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# Serotonin type 1D receptors ($5HT_{1D}R$) are differentially distributed in nerve fibres innervating craniofacial tissues

**AM Harriott**[1] and **MS Gold**[2,3,4]

1 *Department of Biomedical Sciences and Medical Sciences Training Program, University of Maryland, Baltimore, MD*

2 *Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh, Pittsburgh, PA, USA*

3 *Department of Neurobiology, University of Pittsburgh, Pittsburgh, PA, USA*

4 *Pittsburgh Center for Pain Research, University of Pittsburgh, Pittsburgh, PA, USA*

## Abstract

We tested the hypothesis that the $5HT_{1D}R$, the primary antinociceptive target of triptans, is differentially distributed in tissues responsible for migraine pain. The density of $5HT_{1D}R$ was quantified in tissues obtained from adult female rats with Western blot analysis. Receptor location was assessed with immunohistochemistry. The density of $5HT_{1D}R$ was significantly greater in tissues known to produce migraine-like pain (i.e. circle of Willis and dura) than in structures in which triptans have no antinociceptive efficacy (i.e. temporalis muscle). $5HT_{1D}R$-like immunoreactivity was restricted to neuronal fibres, where it colocalized with calcitonin gene-related peptide and tyrosine hydroxylase immunoreactive fibres. These results are consistent with our hypothesis that the limited therapeutic profile of triptans could reflect its differential peripheral distribution and that the antinociceptive efficacy reflects inhibition of neuropeptide release from sensory afferents. An additional site of action at sympathetic efferents is also suggested.

### Keywords

Headache; triptans; trigeminal ganglion neuron; sympathethic postganglionic neuron

## Introduction

Triptans, serotonin type 1B/1D receptor ($5HT_{1B/1D}R$) agonists, are one of the most effective drugs used to treat migraine pain (1). Interestingly, the therapeutic efficacy of these compounds appears to be restricted to migraine pain. For example, in animal behavioural studies, triptans have no impact on baseline nociceptive threshold assessed with application of stimuli to somatic tissue (2) and fail to reverse sciatic nerve injury-induced decreases in paw withdrawal or vocalization thresholds (3). Similarly, naratriptan fails to attenuate noxious stimulation-evoked activity in spinal dorsal horn neurons (4). In contrast, naratriptan dose-dependently inhibited activity in trigeminal dorsal horn neurons evoked with noxious stimuli applied to the dura (4). Moreover, in clinical studies sumatriptan does not alleviate myofacial pain from temporalis muscle (5) and is of little clinical benefit to patients suffering from atypical facial pain (6). The limited therapeutic efficacy of these compounds is striking, however, in light of

Michael S. Gold, PhD, Department of Medicine, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA W1451, USA. Tel. + 1 412 383 5367, fax + 1 412 383 8663, e-mail E-mail: goldm@dom.pitt.edu.

evidence indicating that $5HT_{1D}R$ protein is present in cell bodies of both trigeminal and dorsal root ganglia (7,8).

Mechanisms underlying the therapeutic efficacy of triptans are also debated, with data in support of vasoconstriction, inhibition of neurotransmission in the central nervous system, and inhibition of peripheral neurotransmitter release from primary afferents. Initially, triptans were administered because it was thought that they could reverse the painful vasodilation associated with migraine by producing potent vasoconstriction (1). It was later discovered that the $5HT_{1B}R$ was responsible for this vasoconstriction, and that this receptor subtype was located postsynaptically in vascular smooth muscle (9). However, the absence of a temporal correlation between changes in vascular tone and migraine pain argues against the vascular hypothesis of triptan antinociception and for a dominant role of $5HT_{1D}R$ activation (10). Because the $5HT_{1D}R$ is expressed in primary afferent neurons and not in peripheral tissues or second-order neurons (7–9), the prevailing hypothesis is that the antinociceptive efficacy of triptans reflects the inhibition of transmitter release from primary afferents (11–14), in particular the neuropeptide calcitonin gene-related peptide (CGRP). Consistent with this hypothesis, there is evidence that triptans inhibit inflammation and CGRP release from peripheral nerve terminals (13–15). There is also evidence that triptans may reduce central neurotransmitter release primarily from afferents via a presynaptic mechanism (16).

There is general agreement that migraine pain ultimately reflects increased activity in afferents innervating the dura mater. Dural afferents have received a considerable amount of attention, given evidence that stimulation of fibres innervating the middle meningeal artery (MMA) produces pain that mimics pain felt during a migraine (17) and evidence that dural inflammation and release of CGRP from dural afferents can be blocked by sumatriptan (13,14). However, nerve fibres innervating purely vascular structures may also play a significant role in migraine pain. Stimulation of arteries proximal to the circle of Willis, such as the intracranial internal carotid arteries and middle cerebral arteries, produces migraine-like pain in neurosurgical patients (17) similar to that seen with stimulation of meningeal arteries. There are additional data to suggest that head pain can occur during carotid artery dissection, presumably via activation of trigeminovascular afferents, and that this pain may be treated with sumatriptan (18). Although there is evidence of $5HT_{1D}R$ in fibres innervating the dura (7,8), the presence of this receptor in other migraine-associated tissues (MATs) has yet to be investigated.

In the face of evidence suggesting that the peripheral $5HT_{1D}R$ contributes significantly to the therapeutic efficacy of triptans, the clinical selectivity of these drugs may be explained by differential distribution of this receptor in fibres innervating MATs vs. non-MATs. The observation that the $5HT_{1D}R$ is located in only 20–40% of cell bodies (8,15) is consistent with the possibility that this receptor does not have a uniform peripheral distribution. Therefore, we hypothesized that the selectivity of these drugs reflects a higher density of the $5HT_{1D}R$ in peripheral nerve fibres innervating MATs (common carotid artery, circle of Willis and dura mater) compared with non-migraine-associated vascular (external carotid artery) and non-vascular (temporalis muscle) tissues. To test this hypothesis, the density of $5HT_{1D}R$ was quantified with Western blot analysis of whole-cell lysates taken from dura, temporalis muscle, common, internal, external carotid arteries, circle of Willis and ganglia whose fibres innervate craniofacial structures. To determine where the $5HT_{1D}R$ was located in peripheral tissues, diaminobenzidine (DAB) immunohistochemistry of $5HT_{1D}R$ in whole mounted tissues from dura, extracranial and intracranial arteries was examined. Colocalization of the $5HT_{1D}R$ with CGRP and tyrosine hydroxylase (TH) was also examined in the dura using immunofluorescence.

## Materials and methods

### Animals

Adult female Sprague Dawley rats (Harlan, Indianapolis, IN, USA) weighing 150–250 g were used for all experiments. Rats were housed two per cage in the University of Pittsburgh animal facility on a 12:12 light : dark schedule with food and water freely available. Prior to all procedures, animals were deeply anaesthetized with an intraperitoneal injection (1 ml/kg) of rat cocktail containing ketamine (55 mg/kg), xylazine (5.5 mg/kg) and acepromazine (1.1 mg/kg). Experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals in research.

### Quantification of $5HT_{1D}$ R using Western blotting

The supratentorial dura mater was collected with the MMA, superior sagittal sinus and the nerves that innervate them. Additionally, the entire length of the common carotid artery was isolated from surrounding tissues by blunt dissection and separated from the accompanying jugular vein and vagus nerve. The internal, external carotid arteries and the circle of Willis were collected after these structures were dissected free from large accompanying nerves and surrounding tissues by blunt dissection. The belly of the temporalis muscle was also collected without the attached tendons. Tissues were then homogenized in RIPA buffer [50 mM Tris, 150 mM NaCl, 1 mM ethylenediamine tetraaceticacid, 1% NP 40, 0.1% sodium dodecyl sulphate (SDS)] containing protease inhibitors (6.8 μg/ml apoprotinin, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 4 μg/ml trypsin inhibitor, 4 μg/ml E64, 2 mM phenylmethylsulphonyl fluoride, 2 mM $Na_3VO_4$) on ice. Protein concentration was determined using a BioRad RCDC Protein Assay kit, and 150 μg of total protein per sample was run on 10% SDS–polyacrylamide gel electrophoresis gel. Protein was transferred to nitrocellulose membrane and blots were blocked with 5% milk and then incubated with rabbit polyclonal $5HT_{1D}$R antibody (1:250; Abcam, Cambridge, MA, USA) overnight. Blots were stripped and reprobed with goat polyclonal PGP9.5 antibody (1:5000; Abcam) as a marker for neuronal tissue, mouse monoclonal β-actin (1:5000; Abcam) and mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:5000; Abcam) as loading controls. For immunodetection, blots were incubated in peroxidase-conjugated secondary antibody (1:10 000; Jackson ImmunoResearch, West Grove, PA, USA) and visualized following chemiluminescence reaction (ECL plus; GE Healthcare, Milwaukee, WI, USA) with an LAS3000 CCD camera (FujiFilm Life Science, Stamford, CT, USA).

The band intensity from each tissue in each blot was quantified using MultiGauge software (FujiFilm Life Science). Intensities for $5HT_{1D}$R immunoreactive bands were normalized either to β-actin or to PGP9.5 (as an estimate of $5HT_{1D}$R relative to innervation density). Since comparisons were made between blots, these ratios were also normalized to that for a tissue common to each blot (i.e. dura or internal carotid artery). Data were expressed as the mean ± S.E.M. of the relative ratios.

### Controls for Western blots

To determine the extent of non-specific binding of the primary antibody, the $5HT_{1D}$R antibody was preincubated with peptide fragments corresponding to amino acids 1–18 and 251–267 of the rat $5HT_{1D}$R (GenScript, Scotch Plains, NJ, USA) at a concentration of 20 μg/ml. To determine if there was non-specific binding of the secondary antibody, the primary antibody was omitted from Western blots.

### Localization of $5HT_{1D}R$ in peripheral tissue Immunohistochemistry

Rats were transcardially perfused with ice-cold 1× phosphate-buffered saline (pH 7.2) and ice-cold 4% paraformaldehyde. Tissues were post-fixed with 4% paraformaldehyde from 1 to 12 h. For DAB immunoperoxidase immunohistochemistry, free-floating tissues were incubated in 0.3% peroxide for 30 min, blocked with 2% normal goat serum at room temperature and then incubated in rabbit polyclonal $5HT_{1D}R$ primary antibody (1:500; Abcam) at 4°C for 3 days. Tissues were incubated with biotinylated goat antirabbit IgG (1:200; Vector Laboratories Vectastain kit, Burlingame, CA, USA) for 2 h at room temperature and avidin–biotin–peroxidase complex (Vector Laboratories Vectastain kit) for 30 min at room temperature. DAB (1 mg/ml with 2.5% nickel sulphate) was used to visualize $5HT_{1D}R$ like immunoreactive ($5HT_{1D}R$ LI) fibres. Tissues were mounted on slides and, following ethanol dehydration, cover-slipped with Permount media (Fisher Scientific, Waltham, MA, USA).

For immunofluorescence staining of whole mounts, free-floating tissues were blocked with 10% normal donkey serum at room temperature for 1 h and then incubated in a combination of rabbit polyclonal $5HT_{1D}R$ (1:250; Abcam) and sheep polyclonal CGRP (1:500; Abcam) or sheep TH antibodies (1:500; Chemicon International, Temecula, CA, USA) for 48–72 h at room temperature. Free-floating tissues were then incubated in donkey antirabbit Cy3 (1:200; Jackson ImmunoResearch) and donkey antisheep Cy2 (1:500; Jackson ImmunoResearch)-conjugated secondary antibodies at room temperature for 2 h.

Superior cervical and trigeminal ganglia were removed from animals following perfusion fixation, and tissues were submerged in 30% sucrose overnight, frozen in OCT (Tissue Tek, Torrence, CA, USA) and cut in 16-μm sections. Sectioned tissues were blocked with 5% normal donkey serum, incubated in $5HT_{1D}R$ (1:250), CGRP (1:500) or TH (1:500) antibody overnight at room temperature and then in donkey antirabbit or sheep Cy2 (1:200; Jackson ImmunoResearch)-conjugated secondary antibody for 2 h at room temperature.

For retrograde labelling of trigeminal ganglion neurons innervating the dura, a craniotomy was made in the area overlying the superior sagittal sinus, leaving the underlying dura intact and exposed for application of retrograde tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchloride (DiI; 170 mg/ml in dimethylsulphoxide diluted 1:10 in saline). A single droplet of DiI was applied onto the exposed dura using a 30-G needle attached to a Hamilton syringe by catheter tubing. A dental rubber dam was placed on the exposed dura and secured to the cranium with superglue adhesive. An acrylic cap made from dental orthodontic resin was used to replace the removed cranium and the incision closed with sutures. Animals received intramuscular injections of penicillin G (100 000 U/kg) and buprenorphine (0.03 mg/kg) following surgery. Ten days following application of DiI, animals were sacrificed.

Immunofluorescence staining was visualized on a Leica DM4000B (Bannockburn, IL, USA) microscope and images captured using a Leica DFC300 FX camera. Nerve fibres and cell bodies were considered immunoreactive when the immunofluorescent signal was clearly greater than that obtained when the primary antibody was omitted. For counts of $5HT_{1D}R$ immunoreactive cell bodies from superior cervical ganglia, three sections were examined per animal of tissue obtained from two animals. Data were expressed as the percentage of total cell bodies showing $5HT_{1D}R$ immunoreactivity per section in a field under ×200 magnification and the average percentage was generated for each animal.

### Statistical analysis

One-way analysis of variance (ANOVA) was used to assess differences between tissues. Tukey or Dunn’s tests were used for post-hoc comparisons between these groups. For comparisons

between relative ratios taken from Western blots run for superior cervical and trigeminal ganglia, a *t*-test was performed. $P \leq 0.05$ was considered to be statistically significant.

## Results

### The density of $5HT_{1D}R$ LI does not vary among migraine-associated tissues

To determine if there are differences between MATs with respect to the distribution of $5HT_{1D}R$ protein, Western blots of whole-cell lysates from dura, common carotid artery and circle of Willis were analysed. Total protein concentration was quantified from each sample, and the same amount of protein (150 μg) was loaded per lane (Fig. 1A). Densitometric analysis of blots from eight animals, normalized to the internal carotid artery, revealed no statistically significant difference ($P > 0.05$) in the amount of $5HT_{1D}R$ LI between MATs (Fig. 1B). Use of β-actin as a loading control (Fig. 1A,C) confirmed that this was not due to differences in loading or transfer of proteins.

Data from previous studies suggest that the $5HT_{1D}R$ is present only in neural tissue (7–9). There is also evidence of differences between target tissues with respect to density of innervation (19,20). Therefore, we sought to assess the density of $5HT_{1D}R$ LI among MATs after taking into account differences in innervation density. The neural marker PGP9.5 was used to assess innervation density (Fig. 1A). Among MATs, there was a significantly greater amount ($P < 0.05$, $n = 8$) of PGP9.5 in circle of Willis compared with dura (Fig. 1D). However, when normalized to PGP9.5, there still remained no significant difference between MATs with respect to the $5HT_{1D}R$ : PGP9.5 ratio ($P > 0.05$, $n = 8$, Fig. 1E).

### The density of $5HT_{1D}R$ is greater in MATs than in non-MATs

Since we were able to quantify $5HT_{1D}R$ LI in MATs, we then asked if the clinical selectivity of triptan therapy could reflect differences in the density of $5HT_{1D}R$ between MATs and non-MATs. The external carotid artery was used as a vascular non-MAT and the temporalis muscle was used as a non-vascular non-MAT. One-way ANOVA of the dataset, including non-MATs, indicated a significant ($P < 0.05$) effect of target tissue. Post-hoc analysis revealed that the significant effect of target tissue on $5HT_{1D}R$ LI was due to a significantly lower level of $5HT_{1D}R$ LI in external carotid artery compared with common carotid artery and circle of Willis, and a significantly lower level of $5HT_{1D}R$ LI in temporalis muscle compared with common carotid artery, circle of Willis and dura ($P < 0.05$, $n = 8$, Fig. 1B). The significant differences between external carotid artery, common carotid artery and circle of Willis remained when data were normalized to β-actin (Fig. 1C). Because β-actin was undetectable in striated muscle (Fig. 1A), GAPDH was used as a loading control (data not shown). The relatively high level of GAPDH in striated muscle highlights difficulties associated with assessing relative protein levels between distinct tissue types, but argues against the suggestion that the relatively low density of $5HT_{1D}R$ LI detected in muscle was due to problems with loading or transfer of proteins.

One-way ANOVA of the dataset, including non-MATs, again revealed a significant ($P < 0.05$) effect of target tissue with respect to levels of PGP9.5 LI. Post-hoc analysis indicated the density of PGP9.5 in temporalis muscle was significantly ($P < 0.05$) greater than that in dura (Fig. 1D). Pooled data from eight animals indicated that the relative density of $5HT_{1D}R$ LI normalized to PGP9.5 in the external carotid artery was significantly lower than that in the common carotid artery and circle of Willis ($P < 0.05$; Fig. 1E). Likewise, the density of $5HT_{1D}R$ LI in temporalis muscle was significantly lower than that in the common carotid artery, circle of Willis or dura ($P < 0.05$; Fig. 1E).

Pre-absorption of the $5HT_{1D}R$ antibody with the peptide it was raised against was used to confirm the specificity of the immunoreactivity. This eliminated the two bands present in all tissues (approximately 60 and 62 kDa) and the high-molecular-weight band (approximately 65 kDa) restricted to the dura (control: Fig. 1A). Similar results were obtained following omission of the primary antibody (data not shown).

### $5HT_{1D}R$ LI is primarily localized to neuronal fibres innervating peripheral tissues

To confirm that $5HT_{1D}R$ LI detected with Western blot analysis was associated with neural tissue, localization of the receptor was assessed with immunohistochemistry. We observed $5HT_{1D}R$ LI in nerve fibres innervating the rat dura mater. There did not appear to be any $5HT_{1D}R$ LI in the meningeal arteries or resident mast cells. $5HT_{1D}R$ LI fibres of the dura mater ran along with the MMA. These fibres branched with arterial bifurcations. There were also $5HT_{1D}R$ LI fibres that intersected the MMA and traversed the meninges in a direction perpendicular to the MMA and parallel to the superior sagittal sinus (Fig. 2A). $5HT_{1D}R$ LI was present in large nerve trunks and smaller fibres (Fig. 2B). In addition, we observed $5HT_{1D}R$ LI nerve fibres in segments of the extracranial and intracranial cerebrovasculature, including the external carotid artery and circle of Willis (Fig. 2C,D, respectively).

### $5HT_{1D}R$ LI is colocalized with CGRP LI in the dura mater

Given evidence of $5HT_{1D}R$-mediated inhibition of CGRP release (11,14), we sought to determine the extent to which there is colocalization of the receptor with this primary afferent neuropeptide in peripheral tissue. Double-labelling experiments revealed the presence of both $5HT_{1D}R$ LI and CGRP LI within dural nerve bundles (Fig. 3A,B). However, there were also nerve bundles that displayed $5HT_{1D}R$ LI that did not overlap with CGRP LI (Fig. 3C).

Although CGRP is commonly used a marker for peptidergic sensory neurons, neuropeptide immunoreactivity, including CGRP LI, has been detected in subpopulations of sympathetic postganglionic neurons (SPGNs) (21). Therefore, in order to assess the possibility that a portion of the CGRP LI fibres innervating the dura were from SPGNs, we probed the superior cervical ganglion (SCG) for the presence of CGRP LI. Trigeminal ganglia (TG) from the same rats were collected and run in parallel as a positive control. We were unable to detect the presence of CGRP LI in SCG from two rats, despite CGRP LI in small- and medium-diameter TG neurons (Fig. 4). The absence of CGRP LI in rat SCG is consistent with previous reports indicating that although CGRP LI is present in human, feline and canine SCG, it is undetectable in SCG from rat or guinea pig (21–23).

### $5HT_{1D}R$ LI is present in sympathetic postganglionic neurons innervating the dura mater

Considering recent evidence that sympathetic activity may modulate afferent signalling in the dura mater (24) and the dense innervation of the dural parenchyma and MMA by TH LI fibres, (presumably arising from the superior cervical ganglia: Fig. 5A), we determined if the non-peptidergic $5HT_{1D}R$ LI fibres could be sympathetic efferent. Double-labelling experiments revealed colocalization of $5HT_{1D}R$ LI with TH in a number of dural fibres (Fig. 5B).

To confirm that this staining was indeed from the SCG, the presence of $5HT_{1D}R$ LI was assessed in SCG. Again, TG from the same rats were run in parallel (Fig. 6A). In six sections of SCG from two rats, 9% of cell bodies displayed $5HT_{1D}R$ LI (Fig. 6B). To assess the density of $5HT_{1D}R$ in SCG relative to that in TG, Western blots of whole-cell lysates generated from both tissues were processed. Pooled data from five rats normalized to PGP9.5 suggested that, on average, there is a twofold higher density of $5HT_{1D}R$ in the TG compared with the SCG ($P = 0.012$, Fig. 6C).

Previous reports have indicated that there is a subpopulation of sensory neurons with TH LI (25,26). Therefore, in order to determine the extent to which TH LI fibres innervating the dura could arise from the TG, we probed TG retrogradely labelled from the dura with DiI, for the presence of TH LI. Four sections each from TG obtained from two rats were analysed. TH LI was clearly detectable in a small subpopulation (3–4%) of TG neurons (Fig. 7A). However, none of the 52 TG neurons that were retrogradely labelled with DiI displayed TH LI (Fig. 7B).

## Discussion

The present study was designed to test the hypothesis that the selective therapeutic efficacy of triptans reflects a high density of $5HT_{1D}R$ in tissues underlying migraine pain. We observed that there was no difference between MATs with respect to the relative density of $5HT_{1D}R$. However, the relative density of $5HT_{1D}R$ was lower in non-MATs (i.e. temporalis muscle) than in MATs. $5HT_{1D}R$ LI was restricted to nerve fibres innervating peripheral tissues, where it was colocalized in many fibres with the neuropeptide CGRP. $5HT_{1D}R$ LI was also colocalized with TH in many fibres that appeared to arise from the superior cervical ganglia.

### $5HT_{1D}R$ LI is present in a number of MATs

Our conclusion that the distribution of the $5HT_{1D}R$ is similar between MATs is based on Western blot data showing no significant differences in the amounts of $5HT_{1D}R$ in the common carotid artery, circle of Willis or dura, whether data were analysed as a function of total protein, normalized to loading controls such as β-actin, or normalized to innervation density estimated with PGP9.5. To justify the normalization of $5HT_{1D}R$ to PGP9.5, we explored the possibility that non-neuronal components of peripheral tissues might contain the $5HT_{1D}R$. Consistent with previous reports demonstrating that $5HT_{1D}R$ mRNA and protein are not found in intracranial arteries but rather in the perivascular nerves that innervate them (7,9), our data also indicate that $5HT_{1D}R$ LI is restricted to nerve fibres. It is always possible that we have overestimated density of $5HT_{1D}R$ in the common carotid artery and circle of Willis due to contamination with fibres of passage. However, we suggest that such an overestimation is minimal based on our anatomical results (Fig. 2), which were consistent with a terminal pattern of innervation and the observation that there is no rostral–caudal trend to the pattern of distribution (i.e. higher levels found in proximal structures such as common carotid artery and lower levels found in distal structures such as circle of Willis).

Prior studies have indicated that activation of nerve fibres innervating the dura mater plays a critical role in generating migraine pain. For example, in neurosurgical patients, stimulation of afferents innervating dural blood vessels produced migraine-like pain (17). Furthermore, inflammatory mediators released during the ictal period of migraine are able to increase activity of dural afferents and their second-order neurons in the trigeminal nucleus following exogenous application to the dura (27,28). Importantly, these inflammation-induced changes in dural afferent activity can be blocked by sumatriptan. Sumatriptan is also able to decrease plasma protein extravasation in the dura and CGRP release from dural afferents. Although little attention has been paid to other blood vessels of the head and neck and the afferents that innervate them, our results demonstrating high density of the $5HT_{1D}R$ in nerve fibres innervating the common carotid artery and circle of Willis suggest that other populations of vascular afferents, in addition to those innervating the dura mater, may contribute to migraine pain. This conclusion is supported by immunohistochemistry demonstrating innervation of intracranial arteries by nociceptive-like afferents containing both CGRP and substance P (29), by clinical observations that endovascular procedures resulting in the stimulation of several arteries within the cranial vault are painful (30), and early studies demonstrating that stimulation of proximal branches of the circle of Willis produces migraine-like pain (17). In addition, there are data suggesting that head pain occurs during dissection of the carotid artery,

presumably via activation of trigeminovascular afferents; and that this pain may be treated with sumatriptan (18).

### The density of $5HT_{1D}R$ is greater in MATs than in non-MATs

Although there are few reports to suggest that triptans may inhibit peripheral inflammation irrespective of tissue type, (8,31), as summarized in the Introduction, there are a number of studies demonstrating selectivity in the antinociceptive efficacy of triptans (4–6,32). Our data indicating the density of $5HT_{1D}R$ is lower in temporalis muscle than common carotid artery, circle of Willis or dura are consistent with our hypothesis and suggest that the lower density of receptor in nerve fibres innervating the temporalis muscle may have contributed to failure of triptans in the clinical trial of myofacial pain arising from the temporalis muscle (5).

### There may be unique processing of the $5HT_{1D}R$ within the dura

Interestingly, three distinct $5HT_{1D}R$ LI bands (56, 60 and 62 kDa) were detected in our Western blots of lysates from dura and other vascular tissues. The lower-molecular-weight band was not detected in the temporalis muscle, the TG or the SCG. An additional higher-molecular-weight band (65 kDa) was detected in the dura. All four bands were eliminated by pre-absorption of the primary antibody, suggesting the bands contain a protein with a specific $5HT_{1D}R$ epitope. That the 65-kDa band was absent in the trigeminal ganglion or in lysates taken from the brainstem (data not shown) argues against a splice variant, and for a post-translational modification in the peripheral target. Inspection of the $5HT_{1D}R$ protein sequence reveals several sites for post-translational modification, specifically, N-glycosylation (33). Thus, there may be post-translational processing of the receptor as the receptor is being trafficked or at the peripheral terminal innervating the end organ. The fact that we observed this higher-molecular-weight band only in the dura suggests an alternative mechanism for the therapeutic specificity of triptans.

### $5HT_{1D}R$ LI in peptidergic primary afferent fibres

Previous data suggest that expression of $5HT_{1D}R$ in the cell bodies of sensory neurons is restricted to a subpopulation of neurons expressing CGRP, with 75–90% of $5HT_{1D}R$+ neurons double-labelled with CGRP (8,15,34). Consistent with these reports, we observed colocalization of $5HT_{1D}R$ and CGRP in dural fibres. This overlap in the periphery supports the hypothesis that triptan inhibition of peripheral CGRP release is via a direct mode of action (i.e. on the afferent terminal). However, our data also suggest $5HT_{1D}R$ is present in non-peptidergic fibres. At least some of these fibres appear to be SPGNs, given colocalization of $5HT_{1D}R$ LI with TH LI in the dura mater and the presence of $5HT_{1D}R$ LI in cell bodies of the SCG. That these receptors on SPGNs are functional is suggested by the observation that $5HT_{1D}R$ agonists inhibit sympathetically mediated pressor and tachycardic responses (35, 36).

### A role for SPGNs in migraine?

The presence of $5HT_{1D}R$ LI in SPGNs raises the possibility that the therapeutic efficacy of triptans reflects an action of these neurons innervating the dura mater or other vascular tissues. Given the hypothesis that migraine pain may be caused by painful dilation of dural blood vessels (17), one might predict that an increase in sympathetic efferent activity would result in pain relief in association with vasoconstriction. However, there is also evidence that the SPGN may contribute to peripheral inflammation. For example, sympathetic efferents have been shown to release adenosine triphosphate (37) and prostaglandin (PG) $E_2$ (38), which would contribute to primary afferent activation and sensitization and therefore neurogenic inflammation and pain. Importantly, recent evidence indicates that sympathomimetics increase basal levels of $PGE_2$ in rat dura mater (24).

In summary, there remain a number of other possible factors that might contribute to the clinical selectivity of triptans, including potential differences in bioavailability and pharmacology of peripherally located receptors, contributions of central spinal and supraspinal receptors, and the minimum number of $5HT_{1D}$R-containing fibres needed to appreciate differences in pain perception. Nevertheless, our results are consistent with the hypothesis that the therapeutic efficacy of triptans may reflect, at least in part, a selective pattern of $5HT_{1D}$R expression, with the highest density of receptor in the innervation of structures likely to contribute to migraine pain. Although the location of this receptor in peptide-containing nerve fibres innervating peripheral tissues is consistent with the suggestion that pain relief reflects inhibition of CGRP release from peripheral afferent terminals, the presence of the receptor on SPGN terminals raises the possibility that the therapeutic efficacy of triptans may also reflect an action on efferent fibres. Teasing out the relative importance of these two mechanisms of action may enable the development of more selective and effective treatments for migraine.

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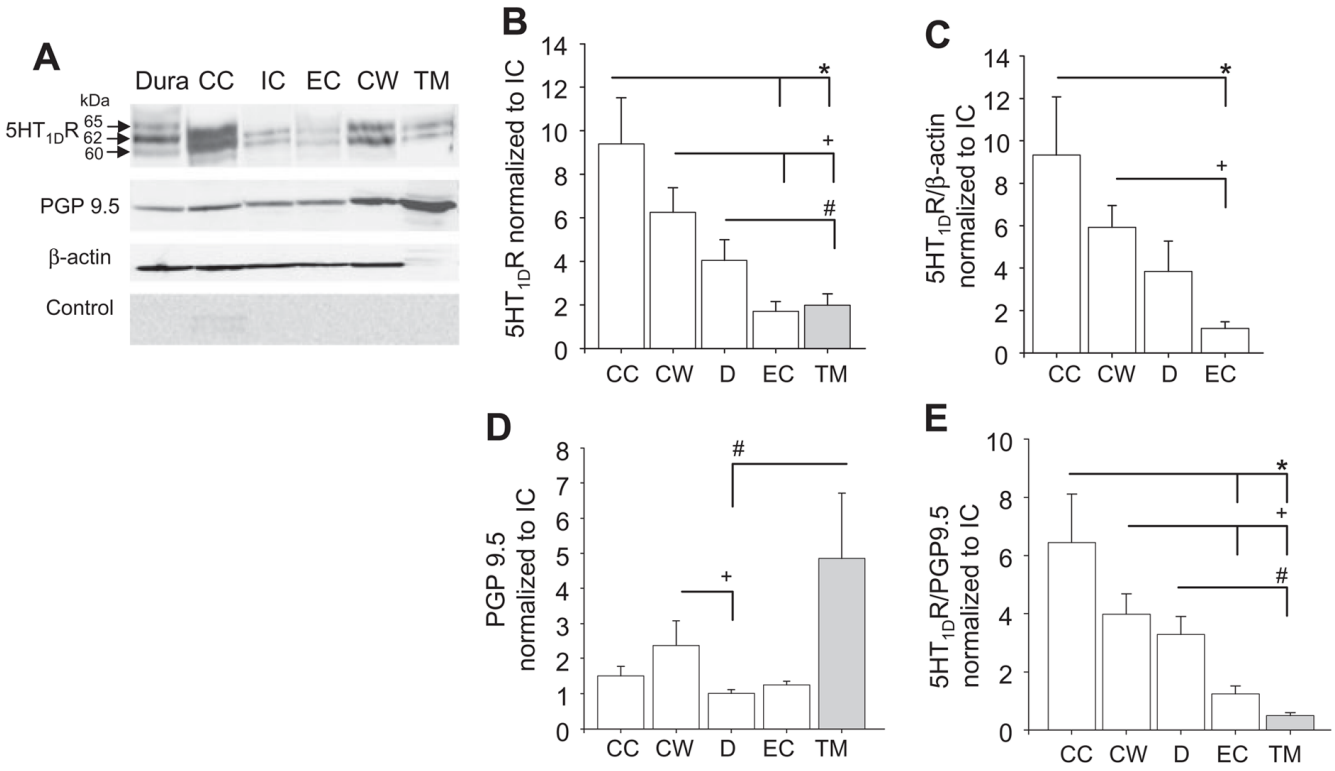


**Figure 1.**

Quantification of $5HT_{1D}R$ in migraine-associated tissues (MATs) and non-MATs. (A) Top panel: Western blot of total protein (150 μg/lane) from whole-cell lysates of dura (D), common (CC), internal (IC), external (EC) carotid arteries, circle of Willis (CW) and temporalis muscle (TM) probed for $5HT_{1D}R$. All structures were obtained from a single rat. Middle panel: blot in A was reprobed with antibodies against PGP9.5 and β-actin. Bottom panel: Control blots were pre-incubated with the peptide used to generate the $5HT_{1D}R$ antibody. (B) Pooled data ($n = 8$) indicate the presence of statistically significant differences between tissues with respect to the amount of $5HT_{1D}R$. (C) Significant differences between CC, CW and EC were also observed when data were normalized to β-actin. (D) There was a statistically significantly difference in the relative amount of PGP9.5 between target tissues. (E) Pooled data indicate the presence of statistically significant differences between tissues with respect to relative levels of $5HT_{1D}R$ normalized to PGP9.5. *Statistically significant differences from CC. +Statistically significant differences from CW. #Statistically significant differences from D where $P < 0.05$.

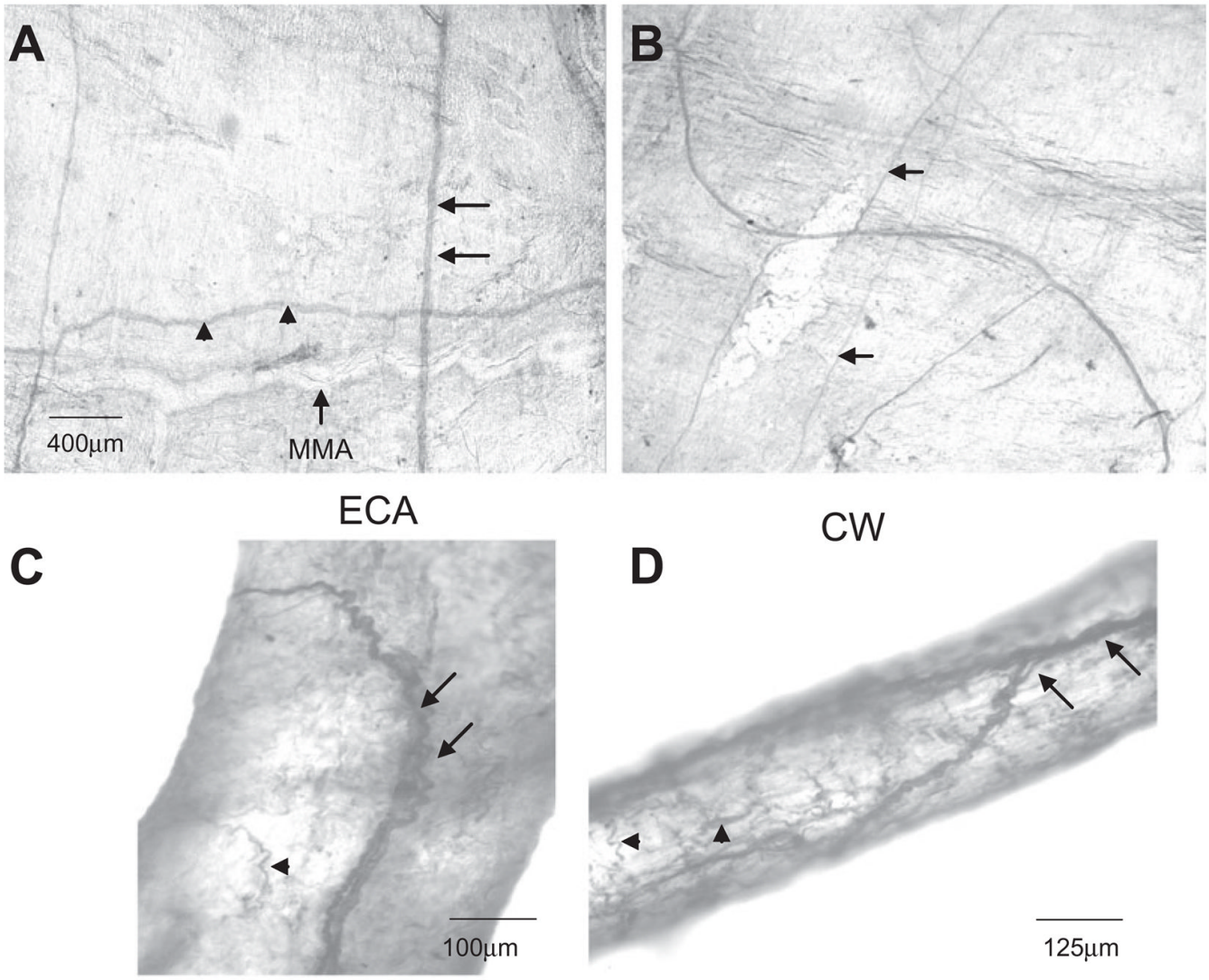


**Figure 2.**

(A) Diaminobenzidene immunohistochemistry of $5HT_{1D}R$ in cerebrovascular tissues. $5HT_{1D}R$ LI is found in nerve fibres innervating dura mater and run parallel (arrowheads) and perpendicular (arrow) to the middle meningeal artery (MMA). (B) There is staining of both larger nerve trunks and smaller fibres (arrow) in the dura. $5HT_{1D}R$ LI is found in nerve fibres innervating the external carotid arteries (C) and branches of the circle of Willis (D) also. There appears to be staining of fine terminal nerve fibres (arrowheads).

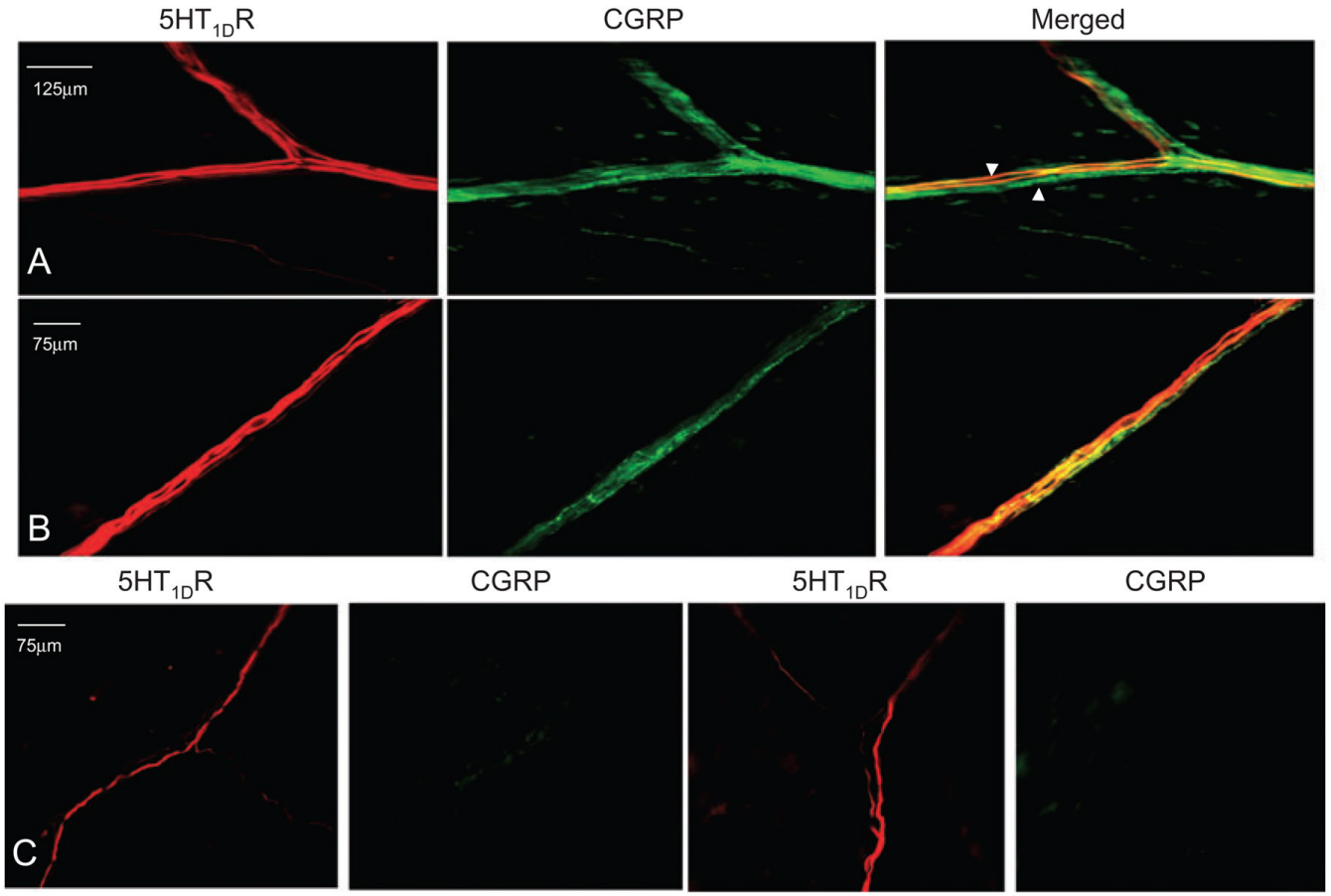


**Figure 3.**

$5HT_{1D}R$ LI was detected in both peptidergic and non-peptidergic fibres in the dura mater. (A,B) Examples of fibres in which both $5HT_{1D}R$ LI and calcitonin gene-related peptide (CGRP) LI were detected. There are also CGRP LI fibres that do not overlap with $5HT_{1D}R$ LI (arrowhead). (C) Examples of fibres in the dura mater in which only $5HT_{1D}R$ LI was detected.

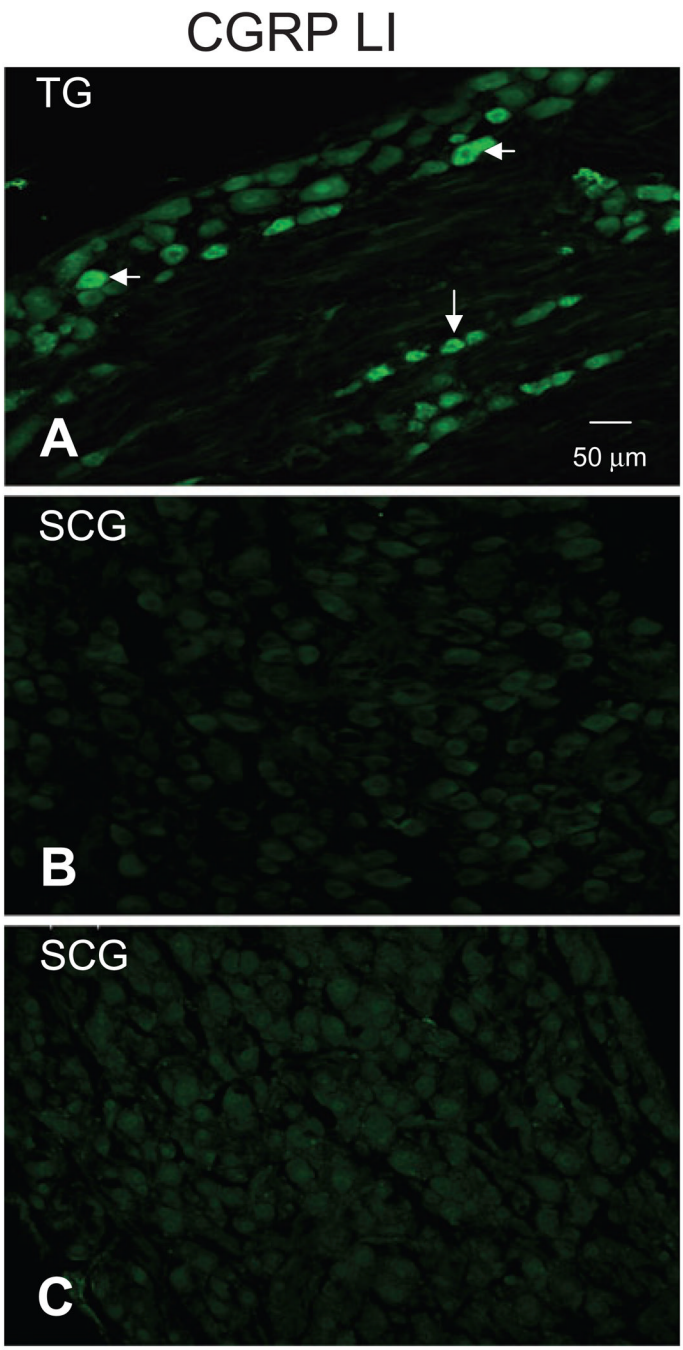


**Figure 4.**
Calcitonin gene-related peptide (CGRP) LI was not detected in rat superior cervical ganglion (SCG). Sections of trigeminal ganglia (TG) (A) and SCG (B) from the same rat were probed in parallel for the presence of CGRP LI. Whereas CGRP-LI was present in a subpopulation of small- and medium-diameter neurons in the TG (arrowheads), CGRP-LI was undetectable in SCG neurons. (C) Omission of primary antibody resulted in a pattern of staining similar to that observed in B.

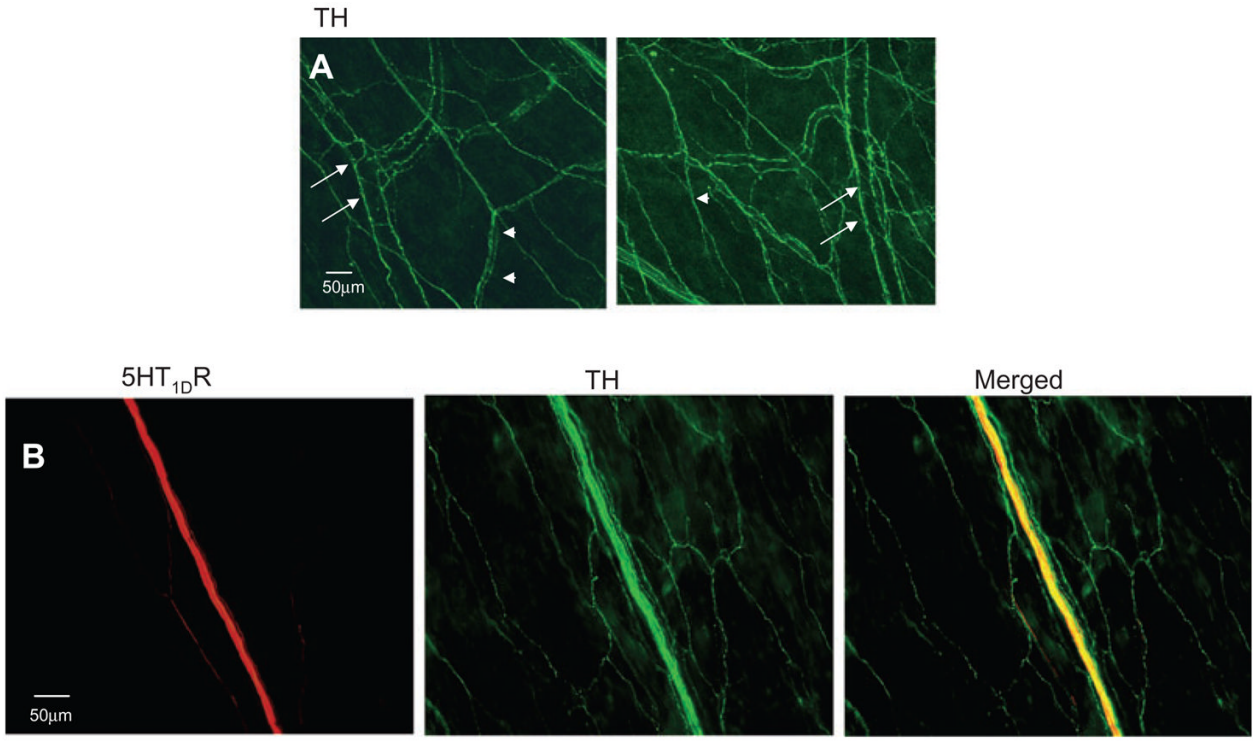


**Figure 5.**
$5HT_{1D}R$ LI was detected in fibres with tyrosine hydroxylase (TH) LI in the dura. (A) TH LI reveals dense sympathetic postganglionic innervation of the dura mater. TH LI appeared in close proximity to meningeal arteries (arrows) and in the parenchyma of the dura (arrows). (B) Example of fibres in which both $5HT_{1D}R$ LI and TH LI were detected in the dura.

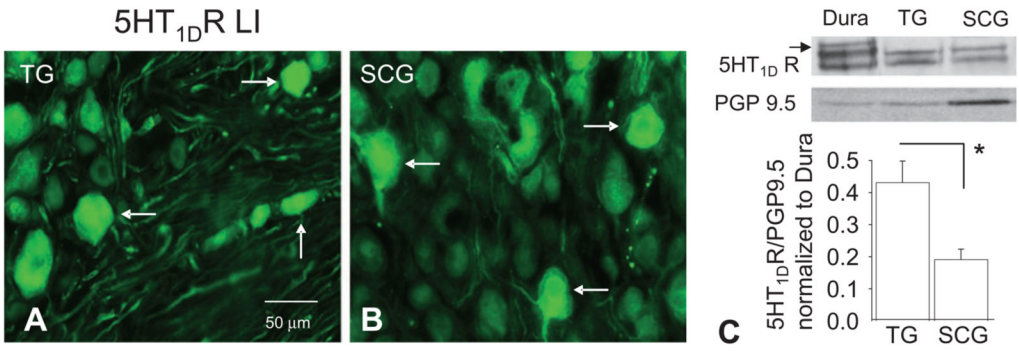


**Figure 6.**

$5HT_{1D}R$ LI (arrows) was present in subpopulations of neurons in trigeminal ganglia (TG) (A) and superior cervical ganglion (SCG) (B). (C) Top panel: Western blot analysis of $5HT_{1D}R$ LI in TG and SCG protein: blot is from tissue obtained from a single rat. The high-molecular-weight band present in the dura (arrow) was present in neither TG nor SCG. Bottom panel: Pooled data ($n = 5$) indicate that relative levels of $5HT_{1D}R$ are greater in TG than in SCG. $^{*}P \leq 0.05$.

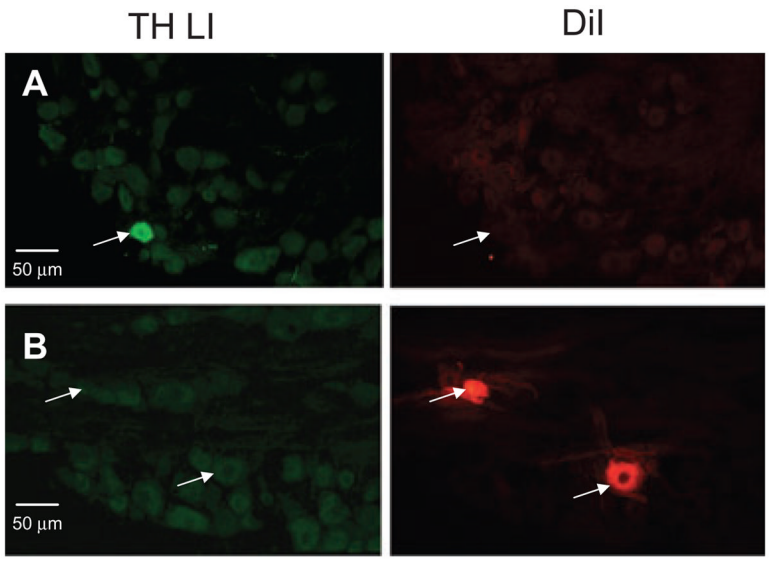


**Figure 7.**
Tyrosine hydroxylase (TH) LI was present in trigeminal ganglia (TG), but not in dural afferents. (A) TH LI was present in a small subpopulation of TG neurons (right panel). None of the TG neurons with TH LI was retrogradely labelled from the dura (middle panel). (B) TH LI was undetectable in TG neurons retrogradely labelled from the dura.