REGULATION OF CGRP GENE EXPRESSION IN A HUMAN NEURONAL-LIKE CELL LINE, DMS 153

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is thought to play an important role in the pathology of migraine and temporomandibular joint disorders. This 37 amino acid neuropeptide is involved in neurogenic inflammation and pain transmission. To investigate CGRP gene expression, I utilized DMS 153 (DMS) cells derived from a small cell carcinoma of human lung. This cell line exhibits a neuroendocrine phenotype, and thus was investigated as a potential human neuronal-like cell model to study gene expression. DMS cells expressed CGRP and SNAP-25, and vanilloid type 1 receptor, tumor necrosis factor alpha type 1 receptor, and serotonin 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} receptors, as determined by immunocytochemistry. CGRP secretion, as measured by radioimmunoassay, was increased following a one-hour treatment with KCl, capsaicin (CAP), nitric oxide (NO), TNF-\textalpha, or protons. The stimulatory effects of CAP and NO on CGRP release were significantly inhibited by co-treatment with botulinum neurotoxin type A. Transient transfection of CGRP/luciferase reporter DNA was used to identify regulatory regions within the 1250 bp fragment of the rat CGRP promoter. Basal CGRP promoter activity was localized to a distal 18 bp enhancer region, while activity of the proximal cAMP-responsive element was stimulated by protein kinase A. In addition, specific cell signaling pathways were investigated using the PathDetect in vivo trans-reporting system. Basal levels of CHOP were much greater than those of Elk1, c-Jun, CREB, NF-\kappaB, or ATF2. Overexpression of the mitogen activated protein kinase MEKK greatly stimulated Elk-1, c-Jun, ATF-2, and NF-\kappaB activity. In conclusion, my results demonstrate that DMS cells can be used as a model of human neuronal cells to study CGRP gene expression in response to inflammatory as well as anti-inflammatory agents.

KEYWORDS: CGRP, DMS 153, migraine, trigeminal, botulinum neurotoxin.
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INTRODUCTION

Calcitonin Gene-Related Peptide

Calcitonin and calcitonin gene-related peptide (CGRP) are alternative products of the calcitonin/CGRP gene of both endocrine and neuronal cells. In thyroid-C cells, alternative splicing of mRNA produces the calcium-regulating hormone calcitonin, while in neurons the mRNA is spliced to produce CGRP. CGRP is a 37-amino acid regulatory neuropeptide found in cells throughout the central nervous system (CNS) and peripheral nervous system (PNS), as well as cells of the immune system (Rosenfeld et al. 1983). Based on its localization, CGRP was initially proposed to be involved in a large number of functions such as maintaining cardiovascular homeostasis (Brain et al. 1985) and nociception (Oku et al. 1987). CGRP is the most potent peptide vasodilator known (Mculloch et al. 1986). Elevated levels of CGRP have been measured in cardiac failure, myocardial infarction (Preibisz 1993), forms of hypertension (Goto et al. 1992), sepsis (Joyce et al. 1990), all forms of headache (Buzzi et al. 1995; Edvinsson and Goadsby 1994; Ferrari and Saxena 1993), and temporomandibular joint (TMJ) disorders (Kopp 2001; Sessle 2001). CGRP is thought to play a key role in the underlying pathology of these conditions due to its ability to regulate blood flow, mediate neurogenic inflammation, and relay nociceptive information to the CNS.

Two isoforms of CGRP have been identified in rats and humans and are referred to as α and β (Amara et al. 1985; Steenbergh et al. 1985). This study will focus only on the regulation of α-CGRP since this isoform is the predominant one found in trigeminal ganglia (Amara et al. 1985) and α-CGRP knockout mice were shown to have reduced
nociceptive hypersensitivity (Zhang et al. 2001). Throughout this thesis, α-CGRP will be referred to as simply CGRP.

**CGRP Gene Expression**

CGRP expression in rat trigeminal ganglia neurons is controlled at the transcriptional level, with no subsequent modifications to the mRNA after it is initially transcribed (Tverberg and Russo 1993). The transcription of CGRP is regulated by a combination of cell-specific enhancer elements (HO) and hormonal stimuli through a calcium-dependent cAMP-responsive element (CRE) (de Bustros et al. 1986; Wahl and Schilling 1993; Yun et al. 1996), and a Ras-responsive element (RRE) (Thiagalingam et al. 1996). The HO enhancer is synergistically activated by the helix-loop-helix upstream stimulating factor and an octamer-binding protein (Lanigan and Russo 1997; Tverberg and Russo 1993). More recent studies indicate that CGRP gene expression is partially mediated by mitogen-activated protein (MAP) kinase (Durham and Russo 1998; Yun et al. 1996; Lander et al. 1996). MAP kinase stimulation of CGRP gene expression is controlled via a proximal RRE (Thiagalingam et al. 1996) and the HO enhancer (Durham and Russo 1998).

MAP kinases are signal-transducing enzymes that connect activated surface receptors to various intracellular activities via a cascade of reversible phosphorylation events (Cohen 1992; Seger and Krebs 1995). There are at least five distinct families of MAP kinases present in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2 and JNK3), p38 kinase proteins (p38α, p38β, p38γ and p38δ), ERK3/ERK4; and ERK5 (Davis 2000; Chang and Karin 2001; Roux and Blenis 2004). Of the five pathways, two (ERK 1/2 and JNK) are investigated
here for their implications in human disease and CGRP gene expression (English and Cobb 2002; Durham and Russo 2000). ERK MAP kinases respond to mitogenic stimuli, such as growth factors and cytokines, to regulate such diverse cellular functions as proliferation, differentiation, cell motility and secretion (Seger and Krebs 1995). The JNK family of proteins are activated in response to cellular stress often attributed to heat shock and elevated osmolarity associated with inflammation.

Reagents such as potassium chloride (KCl), capsaicin, protons, tumor necrosis factor alpha (TNF-α), and nitric oxide (NO) induce CGRP synthesis and secretion in trigeminal ganglia neurons (Iversen et al. 1989; Hou et al. 2002; Durham et al. 2006; Bellamy et al. 2006). The direct mechanism for each of these stimulatory reagents varies, however treatment with each of these reagents resulted in increased CGRP secretion from rat trigeminal neurons (Durham 2006). For example, KCl causes direct depolarization of neurons, while capsaicin and protons have been shown to induce CGRP secretion via transient receptor potential vanilloid (TRPV1) receptor activation of C-fiber afferent nerves (Akerman et al. 2003; Durham et al. 2006). More recently, TNF-α and NO have been shown to stimulate CGRP secretion through MAP kinase pathway activation in trigeminal neurons (Bowen et al. 2006; Bellamy et al. 2006).

In contrast, many reagents have been shown to repress CGRP secretion in trigeminal neurons. For example, studies involving serotonergic antimigraine drugs such as triptans, which have an affinity for 5-HT receptors, have been shown to return elevated levels of CGRP to normal (Edvinsson and Goadsby 1994; Goadsby and Edvinsson 1991; Goadsby and Edvinsson 1993; Stewart et al. 1994). While the mechanisms have not been
fully elucidated, these drugs appear to suppress CGRP production by interrupting stimulatory signaling pathways (Durham and Russo 1999).

Recently, results from my laboratory have shown that botulinum neurotoxin A (BoNT-A) represses stimulated CGRP release from trigeminal neurons (Durham et al. 2004a), making it a potential therapeutic candidate for diseases involving neurogenic inflammation. BoNT-A is a potent neurotoxin that causes muscle paralysis by preventing the release of acetylcholine from motor neurons into the neuromuscular junction. In muscle, the inhibitory effect of BoNT-A is attributed to its ability to block the docking of synaptic vesicles in motor neuron end plates. More specifically, the neurotoxin binds to and cleaves SNAP-25 (synaptosome-associated protein of 25,000 dalton), a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) responsible for synaptic vesicle docking and exocytosis at motor neuron terminals (Blasi et al. 1993). However, in trigeminal neurons, treatment with BoNT-A was shown to repress CGRP release in response to chemical depolarization or the inflammatory agent capsaicin (Durham et al. 2004a; Caputi 2004).

**The Trigeminal Nerve and Migraine**

Among neurons that express CGRP, trigeminal ganglia are of particular interest, due to their function as the primary sensory nerve for the face and neck, and the sole pain-sensing neurons associated with the dura mater surrounding the brain (McCulloch et al. 1986). The trigeminal nerve (the fifth cranial nerve) is composed of 3 major branches or divisions: the ophthalmic branch (V1), the maxillary branch (V2), and the mandibular branch (V3). The three branches converge at the cell bodies within the trigeminal ganglion. The trigeminal ganglia contain pseudounipolar neurons with one process.
projecting to the CNS and the other to the periphery. As the primary source of CGRP in the head and neck, trigeminal ganglia nerve sensitization and activation is a major contributor to the initiation and maintenance of migraine headache (Durham and Russo 2002).

Migraine headaches are a major source of pain and frustration for many Americans. A migraine headache is a severe recurring headache, usually affecting only one side of the head, characterized by sharp pain and often accompanied by nausea, vomiting, and visual disturbances (aura) (Ferrari 1998; Goadsby and Edvinsson 1994a). According the American Medical Association (2003), 6% of men and 18% of women suffer an average of 35 migraine attacks per year. These episodes cause a total of 112 million bed-ridden days a year and cost the American public $13 billion dollars in missed work, annually (AMA 2003). During a migraine episode, disruption of homeostasis within meningeal blood vessels induces afferent release of CGRP, which causes vasodilation and neurogenic inflammation in the dura mater surrounding the brain. The dilated blood vessels exert pressure on the surrounding trigeminal nerve endings, causing the trigeminal neurons to relay nociceptive (pain causing) impulses to the central nervous system (Allen et al. 1996; Edvinsson and Goadsby 1994). Consequently, increased levels of CGRP have been observed in all types of migraine headaches (Buzzi et al. 1995).

Cell Models

Human trigeminal nerves are ideal for research involving neurogenic pain and inflammation associated with CGRP synthesis and release. However, given the ethical, technical, and financial limitations of this type of research, alternative methods have been utilized to gain most of what is known about the cellular mechanisms involved in CGRP
gene regulation. For example, rat in vivo studies and primary cultures of rat trigeminal neurons that secrete CGRP have been powerful models to study disease pathology and treatment processes (Durham and Russo 1999; Mason et al. 1984). In addition, two cell lines, the neuronal-like rat medullary thyroid carcinoma (CA77) and the rat phenochromocytoma (PC-12) have provided model systems for CGRP gene expression and nerve pathology (Russo et al. 1996; Greene and Tischler 1976). Furthermore, the human neuroendocrine cancer cell line, TT has had limited usefulness as a calcitonin secreting cell model (Berger et al. 1984). Although these model systems have been useful tools for the study of CT/CGRP gene expression, none allow study of CGRP gene expression in the context of a human neuronal-like cell. To my knowledge, there are no known human neuronal-like cell models available for research.

**The DMS 153 Cell Line**

The DMS 153 cell line was derived from a human small cell lung carcinoma (SCLC). These aggressive malignant cells had metastasized to the liver where they were isolated and collected during autopsy. SCLC is a neoplasm derived from malignant tumors of neuroendocrine cells found in the lining of the lung. The exact function of this cell type in the lung is unknown; however neuroendocrine cells are distributed throughout the human body. Prevalent in the gastroenteropancreatic system, these cells were thought to derive from neuroectoderm (Sorenson et al. 1983). However, further research indicated a bronchial mucosal origin (Sorenson et al. 1983).

The currently accepted criteria specifying neuroendocrine cells are any cells producing neurotransmitters, neuropeptide hormones, containing dense-core granules and lacking axons or dendrites. DMS 153 (DMS) cells, although not traditionally considered
a neuroendocrine cell line, fit the criteria as a neuropeptide producing cell that exhibits acetylcholine receptors and secretes CGRP (Sorenson et al. 1983). The DMS 153 cell line was used in this study for its potential as a useful human neuronal-like model to determine the cellular mechanisms by which inflammatory and therapeutic agents control CGRP gene expression.

**Goals of the Study**

The initial focus of my studies was to optimize growth conditions and, using immunocytochemistry, determine which neuronal specific proteins are expressed by DMS cells. Next, regulation of CGRP secretion in response to inflammatory stimuli was to be investigated by measuring CGRP levels in the DMS culture media by radioimmunoassay. Transient transfection studies were to be conducted to study rat CGRP promoter activity as well as human CGRP promoter activity under basal, unstimulated conditions and in response to signal kinases. In addition, the basal and stimulated levels of MAP kinase-responsive, cAMP-responsive, and NF-κB-responsive reporter genes were to be investigated. Finally, the effect of BoNT-A on CGRP gene expression was to be evaluated.

Specifically, the goals of the study were to: (1) optimize culture conditions by testing the effects of various substrates and growth factors on DMS cell growth rate and morphology; (2) determine which receptors are expressed in DMS cells and focus on those known to regulate CGRP; (3) determine the effects of inflammatory agents on CGRP synthesis and secretion in DMS cells; (4) determine the effects of BoNT-A on CGRP synthesis and release in DMS cells; (5) identify which signaling pathways are active and responsive to various inflammatory stimuli.
MATERIALS AND METHODS

Cell Culture

DMS cells derived from a small cell carcinoma of human lung were obtained from the American Type Culture Collection (ATCC, Manassas, VA). As per ATCC’s instructions, 1 ml of the DMS cell suspension was rapidly thawed by gentle agitation in a 37°C water bath for ~2 min. The thawed suspension was gently dispensed into a T25 plastic tissue culture flask (Sarstedt, Newton, NC). To dilute the cells to ~60% confluence, 4 ml of Waymouth’s media (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml; Sigma), streptomycin (100 µg/ml; Sigma) and amphotericin B (2.5 µg/ml; Sigma) [henceforth referred to as “growth media”] was added drop wise over a period of 5 min. In addition, gentamicin (100 µg/ml; Sigma) was added to the growth media to prevent *Pseudomonas* contamination. The cultures were maintained in a humidified chamber at 37°C and 7% CO₂.

To maintain a monolayer of adherent clusters, cells were plated on poly-D-lysine (PDL; MW 30,000-70,000; Sigma) coated T25 tissue culture plates. The growth media was replaced on day 3 and day 6. When the cells reached ~90% confluence (~day 8 or day 9), the media was removed and 500 µl of trypsin solution (0.5% trypsin, 0.2% EDTA, Atlanta Biologicals, Lawrenceville, GA) was used to dissociate the cells from the PDL matrix and each other. After 2 min and 4 min, loosely adhered cells were mechanically dislodged by tapping the flask. The cells were resuspended by titration (pipetting up and down > 10 times) in 5 ml of growth media containing 20% FBS and equally divided between two new T25 flasks. Cultures were noticeably healthier in growth media containing 20% FBS as compared to 10%.
Excess DMS cells were frozen and stored in liquid nitrogen. To prepare cells for liquid nitrogen storage, active cultures of DMS cells growing at 70%-90% confluency on PDL-coated T25 tissue culture plates were trypsinized as described above, 24 h prior to freezing. The trypsinized cells were pelleted by centrifugation (1500 rpm for 2-3 min) and resuspended by gentle titration in 90%/10% FBS-DMSO solution. Aliquots (500 µl) were frozen down overnight in an isopropyl alcohol bath at -80ºC and then placed in a liquid nitrogen tank for long-term storage.

**Effects of Extracellular Matrix**

To determine the effects of various extracellular matrix substrates on DMS cell growth rate and morphology, cells were cultured on 6-well tissue culture plates or on plates pre-coated with either PDL, human fibronectin, laminin, or collagen type I (Becton, Dickinson and Company, Bedford, MA). Growth and morphology of DMS cells were monitored using phase contrast microscopy and photographed with a Kodak DC290 Zoom digital camera every 48 h for 7 d.

**Effects of Growth Factors**

The effects of known neuronal growth factors on DMS cell growth and morphology were investigated. Cells were cultured in 3 concentrations of either nerve growth factor (NGF; 1, 10, or 100 ng/ml; Sigma), brain-derived neurotrophic factor (BDNF; 1, 10, or 100 ng/ml; Sigma) or neurotrophin-3 (NT-3; 1, 10, or 100 ng/ml; Sigma). The growth factors were added to the media at the time of plating and on day 4 after media replacement. Growth and morphology of DMS cells were monitored by phase contrast microscopy and photographed every 48 h for 8 d.
**Immunocytochemistry**

For the immunolocalization studies, sterile 13 mm tissue culture coverslips (Sarstedt) were placed in a 24-well tissue culture plate and treated with 100 µl of PDL for 5 min. The coverslips were subsequently rinsed 3 times with 300 µl of 1X phosphate buffered saline solution (PBS, 0.137 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH = 7.4).

DMS cells from a ~80% confluent T25 flask were dissociated as described above and resuspended in 9.6 ml of growth media. To evenly distribute the cells at ~50% confluence, 5-7 X 10⁵ cells were plated on the coverslips in each well of the 24-well tissue culture plate. The cells were incubated at 37° C and 7% CO₂.

After 48 h incubation, cells were rinsed with 500 µl of PBS and fixed with 300µl of 4% paraformaldehyde for 30 min at room temperature. The fixed cells were rinsed 3 times with 500 µl of PBS and treated with 0.1% Triton X-100 in PBS for 15 min at room temperature to permeabilize the cell membranes. Care was taken not to allow the cells to dry between rinses. After permeabilizing, the cells were again rinsed 3 times with PBS and each coverslip to be stained was removed from the well and placed on the lid of a 35 mm plastic Petri dish (Sarstedt). To minimize non-specific Ab-receptor binding, 100 µl of 5% donkey serum diluted in PBS was dispensed onto each coverslip and the cells were incubated for 30 min at room temperature. The cells were subsequently rinsed 3 times with PBS.

The primary and secondary antibodies (Ab) were prepared immediately prior to use by diluting each stock solution to its appropriate concentration in PBS containing 1%
bovine serum albumin (BSA; Sigma) (Table 1). The BSA was added to reduce non-specific Ab-receptor binding.

Once prepared, 100 µl of the appropriate primary Ab was added to the cells and incubated for 1 h at room temperature. When 1 h incubation was insufficient, the primary Ab was incubated with cells overnight at 4°C to increase binding efficiency. Following incubation, the cells were washed 3 times with 0.1% Tween in PBS. After the final wash, 100 µl of the secondary Ab was added to the cells and incubated for an additional 1 h in the dark at room temperature. The cells were subsequently washed 3 times with 0.1% Tween in PBS. As a control, cells were incubated in 1X PBS containing 1% BSA instead of the primary antibody.

The cells were prepared for observation by addition of 4’, 6 diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA). This staining reagent minimizes photobleaching as well as allowing for easy identification of the nuclei. Cells were observed and photographed at 1000X, under oil immersion with an Olympus DP70 camera mounted on an Olympus BX41 inverted fluorescent microscope. Image collection and analysis was performed using Olympus MicroSuite Five image processing software.
<table>
<thead>
<tr>
<th>Antibodies (Ab)</th>
<th>Primary-Ab Concentration</th>
<th>Secondary-Ab Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat CGRP</td>
<td>1:1000</td>
<td>1:100&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human CGRP</td>
<td>1:1000</td>
<td>1:100&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Human TNFR1</td>
<td>1:1000</td>
<td>1:100&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>1:1000</td>
<td>1:100&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1B&lt;/sub&gt;</td>
<td>1:100</td>
<td>1:100&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>5-HT&lt;sub&gt;1D&lt;/sub&gt;</td>
<td>1:100</td>
<td>1:100&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>TRPV1</td>
<td>1:100</td>
<td>1:100&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFkB</td>
<td>1:100</td>
<td>1:100&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
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<sup>1</sup>Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG  
<sup>2</sup>FITC-conjugated donkey anti-goat IgG  
<sup>3</sup>FITC-conjugated donkey anti-mouse IgG
Secretion Studies

To prepare cultures for secretion studies, DMS cells from a ~70% confluent PDL-coated T25 flask were dissociated from the surface with 500 µl of trypsin solution (0.5% trypsin, 0.2% EDTA, Atlanta Biologicals). Following titration, the cells were resuspended in 9.6 ml of growth media. The cell suspension was equally divided into a 24-well PDL-coated tissue culture plate (7-8 X 10^5 cells per well), and the cells were incubated at 37°C and 7% CO₂.

For unstimulated CGRP secretion studies, the growth media was removed after 72 h and replaced with 250 µl of HBS (22.5 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 3.3 mM glucose, and 0.1% BSA, pH 7.4) (Vasko et al, 1994). The plate was incubated for 1 h at 37°C, after which the basal HBS was collected in 1.5 ml Eppendorf tubes and stored at -20°C for analysis by radioimmunoassay (RIA). The amount of unstimulated CGRP secreted in each well was determined, and these values were used to normalize for differences between individual wells and dishes.

For stimulated release studies, cells (cultured for 72 h) were initially incubated for 1 h in 250 µl HBS (7.4 pH) and the basal HBS from unstimulated cells was collected and stored at -20°C. An additional 250 µl of HBS was placed on cells. The cells were then left untreated (control) or stimulated with either KCl (60 mM), capsaicin (2 µM), S-nitroso-N-acetyl-penicillamine (nitric oxide donor, SNAP, 2 µM), TNF-α (100 ng/ml), or protons (HBS pH 5.5). After 1 h of incubation at 37°C, the HBS was collected in 1.5 ml Eppendorf tubes and stored at -20°C for analysis by RIA.

For the BoNT-A studies, 1-day-old cultures were pretreated with either 1.6 or 3.1 units of BoNT-A for 6 h, 24 h or 3 d. Following pretreatment, cells were incubated for
1 h in 250 µl HBS and the basal HBS was collected and stored at -20°C. The basal HBS was replaced with 250 µl HBS (pH 7.4 or pH 5.5) and either KCl (60 mM), capsaicin (2 µM), SNAP (2 µM) or TNF-α (100 ng/ml). The cells were incubated for an additional 1 h at 37°C and the stimulated HBS was collected and stored at -20°C for analysis by RIA.

To determine the effects of BoNT-A on DMS cell viability, 1-day-old cultures were either untreated or pretreated with either 1.6 units or 3.1 units of BoNT-A and incubated for 24 h at 37°C. Following incubation, cells were rinsed once with PBS and treated with 500 µl of trypan blue (Sigma) diluted 1:4 in PBS for 5 min. Cells were then rinsed with PBS and visualized by brightfield microscopy. Cytotoxicity was evaluated by approximating the ratio of viable cells to total cells in three random fields per secretion condition.

**Analysis of CGRP by Radioimmunoassay**

CGRP concentrations were measured, as per manufacturer’s instruction, using a CGRP-specific RIA kit (Peninsula-Bachem, San Carlos, CA). The RIA was a three day assay based on the competitive binding of radioactive versus non-radioactive labeled CGRP molecules. On day one of the RIA, a standard curve was generated using 7 volumes (1 to 128 pg/tube) of standard peptide from the kit. For sample analysis, 50 µl of HBS from each secretion condition was added to a 50 µl solution of primary CGRP antibody. All tubes were vortexed and incubated for 24 h at 4°C.

On day two of the RIA, a competition assay between radioactive and non-radioactive peptide was established by adding 50 µl of the appropriately diluted ¹²⁵I-CGRP conjugate to each tube from day 1. All tubes were then incubated for 24 h at 4°C. On the third day, antibody-peptide complexes were immunoprecipitated in each sample.
by adding 50 µl of goat anti-rabbit IgG serum and 50 µl of normal rabbit serum. All samples were then centrifuged for 20 min at 3,000 rpm and 4°C to pellet the antibody-CGRP complexes. The supernatant containing unbound radioactive CGRP was aspirated, and the amount of radioactivity remaining in immunoprecipitated pellets was determined using a gamma counter (PerkinElmer Wizard, 1470 Automatic Gamma Counter). The level of radioactivity in the pellet was inversely proportional to CGRP levels in each well.

**Transient Transfection**

DMS cells were transiently transfected with Lipofectamine 2000 (LF2000, Invitrogen, Gaithersburg, MD) as described previously (Durham and Russo, 2003). The rat CGRP-luciferase and cytomegalovirus β-galactosidase (CMV-β-gal) reporter plasmid constructs used in these experiments have been previously described (Tverberg and Russo, 1993; Lanigan and Russo, 1997) (Figure 1 and 2).

To prepare cultures for transient transfection, DMS cells from a ~90% confluent PDL-coated T25 flask were dissociated from the surface with 500 µl of trypsin (10X, Atlanta Biologicals) and resuspended by titration in 9.6 ml of growth media. The cell suspension was equally divided into a 24-well PDL-coated tissue culture plate (8-9 X 10⁵ cells per well), and the cells were incubated at 37°C and 7% CO₂ for 24 h. Immediately prior to transfection, the media was replaced with 400 µl of new growth media.

Following incubation, the cells were transfected with 0.25 µg of the appropriate trans-reporting or expression plasmid(s) (Figure 1 and 2). To prepare the transfection cocktail for each well, the appropriate DNA was combined with the LF2000 reagent (ratio of 1 µg:2 µl) in 100 µl of Waymouth’s medium (without supplements) and
incubated for 20 min. The DNA-LF2000 complex was then added to the cells maintained in 400 µl growth media and incubated for 24 h at 37°C and 7% CO₂.

After 24 h, the cells were harvested by scraping each well, collecting the cellular suspension in 1.5 ml Eppendorf tubes (one well per tube), and pelleting the cells by centrifugation (2 min at 13,000 rpm). The media was then aspirated and the cell pellet was resuspended by titration in 40 µl lysis buffer (Applied Biosystems, Foster City, CA). The lysed cell suspensions were stored at -20°C for analysis for luciferase and CMV-β-gal activity.
Figure 1. Trans-reporting plasmids used for signal transduction experiments. A, The pFR-Luc reporter plasmid promoter, containing GAL4 binding sites, controls expression of firefly luciferase. Transcription of luciferase is induced by phosphorylation of the transcription activator bound to the reporter. The resulting luciferase activity can then be measured to determine the activation status of the signaling events. B, The trans-activator plasmid includes the human cytomegalovirus (CMV) promoter active in most mammalian cells. The CMV promoter is used to control expression of the activation domain of the transcription activator fused with the yeast GAL4 DNA binding domain. Figures adapted from www.stratagene.com.
Figure 2. CGRP-luciferase reporter constructs. The ~2.0 kb human CGRP promoter contains a proximal cyclic AMP response element (CRE; -104 to -109 and -253 to -260) and a distal enhancer element (-826 to -843). The 1.25 kb rat CGRP promoter also contains a proximal CRE and a distal enhancer element. The distal enhancer elements of both the rat and the human contain noncell-specific elements (striped box) and a cell-specific enhancer (Bgl; solid box). The cell-specific enhancer contains an 18 bp binding site for HLH and forkhead proteins. The CGRP promoter (rat and human) contains a heterologous promoter comprised of thymidine kinase promoter and a luciferase reporter gene. The 18 bp plus A forkhead mutation in the rCGRP is used to confirm binding at the 18 bp binding site (Tverberg and Russo 1993; Lanigan and Russo 1997).
Analysis of Luciferase Activity

Gene expression was analyzed, as per manufacture’s instructions, by quantifying luciferase activity in each thawed sample using a trans-reporting system (PathDetect, Stratagene, La Jolla, CA) with a Gal4-luciferase reporter plasmid, and plasmids encoding fusion proteins of the Gal4-DNA-binding domain and transactivation domains of either CHOP, c-Jun, Elk1, CREB, NF-κB, or ATF2 (Figure 1). To determine reporter activity in each sample, 10 µl of the lysed cell suspension from each tube was added to 50 µl of luciferase substrate. The light produced from the luciferase-luciferin substrate reaction was measured with a luminometer.

Analysis of β-Galactosidase Activity

CMV-β-gal activity was measured using the Galacto-light Plus β-Galactosidase Reporter Gene Assay System (Tropix, Bedford, MA). Since β-gal gene expression is known to be high in all mammalian cell types, this assay was performed to normalize transfection efficiencies in all transient transfection experiments.

The lysed cell samples were thawed to room temperature and centrifuged (1 min, 13,000 rpm) to pellet cellular debris. For each assay, 5 µl of lysed cell suspension was added to 50 µl of buffered Galacto-Plus substrate and the complex was incubated for 30 min. Following incubation, 75 µl of Accelerator was added, and the light emitted was measured with a luminometer.

Protein Analysis by the Bradford Method

The Bradford method (Bradford 1976; Biorad Laboratories, Hercules, CA) was used to determine protein concentrations in each transfected sample. These data were then used to report gene expression in light units per 10 µg of protein.
Following the Bradford method protocol, a standard curve was established using BSA (0.25 mg/ml) in eight duplicate volumes ranging from 0-14 µl. Protein concentration in each sample was determined in duplicate using 1.5 ul of lysed cell suspension in each well of a 96-well plate. Diluted 1X Coomassie Brilliant Blue G-250 (CBBG) was added to each sample and the complex was incubated for 5 min. Following incubation, absorbance was measured at 595 nm (PerkinElmer Victor³V, 1420 Multilabel Counter). The standard curve was generated by the system software and final protein concentrations were expressed in µg/µl.

**Statistical Analysis**

Each experimental condition was repeated in at least three independent experiments performed in duplicate. Statistical analyses for secretion and transfection studies were done using the nonparametric test, Mann-Whitney U test (due to small sample sizes the data collected failed tests for normality). All comparisons were tested for statistical significance at p < 0.05.
RESULTS

Effects of Matrix Substrates on DMS 153 Morphology

DMS cells were grown on different matrix substrates to determine optimal growth conditions for the cell line (Figure 3A-3O). On day 4 after plating cells grown on plastic only, collagen, fibronectin, or laminin exhibited large, multilayer clusters (Figure 3F-3I). In contrast, the cells plated on PDL-coated plastic were growing as a monolayer of adherent clusters (Figure 3J). On day 7 the cells grown on plastic only, collagen, fibronectin, or laminin were lifting off the surface in large multilayer clusters, while the cells on PDL maintained growth as a monolayer of adherent clusters (Figure 3K-3O). No cell processes were observed in cells grown on plastic only, collagen, fibronectin or laminin substrates. However, cell processes were present on ~5% of cells plated on PDL after 4 days. Cell growth rate appeared to be declining by day 7 in cultures maintained on plastic only, collagen, fibronectin, or laminin (Figure 3K-3N). Although, cells plated on PDL appeared to be growing at a constant rate through day 7 (Figure 3O).

Effects of Growth Factors on DMS 153 Cells

To further optimize growth conditions and promote neuronal differentiation, DMS cells were cultured for 6 d in media containing increasing concentrations of brain-derived neurotrophic factor, nerve growth factor, and neurotropin-3. Upon visual inspection, growth rate appeared to be slightly accelerated in media containing growth factors at day 1 and day 6 when compared to control rates (Figures 4 and 5). Otherwise, cell morphology did not appear to vary from the control cultures. Similar to the matrix substrate studies, ~5% of cells cultured in all culture conditions exhibited processes at day 4. Thus, somewhat surprisingly, it appears that the addition of known neuronal
growth factors does not stimulate DMS cell growth nor does it promote a neuronal morphology, which is characterized by neuronal processes.

**Expression of Neuronal Proteins in DMS 153 Cells**

Immunocytochemistry was used to characterize the morphology and biochemistry of the DMS 153 cell line. DMS cells grow on PDL-coated flasks as a monolayer of adherent clusters with large round nuclei and very little cytoplasm (Figure 6A, 6B, 6D, and 6E). In my culture conditions, >95% of the DMS cells express high levels of CGRP in a punctate pattern in the cytoplasm (Figure 6C and 6F).

The synaptic membrane fusion protein, SNAP-25 is present in a punctate pattern in >90% of the cell bodies and processes of the DMS cells (Figure 7D). DMS cells also express abundant levels of the TNF-α receptor, TNFR1 (Figure 7E) and the vanilloid receptor, TRPV1 (Figure 7F) in a punctate pattern in the cytoplasm and on the cell membrane. The serotonin receptors, 5-HT\textsubscript{1B} (Figure 8C) and 5-HT\textsubscript{1D} (Figure 8D) are present in moderate levels in the cytoplasm and cell membrane of DMS cells. No staining was observed in the absence of primary antibodies (Figure 9C, 9F, and 9I).

**Effects of Inflammatory Agents on CGRP Secretion in DMS 153 Cells**

To investigate the stimulatory effects of various inflammatory agents on CGRP secretion, DMS cells were incubated for 1 h in the presence of either 60 mM KCl, 2 µM of capsaicin, 40 ng of TNF-α, 2 µM of SNAP, protons (pH 5.5) or 2 µM SNAP and protons (pH 5.5) in 250 µl HBS. KCl, capsaicin, and NO stimulated secretion of CGRP by ~3-fold when compared to unstimulated control levels (Figure 10). In contrast, TNF-α and protons did not increase relative levels of CGRP above that of the control (Figure 10). The lack of effect of TNF-α on CGRP release was somewhat surprising given that
DMS cells express TNFR1 receptors. It is possible that higher concentrations of TNF-α are required to stimulate CGRP release from DMS cells than what was used in this preliminary screening. Interestingly, cotreatment with SNAP and protons resulted in a much greater increase (~7-fold) in CGRP secretion than seen with either agent alone (Figure 10).

As a control, untreated DMS cells were incubated in 250 μl HBS for 1 h prior to secretion (basal) and 1 h during treatment (experimental). Secreted CGRP levels in the basal samples were not significantly different than those in the experimental samples (Figure 11).

**Effects of BoNT-A on CGRP Secretion**

To determine the effects of BoNT-A on basal and stimulated CGRP secretion, DMS cells were pretreated for 24 h with either 1.6 or 3.1 units of BoNT-A followed by a 1 h incubation with either 60 mM of KCl, 2 μM of capsaicin, 2 μM of SNAP, or 2 μM of SNAP and protons (pH 5.5) in 250 μl HBS. While BoNT-A did not inhibit basal, unstimulated CGRP release (Figure 12), BoNT-A slightly, but significantly, repressed capsaicin-stimulated CGRP secretion from a 3-fold increase to a ~2-fold increase (Figure 13). A more robust effect of BoNT-A was seen on NO stimulated cultures, where BoNT-A greatly repressed NO-stimulated CGRP secretion to near basal levels (Figure 14). Curiously, BoNT-A had no significant effect on NO/protons-stimulated CGRP secretion in DMS cells (Figure 15). In addition, BoNT-A had no effect on KCl-stimulated CGRP secretion (data not shown).

Following the secretion studies, trypan blue exclusion was used to evaluate the effects of BoNT-A on DMS cell viability. There was no appreciable difference in cell
viability (>90%) between those treated with either 1.6 or 3.1 units of BoNT-A (Figure 16B and 16C) and the control (Figure 16A).

CGRP Promoter Activity in DMS 153 Cells

Preliminary experiments have shown that the rat CGRP promoter functions similarly to the human CGRP promoter in DMS cells (Abbey 2006). As previously described, the rat CGRP promoter-luciferase construct includes the TATA box, a proximal cAMP response element, and a distal enhancer containing a cell-specific 18 bp element (Figure 17A) (Durham and Russo, 1998). To evaluate CGRP promoter activity in the DMS 153 cell line, cells were transiently transfected with various rat CGRP promoter fragment-luciferase plasmids, including: a 200 bp Bgl fragment; the 18 bp element; the 18 bp element that contained an additional adenosine nucleotide (+A mutation) or a BamHI restriction enzyme site mutation (ΔBAM); or the CRE, and the luciferase activity was measured (Figure 17B). A TK-luciferase construct was used as a control. Luciferase activity was highest with the 18 bp fragment, closely followed by Bgl and then the entire promoter sequence (Figure 17B). Transfection with the CRE and ΔBAM resulted in significantly lower luciferase activity than the 18 bp fragment. While transfection with the 18 bp+A mutation-luciferase construct and the TK-luciferase yielded similar minimal activity (Figure 17B).

Effect of PKA on CGRP Promoter Activity in DMS 153 Cells

Rat CGRP Promoter Activity. When transfected into rat primary trigeminal ganglion cultures, the expression vector containing the gene for protein kinase A or PKA has been shown to have a stimulatory effect on CGRP promoter activity (Durham and Russo 1999). To evaluate the stimulatory effects of overexpressing PKA on the rat
promoter in human DMS cells, cells were cotransfected with a PKA expression vector, and either the entire rat CGRP promoter-luciferase reporter or the CRE-luciferase reporter. PKA significantly stimulated both the rat promoter (>20-fold) and the CRE reporter (~15-fold) (Figure 18).

**Human CGRP Promoter Activity.** To compare human CGRP promoter activity to rat CGRP promoter activity, DMS cells were cotransfected with the human CGRP promoter-luciferase reporter and a PKA expression vector. The human promoter sequence contains many regulatory sites not found in the rat promoter. For example, the human promoter contains 2 CRE sites instead of just one as found in the proximal region of the rat promoter (Figure 19A). Similar to that of the rat promoter, the human promoter was stimulated by PKA (>15-fold) (Figure 19B). As a control, DMS cells were cotransfected with a minimal human promoter-luciferase construct that contains the TATA box but no other known regulatory sites, and a PKA expression vector. PKA did not stimulate minimal promoter activity (Figure 19B).

**Signal Transduction Pathway Activity in DMS 153 Cells**

**CREB.** To investigate signal transduction pathway activity in the DMS 153 cell line, cells were transiently transfected with transcription factor reporter plasmids and plasmids overexpressing upstream activators within the signal transduction pathway. Relatively low CREB reporter activity (4373 light units/10 µg protein) was greatly stimulated by PKA (>70-fold) (Figure 20).

**Elk1.** Unstimulated Elk1 activity was low (4683 light units/10 µg protein). However, when cotransfected with vectors expressing the MAP kinase enzymes MEK1 or MEKK, luciferase activity was greatly increased (>400-fold) (Figure 21).
**cJun.** Like Elk1, unstimulated cJun activity was low (5947 light units/10 µg protein). MEKK moderately stimulated activity (>6-fold), while MEK1 had no stimulatory effect (Figure 22).

**ATF2.** Overexpression of MEK1, MEK3, or MEK 6 did not stimulate basal ATF2 reporter activity (16,149 light units/10 µg protein). In contrast, MEKK greatly stimulated ATF2 activity (>100-fold) (Figure 23).

**CHOP.** Under my culture conditions, unstimulated levels of CHOP are much higher than other MAP kinase–responsive transcription factors. Neither MEK1 nor MEK6 significantly stimulated constitutively high CHOP activity (757,618 light units/10 µg protein) (Figure 24). MEK3 only slightly, although significantly, stimulated CHOP reporter activity (~0.5-fold) (Figure 24).

**NF-κB.** Basal NF-κB reporter activity (19,316 light units/10 µg protein) was not significantly stimulated by MEK6, MEK1, or MEK3. However, similar to cJun and ATF2 stimulation results, NF-κB activity was strongly stimulated by MEKK (>40-fold) (Figure 25). Over expression of PKA resulted in moderate stimulation of NF-κB (~9-fold) (Figure 25).
Figure 3: DMS 153 cells grown on various matrix substrates.  

A-E, Day 1 DMS cells on plastic, collagen, fibronectin, laminin, or poly-D-lysine, respectively.  

F-J, Day 4 DMS cells on plastic, collagen, fibronectin, laminin, or poly-D-lysine, respectively.  

K-O, Day 7 DMS cells on plastic, collagen, fibronectin, laminin, or poly-D-lysine, respectively.
Figure 4: Day 1 DMS 153 cells grown on PDL-coated flasks in media with increasing concentrations of BDNF, NGF, or NT3. A, Control. B-D, DMS cells with 1ng/ml of BDNF, NGF, or NT3, respectively. E-G, DMS cells with 10ng/ml of BDNF, NGF, or NT3, respectively. H-J, DMS cells with 100ng/ml of BDNF, NGF, or NT3, respectively.
Figure 5: Day 6 DMS 153 cells grown on PDL-coated flasks in media with increasing concentrations of BDNF, NGF, or NT3.  

A, Control.  
B-D, DMS cells with 1ng/ml of BDNF, NGF, or NT3, respectively.  
E-G, DMS cells with 10ng/ml of BDNF, NGF, or NT3, respectively.  
H-J, DMS cells with 100ng/ml of BDNF, NGF, or NT3, respectively.
Figure 6. Expression of CGRP in DMS 153 cells. A and D, Photomicrograph of DMS cells viewed with phase contrast microscopy 48 h after plating on poly-D-lysine coated coverslips. B and E, The same field of cells stained with DAPI to identify nuclei. C and F, DMS cells visualized after immunostaining with anti-CGRP antibodies and FITC-conjugated anti-rabbit IgG antibodies. Panels A-C were taken at 100X while panels D-F were taken at 1000X magnification.
Figure 7: Expression of SNAP-25, TNFR1, or TRPV1 in DMS 153 cells. A-C, DMS cells stained with DAPI. D-F, The same field of cells photographed after immunostaining with anti-SNAP-25, anti-TNFR1, or anti-TRPV1 and FITC-conjugated IgG antibodies, respectively.
Figure 8: Expression of 5-HT_{1B} and 5-HT_{1D} in DMS 153 cells.  

*A and B*, DMS cells stained with DAPI. *C and D*, The same field of cells photographed after treatment with anti-5-HT_{1B}, or 5-HT_{1D} and FITC-conjugated IgG antibodies, respectively.
Figure 9. DMS 153 no primary antibody controls. A, D, and G, Photomicrograph of DMS cells viewed with phase contrast microscopy 48 hrs after plating on poly-D-lysine coated coverslips. B, E, and H, Same field of cells stained with DAPI. C, F, and I, DMS cells immunostained with FITC-conjugated anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG, respectively.
Fig 10. Stimulation of CGRP release from DMS 153 cells. The relative amount of CGRP secreted into media per h from untreated cells or cells treated with 60 mM KCl, 2 µM capsaicin, 40 ng TNF-α, 2 µM SNAP, protons (pH 5.5) or SNAP (2 µM) and protons. The mean basal rate of CGRP secretion was 19.8 pg/h/well +/- 4.1 SEM. The secretion rate for each condition was normalized to the basal rate for each well. n > 3, *p < 0.01, **p < 0.01, #p < 0.01, and ##p < 0.01 when compared to CON.
Fig 11. Unstimulated release of CGRP from DMS 153 cells remained unchanged during experimental treatments. The amount of CGRP secreted into media (pg/h/well) from untreated DMS cells 1 h prior to treatment (Basal) and 1 h after treatment (Experimental). n > 10. p >0.8 when compared to Basal.
Fig 12. BoNT-A does not repress unstimulated CGRP secretion from DMS 153 cells. The relative amount of CGRP secreted into media per h from untreated cells or cells pretreated with BoNT-A for 24 h. The mean basal rate of CGRP secretion was 14 pg/h/well +/- 2.85 SEM. The secretion rate for each condition was normalized to the basal rate for each well. n > 4, p > 0.1 when compared to CON.
Fig 13. BoNT-A represses capsaicin-stimulated CGRP secretion from DMS 153 cells. The relative amount of CGRP secreted into media per h from untreated cells or cells pretreated for 24 h with BoNT-A (3.1 units) before the addition of 2µM capsaicin (CAP). The mean basal rate of CGRP secretion was 10.8 pg/h/well +/- 3.3 SEM. The secretion rate for each condition was normalized to the basal rate for each well. n > 4, *p < 0.005 when compared to CON, **p < 0.04 when compared to CAP-stimulated levels.
**Fig 14.** BoNT-A represses NO-stimulated CGRP secretion from DMS 153 cells. The relative amount of CGRP secreted into media per h from untreated cells or cells pretreated for 24 h with BoNT-A (3.1 units) before the addition of 2μM SNAP. The mean basal rate of CGRP secretion was 13.2 pg/h/well +/- 3.0 SEM. The secretion rate for each condition was normalized to the basal rate for each well. n > 4, *p < 0.004 when compared to CON, **p < 0.01 when compared to NO-stimulated levels.
Fig 15. BoNT-A does not repress NO + proton-stimulated CGRP secretion from DMS 153 cells. The relative amount of CGRP secreted into media per h from untreated cells or cells pretreated for 24 h with BoNT-A (3.1 units) before the addition of 2µM SNAP in pH 5.5-buffered HBS. The mean basal rate of CGRP secretion was 15.6 pg/h/well +/- 3 SEM. The secretion rate for each condition was normalized to the basal rate for each well. n > 4, *p < 0.03 when compared to CON, **p > 0.45 when compared to NO+pH-stimulated levels.
Figure 16: Effect of BoNT-A on DMS 153 cell viability. Cells were left either untreated (A) or treated with 1.6 units BoNT-A (B) or 3.1 units BoNT-A (C) for 24 h. Cell viability was determined by trypan blue exclusion. Arrows identify cells stained with trypan blue that are no longer viable. Cell viability was similar for all conditions (>90%).
Figure 17. Rat CGRP promoter fragments, but not the 18 bp+A mutations or TK fragments enhance reporter activity in DMS 153 cells. A, Schematic of the rat CGRP-luciferase construct showing the TATA box, proximal cAMP-response elements, and the distal enhancer containing a cell-specific 18 bp element. B, DMS cells were transfected with the rCGRP-luciferase reporter construct or various fragments of the rCGRP-luciferase reporter construct (Bgl-luciferase, 18 bp-luciferase, 18 bp+A mutation-luciferase, ΔBAM-luciferase, CRE-luciferase, TK-luciferase), and reporter activity measured after 24 h after transfection. The mean light units/9 X 10^5 cells, normalized to cotransfected β-galactosidase activity, are shown.

Figure 17. Rat CGRP promoter fragments, but not the 18 bp+A mutations or TK fragments enhance reporter activity in DMS 153 cells. A, Schematic of the rat CGRP-luciferase construct showing the TATA box, proximal cAMP-response elements, and the distal enhancer containing a cell-specific 18 bp element. B, DMS cells were transfected with the rCGRP-luciferase reporter construct or various fragments of the rCGRP-luciferase reporter construct (Bgl-luciferase, 18 bp-luciferase, 18 bp+A mutation-luciferase, ΔBAM-luciferase, CRE-luciferase, TK-luciferase), and reporter activity measured after 24 h after transfection. The mean light units/9 X 10^5 cells, normalized to cotransfected β-galactosidase activity, are shown.
Figure 18. The expression vector, PKA enhances rat CGRP-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with either rCGRP-luciferase, CRE-luciferase, or TK-luciferase reporters and the expression vector, PKA. Reporter activity was measured after 24 h. The data in each experiment were normalized to either the rCGRP-luciferase, CRE-luciferase or TK-luciferase reporter activity, 27,696 +/- SEM 4250; 24,233 +/- SEM 8038; 9339 +/- SEM 1193 (light units/10µg protein), respectively. n > 5, * p < 0.03 and **p < 0.03 when compared to CON.
Figure 19. The expression vector, PKA enhances hCGRP-luciferase activity in DMS 153 cells. A, Schematic of the human CGRP-luciferase reporter construct showing the TATA box (-25 to -29), proximal cAMP-response elements (-104 to -109 and -253 to -260), and the distal enhancer that contains a cell-specific 18 bp element (-826 to -843). B, DMS cells were transfected with the CGRP-luciferase reporter gene (control, CON) or the minimal human promoter (MHP) containing the TATA box. Cells were co-transfected with the constitutively active expression vector, PKA, and reporter activity measured after 24 h. The data in each experiment were normalized to the CGRP-luciferase reporter activity, 142,740 +/- SEM 11,705 (light units/10µg protein) or the MHP-luciferase reporter activity, 13,378 +/- SEM 6602 (light units/10 µg protein) only. n > 6, *p < 0.005 when compared to CON.
Figure 20. The PKA expression vector enhances CREB-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with a CREB-luciferase reporter gene and the PKA expression vector and reporter activity measured after 24 h. The data were normalized to the mean unstimulated CREB activity, 4373 +/- SEM 1704 (light units/10 µg protein). n > 4, p < 0.03 when compared to CON.
Figure 21. Expression vectors MEK1 and MEKK enhance Elk1-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with Elk1-luciferase reporter and MEK1 or MEKK expression vectors and reporter activity measured after 24 h. The data were normalized to the mean unstimulated Elk1 activity, 4683 +/- SEM 1434 (light units/10 µg protein). n > 4, *p < 0.05 and **p < 0.05 when compared to CON.
Figure 22. The expression vector MEKK, but not MEK1 enhances c-Jun-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with c-Jun-luciferase reporter and MEK1 or MEKK expression vectors and reporter activity measured after 24 h. The data were normalized to the mean unstimulated c-Jun activity, 5947 +/- SEM 2307 (light units/10 µg protein). n > 6, *p < 0.02 when compared to CON.
Figure 23. The expression vector MEKK, but not MEK, MEK1, MEK3, or MEK6 enhances ATF2-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with ATF2-luciferase reporter and MEK1, MEK3, MEK6, or MEKK expression vectors and reporter activity measured after 24 h. The data for each experiment were normalized to the mean unstimulated ATF2 activity, 16,149 +/- SEM 8886 (light units/10 µg protein). n > 8, *p < 0.01 when compared to CON.
Figure 24. The expression vector MEK3, but not MEK6 or MEK1 enhances Chop-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with Chop-luciferase reporter and MEK1, MEK3, or MEK6 expression vectors and reporter activity measured after 24 h. The data for each experiment were normalized to the mean unstimulated Chop activity, 757,618 +/- SEM 85,869 (light units/10 µg protein). n > 6, *p < 0.03 when compared to CON.
Figure 25. The expression vector MEKK, but not MEK1, MEK3, MEK6, or PKA enhances NFκB-luciferase reporter activity in DMS 153 cells. Cells were co-transfected with NFκB-luciferase reporter and MEK1, MEK3, MEK6, MEKK, or PKA expression vectors and reporter activity measured after 24 h. The data for each experiment were normalized to the mean unstimulated NFκB activity, 19,316 ± SEM 2734 (light units/10 µg protein). n > 6, *p < 0.02 and **p < 0.02 when compared to CON.
DISCUSSION

Given the importance of CGRP in human disease, the primary goal of this study was to determine whether DMS cells could be used as a human neuronal cell model to study CGRP gene expression. It had previously been reported that DMS cells express CGRP (Sorenson et al. 1983), but very little information was available about whether DMS cells expressed other neuronal specific proteins. Hence, I initially wanted to further characterize this cell line by determining which neuronal proteins are expressed by DMS cells. However, my initial studies focused on optimizing the growth conditions for these cells since I had difficulty maintaining them in culture following the guidelines provided by ATCC. In fact, it took several attempts before I was able to propagate the cells to have enough to preserve samples in liquid nitrogen for long-term storage.

There are several key variables to consider when optimizing growth conditions for a particular cell line. These include temperature, CO₂ concentration (which maintains physiological pH), growth medium, substrate, and inclusion of specific growth factors. Based on the recommendation of ATCC, the cells were initially incubated at 37°C with 7% CO₂ in Waymouth’s supplemented media as described in the material and methods section. The cells were plated at a high density (>60%) on tissue culture treated plastic dishes. However, the cells grew very slowly and formed large clumps of cells. I then began testing different types of substrates since cellular morphology is often dependent on the extracellular matrix. I found that the substrate which allowed for maximum growth as a uniform monolayer of cells with a neuronal morphology was poly-D-lysine or PDL. This substrate was superior to other extracellular matrices, including tissue culture plastic, human fibronectin, laminin, or collagen type I. Given the neuronal
phenotype exhibited by DMS cells, it was surprising that laminin did not provide a suitable substrate even though it has been reported to promote neuronal differentiation and process formation (Sephel et al. 1989; Weeks et al. 1990). Unlike other neuronal-like cell lines, addition of neuronal growth factors did not promote a neuronal phenotype (Brodski et al. 2000). Importantly, under my culture conditions, almost all DMS cells (>95%) express CGRP.

Using immunocytochemistry, I discovered that DMS cells also express several other neuronal specific proteins. For example, DMS cells express the TRPV1 receptor, which is activated by capsaicin (the hot ingredient in chili peppers) (Liapi and Wood 2005), acidic pH (protons) (Steen et al. 1992), and elevated temperatures (>40°C) (Caterina et al. 1997). Neurons that express TRPV1 are nociceptive, which means they are involved in transmitting painful stimuli from the peripheral tissue to the CNS. DMS cells also express TNFR1 receptors that are activated by the inflammatory molecule TNF-α. Levels of TNF-α are elevated in painful joint conditions including TMJ pathology and arthritis (Nordahl et al. 2000; Fu et al. 1995). In addition, DMS cells express 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} receptors. These serotonin type 1 receptors are targets for the triptan drugs commonly used to treat migraine headache (Ferrari and Saxena 1993; Edvinsson and Goadsby 1994; Buzzi et al. 1995). It is significant to note that all of these receptors have previously been shown to regulate rat CGRP gene expression in trigeminal ganglion neurons (Durham et al. 1997; Durham and Russo 2002; Bellamy et al. 2006).

Based on my results, DMS provides a novel human cell line to study CGRP gene expression by inflammatory and anti-inflammatory mediators. CGRP is thought to play an important role in a number of chronic painful disorders including migraine, allergic
rhinitis (Bellamy et al. 2006), TMJ pathology (Sato et al. 2004), arthritis (Appelgren et al. 1993), and most recently cancer (Suzuki et al. 2006). Previous studies have shown that CGRP secretion from primary afferent nerve fibers is increased in response to chemical depolarization by KCl, capsaicin, and nitric oxide/pH 5.5 (protons) (Durham et al. 2004b; Bellamy et al. 2006). NO and proton stimulated release of CGRP is particularly relevant to migraine since both are released in response to cortical spreading depression, which can activate trigeminal sensory neurons causing both peripheral and central CGRP release (Scheller et al. 1992; Buzzi and Moskowitz 2005). Peripheral release leads to neurogenic inflammation in the meninges (Limmroth et al. 2001), while central release leads to pain, central sensitization, and allodynia (Bennett et al. 2001). Thus, DMS cells can be used to screen novel drugs thought to target CGRP expression. In addition, the DMS 153 cell line could be used to study receptor coupling of the 5-HT\textsubscript{1B} and 5-HT\textsubscript{1D} serotonin receptors. Because these receptors are targets for the anti-migraine triptan drugs, the use of this model could have important implications for understanding migraine therapies. Although current research has elucidated many of these mechanisms, the work has been done primarily in animal models such as rat (Durham et al. 1997) and cat (Storer and Goadsby 1999). The DMS 153 cell line has the distinction of being the only human neuronal-like model available for study.

Similar to results involving rat trigeminal nerves and the rat neuronal-like cell line CA77, BoNT-A repressed NO and capsaicin stimulated CGRP secretion in DMS cells (Durham et al. 2006; Bellamy et al. 2006). BoNT-A, as previously discussed, prevents neurotransmitter release by interfering with the elaborate docking mechanisms involving SNAP-25 and other SNARE proteins at the presynaptic cleft (Blasi et al. 1993). Since
SNAP-25 was localized to the surface of the DMS cells, I hypothesize that similar mechanisms are active in this cell line. Recent preliminary studies in my laboratory using the DMS 153 cell line have shown that BoNT-A decreases CGRP gene expression by inhibiting CGRP promoter activity (data not shown). Thus, it appears that BoNT-A can regulate both the synthesis and release of CGRP, providing evidence that DMS cells can monitor the vesicle pool size and slow synthesis. This type of coupled regulation has been reported previously in other cells (Durham et al. 1997). However, this is the first evidence that BoNT-A can repress the stimulated release of CGRP from a human cell line. Because the DMS 153 cell line is of human origin, use of this model would provide more clinically significant results on human CGRP expression than any other model currently available.

Interestingly, BoNT-A did not block the stimulated release of CGRP from DMS cells in response to both NO and protons. This finding is in agreement with data obtained using primary cultures of rat trigeminal ganglia. Studies in my laboratory have shown that while pretreatment with BoNT-A can inhibit CGRP secretion from trigeminal neurons in response to KCl, NO, and capsaicin, stimulation with protons alone or in combination with NO is not blocked by BoNT-A (data not shown). This raises the important question of how this is possible? One possible explanation is that proton stimulation activates a different vesicle pool within the cell that is secreted via a SNARE (SNAP-25) independent mechanism. Clearly, more studies are required to understand the signaling mechanisms utilized by cells in response to proton stimulation.
Future Studies

DMS cells should provide an excellent model to study CGRP promoter activity as well as signal transduction pathways. Data from my studies demonstrate that the activity of both the rat and human promoters can be studied under basal and stimulated conditions. The advantage of using the rat promoter sequence is that the regulatory sequences have already been determined in rat cells and thus, results using the DMS 153 cell line can be compared. My results showed that the 18 bp region, which contains the cell-specific enhancer region, and proximal CRE are active in DMS cells. These data are suggestive that similar transcription factors are expressed in this human cell line that are involved in regulating CGRP promoter activity. Furthermore, this cell line provides the first opportunity to identify the key regulatory sites within the human CGRP promoter required for basal and stimulated activity in a neuronal-like cell. This information could be very important clinically since there are many more regulatory sites identified in the human promoter compared to the rat. Thus, it is likely that control of the human CGRP gene may be more complex than that of the rat. Finally, since DMS cells exhibit low basal level expression of several MAP kinases, this cell line should provide a good model to study signal transduction pathways activated by inflammatory stimuli and inhibited by serotonergic anti-migraine drugs, steroids, and other novel anti-inflammatory agents.
REFERENCES CITED


