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Investigation of Nocifensive and Cellular Effects of Dihydroergotamine in a Model of Chronic Migraine

Jennifer Elise Denson

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**INVESTIGATION OF NOCIFENSIVE AND CELLULAR EFFECTS OF
DIHYDROERGOTAMINE IN A MODEL OF CHRONIC MIGRAINE**

A Masters Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Jennifer E. Denson

July 2015

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INVESTIGATION OF NOCIFENSIVE AND CELLULAR EFFECTS OF DIHYDROERGOTAMINE IN A MODEL OF CHRONIC MIGRAINE

Biology

Missouri State University, July 2015

Master of Science

Jennifer E. Denson

ABSTRACT

The goal of my study was to investigate the nocifensive behavioral and cellular effects of dihydroergotamine (DHE) and sumatriptan in a model of sustained trigeminal activation. Trigeminal nerves provide sensory innervation to the head and face and their activation has been implicated in migraine pathology. The most commonly prescribed medications for migraine treatment are the triptan class of drugs. However, triptans are not an effective therapy for all migraine patients and some develop triptan resistance. Ergot-derivatives, including DHE, could be a potential alternative for this subpopulation of patients. To cause activation of the trigeminal system, adult male Sprague Dawley rats were given an inflammatory injection of complete Freund's adjuvant (CFA) or saline as a vehicle control in the temporomandibular joint capsule. Three and four days post-CFA-injection, intraperitoneal injections of DHE, sumatriptan, or vehicle control were delivered. I found that DHE, but not sumatriptan, was able to transiently inhibit CFA-mediated increases in nocifensive withdrawal behavior. Within the spinal cord, DHE was shown to inhibit CFA stimulated levels of the signaling protein PKA, which is known to promote central sensitization, and Iba1, a marker of activated microglial cells. However, both DHE and sumatriptan were able to inhibit stimulated levels of GFAP, a protein used as a marker of activated astrocytes. Results from my study provide evidence that DHE, but not sumatriptan, can inhibit nociception caused by prolonged activation of trigeminal neurons and the inhibitory effect is likely to involve suppressing development of central sensitization. I propose that DHE may be therapeutically beneficial by blocking ongoing peripheral and central sensitization as characteristic of frequent episodic and chronic migraine.

KEYWORDS: migraine, trigeminal, central sensitization, inflammation, nociception.

This abstract is approved as to form and content

Paul L. Durham, Ph.D.
Chairperson, Advisory Committee
Missouri State University

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Approved:

Paul Durham, Ph.D.

Kyoungtae Kim, Ph.D.

Laszlo Kovacs, Ph.D.

Julie Masterson, PhD: Dean, Graduate College

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INTRODUCTION

Pathology of Migraine

Migraine is characterized by a severe, debilitating headache that can be accompanied by somatic symptoms such as nausea, vomiting, sensitivity to light, sound, or odors, and visual disturbances known as auras. Migraine affects 16.6-22.7% of adults in the United States, occurring three times more often in females than males (Schwedt, 2014). Chronic migraine (defined as having a headache for 15 days a month for the previous three months) comprises 7.7% of all migraine cases and, as compared with episodic migraine, results in significantly more severe disability for the patient as well as a higher co-morbidity with depression and anxiety (Smitherman et al., 2013). Risk factors for episodic migraine progressing into chronic migraine include caffeine use, medication overuse, obesity, snoring or sleep apnea, psychiatric conditions, and stressful life events (Dodick, 2009).

Historically, migraine has been viewed as a vascular disease but research in the last few decades suggests that migraine has a strong neurological basis and it is now believed that sensitization and activation of the trigeminal nerve, the fifth cranial nerve, which provides sensory innervation of much of the head and face, underlies migraine pathology (Pietrobon and Moskowitz, 2013). One proposed mechanism for this activation begins with cortical spreading depression (CSD), a period of enhanced neuronal activity that moves across the cortex, followed by a period of very low cortical neuronal activity (Xu et al., 2010). The enhanced neuronal activity results in excessive extravasation of proteins and ions into the perivascular space that triggers vasodilation of

the meninges, including the middle meningeal artery (MMA), and subsequently causes excitation of trigeminal afferent neurons. Upon activation, these neurons release inflammatory neuropeptides like calcitonin gene-related peptide (CGRP) and substance P into the meninges as well as transmitting nociceptive signals centrally to the spinal trigeminal nucleus (STN). Within the spinal cord, these neuropeptides activate parasympathetic pathways to release additional vasoactive and inflammatory molecules into the meninges, further increasing blood flow and sustaining neurogenic inflammation (Bolay et al., 2002). These events can lead to both peripheral and central sensitization, which are characterized by the lowering of the stimulus threshold required to elicit a neuronal response. Sensitization of nociceptive neurons disproportionately increases migraine pain (hyperalgesia), causes pain responses to normally non-painful stimuli (allodynia), and prolongs the migraine attack (Dodick and Silberstein, 2006).

The Trigeminal System

The afferent sensory neurons of the trigeminal nerve are pseudounipolar with their cell bodies located in the trigeminal ganglion (TG). These neurons have peripheral processes that extend to the head and face and central processes that synapse with second order neurons in the spinal trigeminal nucleus (STN) of the upper spinal cord (Fig. 1). These fibers are either lightly myelinated A δ fibers or unmyelinated C fibers that respond to thermal, mechanical, and chemical stimuli to relay nociceptive information to the central nervous system (CNS) (Devor, 1999, Rathee et al., 2002). These peripheral neurons are associated with several types of glial cells including Schwann cells, which provide the myelination that increases conduction speeds, as well as satellite glial cells,

which are found in close association with the neuronal cell bodies and help to modulate the excitability state of the trigeminal neurons (Thalakoti et al., 2007, Durham and Garrett, 2010b). A single neuronal cell body is surrounded by several satellite glia, forming a functional unit that develops during the first few weeks of neonatal development (Hanani, 2005, Durham and Garrett, 2010a). Importantly, increased paracrine and gap junction signaling between neurons and glia facilitates and maintains nociceptor sensitization and is thus implicated in the underlying pathology of migraine (Durham and Garrett, 2010b).

As the name implies, the TG has three branches that include the ophthalmic, the maxillary, and the mandibular (Lazarov, 2002) (Fig. 2). The ophthalmic branch (V1) contains only sensory neurons and serves many structures of the eye, the eyebrow and forehead region, and the upper sinuses (Shankland, 2001a). The maxillary branch (V2) is also only sensory in function and innervates all the structures surrounding the maxillary bone including the lower eyelid, skin of the midfacial region, the mucus membrane of the nasopharynx, the maxillary sinus, the soft palate and the roof of the mouth (Shankland, 2001b). The mandibular branch (V3) is the largest of the three trigeminal nerve branches and contains both sensory fibers that innervate the lower jaw and motor fibers that innervate and control the muscles of mastication (Shankland, 2001c).

The STN receives fibers from all three branches of the TG and contains three subnuclei: the oralis, interpolaris, and caudalis (Bereiter et al., 2000). The most inferior of these, the trigeminal nucleus caudalis (TNC) contains neurons involved in processing nociception from the head and face and is thus implicated in migraine pathology (Akerman et al., 2011). The second order neurons located in the outer laminae in the TNC

carry nociceptive signaling received from the TG and transmit it to the thalamus and cortex (Shankland, 2000). The TNC contains a large glial population, including astrocytes and microglia. In addition to rapid proliferation following injury, activated glia produce and release pro-inflammatory molecules that modulate the function and excitability of nearby neurons by changing the expression of ion channels and receptors within the plasma membrane (Guo et al., 2007). Throughout the CNS, astrocytes form networks with each other connected by gap junctions, primarily formed by connexin 43, which allow the exchange of Ca^{2+} and other small molecules. Astrocytes have extensive contact with neuronal synapses as well as cerebral blood vessels, leading to a “tripartite synapse” theory which suggests that synapses are made up of pre- and post- synaptic neuronal processes as well as astrocytic processes, allowing the astrocyte network to modulate neuronal signals (Ji et al., 2013). Microglia, when activated, proliferate and migrate, undergoing morphological changes that allow them to become phagocytic (Ohsawa et al., 2004).

Proteins Implicated in Inflammation

Migraine and other neuroinflammatory pathologies involving nociception are associated with an increased expression of mitogen activated protein (MAP) kinases, which activate signaling cascades in order to alter gene expression and release inflammatory mediators. MAP kinase pathways are involved in a diverse range of cell functions, including differentiation, division, movement, and death, and are thus common research targets (Schaeffer and Weber, 1999). Two of the most well studied groups of MAP kinases are extracellular signal-regulated kinase (ERK1/2) and p38. These proteins

are activated via phosphorylation and then translocate to the nucleus where they activate transcription factors that directly regulate expression of inflammatory genes involved in production of cytokines, chemokines, and nitric oxide (Raman et al., 2007).

ERK is strongly activated by nerve growth factor (NGF), which maintains the nociceptor phenotype and also sensitizes nociceptors and ion channels (Nicol and Vasko, 2007). It has also been suggested that ERK plays an important role in neural plasticity, which can lead to tactile allodynia or movement-induced pain, both of which are important features of chronic pain (Ji et al., 2009). ERK may also be involved in long term potentiation (Xin et al., 2006), an important feature of central sensitization (Ji et al., 2003).

The signaling protein p38 is activated by cellular stress and proinflammatory cytokines (Ji et al., 2009). Once activated, p38 translocates to the nucleus and induces the phosphorylation of transcriptional factors that increase the biosynthesis of inflammatory mediators such as TNF- α and IL-1 β (Ji and Woolf, 2001). In addition, p38 can sensitize nociceptors posttranslationally, a much faster process than acting on gene expression (Jin and Gereau, 2006).

Another signaling cascade implicated in the production of inflammatory mediators involves activation of adenylyl cyclase-cAMP-protein kinase A (PKA) pathway by numerous inflammatory mediators including the prostaglandin E₂ (PGE₂) receptor, the adenosine receptor, and the serotonin (5-hydroxytryptamine or 5-HT) receptors (Taiwo and Levine, 1991). PKA then modulates and enhances the tetrodotoxin-resistant voltage-gated sodium currents that are found primarily in nociceptors, leading to

sensitization of those nociceptors (Aley and Levine, 1999) and hyperalgesia (Fitzgerald et al., 1999).

The class of P2X purinoceptors mediate vasoconstriction and contraction of visceral smooth muscle and are activated by α,β -methylene ATP (Burnstock, 2000). ATP functions in neuronal signaling by eliciting both fast- and slow-inactivating inward currents which depolarizes neurons (Xu and Huang, 2002). The subtype P2X₃ is located in both sensory neurons and glia and is expressed in structures associated with pain signal processing, and thus has been implicated in cranial nociception, particularly through its association with Transient Receptor Potential V1 (TRPV1), which functions as a signal integrator downstream of several pro-inflammatory G-protein coupled receptors (Saloman et al., 2013).

The calcium binding adapter protein Iba1 is often utilized as a marker of activated microglia within the CNS and a marker of phagocytes within the PNS (Ohsawa et al., 2004). Elevated levels of Iba1 are associated with inflammatory events such as increased proliferation and migration as well as an increase in the production and secretion of pro-inflammatory cytokines and chemokines (Ito et al., 1998), all of which are required for repairing cellular damage (Kreutzberg, 1996). Microglia are often described as macrophage-like as they rapidly proliferate and assume immune roles in both the central and peripheral nervous systems after injury (Ji et al., 2013) and have been shown to play a role in inflammatory pain (Zhou et al., 2008).

Glial fibrillary acidic protein (GFAP), an intermediate filament protein, is expressed in astrocytes, which are specialized glial cells that perform multiple functions within the central nervous system including formation of blood brain barrier and

modulating synaptic connections between neurons. With respect to nociception, upregulation of GFAP expression correlates with the state of reactive astrogliosis, or the astrocyte's response to injury and as such is used as a biomarker of these cells (Anderson et al., 2014). Importantly, changes in GFAP are associated with morphological and biochemical events within astrocytes, which are found in close proximity to neurons and help to regulate the local extracellular environment and thus, the state of neuronal excitability.

Migraine Therapies – Dihydroergotamine and Sumatriptan

Ergotamine has been used for the clinical treatment of migraine since the 1920's, but its medicinal effects have been known and utilized since the middle ages (Hofmann, 1978). The original source of ergot was the ascomycetous fungus *Claviceps purpurea*, an obligate pathogen that infects cultivated cereals, primarily rye. When bread made from contaminated grains is consumed, the parasite can cause ergotism (also known as St. Anthony's Fire), a neurological disease that can manifest as either convulsions and hallucinations or gangrene and necrosis of the extremities, depending on the strain of ergot contamination (De Costa, 2002). However, its vasoconstrictive effects were often utilized by midwives to speed labor and manage post-partum hemorrhages. Ergot extract was first recommended for migraine treatment in 1868, when it was thought that migraine was due to hypersympathetic activity, and ergotamine was first isolated in 1918, followed by many clinical trials showing its efficacy throughout the 1920's and '30's (Tfelt-Hansen and Koehler, 2008). Ergotamine and its derivatives were then the standard anti-migraine drug until the development of the triptan class of drugs in the 1990's and even

after the advent of triptans, ergot derivatives may be chosen over a triptan due to their ability to lower the rate of headache recurrence (Tfelt-Hansen et al., 2000).

Dihydroergotamine (DHE) was synthesized in 1943 and, though originally meant as an anti-hypertensive agent, was first used to treat migraine in 1945. DHE was shown to be effective for the treatment of both intermittent and intractable migraine in 1986, with less nausea and vomiting as well as fewer vasoconstrictor effects than ergotamine (Silberstein and McCrory, 2003).

Dihydroergotamine belongs to the tetracyclic ergoline family and differs from ergotamine by the hydrogen found at the 10 position on the tetracyclic ergoline ring (Fig. 3) (Maurer and Frick, 1984). DHE acts as an agonist upon multiple 5-HT (serotonin) receptor subtypes, α -adrenoreceptors, and dopamine receptors (Perrin, 1985, Cook et al., 2009, Dahlof and Maassen Van Den Brink, 2012), with more receptors activated when it is administered via IV vs. an inhaled form (MAP0004). DHE has a much longer dissociation half-life from serotonin receptors than sumatriptan (Kori et al., 2012), possibly contributing the lower rate of headache recurrence seen with ergot derivatives.

Dihydroergotamine has been found to inhibit second-order trigeminocervical neurons in experiments with cats, similar to other proven anti-migraine agents including several triptans (Hoskin et al., 2004). DHE has also been found to inhibit central sensitization both when administered at the same time as a prolonged inflammatory stimulus as well as when administered 2.5 hours after the stimulus (Oshinsky et al., 2012). This study contrasted DHE with zolmitriptan, which could not inhibit central sensitization in that model.

Developed in the early 1990's as a specific anti-migraine therapy, sumatriptan was the first of the triptan class of drugs designed to abort an episodic migraine attack. It is a derivative of a non-ergot alkaloid, called an indole, and was initially developed as a selective constrictor of intracranial blood vessels (Moskowitz, 1993). Sumatriptan shows a high specificity for 5-HT₁ receptors, specifically the 5-HT_{1B} and 5-HT_{1D}, with a lower affinity for 5-HT_{1F} receptors (Dahlof and Maassen Van Den Brink, 2012). At concentrations above that found at clinically relevant doses, sumatriptan will display a weak affinity for 5-HT_{1A} and 5-HT_{1E} receptors. Sumatriptan has no significant activity at other 5-HT receptor subtypes or at other non-5-HT receptor types such as adrenergic, dopaminergic, or histamine receptors.

Along with their vasoconstrictive effects, sumatriptan, DHE, and other 5-HT₁ agonists are thought to work through three possible pathways. The first is through activation of the 5-HT_{1D} receptors on trigeminal nerve terminals that innervate the meningeal blood vessels, blocking the release of inflammatory neuropeptides. The second route is through activation of central 5-HT_{1B//1D//1F} receptors in the trigeminal nuclear caudalis, which blocks transmission of nociception from first order to second order neurons. The third possible mode of action is via stimulation of 5-HT_{1B//1D} receptors in the ventroposteromedial thalamus, preventing the processing of nociceptive input from second order to third order neurons within the thalamus. (Dahlof and Hargreaves, 1998, Tepper et al., 2002, Dahlof and Maassen Van Den Brink, 2012).

Goals of Study

The goal of my study was to determine the effect of DHE in an animal model which mimics trigeminal system pathology associated with chronic migraine and to compare those effects to the effects of a well-known and widely accepted migraine treatment, sumatriptan. I wanted to test the hypothesis that DHE, due to its less specific receptor-binding profile, would be more effective than sumatriptan in blocking nocifensive behaviors and then determine through immunohistochemistry which signaling pathways were most effected.

To test this hypothesis, I utilized behavioral and molecular techniques to investigate the effects of both of these drugs in an established model of chronic trigeminal nerve activation. Briefly, an inflammatory agent, complete Freund's adjuvant (CFA) was injected into the temporomandibular joint capsule to cause sustained inflammation and activation of the trigeminal system, followed by an intraperitoneal injection of each of the drugs of interest and then both nocifensive behaviors and cellular changes were examined. Results from this study suggest that DHE is more effective than sumatriptan in blocking nocifensive responses and does so by uniquely attenuating cellular changes in nociceptive pathways. Based on my findings, I propose that DHE could be a useful alternative for those migraneurs dissatisfied or unresponsive to triptans.

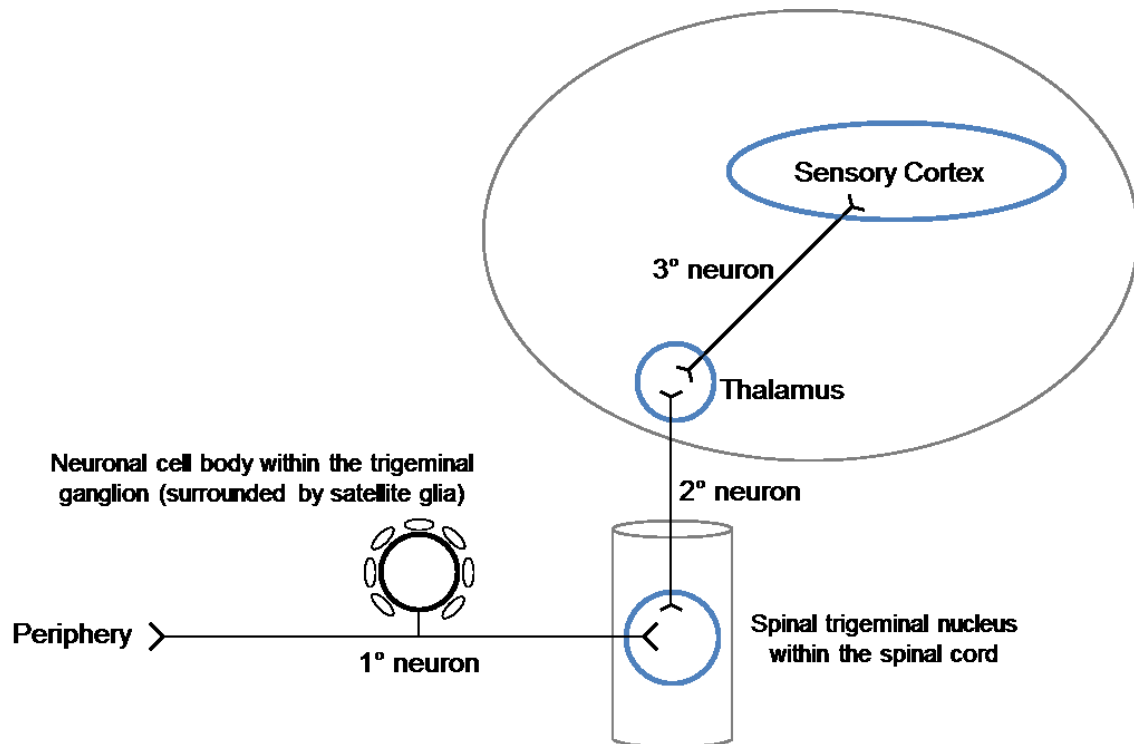


Figure 1. Nociception pathway through the trigeminal system. From the periphery, signals are transmitted up primary neurons to the STN in the spinal cord, where the primary neurons synapse with the secondary neurons. From the STN, the signal is transmitted to the thalamus and then up tertiary neurons to the sensory cortex.

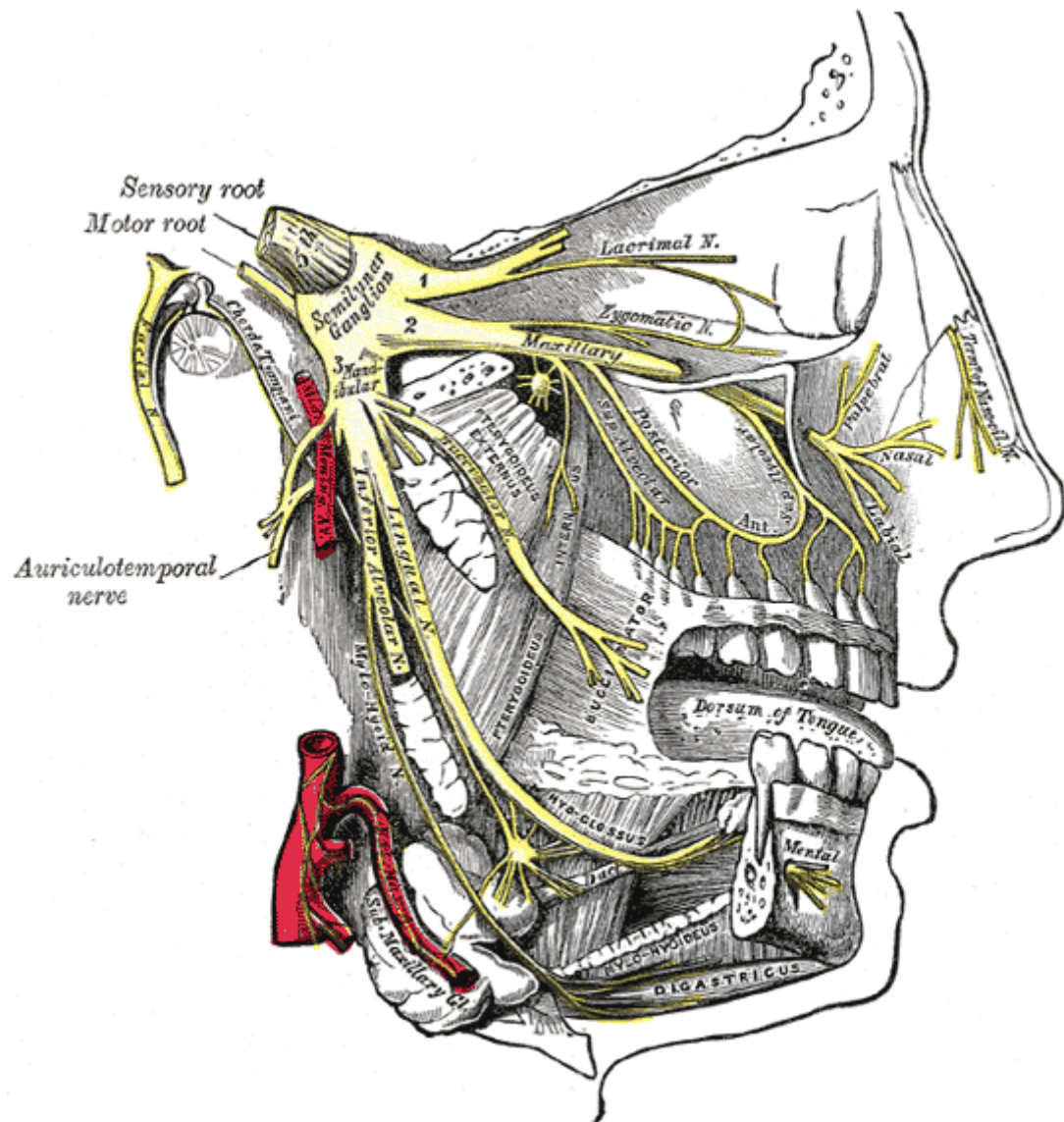


Figure 2. The trigeminal nerve. See text for details.
<https://commons.wikimedia.org/wiki/File:Gray778.png#/media/File:Gray778.png>

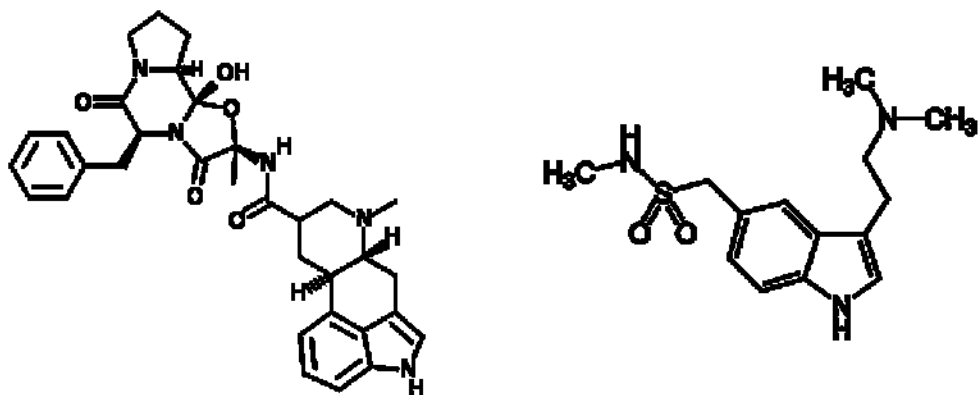


Figure 3. Chemical structures of DHE (left) and sumatriptan (right).

MATERIALS AND METHODS

Animals and Chronic Inflammation Model

All animal procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Missouri State University (protocol # 14-019.0A approved on 1/27/2014) in accordance with the guidelines established in the Animal Welfare Act and National Institutes of Health. Every effort was made to minimize animal suffering and to use the minimum number of animals. Adult male Sprague-Dawley rats (300–400 g; Charles River Laboratories, Wilmington, MA) were individually housed in clean plastic cages exposed to a 12-h light/dark cycle (light from 7:00 a.m. to 7:00 p.m.) and allowed unrestricted access to food and water.

As a model to study prolonged trigeminal nerve activation as reported in frequent and chronic migraine, some rats received bilateral injections in the temporomandibular joint capsule of 50 μ L complete Freund's adjuvant (CFA; 1:1 CFA/saline; Sigma-Aldrich, St. Louis, MO). As a control, some rats were injected only with 50 μ L of saline. Injections were administered using a 26½ G needle (Becton Dickinson, Franklin Lakes, NJ) attached to a 100 μ L glass syringe (Hamilton Company, Reno, NV) while the animals were anaesthetized via inhalation of 3-5% isoflurane (Patterson Veterinary, Devens, MA).

Three days post-injection of CFA or saline, rats were given intraperitoneal (i.p.) injections of either dihydroergotamine [DHE; Tocris Bioscience, Bristol, UK; prepared at a concentration of 50 mg/mL in dimethyl sulfoxide (DMSO) and administered at a dosage of 10 mg/kg], an equivalent volume of the vehicle DMSO (Sigma-Aldrich), or

sumatriptan (brand name Alsuma; manufactured at a concentration of 6 mg/0.5 mL and administered at a dosage of 0.3 mg/kg; Pfizer, New York, NY). See Table 1 for a summary of experimental conditions and number of animals utilized in my study.

Five days post-injection, animals were sacrificed via inhalation of CO₂ followed by decapitation and tissues were collected for molecular analysis. The animals used for behavioral testing were sacrificed in the same manner fourteen days post CFA injection. See Figure 6 for overall timeline of study.

Nocifensive Responses to Mechanical Stimuli

For behavioral testing, animals were first acclimated to the Durham Animal Holder (Ugo Basile, Varese, Italy) (Fig. 4) for five minutes on three consecutive days then allowed two days to rest undisturbed before experimental readings were begun. Acclimation involved placing the animal in the holder and gently rubbing that rat's face in the area to be tested with a plastic filament similar to the von Frey filaments so as to condition the animal to a mechanical stimulus. This minimizes false reactions due to the animal being startled when measurements are taken at later timepoints during the experiment.

Nocifensive responses were determined using a series of calibrated von Frey filaments (60, 100, and 180 g; Ugo Basile) (Fig. 5) applied in increasing force to the cutaneous area directly over the TMJ. Base line readings were taken on the day prior to CFA or saline injections and further readings were taken two hours post-injection and days 3 (prior to i.p. injection), 5, 7, 10, and 14 post-injection. Measurements were obtained between 8:00 and 10:00 a.m. (with the exception of the two hour post-injection

time-point). The researcher responsible for the direct application of the filament was blinded to the experimental conditions.

Data are reported as the mean number of positive responses (a withdrawal of the animal's head) out of five applications to each side of the face of 100 g force \pm SEM. Statistical analysis was performed using a factorial ANOVA followed by Tukey's post hoc with SPSS 21 software. Differences were considered to be significant at $p < 0.05$.

Tissue Collection, Preparation, and Immunostaining

Following sacrifice, the upper spinal cord and the trigeminal ganglia were removed. The spinal cord was trimmed at the obex and 5 mm posterior to the obex, allowing the area encompassing the spinomedullar junction (Vc/C1) transition zone, which contains the spinal trigeminal nucleus (STN), to be kept. Tissues were placed in 4% paraformaldehyde for fixation and incubated overnight at 4°C. Tissues were then cryoprotected by placing them in a 15% sucrose solution and incubated for one hour at 4°C before being moved to a 30% sucrose solution and incubated overnight at 4°C.

Tissues were quickly frozen in Optimal Cutting Temperature medium (OCT; Sakura Finetek, Torrance, CA) with the dorsal side (ganglia) or posterior side (spinal cord) facing up. Fourteen micron longitudinal (ganglia) or transverse (spinal cord) sections were taken using a cryostat (Microm HM 525, Thermo Scientific, Waltham, MA) set at -24°C and sections were mounted on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) then stored at -20°C until utilized for immunohistochemistry. Each slide contained one section of each experimental condition (Table 1) for that round.

To minimize non-specific binding of antibodies and permeabilize the tissues, sections were incubated for twenty minutes in a solution of phosphate buffered saline (1X PBS; Sigma-Aldrich) that contained 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.1% Triton-X (Fisher Scientific, Pittsburgh, PA). Slides were rinsed with PBS and incubated with primary antibodies diluted in 5% donkey serum for either three hours at room temperature or overnight at 4°C in a humidity chamber constructed using a damp paper towel, parafilm, and a pipet tip box. A summary of the antibody sources, dilutions, and incubation times is provided in Table 2. Slides were then rinsed with 0.1% Tween 20 solution (Sigma-Aldrich) and incubated with appropriate secondary Alexa antibodies diluted in PBS for one hour at room temperature. Slides were then rinsed with 0.1% Tween again, covered with Vectashield Mounting Media (H-1200) containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to co-stain cell nuclei, and covered with a glass coverslip (Fisher Scientific, Pittsburgh, PA). Slides were stored at 4°C prior to image collection.

Microscopy and Image Analysis

Images (200x) of the medullary horn of the STN and the V3 region of the TG were collected using a Zeiss AxioCam mRm camera mounted on a Zeiss Imager Z1 fluorescent microscope equipped with an ApoTome. Image acquisition and analysis was performed using Zeiss Zen Blue 2012 (Thornwood, NY). All images within a set (meaning those images from sections on a single slide that are from the same round and immunostained with the same antibody) were balanced so that the non-staining, acellular regions of the STN or the fiber tracks of the TG appeared to be the same brightness. The

images were then evaluated using a +/- system as follows: the control tissue was assigned a value of 0 and all other images within that set were compared to the level and number of cells positively stained and designated according to the following list:

- 0 = No change from control
- + = Increased expression from control (approximately 50%)
- ++ = Dramatically increased expression from control (approximately 100%)
- - = Decreased expression from control (approximately 50%)
- -- = Dramatically decreased expression from control (approximately 100%)

Table 1. Experimental Conditions and Number of Animals Utilized in Study

TMJ Injection	i.p. Injection	Behavioral <i>n</i> Number	Molecular <i>n</i> Number
Saline	DMSO	6	2
Saline	DHE	6	2
Saline	Sumatriptan	6	2
CFA	DMSO	6	2
CFA	DHE	6	2
CFA	Sumatriptan	6	2

Table 2. Summary of Antibodies and Incubation Conditions

Antibody	Company	Product #	Species	Dilution	Incubation
PKA	Epitomics	2113-1	Rabbit	1:500	3 hours, RT
P-p38	Cell Signaling Technology	4511S	Rabbit	1:200	Overnight, 4°C
P-ERK	Bioworld	BS5016	Rabbit	1:200	Overnight, 4°C
GFAP	Abcam	Ab53554	Goat	1:5000	3 hours, RT
Iba1	Wako	019-19741	Rabbit	1:1000	3 hours, RT
P2X3	ThermoScientific	PA1-30936	Rabbit	1:500	Overnight, 4°C
Alexa 488	Invitrogen	A21206	Rabbit	1:200	1 hour, RT
Alexa 568	Invitrogen	A11057	Goat	1:200	1 hour, RT



Figure 4. A Sprague Dawley rat in the Durham Animal Holder. Slits were cut into the original holder to facilitate testing sensitivity of trigeminal neurons to a mechanical stimulus applied in the orofacial region of the rat. The red color of the device prevents the animal from seeing the approach of the von Frey filaments and thus reduces non-evoked withdrawal responses.

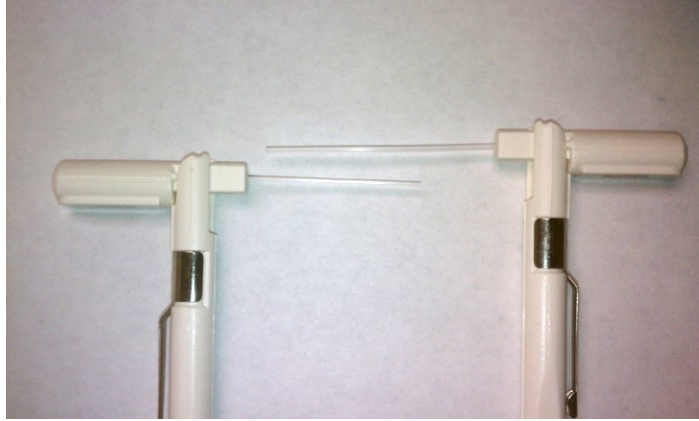


Figure 5. A 60 gram (left) and 100 gram (right) Von Frey filament. These filaments were used to evoke a withdrawal response when applied to the orofacial region of the rat. A positive withdrawal response was associated with bending of the filament.

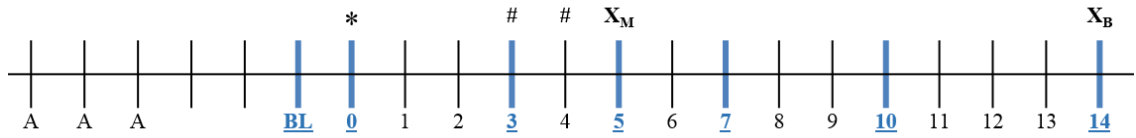


Figure 6. Experimental timeline. * = CFA or saline injection in TMJ, # = i.p. treatment injection, **X_M** = animals sacrificed for molecular studies, **X_B** = animals from behavioral studies sacrificed, A = behavioral acclimations, BL = base line behavioral readings taken, underlined numbers = days on which further behavioral readings were taken (note that the day 3 readings were taken prior to administration of treatment injections).

RESULTS

Effect of Dihydroergotamine and Sumatriptan on Nocifensive Response

Administration of DMSO, which served as a vehicle control for drug delivery, on days 3 and 4 post-saline injection did not elevate mean nocifensive responses above 2 out of 5 (Fig. 7). Responses of animals that were injected with saline, which served as a vehicle control of CFA, and then administered sumatriptan did not differ significantly from those of the DMSO group at any timepoint. In contrast, animals that received the saline injection and were then administered DHE did have significantly fewer nocifensive responses than the DMSO group on day 5 ($p = 0.040$), which corresponds to the timepoint on the day immediately following the DHE administration. No further significant differences were found between experimental conditions at any later timepoints.

Following injection of CFA into the TMJ, nocifensive responses were significantly elevated as compared to the group that received saline injections ($p = 0.005$ on day 5, $p < 0.001$ at all other timepoints after CFA injection) (Fig. 8). Administration of sumatriptan on days 3 and 4 post CFA injection did not significantly affect nocifensive responses as compared to those that were administered DMSO at any timepoint. Administration of DHE on days 3 and 4 significantly lowered nocifensive responses on day 5 ($p = 0.005$) as compared to the DMSO group. However, this effect was transient as no significant differences were seen for the remainder of the timepoints investigated.

Suppression of PKA Expression in the STN by Dihydroergotamine

All sections were examined using the nuclear stain DAPI, which fluorescently marks the nuclei of all cells. No differences in DAPI distribution were seen between any treatment conditions (data not shown). A representative image is shown in Figure 9, illustrating the overall cellular morphology as well as the area of interest to which all further STN images will be confined.

Within the control tissue, active PKA expression was localized in a narrow, somewhat diffuse band along the dorsal horn of the STN (Fig. 10). Injection of DHE following saline injection within the TMJ resulted in a much lighter and more diffuse band, suggesting a downregulation of basal PKA expression. Injection of sumatriptan following saline injection had no effect, with PKA staining pattern similar to that of control tissue.

Injection of CFA in the TMJ resulted in a robust increase in active PKA expression with a bright, discrete band along the medullary horn (outer laminae) surrounded by a wider band of bright and diffuse staining. Injection of DHE following CFA injection greatly lowered PKA expression to below that of even control levels, with no discernable discrete band and only dim, diffuse staining along the horn. In contrast, injection of sumatriptan following CFA injection resulted in a level of PKA expression that appears lower than that seen in the CFA and DMSO tissues, but is still more intense than the levels in control tissue.

P2X₃ Expression is Repressed in the STN by Dihydroergotamine and Sumatriptan

Within the control tissue, P2X₃ expression was limited to a diffuse band along the horn with sporadic brighter areas of intensity (Fig. 11). Treatment with DHE following saline injection in the TMJ diminishes those bright areas, leaving only a diffuse band of staining. Treatment with sumatriptan following saline injection reduced the intensity of staining in the outer laminae as compared to control tissue.

Injection of CFA in the TMJ resulted in a very bright band of staining along the dorsal horn with numerous bright areas of staining. Treatment with DHE following the CFA injection brought the expression level down to near control levels, with a diffuse band of staining and sporadic bright areas. Treatment with sumatriptan following CFA injection inhibited the staining intensity, leaving only a diffuse band in the outer laminae.

P-ERK Expression is Downregulated in the STN by Dihydroergotamine

Within the control tissue, there was little to no P-ERK staining visible, with only a few neurons expressing active levels of P-ERK (Fig. 12). Treatment with DHE or sumatriptan following saline injection did not change the staining appearance.

Injection of CFA in the TMJ resulted in a robust increase in the number of neuronal cells expressing elevated levels of P-ERK within the dorsal horn. Treatment with DHE following CFA injection reduced the number of P-ERK-positive neurons, but there was still a greater number than in the control tissues. Treatment with sumatriptan following CFA injection appeared to have no effect on P-ERK expression as the staining pattern was similar to that seen in the CFA sample.

Repression of CFA-Stimulated Iba1 Expression in the STN by Dihydroergotamine

Within the control tissue, Iba1 expression, which is a biomarker of activated microglia, was faint, limited to a handful of glial cells along the dorsal horn (Fig. 13). Treatment with DHE following saline injection moderately increased Iba1 expression, as did treatment with sumatriptan following saline injection.

Injection of CFA in the TMJ greatly increased the number of activated microglial cells in the dorsal horn. Treatment with DHE following CFA injection repressed Iba1 levels down to near control levels. Treatment with sumatriptan following CFA injection somewhat reduced the number of activated glial cells as compared to the CFA sample, but to a lesser extent than did the DHE treatment.

GFAP Expression is Inhibited in the STN by Dihydroergotamine and Sumatriptan

Within the control tissue, GFAP expression was observed in a sparse band of activated astrocytes within the dorsal horn (Fig. 14). Treatment with DHE following saline injection greatly reduced basal GFAP staining intensity and number of GFAP expression astrocytes, as did treatment with sumatriptan following saline injection.

Injection of CFA in the TMJ increased the number of active astrocytes, while treatment with DHE and sumatriptan both greatly reduced the intensity of staining to below control levels.

Repression of P2X₃ Expression in the TG by Dihydroergotamine and Sumatriptan

All sections were examined using the nuclear stain DAPI, which fluorescently marks the nuclei of all cells. No differences in DAPI distribution were seen between any

treatment conditions (data not shown). A representative image is shown in figure 15, illustrating the overall cellular morphology as well as the area of interest to which all further TG images will be confined.

Within the control tissue, there was very little P2X₃ staining (Fig. 16A). Neither administration of DHE nor sumatriptan had an effect on the staining intensity.

CFA injection in the TMJ dramatically increased the amount of P2X₃ staining, with numerous large- and small-diameter neurons showing fluorescent staining (Fig. 16B). Treatment with DHE and sumatriptan each reduced the amount of staining as compared to the CFA sample, but not to the low levels of the control tissue.

Dihydroergotamine Inhibits CFA-Induced P-ERK Expression in the TG

There was very little P-ERK staining in the control tissue, mainly faint fluorescence in the cytoplasm of neurons (Fig. 17A). Treatment with DHE following saline injection had no effect on ERK expression. Treatment with sumatriptan following saline injection, on the other hand, slightly upregulated ERK expression with some neurons showing ERK expression within the nucleus.

Following CFA injection, ERK expression was upregulated, with several neurons showing ERK expression within the nucleus (Fig. 17B). Treatment with DHE following CFA injection resulted in ERK expression similar to that of the control tissue. Treatment with sumatriptan following CFA injection had no effect on ERK expression, resulting in a staining pattern and intensity similar to that of the CFA sample.

Active p38 Expression is Downregulated in the TG by Dihydroergotamine

Within the control tissue, a small amount of neurons show P-p38 staining within the nucleus, a sign of activated P-p38 (Fig. 18A). Treatment with dihydroergotamine reduced this staining, with no visible neurons showing nuclear staining. Treatment with sumatriptan following saline injection resulted in a staining pattern similar to that seen in the control tissue.

Injection of CFA in the TMJ upregulated P-p38 expression, with the majority of neurons showing nuclear staining (Fig. 18B). Treatment with DHE and sumatriptan resulted in staining patterns similar to that seen in the control tissue, with only a few neurons showing nuclear staining.

Iba1 Expression is Downregulated in the TG by Dihydroergotamine and Sumatriptan

Within the control tissue, a moderate number of glial cells brightly fluoresced (Fig. 19A). Treatment with DHE and sumatriptan each reduced the intensity of fluorescence in the glial cells.

Following CFA injection in the TMJ, more glial cells brightly fluoresced as compared to the control tissue (Fig. 19B). Administration of DHE following CFA injection greatly reduced the number of glial cells showing fluorescence as well as the intensity of fluorescence. Administration of sumatriptan following CFA injection slightly reduced the intensity of fluorescence, displaying a similar pattern and brightness to that of the samples administered sumatriptan following saline injection.

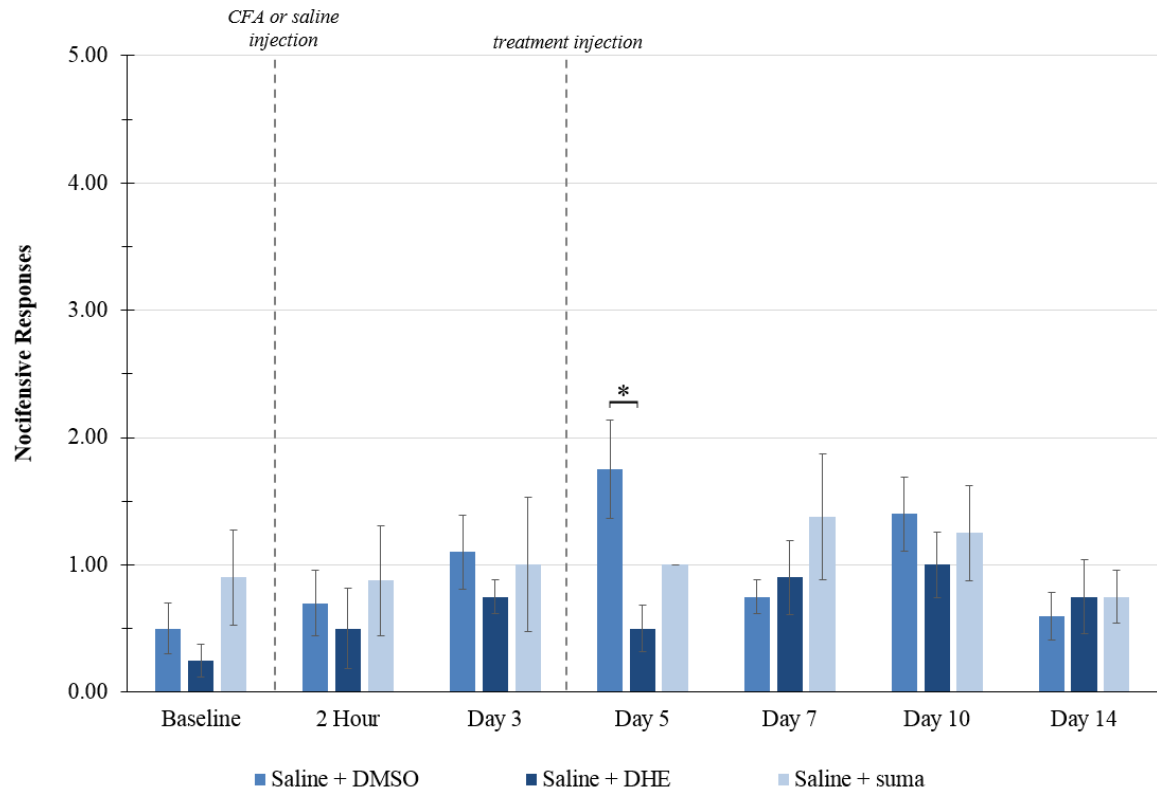


Figure 7. Effect of DHE and sumatriptan on basal responses. Treatment with DHE significantly inhibited nocifensive responses as compared to vehicle only on day 5. Values are reported as the average number of withdrawal responses to mechanical stimulation out of 5 applications to each side using a 100 g von Frey filament. * $p = 0.040$.

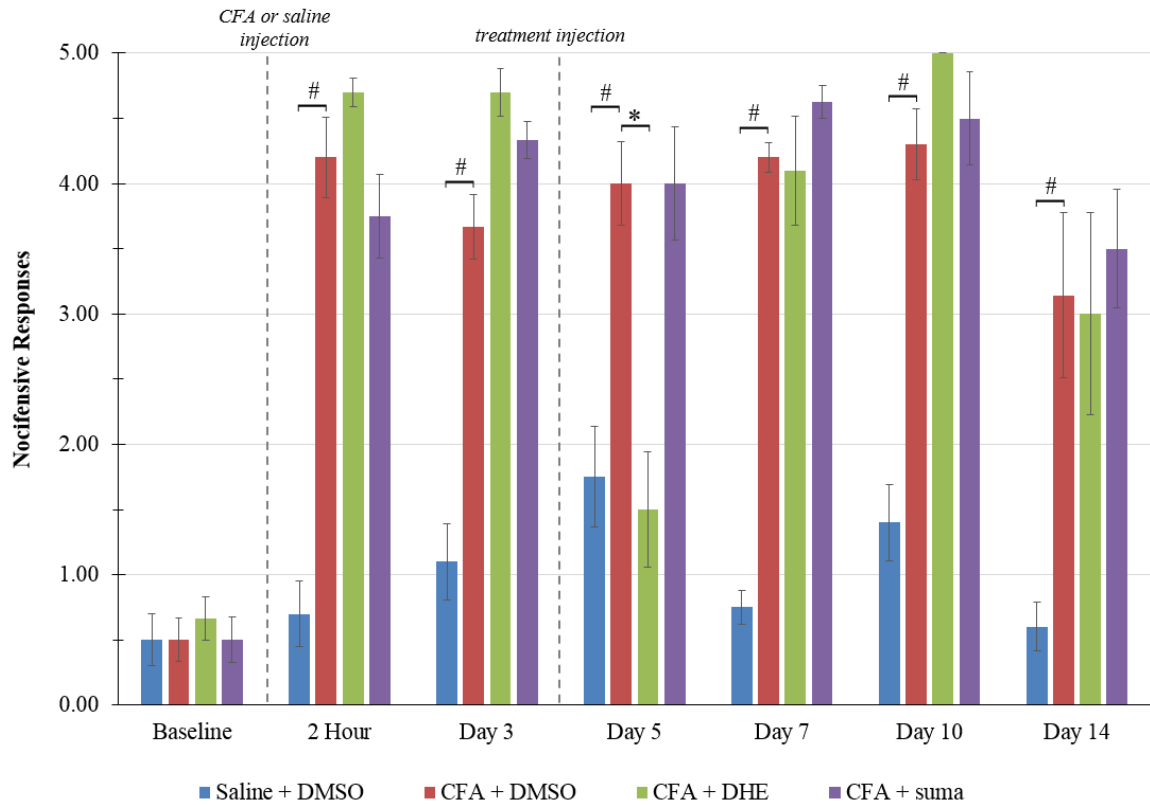


Figure 8. Effect of DHE and sumatriptan on nocifensive responses following CFA injection. CFA mediated sustained and significant nocifensive responses beginning 2 hours after injection and continuing for 14 days. Treatment with DHE following CFA injection significantly inhibited nocifensive responses on day 5. Values are reported as the average number of withdrawal responses to mechanical stimulation out of 5 applications to each side using a 100 g von Frey filament. # $p < 0.05$. * $p = 0.005$.

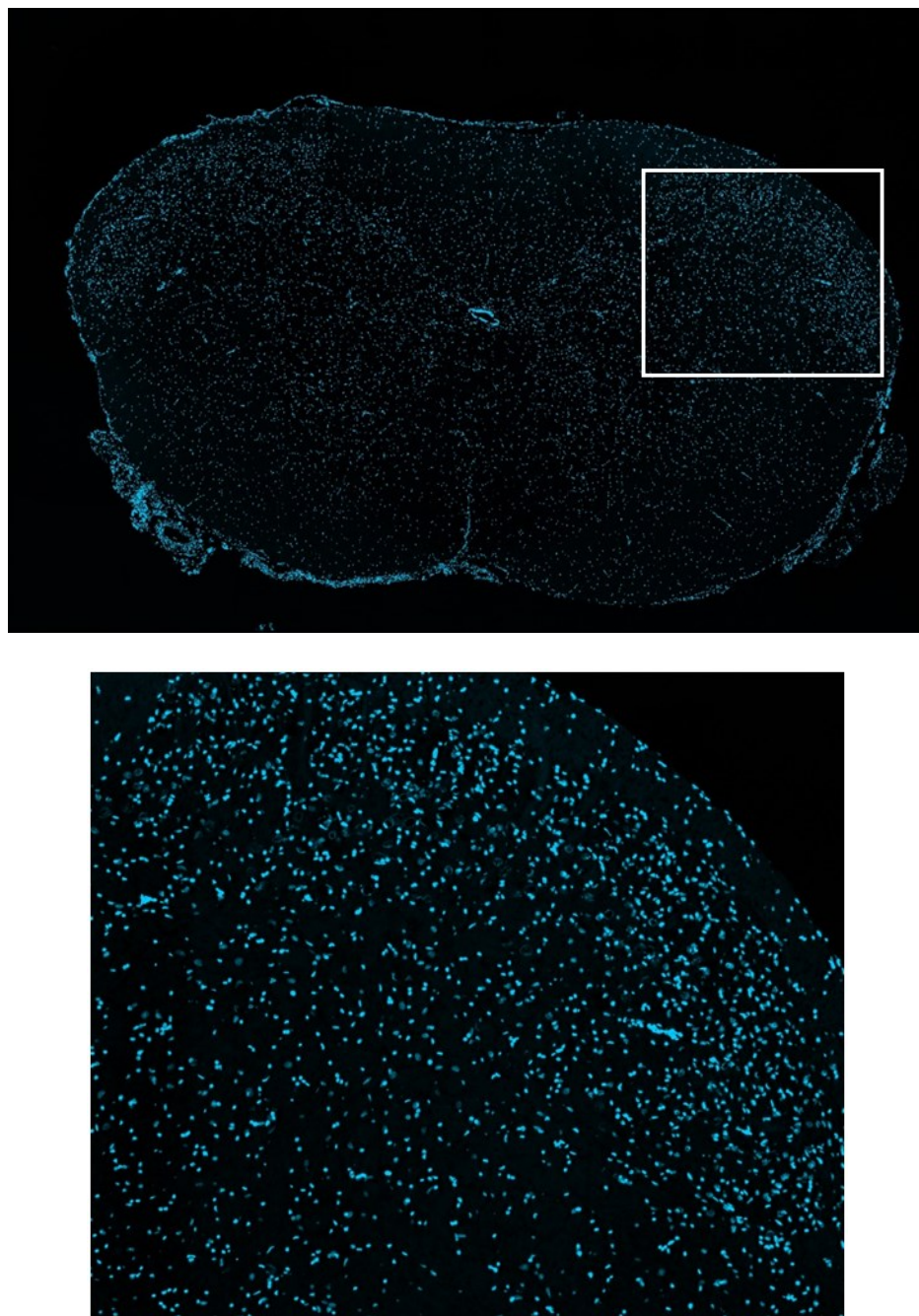


Figure 9. TNC morphology. A view of a full TNC slice stained with DAPI (top) and an enlarged view of the area of interest (bottom), which is outlined in white in the top image.

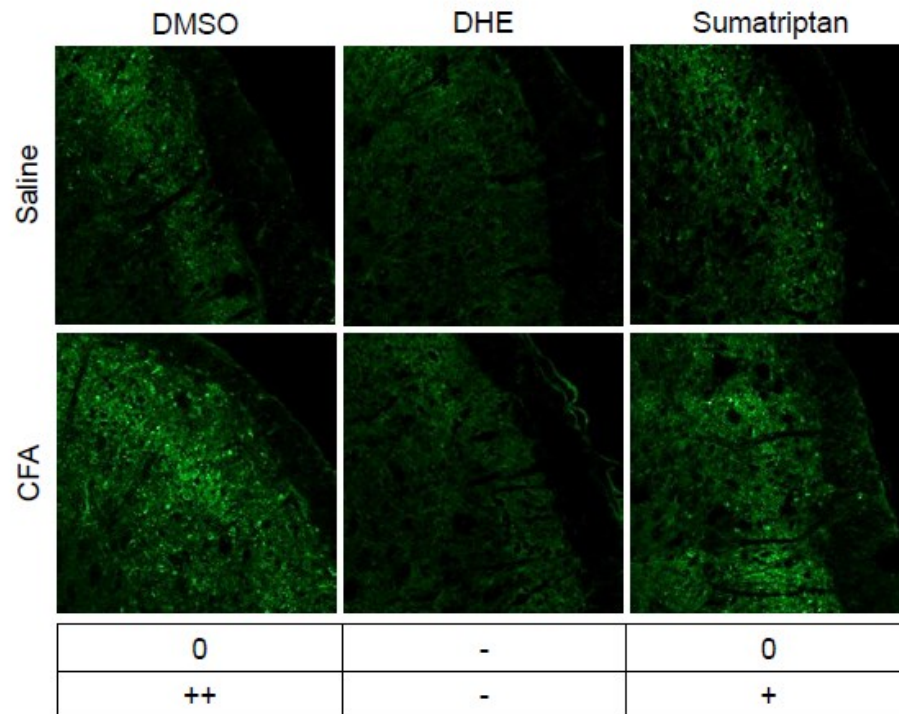


Figure 10. PKA expression in the STN. DHE inhibits both basal levels of PKA expression as well as CFA-induced increases in PKA expression. Sumatriptan had no effect on basal PKA expression and only somewhat inhibited CFA-induced increases in PKA expression. Spinal cords were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

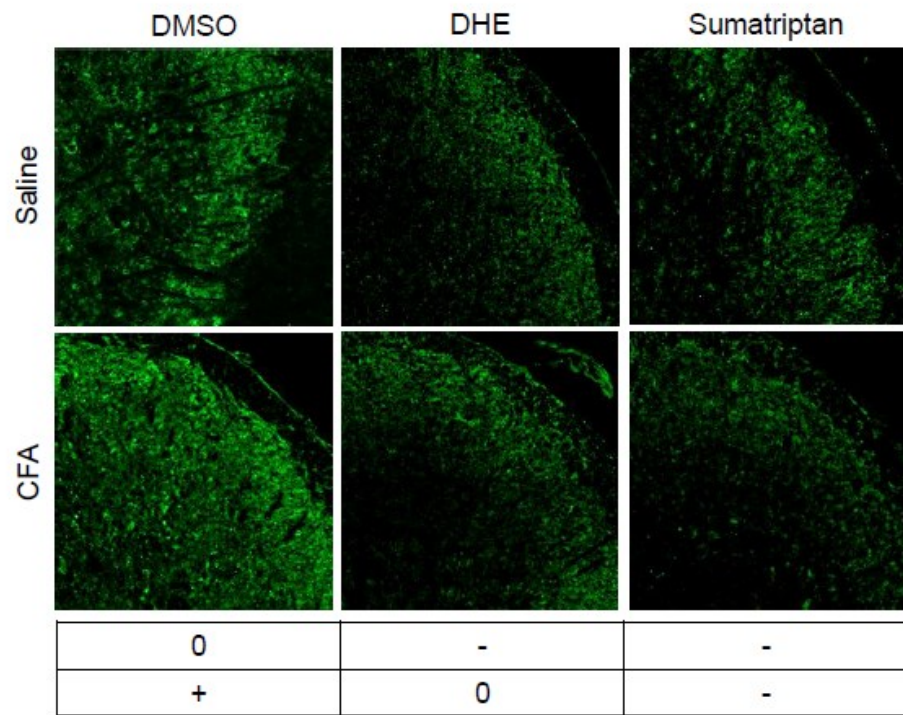


Figure 11. P2X₃ expression in the STN. DHE and sumatriptan each inhibited basal P2X₃ expression. Sumatriptan inhibited CFA-induced increases in P2X₃ expression to a greater extent than DHE. Spinal cords were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

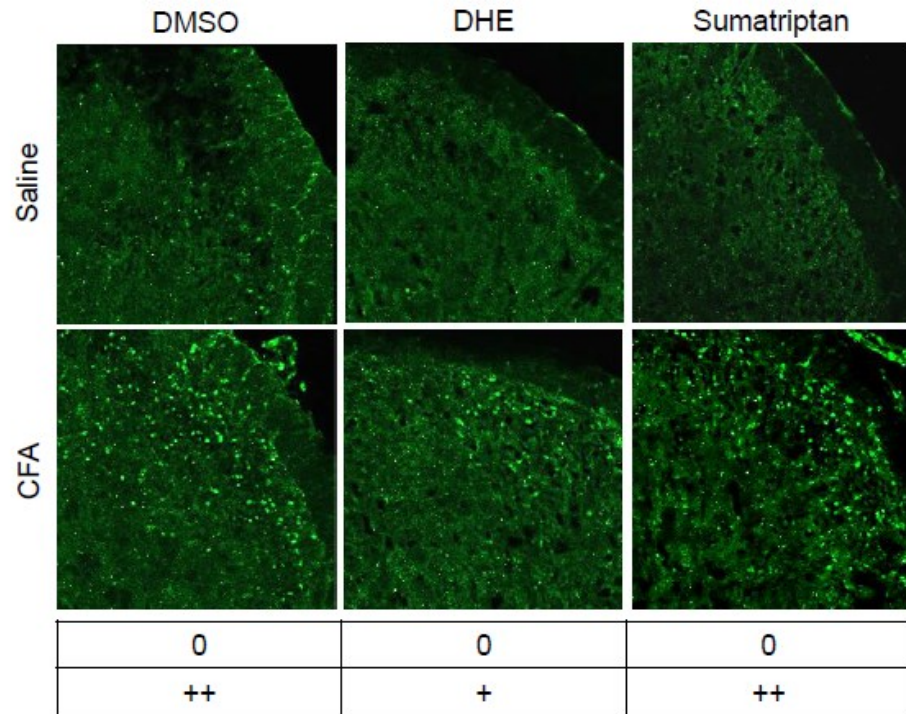


Figure 12. P-ERK expression in the STN. Neither DHE nor sumatriptan had an effect on basal P-ERK expression. DHE somewhat inhibited CFA-induced increases in P-ERK expression while sumatriptan had no effect. Spinal cords were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

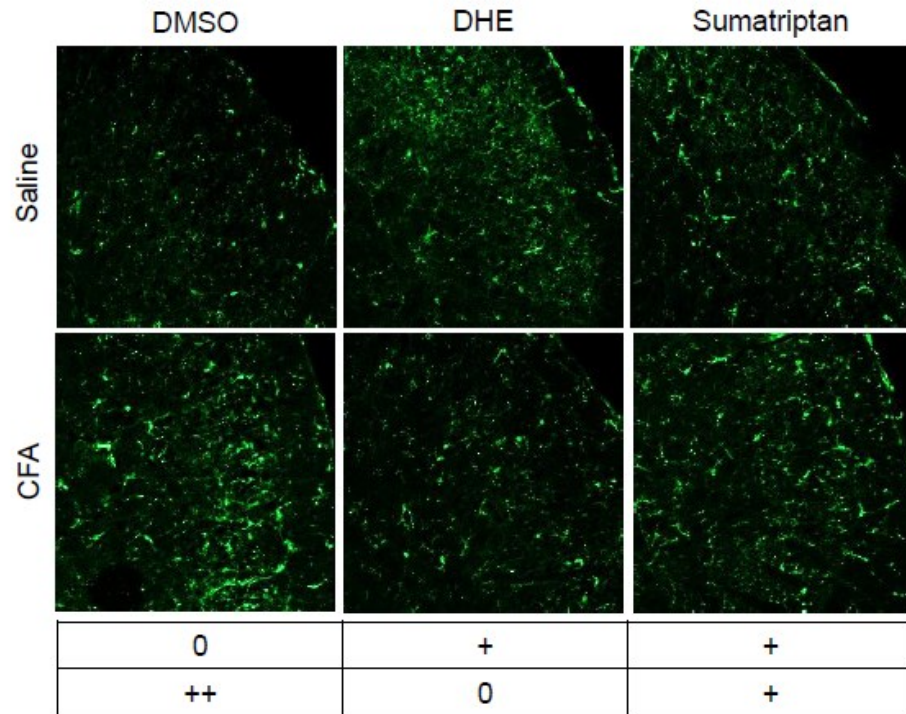


Figure 13. Iba1 expression in the STN. DHE and sumatriptan treatment resulted in a slight increase in basal Iba1 expression. DHE greatly inhibited CFA-mediated Iba1 increases while sumatriptan only somewhat inhibited CFA-mediated Iba1 increases. Spinal cords were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

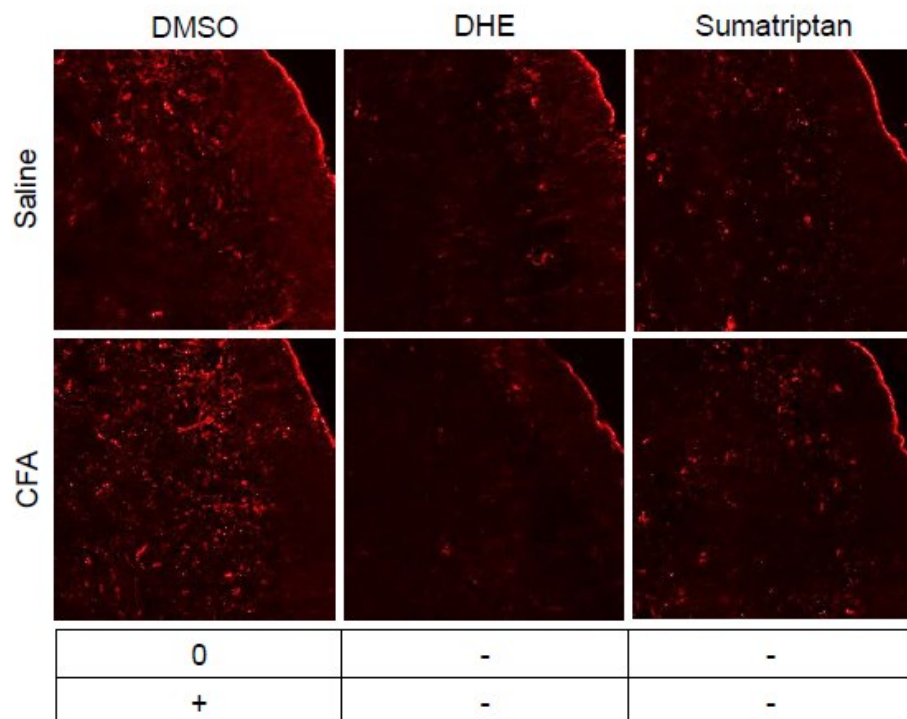


Figure 14. GFAP expression in the STN. DHE and sumatriptan each greatly inhibited both basal GFAP expression as well as CFA-mediated increases in GFAP expression. Spinal cords were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

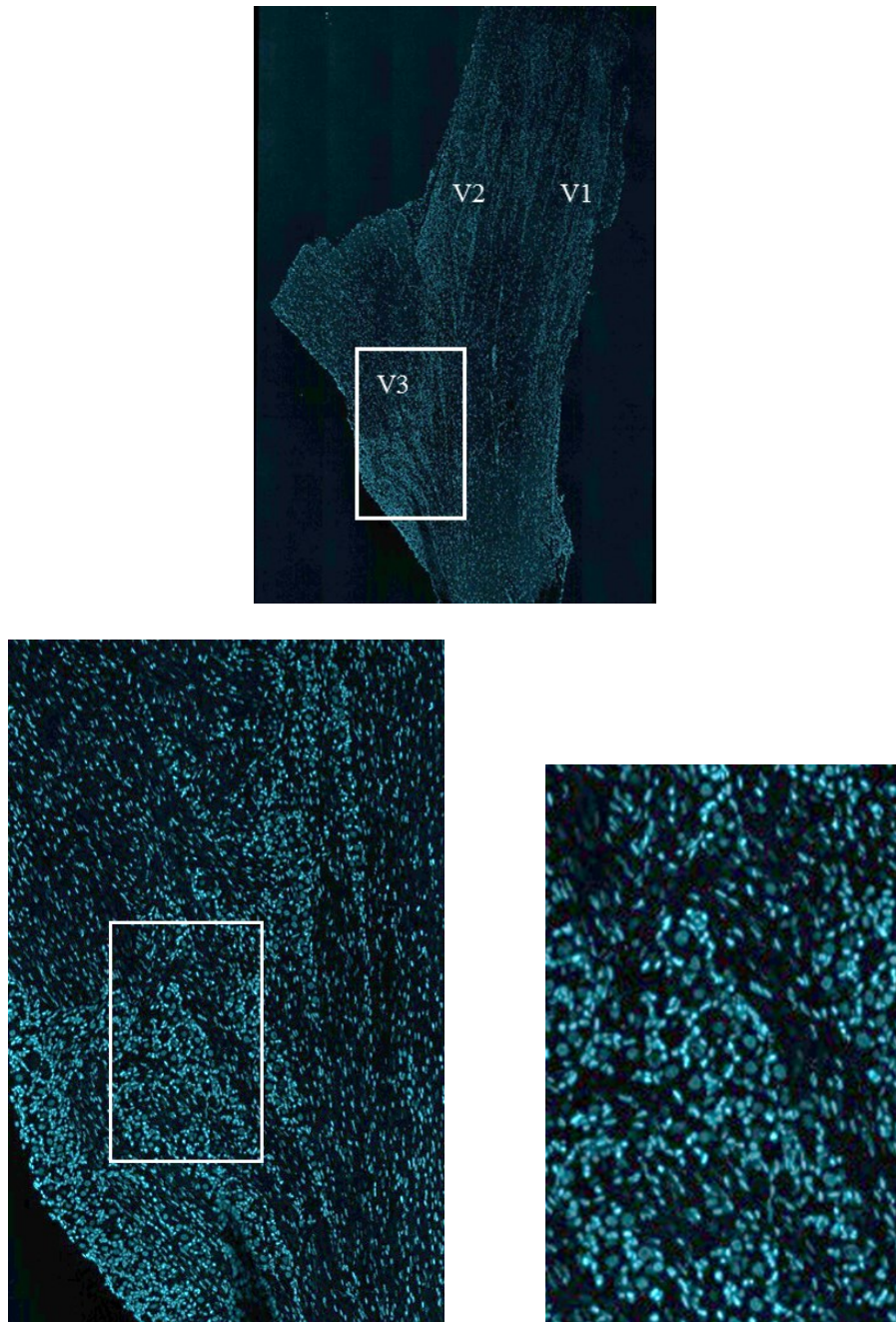


Figure 15. TG morphology. Full image of a TG slice stained with DAPI (top). Area outlined in white is enlarged in bottom left. Area outlined in white in bottom left image is enlarged in bottom right.

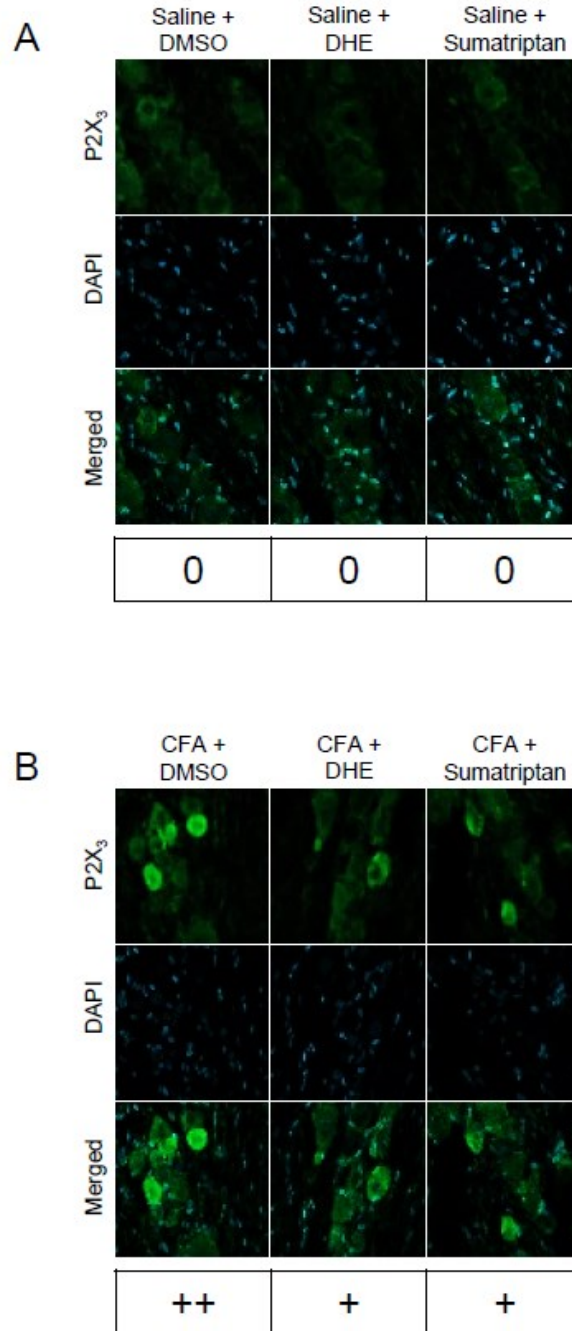


Figure 16. P2X₃ expression in the TG. (A) Neither DHE nor sumatriptan had an effect on basal P2X₃ expression in the TG. (B) DHE and sumatriptan each somewhat inhibited CFA-mediated increases in P2X₃ expression in the TG. Ganglia were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

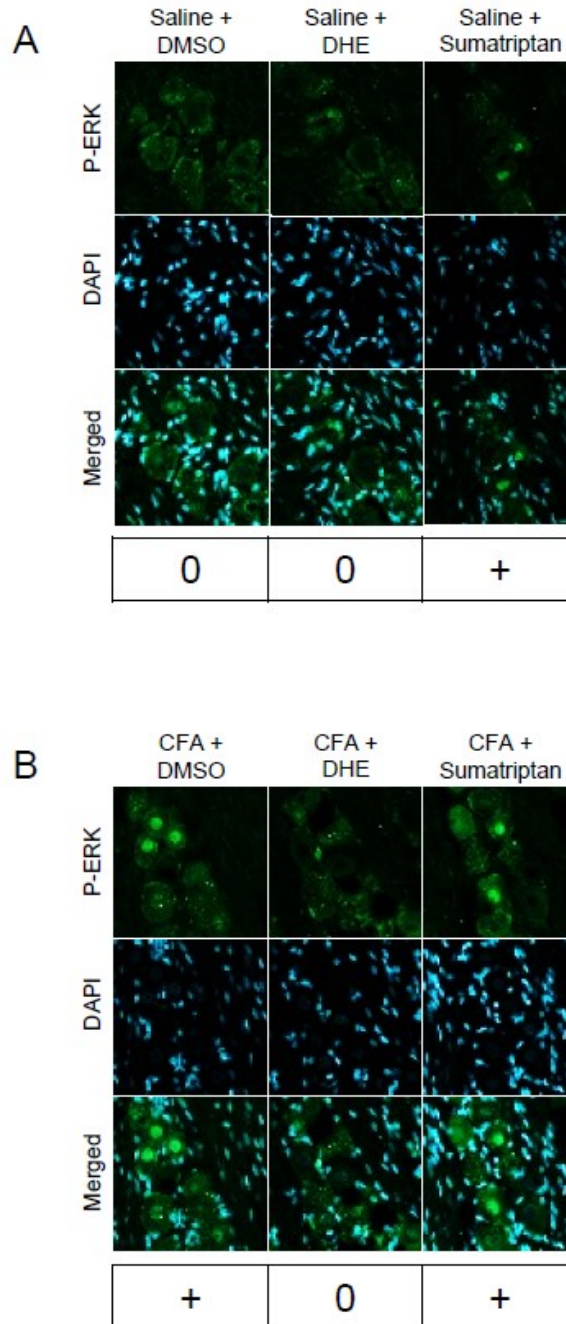


Figure 17. P-ERK expression in the TG. (A) DHE had no effect on basal P-ERK expression while sumatriptan slightly upregulated basal P-ERK expression. (B) DHE inhibited CFA-mediated increases in P-ERK expression. Sumatriptan had no effect on CFA-mediated increases. Ganglia were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity (n = 2 independent experiments).

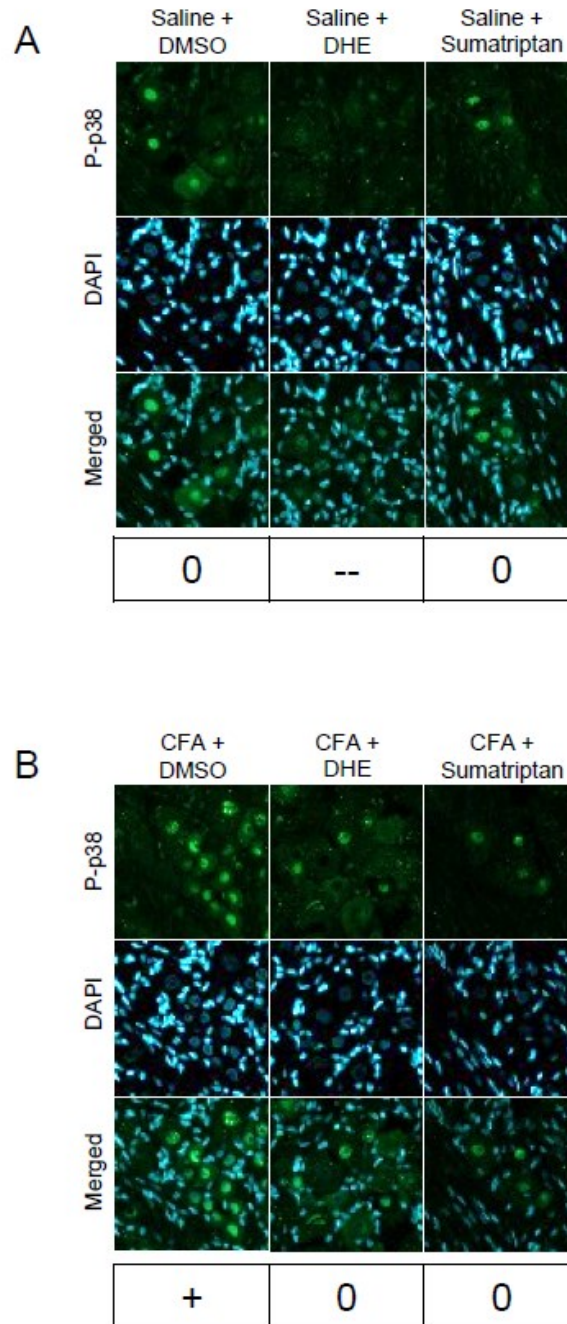


Figure 18. Active p38 expression in the TG. (A) DHE greatly decreased basal P-p38 expression while sumatriptan had no effect. (B) DHE and sumatriptan each inhibited CFA-mediated increases in P-p38 expression. Ganglia were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower tables display staining intensity as determined by previously described guidelines.

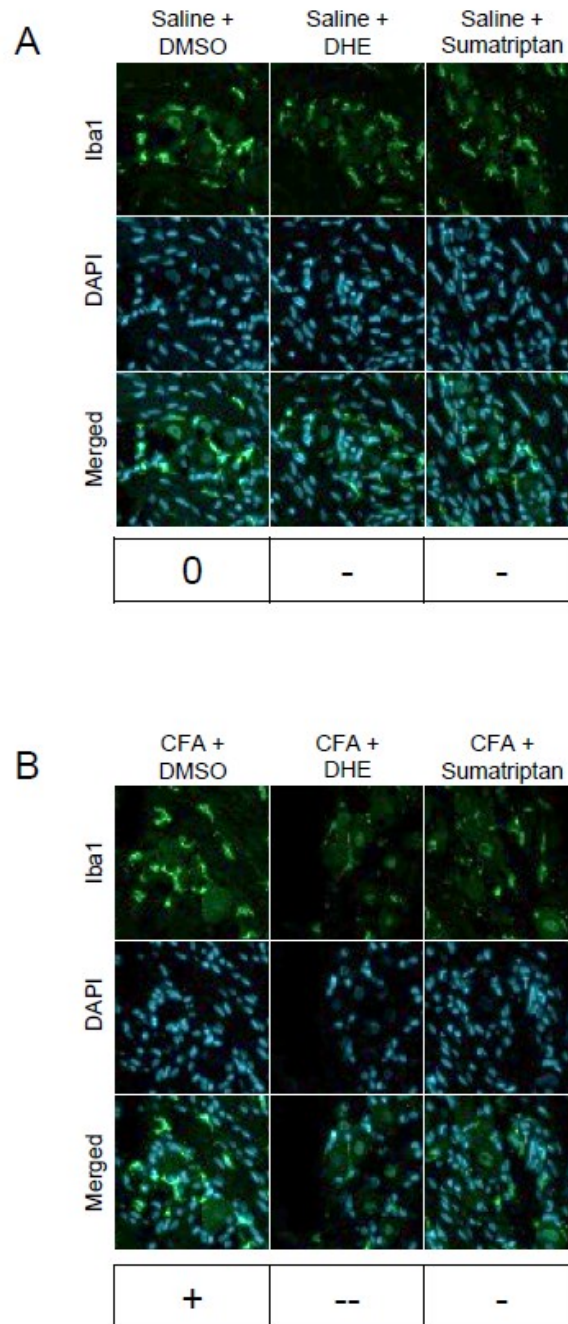


Figure 19. Iba1 expression in the TG. (A) DHE and sumatriptan each decreased basal Iba1 expression in the TG. (B) DHE greatly inhibited CFA-mediated increases in Iba1 expression, while sumatriptan only somewhat decreased it. Ganglia were removed as described five days after saline or CFA injection in the TMJ with DMSO, DHE, or sumatriptan i.p. injections occurring on days 3 and 4 post-saline/CFA injection. Lower table displays changes in relative staining intensity ($n = 2$ independent experiments).

DISCUSSION

DHE Represses Nocifensive Responses to Mechanical Stimulation

The major finding from my study is that DHE, but not sumatriptan, was effective in blocking nociception associated with prolonged inflammation and sustained trigeminal neuron activation. I hypothesized based on receptor binding profiles and a previous study in our laboratory (Masterson and Durham, 2010) that DHE may function by a different mechanism of action than sumatriptan, and therefore may be beneficial in decreasing nociception caused by persistent trigeminal nociceptor activation. The differential ability of DHE and sumatriptan to modulate responses to mechanical stimulation likely lies in their different pharmacological profiles. DHE predominantly acts as a pan-amine receptor agonist while sumatriptan binds selectively and with high affinity to the 5-HT_{1B} and 5-HT_{1D} receptors. Similar to the triptans, DHE is known to bind to the serotonin 5-HT_{1B} and 5-HT_{1D} receptors (Goadsby, 2000, Cook et al., 2009). However, the potency of DHE binding to the different receptors depends on maximum plasma concentration (C_{max}) achieved by the different routes of administration used for therapeutic purposes. When 1 mg of DHE is administered via IV, C_{max} is about 45,289 pg/mL, effective binding includes 8 serotonin receptors, 4 α -adrenoceptors, and 2 dopamine receptors and, as a result, DHE IV treatment is associated with a number of unwanted side effects. When administered through oral inhalation (MAP0004, 6 actuations, 1.8 mg per dose), the C_{max} is about 5,241 pg/mL and effective bindings include only 4 serotonin receptors, 3 α -adrenoceptors, and 1 dopamine receptor and, as a result, some of the common side effects of the IV dosing were not reported. My finding

that sumatriptan was not inhibitory in the CFA-induced trigeminal activation model was unexpected. It should be noted that DHE only mediated a transient inhibitory effect on mechanical sensitivity of trigeminal primary neurons with a significant decrease in withdrawal responses on day 5 following DHE treatment on days 3 and 4. On day 7, the average number of nocifensive responses was similar to that observed in the CFA alone treated animals. Thus, it appears that a sustained inflammatory stimulus, as occurs in response to CFA injection into the TMJ capsule, results in a pathological condition of trigeminal activation that cannot be reversed by only two treatments of DHE. Based on my results, I can only speculate that DHE would need to be administered on a daily basis to be an effective therapy for frequent episodic or chronic migraine.

My finding that sumatriptan could not inhibit ongoing peripheral and central sensitization of trigeminal nociceptive neurons differs from the results published by Burstein and Jakubowski (Burstein and Jakubowski, 2004). In their experiment, the investigators used a rat model in which intracranial pain is used to induce cutaneous allodynia, a manifestation of central sensitization of the STN, to measure the effects of treatment with triptans given either before initiation of central sensitization or after it is established. Using this model, these researchers found that concomitant administration of sumatriptan and an inflammatory soup (IS), containing 1 mM histamine, serotonin, bradykinin, and 0.1 mM prostaglandin E2 in 10 mM HEPES buffer, pH 5.5, is applied to the dura mater effectively blocked the development of central sensitization. Specifically, experimental evidence included the lack of expansion of dural receptive fields or increase in dural indentation sensitivity, a lack of long-term spontaneous firing increases, and no increase in the magnitude of response to mechanical or thermal skin stimulation in central

trigeminovascular neurons. Sumatriptan, administered after the establishment of central sensitization, reversed intracranial sensitivity of central trigeminovascular neurons, as evidenced by a reversal of IS-induced dural receptive field expansion and neuronal sensitivity to dural stimulation; however, it did not reverse the increased neuronal sensitivity to mechanical or thermal stimulation at the periorbital skin or the increased spontaneous firing. These findings suggest that the anti-nociceptive effects of triptans are mediated largely through pre-synaptic inhibition of transmitters release in the dorsal horn by the peripheral nociceptors, rather than post-synaptic inhibition of receptors on central trigeminovascular neurons (Oshinsky et al., 2012). In another study using the same animal model, the effects of zolmitriptan and DHE were compared on the generation and maintenance of central sensitization. Zolmitriptan administered 1 hour before or 2.5 hours after application of an IS to the dura mater did not inhibit central sensitization, as measured electrophysiologically, in the STN. In contrast, DHE given either before or at the time of IS application to the dura mater was able to block the induction of central sensitization. When given after central sensitization of the TNC was established, DHE was able to reverse sensory thresholds to brush, pin, and pressure stimulation. These results suggest that DHE and triptans act by different mechanisms of action once central sensitization is established and that DHE, unlike a triptan, reverses central sensitization (Oshinsky et al., 2012). The difference in the ability of DHE to reverse central sensitization is most likely due to the transient nature of the stimulus used in their study when compared to the persistent stimulus used in my model of trigeminal activation.

DHE Differentially Regulates Cellular Pathways

Results from my study provide evidence at the cellular level to help explain why administration of DHE on days 3 and 4 was sufficient to inhibit the nocifensive effect of CFA in response to mechanical stimulation. DHE was found to inhibit the CFA-stimulated increase in STN expression of PKA, P2X₃, P-ERK, Iba1, and GFAP, which are all proteins implicated in the development and maintenance of central sensitization. In addition, DHE also repressed the stimulatory effect of CFA on P2X₃, P-ERK, P-p38, and Iba1 in the trigeminal ganglion. Each of these proteins is involved in modulating the excitability state of primary sensory trigeminal neurons. For example, increased expression of the active phosphorylated form of the MAP kinase signaling protein ERK is associated with sensitization of primary and secondary nociceptive neurons that facilitates prolonged nociception (Dai et al., 2002, Ji, 2004). Elevated levels of MAP kinases such as ERK and P38 promote and sustain neuronal sensitization by modulating the expression of receptors and ion channels, stimulating release of inflammatory molecules, and facilitating interactions with glial cells (Ji et al., 2009). Similarly to our previous observations (Cady et al., 2014), I found that CFA injection in the TMJ increased the expression of Iba1, a protein used as a biomarker of activated macrophage cells (Nakamura et al., 2013), within the functional units of the ganglia. Our finding of increased activation of macrophages in response to CFA-induced inflammation within the TMJ is in agreement with the results of previous studies involving trigeminal nerve activation caused by inflammatory stimuli (Villa et al., 2010, Franceschini et al., 2013). An increase in the activity level of macrophages within the ganglion is likely to facilitate the inflammatory response via the release of cytokines and other mediators and to

function as scavengers to remove cellular debris caused during pathology. CFA also mediated an increase in Iba1 expression in microglia within the STN. Activated microglial cells are known to increase the synthesis and release of pro-inflammatory mediators including cytokines and chemokines.

In agreement with results from our previous study (Cady et al., 2014), I found that CFA injection in the TMJ resulted in an upregulation of PKA expression within the STN. Elevated levels of PKA modulate nociceptive responses of primary neurons by enhancing sodium currents, sensitizing nociceptors. This pathway is activated by CGRP, which has been shown to be released in response to peripheral tissue injury (Seybold, 2009). Once CGRP binds to its receptor, which is located on neuronal cell bodies, astrocytes, and microglia, the concentration of the secondary messenger cAMP increases, resulting in activation of PKA. Activated PKA promotes and sustains central sensitization by phosphorylating proteins that regulate the excitability state of both neurons and glia. I also observed an increase in the level of P2X3 expression in both the STN and TG. P2X3 receptors, which are abundantly expressed by TG neurons projecting to the dura (Durham and Garrett, 2010b) and upon activation by ATP are known to mediate acute and chronic pain (Durham and Russo, 2003, Olesen et al., 2004, Levy et al., 2005, Bowen et al., 2006, Bigal et al., 2007). Similarly to my finding with DHE, results from another study using the selective P2X3 receptor antagonist A-317491 have provided evidence that blocking P2X3 receptors was effective in repressing nociception in inflammatory and neuropathic pain models (Vause et al., 2007). This effect is not surprising since ATP activation of P2X3 receptors was shown to promote sensitization of primary afferent nociceptors involved in development of hyperalgesia (Cady et al., 2010). Finally, I also observed an

increase in the expression of the structural protein GFAP, which is associated with activated astrocytes (Anderson et al., 2014). The development and maintenance of peripheral and central sensitization of nociceptive neurons has been shown to be dependent on activated astrocytes (Watkins et al., 2001).

Although I found that all of these proteins were downregulated by DHE following CFA injection, the expression levels of many of them were also inhibited by sumatriptan. Only ERK and Iba1 were downregulated to a greater extent by DHE than by sumatriptan in both the ganglion and the spinal cord, while PKA was downregulated to a greater extent by DHE in the spinal cord. Thus, I propose that decreased expression of these particular proteins are likely responsible for the inhibitory effect of DHE on nocifensive withdrawal responses following prolonged trigeminal activation. In support of this notion, blocking ERK and PKA activation in nociceptive neurons and activation of microglia are associated with a decrease in the inflammatory response and promotes an anti-nocifensive state within animals (Aley and Levine, 1999, Watkins and Maier, 2002, Ji et al., 2009).

My results provide evidence that DHE induces cellular changes that are distinct from those induced by sumatriptan. This observation is in agreement with previous results from our laboratory in which DHE, but not sumatriptan, repressed ATP-mediated sensitization of trigeminal ganglion neurons (Masterson and Durham, 2010). In that study, neonatal rat trigeminal ganglia cultures were used to investigate effects of ATP, alpha, beta-methyl ATP (α,β -meATP), and DHE on intracellular calcium levels and calcitonin gene-related peptide (CGRP) secretion. Pretreatment with ATP or α,β -meATP caused sensitization of neurons, via P2X3 receptors, such that a subthreshold amount of

potassium chloride (KCl) significantly increased intracellular calcium levels and CGRP secretion. Pretreatment with DHE repressed increases in calcium and CGRP secretion in response to ATP-KCl or α,β -meATP-KCl treatment. Importantly, these inhibitory effects of DHE were blocked with an α_2 -adrenoceptor antagonist and unaffected by a 5HT1B/D receptor antagonist. DHE also decreased neuronal membrane expression of the P2X3 receptor. Results from that study provided evidence for a novel mechanism of action for DHE that involves blocking ATP mediated sensitization of trigeminal neurons, repressing stimulated CGRP release, and decreasing P2X3 membrane expression via activation of α_2 -adrenoceptors. Further evidence that DHE may function differently from the triptans was provided by data showing that DHE treatment of trigeminal neurons did not change intracellular calcium levels as previously reported for the triptans. (Durham and Russo, 1999, 2003) While DHE repression of potassium chloride–stimulated release of CGRP from ATP-sensitized trigeminal neurons involved inhibition of intracellular calcium levels by decreasing membrane expression of the P2X3 receptor (Masterson and Durham, 2010), studies on sumatriptan demonstrated that its inhibitory effects were mediated via activation of 5-HT1 receptors. (Durham and Russo, 1999, 2003).

Summary and Future Directions

In summation, my study showed that DHE but not sumatriptan can transiently block nocifensive responses to mechanical stimulation following CFA-mediated chronic inflammation. This is likely due to a different mechanism of action, as suggested by my

immunohistochemical results, which showed that DHE differentially regulates ERK, Iba1, and PKA.

Due to the transitory nature of its effects, DHE may be more helpful in treating frequent episodic migraine than chronic migraine, and thus prevent patients from progressing to chronic migraine. In fact, the best indicator of the likelihood of a patient developing chronic migraine is the frequency of their episodic migraines (Schwedt, 2014). Once a patient's migraines become chronic, the only currently FDA-approved treatment is injection with Botox, so preventing that chronification should be a priority for sufferers of episodic migraine. Currently, sumatriptan is the most commonly prescribed migraine treatment, but approximately 30% of patients do not get satisfactory results from it (Mathew, 2001). My results suggest that since DHE works through different pathways than sumatriptan, it could be a good potential alternative for that subpopulation of patients. Another advantage of DHE over the use of triptans is that DHE has been reported to cause minimal constriction of human cerebral arteries in vivo—significantly less than caused by triptans (Nilsson et al., 1999). DHE did not have any vasoconstrictor effects on the porcine middle meningeal artery (Mehrotra et al., 2006) but did cause vasoconstriction of the perfused middle cerebral artery of rats (Tfelt-Hansen et al., 2007). DHE was reported to increase resistance of the meningeal vascular bed in anesthetized cats (Lambert and Michalick, 1996). Evaluation of the effect of DHE in vitro on distal and proximal coronary arteries demonstrated no significant vasoconstriction (Labruijere et al., 2015). Taken together, the results of human and animal studies show that DHE has variable and condition-dependent effects on cerebral and peripheral arteries and veins. As with other acute migraine therapies, there is no

direct evidence that vasoconstriction plays a primary role in the therapeutic effects of DHE.

In the future, it may be beneficial to look at several different iterations of the model presented in my study to determine the best efficacy of DHE. Some possibilities include pre-treating with DHE prior to initiation of inflammation, continuing daily treatment throughout the course of the study, or increasing the doses of DHE and sumatriptan.

Given the increasing prevalence and socio-economic impact of more frequent migraine attacks and the lack of effective medications to inhibit the progression from frequent to chronic migraine, more effective therapeutic approaches are in great need for this group of migraine sufferers. Towards this end, a new method to deliver DHE in an aerosolized form that is rapidly absorbed by the lungs and minimizes unwanted side effects caused by oral or injectable administration of DHE is in development by the pharmaceutical company Allergan, and awaiting approval from the Food and Drug Administration.

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