain and effusion and nitric oxide concentration in the joint fluid. *Dentomaxillofac Radiol*. 2001; 30:214–218. [PubMed: 11681483]
- Takahashi T, Homma H, Nagai H, Seki H, Kondoh T, Yamazaki Y, Fukuda M. Specific expression of inducible nitric oxide synthase in the synovium of the diseased temporomandibular joint. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2003; 95:174–181. [PubMed: 12582357]
- Takahashi T, Kondoh T, Ohtani M, Homma H, Fukuda M. Association between arthroscopic diagnosis of temporomandibular joint osteoarthritis and synovial fluid nitric oxide levels. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1999; 88:129–136. [PubMed: 10468453]
- Takeda M, Tanimoto T, Kadoi J, Nasu M, Takahashi M, Kitagawa J, Matsumoto S. Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation. *Pain*. 2007; 129:155–166. [PubMed: 17127002]
- Thalakoti S, Patil V, Damodaram S, Vause C, Langford L, Freeman S, Durham P. Neuron-Glia signaling in trigeminal ganglion: Implications for migraine pathology. *Headache*. 2007; 47:1008–1023. [PubMed: 17635592]
- Ulrich-Lai YM, Flores CM, Harding-Rose CA, Goodis HE, Hargreaves KM. Capsaicin-evoked release of immunoreactive calcitonin gene-related peptide from rat trigeminal ganglion: evidence for intraganglionic neurotransmission. *Pain*. 2001; 91:219–226. [PubMed: 11275377]
- Van Rossum D, Hanisch U, Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. *Neuroscience and Biobehavioral Reviews*. 1997; 21:649–678. [PubMed: 9353797]
- Wang MJ, Jeng KC, Kuo JS, Chen HL, Huang HY, Chen WF, Lin SZ. c-Jun N-terminal kinase and, to a lesser extent, p38 mitogen-activated protein kinase regulate inducible nitric oxide synthase expression in hyaluronan fragments-stimulated BV-2 microglia. *J Neuroimmunol*. 2004; 146:50–62. [PubMed: 14698847]
- Watkins L, Maier S. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiol Rev*. 2002; 82:981–1011. [PubMed: 12270950]
- Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev*. 1999; 79:143–180. [PubMed: 9922370]
- Wimalawansa S. Calcitonin gene-related peptide and its receptors: Molecular genetics, physiology, pathophysiology, and therapeutic potentials. *Endocrine Reviews*. 1996; 17:533–585. [PubMed: 8897024]
- Xie Q, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem*. 1994; 269:4705–4708. [PubMed: 7508926]

- Yamaza T, Masuda K, Tsukiyama Y, Nishijima K, Murakami R, Kido M, Koyano K, Tanaka T. NF- κ B activation and iNOS expression in the synovial membrane of rat temporomandibular joints after induced synovitis. *J Dent Res.* 2003; 82:183–188. [PubMed: 12598546]
- Yu XJ, Li CY, Wang KY, Dai HY. Calcitonin gene-related peptide regulates the expression of vascular endothelial growth factor in human HaCaT keratinocytes by activation of ERK1/2 MAPK. *Regul Pept.* 2006; 137:134–139. [PubMed: 16904202]
- Yun H-Y, Dawson V, Dawson T. Neurobiology of nitric oxide. *Crit Rev Neurobiol.* 1996; 10:291–316. [PubMed: 8978984]
- Zhang Z, Winborn C, Marquez de Prado B, Russo A. Sensitization of calcitonin gene-related peptide receptors by receptor activity-modifying protein-1 in the trigeminal ganglion. *J Neurosci.* 2007; 27:2693–2703. [PubMed: 17344407]

Fig. 1.

CGRP stimulation of MAP kinase reporter genes in trigeminal glial cells. Primary cultures of trigeminal ganglion glial cells (day 2) transfected with reporter plasmids were treated for 2 hours with 1 μ M CGRP. Data are reported as fold change $\pm$ SEM over basal luciferase expression levels for Elk (6760 ± 197 relative light units (RLU)/50 μ g total protein), ATF-2 (9520 ± 812 RLU/50 μ g total protein), and CHOP (28050 ± 562 RLU/50 μ g total protein), whose means ($n = 4$ performed in duplicate) were set equal to one. * $p < 0.01$.

Fig. 2.

CGRP increases P-ERK levels in trigeminal glial cells. Trigeminal ganglion glial cells were costained with the nuclear dye DAPI and for GFAP expression (A). In panels B and C, cells that were left untreated (control, CON), treated with 1 μ M CGRP, or pretreated with 10 μ M CGRP₈₋₃₇ prior to CGRP stimulation were costained with DAPI and a primary antibody directed against the active, phosphorylated form of ERK (PERK). As a control, cells were costained with DAPI and only anti-rabbit secondary antibodies (D). Scale bar = 20 μ m.

Fig. 3. CGRP increases P-JNK and P-p38 levels in trigeminal glial cells. Trigeminal ganglion glial cells were costained with DAPI and a primary antibody directed against the active, phosphorylated form of JNK (P-JNK) (A) or p38 (P-p38) (B). Cells were left untreated (CON), treated with 1 μ M CGRP or pretreated with 10 μ M CGRP₈₋₃₇ prior to CGRP stimulation. Scale bar = 20 μ m.

Fig. 4.

Effect of MAP kinase inhibitors on CGRP regulation of iNOS. Trigeminal ganglion glial cells were costained with DAPI and a primary antibody directed against iNOS. Cells were left untreated (A), or treated with 1 μ M CGRP (B). Other cells were pretreated with the ERK inhibitor U0126 (1 μ M) (C), pretreated with JNK inhibitor SP600125 (1 μ M) (D), or pretreated with p38 inhibitor SB239063 (10 μ M) prior to CGRP stimulation (E). As a control, cells were costained with DAPI and only anti-mouse secondary antibodies (F). Scale bar = 20 μ m.

Fig. 5.

Effect of MAP kinase inhibitors on CGRP regulation of NO release from trigeminal glial cells. NO levels were determined by the Griess reaction in media collected from untreated control cultures (CON) or 48 hours after treatment with CGRP (1 μ M), or pretreatment with the p38 inhibitor SB239063 (SB; 10 μ M), JNK inhibitor SP600125 (SP; 1 μ M), or ERK inhibitor U0126 (1 μ M) prior to CGRP stimulation. Data are reported as the mean concentration/mg total protein $\pm$ SEM (n = 5). * p<0.05

Fig. 6.

Effect of over-expressing MAP kinases in trigeminal ganglion glial cells on iNOS expression. Cells that were left untreated (A) or transfected with expression plasmids for MEK1 (B), MEKK (C), MEK3 (D), or MEK6 (E) were costained with DAPI and a primary antibody directed against iNOS. Scale bar = 20 μ m.

Fig. 7.

Effects of MAP kinase over-expression on the regulation of NO release from trigeminal glial cells. NO levels were determined by the Griess reaction in media collected from untreated control cultures (CON) or 48 h after transient transfection of expression plasmid DNA for MEK1, MEKK, MEK3, or MEK6 or treatment with LPS (1 $\mu\text{g}/\text{mL}$). * $p < 0.05$; # $p < 0.01$

Table 1

Summary of CGRP Mediated Increases in MAP Kinase Immunoreactivity in Trigeminal Glial. Data are presented as percent immunoreactive cells compared to total number of cells $\pm$ SEM. P-values are reported relative to control levels.

P-ERK

Treatment	Percent Positive	P-value	N
Control	13.0 $\pm$ 4.62		168
CGRP	71.35 $\pm$ 3.37	0.027	132
CGRP ₈₋₃₇	5.37 $\pm$ 4.72	NS	120

P-JNK

Treatment	Percent Positive	P-value	N
Control	18.67 $\pm$ 4.75		105
CGRP	72.29 $\pm$ 5.54	0.047	108
CGRP ₈₋₃₇	4.16 $\pm$ 1.95	NS	100

P-p38

Treatment	Percent Positive	P-value	N
Control	18.0 $\pm$ 4.55		100
CGRP	92.68 $\pm$ 1.35	0.001	117
CGRP ₈₋₃₇	3.25 $\pm$ 1.35	NS	120

Table 2

Summary of CGRP Mediated Increases in iNOS Immunoreactivity in Trigeminal Glial. Data are presented as percent immunoreactive cells compared to total number of cells $\pm$ SEM. P-values are reported relative to control levels.

iNOS			
Treatment	Percent Positive	P-value	N
Control	19.69 $\pm$ 6.13		100
CGRP	85.11 $\pm$ 1.82	0.001	135
CGRP + U0126	14.62 $\pm$ 4.83	NS	120
CGRP + SP600125	56.92 $\pm$ 2.57	NS	102
CGRP + SB239063	23.61 $\pm$ 2.70	NS	136
MEK1	82.17 $\pm$ 5.99	0.001	117
MEKK	93.75 $\pm$ 3.12	0.001	105
MEK3	72.26 $\pm$ 4.27	0.001	108
MEK6	92.0 $\pm$ 2.62	0.001	100