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Serotonin in Trigeminal Ganglia of Female Rodents: Relevance to Menstrual Migraine

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Objectives.—We examined changes in the serotonin system across the estrous cycle in trigeminal ganglia of female rodents to determine which components are present and which are regulated by the variations in levels of ovarian steroids that occur during the estrous cycle.

Background.—Migraine is 2–3 times more prevalent in women than in men and attacks are often timed with the menstrual cycle, suggesting a mechanistic link with ovarian steroids. Serotonin has been implicated in the pathogenesis of migraine, and the effectiveness of triptans, selective 5HT-1B/D/F agonists, has provided further support for this concept. It is not known whether serotonin, its rate-limiting enzyme tryptophan hydroxylase (TPH), or its receptors are regulated by ovarian steroids in trigeminal ganglia.

Methods.—We used reverse transcription-polymerase chain reaction to examine gene expression in cycling mice, Western blots to examine protein expression, double-labeling immunohistochemistry using markers of nociceptors and nonnociceptors and confocal microscopy to identify specific types of neurons, and primary tissue culture to examine effects of estrogen on trigeminal neurons in vitro.

Results.—In C57/BL6 mice mRNA levels of TPH-1, the rate-limiting enzyme in serotonin synthesis, were over 2-fold higher and protein levels were 1.4-fold higher at proestrus, the high estrogen stage of the cycle than at diestrus, the low estrogen stage. TPH protein also was present in primary trigeminal cultures obtained from female Sprague-Dawley rats, but levels were not affected by 24-hour treatment with physiological levels (10^{-9} M) of 17β-estradiol. Gene expression of 5HT-1B and 5HT-1D receptors in trigeminal ganglia was not regulated by the estrous cycle. Serotonin was present in trigeminal neurons containing CGRP, a potent vasoactive neuropeptide, peripherin, an intermediate filament present in neurons with unmyelinated axons, neurofilament H, which is present in neurons with myelinated axons, and in neurons binding IB4, a marker of nonpeptidergic nociceptors. Serotonin was also present in neurons containing 5HT-1B. The serotonin-positive population was significantly larger in diameter than the serotonin-negative population.

Conclusions.—Expression of the rate-limiting enzyme required for serotonin synthesis is regulated during the natural estrous cycle, and serotonin is present in larger trigeminal neurons of all the major subtypes. Colocalization of serotonin with 5HT-1B suggests that this receptor functions as an autoreceptor to regulate serotonin release. Cyclical changes in serotonin levels in trigeminal ganglia could contribute to the pathogenesis of menstrual migraine.

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Abbreviations: RT-PCR reverse transcription-polymerase chain reaction, 5HT-1B 5 hydroxytryptophan receptor type 1B, 5HT-1D 5 hydroxytryptophan receptor type 1D, 5HT-1F 5 hydroxytryptophan receptor type 1F, C57/BL6 C57 black 6 mouse strain, TPH tryptophan hydroxylase, IB4 isolectin B4, FBS fetal bovine serum, PB phosphate buffer, PBS phosphate-buffered saline

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Serotonin has been implicated in the pathogenesis of migraine since early studies showed that serum serotonin levels were altered during migraine headaches[1] and that administering serotonin can abort a migraine attack.[2-4] The effectiveness of triptans, selective 5HT-1B,D,F agonists that act on the trigeminovascular system to bring about migraine relief,[5] supports a role for serotonin in migraine pathogenesis, but the endogenous source of serotonin that activates 5HT-1B,D,F receptors in trigeminal neurons or cerebral vessels remains open to question.

Migraine is 2–3 times more prevalent in women than in men,[6] and severity of pain often varies with the menstrual cycle, peaking around the time of menstruation.[7,8] Although clinical data suggest that estrogen increases the probability of suffering from migraine, paradoxically, the sudden decreases in estrogen levels at the time of menstruation increase the probability of an attack. Trigeminal neurons contain estrogen receptor α (ERα),[9-11] providing a potential mechanism for estrogen to affect their function directly.

The goal of this study was to examine trigeminal ganglia of female rodents to determine which components of the serotonin system are present and to determine whether there are any links between the natural hormonal cycle and expression of genes relevant to serotonin function. Specifically, we examined serotonin, expression of the rate-limiting synthetic enzyme tryptophan hydroxylase (TPH), and the 5-HT-1B and 5-HT-1D receptors in trigeminal ganglia of female mice and rats. We also assessed effects of β-estradiol treatment on female rat trigeminal neurons in vitro.

MATERIALS AND METHODS

Estrous Cycle.—Estrous cycle studies were conducted using C57/BL6 mice, aged 6 to 8 weeks, obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in the Laboratory Animal Resources Center at the University of Kansas Medical Center. Mice were exposed to a 12:12 hours light:dark schedule and provided with food and water ad libitum. Stages of the estrous cycle were determined by daily (0900 hours) assessment of cells retrieved after vaginal lavage for at least 15 consecutive days. Day of the cycle was based on the majority of cells present in vaginal lavage as follows, briefly: proestrus, presence of rounded nucleated cells; estrus, presence of cornified cells; diestrus, presence of leukocytes. Mice were studied at 3 stages of the estrous cycle, proestrus, when estrogen levels peak, early estrus, just before estrogen levels fall precipitously, and diestrus, when estrogen levels are low.[12]

Because metestrus is very short in mice,[13] that stage was not sampled. NIH guidelines of Laboratory Animal Care were followed.

RNA Methods.—Immediately following the final vaginal smear procedure, mice for RNA studies were killed at appropriate stages of the cycle, brains were removed, and trigeminal ganglia were preserved in RNA later (Ambion, Austin, TX, USA). Tissue was homogenized in TRIZOL Reagent (Life Technologies, Rockville, MD, USA). Total RNA was extracted following manufacturer's specifications, precipitated, and dissolved in RNase-free water. Integrity of RNA was tested by viewing denatured ethidium bromide-stained samples in a 1% agarose/formaldehyde gel. For semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription was performed on mRNA (1.0 μg) as directed by the manufacturer using the superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). The cDNA pools from individual mice were divided into aliquots for separate PCRs for a specific gene product and the housekeeping gene rig/S15.[14,15] Invitrogen Life Technologies synthesized the primers. Primer sequences

Table 1.—Primer Sequences

Gene	Primer Sequences	Product
Tryptophan hydroxylase	Sense-(5′-3′)-CTG GAC ATG CCA AAG TCA A Antisense-(5′-3′)-CAA GGT CAT ACC GCA ACT CA	250 bp
5HT-1B	Sense-(5′-3′)-TGA TCG TGC TGG TGT GGG TCT TCT C Antisense-(5′-3′)-GGG AGT TAA TGG AGG TGA CCG AGG	321 bp
5HT-1D	Sense-(5′-3′)-GTC ATC ACA CTG GCC ACT GTC CTC Antisense-(5′-3′)-CAA GAG CAA TGA CAC AGA GAT GCA AG	262 bp
rig/S15	Sense-(5′-3′)-TTC CGC AAG TTC ACC TAC C Antisense-(5′-3′)-CGG GCC GGC CAT GCT TTA CG	361 bp

and predicted product sizes are provided in Table 1. Each reaction was run for 3 minutes at 94°C for 1 cycle, followed by cycles of 1 minute at 94 °C and 1 minute at 55 °C, 2 minutes at 72 °C for 25 to 30 cycles, and 7 to 10 minutes at 68 °C for 1 cycle in a Hybaid PCR Sprint (Middlesex, UK) thermocycler. The cycle number for each primer set was optimized by empirically determining the number of cycles to reach the plateau phase and by carrying out hybridizations within the linear range of amplification. The amplification products were visualized by electrophoresis using a 2% ethidium bromide-stained agarose gel, then digitized using a ChemiIimager 4400 (Alpha Innotech Corp., San Leandro, CA, USA) and analyzed using Scion Image. All results were verified using at least 6 independent samples. A ratio of the gene of interest to the housekeeping gene Rig S/15[14,15] was determined for each sample. Ratios were averaged to obtain mean gene levels for each stage of the cycle and differences were assessed using the Kruskal-Wallis test.

Tissue Culture.—For each experiment, trigeminal ganglia from 10 to 12 random cycling female rats 50 to 60 days old were dissected from the bony base of the brain, rinsed in Hank's balanced salt solution, minced and suspended in digestion medium containing Leibovitz's L15 with 1 mg/mL bovine serum albumin (BSA), 250 U/mL of CLSPA collagenase, 1 U/mL of ESL elastase, 5 U/mL of PAPL papain (enzymes from Worthington Biochemical Corp., Lakewood, NJ, USA). An equal volume of 30% Stractan (Larex, White Bear Lake, MN, USA) in Leibovitz's L15 was added to the digest. After mixing by inversion, samples were centrifuged in a swinging bucket rotor at 4 °C or 10 minutes at 500*g* to enrich the culture for neuronal cells. The pellet of trigeminal cells was suspended in a small volume of DMEM F-12 containing 10% fetal bovine serum, 50 ng/mL nerve growth factor, and 20 μM cytosine arabinoside. Finally, the cells were plated onto matrix-coated (Biomedical Technologies Inc., Stoughton, MA, USA) 24-well plate. The procedure yielded cultures having at least 60% to 70% neurons. Three separate cultures from 10 to 12 rats each were prepared for protein studies.

We used synthetic 17β-estradiol (Sigma E-2758, St. Louis, MO, USA) the most potent and receptor-specific form of estrogen to study the effects of estrogen.[16] After waiting for 3 days to allow any endogenous estrogen to be metabolized, 17β-estradiol was added to the cultures at a final concentration of 10^{-9} M.[17] The 17β-estradiol was initially dissolved in ethanol at a concentration of 10^{-1} M, then further dilutions were performed in phosphate-buffered saline (PBS) + 0.1% BSA to achieve a final concentration of 10^{-9} M, which corresponds to plasma estrogen levels in a physiological range.[17] Cells in the control wells received the diluent only. The cells were treated with 17β-estradiol for 24 hours, a sufficient time to induce changes in gene expression,[18] after which the cultures were used for protein analysis.

Western Blot Analysis.—The Western blot analysis was carried out using tissue obtained from cycling mouse trigeminal ganglia and brain, and tissue culture lysates from rat trigeminal ganglia. Tissue lysates were prepared from trigeminal ganglia or whole brain with radioimmunoprecipitation (RIPA) buffer. In brief, tissues were homogenized using lysis buffer consisting

of 10 mM Tris HCl buffer (pH 7.4) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl. Protease inhibitors, 1 mM phenylmethyl sulfonyl fluoride, 3 mM benzamidine, 5 mM EDTA, 2 mM EGTA, 5 μM pepstatin A, and 10 μM leupeptin were added to the lysis buffer. The tissue lysates were centrifuged at 12,000*g* for 30 minutes at 4°C. The clear lysates were used for Western blots. Prior to Western blot analysis, protein content was determined using BCA protein quantitation kits (Pierce, Rockford, IL, USA). Samples containing 10 μg proteins were separated in 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. Membranes were incubated in blocking buffer containing 4% BSA in TBS with 0.1% Tween 20 (TBS-T, and then probed with anti-sheep TPH antibody (Novus Biologicals, Littleton, CO, USA) in blocking buffer at room temperature for 3 hours at 1:400 dilution, respectively. Membranes were washed 3 times in TBS-T before detection with a biotinylated donkey anti-sheep secondary antibody. Vectastain ABC-AmP kits (Vector Laboratories, Burlingame, CA, USA) were used for Western blot immunodetection. Blots were scanned and quantitated using a Biorad ChemiDoc XRS system.

Immunohistochemistry and Lectin Binding.—To localize serotonin, 5HT-1B receptors, calcitonin gene-related peptide (CGRP), neurofilament H (NF-H), IB4, and peripherin in trigeminal neurons, random cycling female Sprague-Dawley rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2, and trigeminal ganglia removed. Tissue was cryoprotected in 30% sucrose in PBS, and frozen sections through the trigeminal ganglia were prepared at 20 μm using a cryostat. The sections were permeabilized using 0.2% Triton-X100 in 10% normal goat serum (NGS), followed by blocking with Background Buster™ (Innovex Biosciences, Richmond, CA, USA). Primary antibodies were guinea pig anti-5HT1B (1:800, AB5410, Chemicon, Temecula, CA, USA), chicken anti-CGRP (1:300, AB5705, Chemicon), chicken anti-NF-H (N52) antibody (1:900, AB5539, Chemicon), chicken anti-peripherin (1:500, AB9282, Chemicon), and rabbit anti-serotonin (1:400 S5545, Sigma-Aldrich, St. Louis, MO, USA). The 5HT-1B antibody was made using a synthetic peptide corresponding to a region of the large third intracellular loop of the rat and mouse 5HT-1B receptor. The CGRP antibody was made using synthetic rat CGRP. The NF-H antibody was made against purified bovine NF-H and recognizes a 200 to 220 kDa band in Western blots. The peripherin antibody was made against full length rat peripherin and reacts with the 57-kDa peripherin protein. The serotonin antibody was made against serotonin creatinine sulfate conjugated with formaldehyde to BSA. For serotonin, the secondary antibody was Alexafluor 568 (red color) goat anti-rabbit (1:400, Invitrogen/Molecular Probes, Eugene, OR, USA). For 5HT-1B receptors, the secondary antibody was biotinylated goat anti-guinea pig (1:200, Vector Laboratories). For NF-H, peripherin, and CGRP, secondary antibodies were biotinylated goat anti-chicken followed by streptavidin labeled with Alexafluor 488 (1:500, Invitrogen/Molecular Probes). To identify IB4-binding neurons, sections were incubated for 45 minutes with isolectin B4 from *Griffonia simplicifolia* (1:50, IB4 conjugated to FITC, Vector Laboratories) in 10% normal goat serum. Sections were counterstained with 4′6′-diamidino-2-phenylindole, dihydrochloride (DAPI) (1:2000, Invitrogen/Molecular Probes) to visualize nuclei, washed and coverslipped with Slow Fade antifade media (Invitrogen/Molecular Probes).

Fluorescent digital images were obtained using a Zeiss LSM510 confocal microscope equipped with a UV laser (80 mW) for the excitation (364 nm, 50% laser power) and detection (band pass 385 to 470 nm filter; BP385-470) of the DAPI (Hoeschst 33258), and with an Argon/2 laser (25 mW) for the excitation (488 nm, 50% laser power) and detection (BP505-550) of the DTAF fluorescein or Alexafluor 488, a HeNe laser (1 mW) for the excitation (543 nm, 100% laser power) and detection (BP560-615) of the Alexafluor 568. Images were acquired in Multitrack channel mode (sequential excitation/emission) with LSM510 (v 3.2) software and a Plan-Apochromat 25X/0.8 Oil DIC objective with a frame size of 1024 $\times$ 1024 pixels and a zoom factor of 0.7 (field size of 0.512 mm $\times$ 0.512 mm, effective magnification 18$\times$). Detector gain was set initially to cover the full range of all the samples and background corrected by setting the amplifier gain in comparison to the relevant

control slides, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. Control slides consisted of (1) mounted tissue only, no secondary antibodies for autofluorescence, (2) single color only stained samples check for bleedthrough into all the other channels, and (3) secondary antibodies only to check for nonspecific binding.

Single and double-labeled neurons were counted from digital images to obtain a percent score for colocalization of serotonin with each phenotype marker. The percentage of the double-labeled neurons was determined by counting the total number of neurons containing serotonin immunoreactivity and identifying the number of cells labeled with the second neuronal marker. For example, the Alexafluor 488 and DAPI layers were hidden, and the individual digital image frames of the Alexafluor 568 labeled cells were converted into tiff files (standard 24-bit tiff file format in 24-bit color) and merged by image stitching into a single high-resolution full-sized tissue section digital image using Adobe Photoshop.[19] This method prevents double counting of cells on the edges of the individual digital image frames. The same procedure was repeated to count the Alexafluor 488 labeled cells.

We determined the percentage of IB4 binding, peripherin, NF-H, CGRP, and 5HT-1B immunoreactive neurons that also contain serotonin by dividing the percentage of double-labeled neurons by the total number of neurons that were immunoreactive for each marker. Unlabeled cells were counted the same way from the grayscale transmitted light image collected simultaneously with the fluorescent digital images on a separate multitrack channel. The percentage of unlabeled cells was calculated by dividing the number of unlabeled cells by the total number of cells present in the tissue. Counting all 4 groups of cells (red, green, both, and neither) allowed determination of 4 combinations of labeling. The experiment was repeated using two sets of independent samples. Cell diameters were measured using the overlay/ruler function of the Zeiss LSM Image browser. The length of the short and long axes of all the neurons in every selected file was measured. Cells with DAPI-stained nuclei were measured. The diameter of each cell was calculated as the square root of its length multiplied by its width. The diameter was used for the calculation of the area of the cell and corrects the elliptical shape of most of the neurons.

Data were analyzed using Graph Pad Instat (San Diego, CA, USA) version 3.0 software. For mRNA data, differences between groups with respect to stages of the cycle were analyzed using Mann-Whitney or Kruskal-Wallis tests. Data are reported as mean ± standard error with a P value less than .05 considered significant.

RESULTS

Tryptophan Hydroxylase.—Figure 1A demonstrates expression of TPH-1 mRNA in female mouse trigeminal ganglia at diestrus, proestrus, and estrus, compared with a constitutive expressed gene, Rig S/15.[15] TPH-1 mRNA is present at all stages of the estrous cycle, but is upregulated by 2.2-fold at proestrus when compared with diestrus (Kruskal-Wallis test, $P = .03$).

To determine whether the same cylical changes are also present at the protein level, we quantitated TPH protein in trigeminal ganglia at the low estrogen stage (diestrus) and the high estrogen stage (proestrus) using Western blots. Figure 2A shows Western blots of trigeminal ganglia obtained from 4 mice at diestrus and 4 mice at proestrus. Quantitation of chemiluminescence shown in Figure 2B demonstrates that TPH levels were 1.4-fold higher at proestrus than at diestrus,

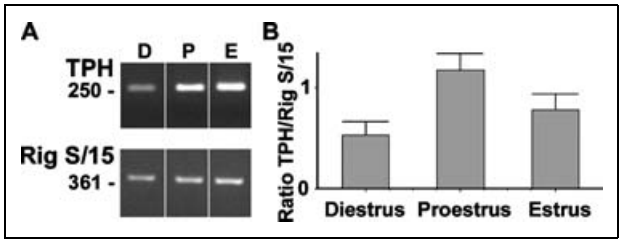

Fig 1.—(A) Semiquantitative analysis of tryptophan hydroxylase-1 mRNA at the low estrogen stage diestrus (D), and at the high estrogen stages proestrus (P), and early estrus (E) in cycling female C57/BL6 mice. Expression of the housekeeping gene (Rig S/15) in the same samples is shown below. (B) Quantitation of mRNA ratios of target gene (tryptophan hydroxylase (TPH)) to housekeeping gene (Rig S/15) from 6 diestrus samples, 6 proestrus samples, and 7 estrus samples, mean ± standard error. The differences among the samples were significant (Kruskal-Wallis test).

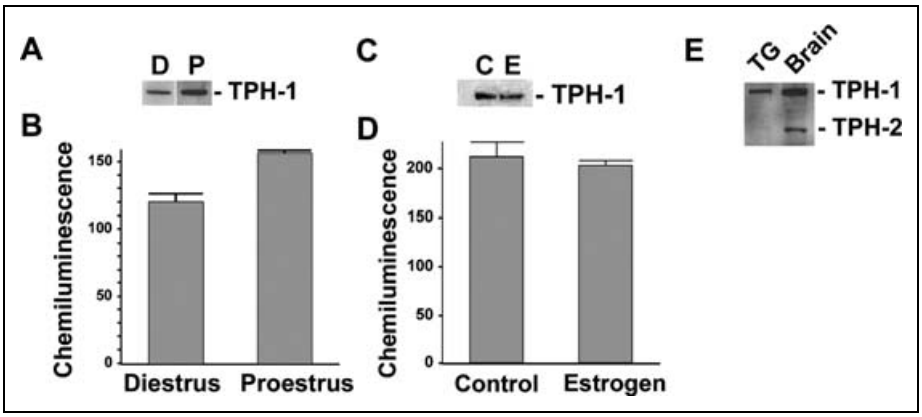

Fig 2.—Tryptophan hydroxylase protein in trigeminal ganglia. (A) Western blot of TPH-1 protein obtained from trigeminal ganglia of female mice at diestrus (D, low estrogen stage) and proestrus (P, high estrogen stage). (B) Quantitation of protein using chemiluminescence, mean ± standard error. Protein levels were 1.4-fold higher at proestrus than at diestrus and the difference was significant (Mann-Whitney test). (C) Western blot analysis of tryptophan hydroxylase (TPH) protein obtained from control (C) and estrogen-treated (E) rat trigeminal neurons in culture. (D) Quantitation of protein using chemiluminescence, mean ± standard error. Estrogen, 10^{-9} M β-estradiol, 24-hour treatment did not increase the amount of TPH-1 protein. (E) Western blot analysis of TPH protein obtained from trigeminal ganglia (TG) and brain of mice at diestrus. In the brain, 2 bands are present corresponding to TPH-1 and TPH-2, while in trigeminal ganglia, only 1 band corresponding to TPH-1 is present.

and the difference was significant (Mann-Whitney test, $P = .014$). To further confirm that the TPH protein seen in trigeminal ganglia is TPH-1, we assessed TPH protein in trigeminal ganglia and whole brain from the same mice using an antibody that recognizes both forms of TPH. Figure 1C shows those data. In brain, 2 bands, corresponding to TPH-1 and TPH-2 were present, while in trigeminal ganglia, only 1 band, corresponding to TPH-1, was present.

Increased expression of TPH mRNA and protein at proestrus suggests direct regulation by estrogen. To examine this possibility, we maintained neurons from female rat trigeminal ganglia for 24 hours in vitro, and then treated the cultures with physiological doses of β-estradiol. Protein was extracted and examined by Western blotting. Results are shown in Figure 2D. Cultures treated with β-estradiol and untreated cultures demonstrated similar amounts of TPH-1 protein. Therefore, treatment of trigeminal neurons with a physiological dose of estrogen for 24 hours did not induce TPH-1.

Serotonin Receptors 5HT-1B and 5HT-1D.—We examined gene expression of serotonin receptors 5HT-1B and 5HT-1D at diestrus, proestrus, and estrus using RT-PCR. Figure 3 demonstrates that both receptors are expressed in mouse trigeminal ganglia. Expression of 5HT-1B was more variable than expression of 5HT-1D (Fig. 3), but neither showed significant variation with stage of the estrous cycle (Fig. 3C).

Serotonin.—The presence of TPH suggests that serotonin may be present in trigeminal ganglia of female rodents. Immunohistochemical studies in female Sprague-Dawley rats showed that proportion of trigeminal neurons containing serotonin (column 1 in all subtables of Table 2) varied from 41% to 69%, with an overall mean of 56%. Figure 4 provides diameter measurements of a sample of the 2 populations and demonstrates that smallest unmyelinated nociceptors, 10 to 15 μm in diameter, do not contain serotonin while the largest neurons, 35 μm and larger in diameter and presumed nonnociceptors, were serotonin-positive. This difference is confirmed by cell diameter measurements of the whole population of trigeminal neurons. The average diameter of serotonin-positive cells (26.9 ± 0.8 μm) was significantly (t-test $P < .01$) larger than the average diameter of serotonin-negative cells (22 ± 1.04 μm).

Figure 5A-C demonstrates colocalization of serotonin and 5HT-1B. In this figure, the center panel shows merged images of 2 labels seen in the right and left panel. Double-labeled cells are visible in the center panel, and examples are marked with arrows. Table 2A provides counts of neurons that contained serotonin, 5HT-1B, or were double-labeled. Seventy percent of

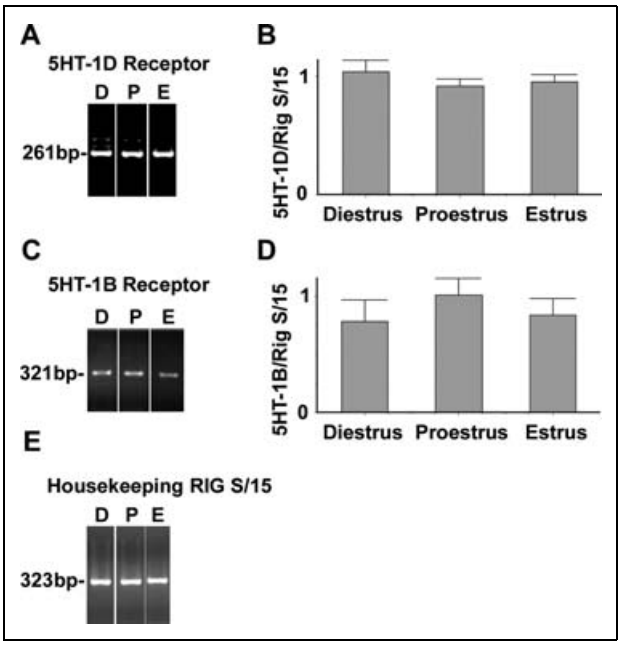

Fig 3.—(A) Semiquantitative analysis of 5HT-1D mRNA at diestrus (D), proestrus (P), and estrus (E) in female C57/BL6 mice. (B) Quantitation of mRNA ratios of target genes 5HT-1D to housekeeping gene Rig S/15 in 8 diestrus, 9 proestrus, and 11 estrus samples, mean ± standard error. The difference among the samples was not significant for either receptor (Kruskal-Wallis test). (C) Semiquantitative analysis of 5HT-1B mRNA at diestrus (D), proestrus (P), and estrus (E) in female C57/BL6 mice. (D) Quantitation of mRNA ratios of 5HT-1D to housekeeping gene Rig S/15 in 5 diestrus, 6 proestrus, and 7 estrus samples. The difference among the samples was not significant for either receptor (Kruskal-Wallis test). (E) Expression of the housekeeping gene (Rig S/15) in the same samples.

serotonin-positive neurons contained 5HT-1B, while 41% of 5HT-1B-positive neurons contained serotonin. The presence of neurons that colocalize serotonin and 5HT-1B suggests that 5HT-1B may function as an autoreceptor, while the presence of neurons containing 5HT-1B without serotonin suggests that 5HT-1B may also function as a heteroreceptor.

Specific functional subsets of trigeminal neurons are also identified by markers that correlate with function, including CGRP, NF-H, the lectin Bandeiraea simplicifolia isolectin 4 (IB4), and peripherin.[20-25] Figure 5 demonstrates serotonin labeling in subpopulations of trigeminal neurons identified using these markers. Counts of single- and double-labeled neurons using these markers are provided in Table 2. Figure 5E shows colocalization of serotonin and CGRP, and counts are presented in Table 2A. The percent of serotonin-positive neurons that contained CGRP was 66%, while the percent of CGRP-positive neurons that contained serotonin was 52%. The proportion of all cells that contained CGRP was 75%. Figure 5N demonstrates colocalization of serotonin and NF-H, and counts provided in Table 2B indicate that 61% of serotonin-positive neurons contained NF-H, while 65% of NF-H-positive neurons contained serotonin. Figure 5N demonstrates colocalization of serotonin and peripherin. Counts provided in Table 2D indicate that 45% of serotonin-positive neurons contain peripherin, while 67% of peripherin-positive neurons contain serotonin. Figure 5K demonstrates colocalization of serotonin and IB4. Counts provided in Table 2C indicate that 23% of serotonin-positive cells are IB4 positive, while 75% of IB4 positive cells contain serotonin.

DISCUSSION

This study has demonstrated the presence of serotonin and its rate-limiting synthetic enzyme, tryptophan hydroxylase,[26] in trigeminal ganglia of female rodents. There are 2 distinct molecular forms of TPH, TPH-1 and TPH-2. TPH-1 is expressed in peripheral tissues while both TPH-1 and TPH-2 are expressed in the brain.[27-29] In addition, in female mice, both TPH-1 mRNA and protein were present at higher levels at proestrus, the high estrogen phase of the estrous cycle, than at diestrus, the low estrogen phase of the cycle. Serotonin receptors 5HT-1B and D were expressed in trigeminal ganglia, but their mRNA levels did not vary with the estrous cycle in female mice. Serotonin was present in about half of all trigeminal neurons, and many of them also contained 5HT-1B. Serotonin was present in medium-sized trigeminal neurons of the major functional subtypes including those containing CGRP, a potent vasodilator present in sensory neurons innervating cerebral vessels,[30-33] IB4, a marker of nociceptors that are less likely to contain CGRP but may contain other peptides,[20,34-37] NF-H, a marker of myelinated primary afferents,[38] and peripherin, an intermediate filament present in unmyelinated axons. Finally, rat trigeminal neurons maintained in vitro contained TPH-1 protein, but levels of this protein were not changed by estrogen treatment.

Table 2.—Counts of Single and Double-Labeled Neurons From 2 Independent Experiments

A. Serotonin + 5HT1B

	A Serotonin only	B 5HT1B only	C Both	D Neither	E Total	F C/(A+C)	G C/(B+C)	H (A+C)/E	I (B+C)/E
First experiment	67	185	125	13	390	0.65	0.40	0.49	0.79
Second experiment	89	374	262	125	850	0.74	0.41	0.41	0.75
Mean						0.70	0.41	0.45	0.77

F = proportion of serotonin-positive cells that are also 5HT1B-positive.
G = proportion of 5HT1B-positive cells that are also serotonin-positive.
H = proportion of all cells that are serotonin-positive.
I = proportion of all cells that are 5HT1B-positive.

B. Serotonin + CGRP

	A Serotonin only	B CGRP only	C Both	D Neither	E Total	F C/(A+C)	G C/(B+C)	H A+C)/E	I (B+C)/E
First experiment	171	502	262	23	958	0.60	0.34	0.45	0.79
Second experiment	115	128	298	60	601	0.72	0.69	0.68	0.71
Mean						0.66	0.52	0.57	0.75

F = proportion of serotonin-positive cells that are also CGRP-positive.
G = proportion of CGRP-positive cells that are also serotonin-positive.
H = proportion of all cells that are serotonin-positive.
I = proportion of all cells that are CGRP-positive.

C. Serotonin + NF-H

	A Serotonin only	B NF-H only	C Both	D Neither	E Total	F C/(A+C)	G C/(B+C)	H (A+C)/E	I (B+C)/E
First experiment	133	97	97	120	447	0.42	0.50	0.51	0.43
Second experiment	104	107	424	327	962	0.80	0.79	0.55	0.55
Mean						0.61	0.65	9.53	0.49

F = proportion of serotonin-positive cells that are also NF-H-positive.
G = proportion of NF-H-positive cells that are also serotonin-positive.
H = proportion of all cells that are serotonin-positive.
I = proportion of all cells that are NF-H-positive.

D. Serotonin + IB4

	A Serotonin only	B IB4 only	C Both	D Neither	E Total	F C/(A+C)	G C/(B+C)	H (A+C)/E	I (B+C)/E
First experiment	320	39	76	136	571	0.19	0.66	0.69	0.20
Second experiment	263	19	97	193	572	0.27	0.83	0.62	0.20
Mean						0.23	0.75	0.66	0.20

F = proportion of serotonin-positive cells that are also IB4-positive.
G = proportion of IB4-positive cells that are also serotonin-positive.
H = proportion of all cells that are serotonin-positive.
I = proportion of all cells that are IB4-positive.

Continued.

Table 2.—Counts of Single and Double-Labeled Neurons From 2 Independent Experiments

	E. Serotonin + Peripherin								
	A Serotonin only	B Peripherin only	C Both	D Neither	E Total	F C/(A+C)	G C/(B+C)	H (A+C)/E	I (B+C)/E
First experiment	226	102	159	213	700	0.41	0.61	0.55	0.37
Second experiment	146	54	140	143	483	0.48	0.72	0.59	0.40
Mean						0.45	0.67	0.57	0.39

F = proportion of serotonin-positive cells that are also peripherin-positive.
G = proportion of peripherin-positive cells that are also serotonin-positive.
H = proportion of all cells that are serotonin-positive.
I = proportion of all cells that are peripherin-positive.

Columns A show and B indicate counts of single-labeled neurons, columns C indicate double-labeled neurons, while columns D indicate neurons identified using transmitted light that contained neither label. Columns E indicate the total number of neurons counted in each experiment. Proportions were calculated by the formulas indicated in columns F, G, H, and I. Columns F show the proportion of serotonin-positive neurons that contain 5HT-1B or each of the functional markers CGRP, NF-H, IB4, and peripherin, columns G show the proportion of each of these subpopulations that contain serotonin, and columns H and I show the proportions of all neurons that contain either serotonin or the specific marker.

Relevance to Previous Work.—Given the widespread use of selective serotonin agonists for migraine, it is surprising that neither serotonin nor TPH has been identified previously in trigeminal neurons. Nevertheless, serotonin or TPH-immunoreactive fibers have been described in close proximity to cerebral vessels and coursing along meninges. The serotonin innervation of cerebral blood vessels and pia has been documented many times.[39-43] Some of these axons originate in the dorsal raphe nucleus,[43,44] but other sources including the superior cervical ganglion have been thought to provide these axons.[45] The absence of serotoninergic neurons in superior cervical ganglia, however, led to the conclusion that the serotonin in axons innervating cerebral vessels is not synthesized in the superior cervical ganglion,[46] leaving the origin of the serotonin in question.[47] Other studies, however, showed that cerebrovascular fibers innervating the middle cerebral and basilar arteries, as well as those innervating pial and dural vessels, originate in

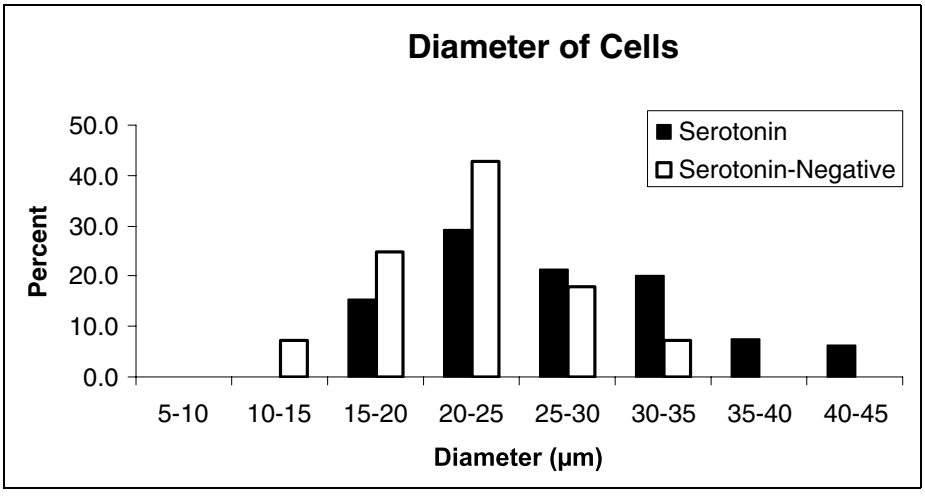

Fig 4.—Cell size measurements of 65 serotonin-positive and 28 serotonin-negative neurons in female Sprague-Dawley rat trigeminal ganglia.

COLOUR FIG.

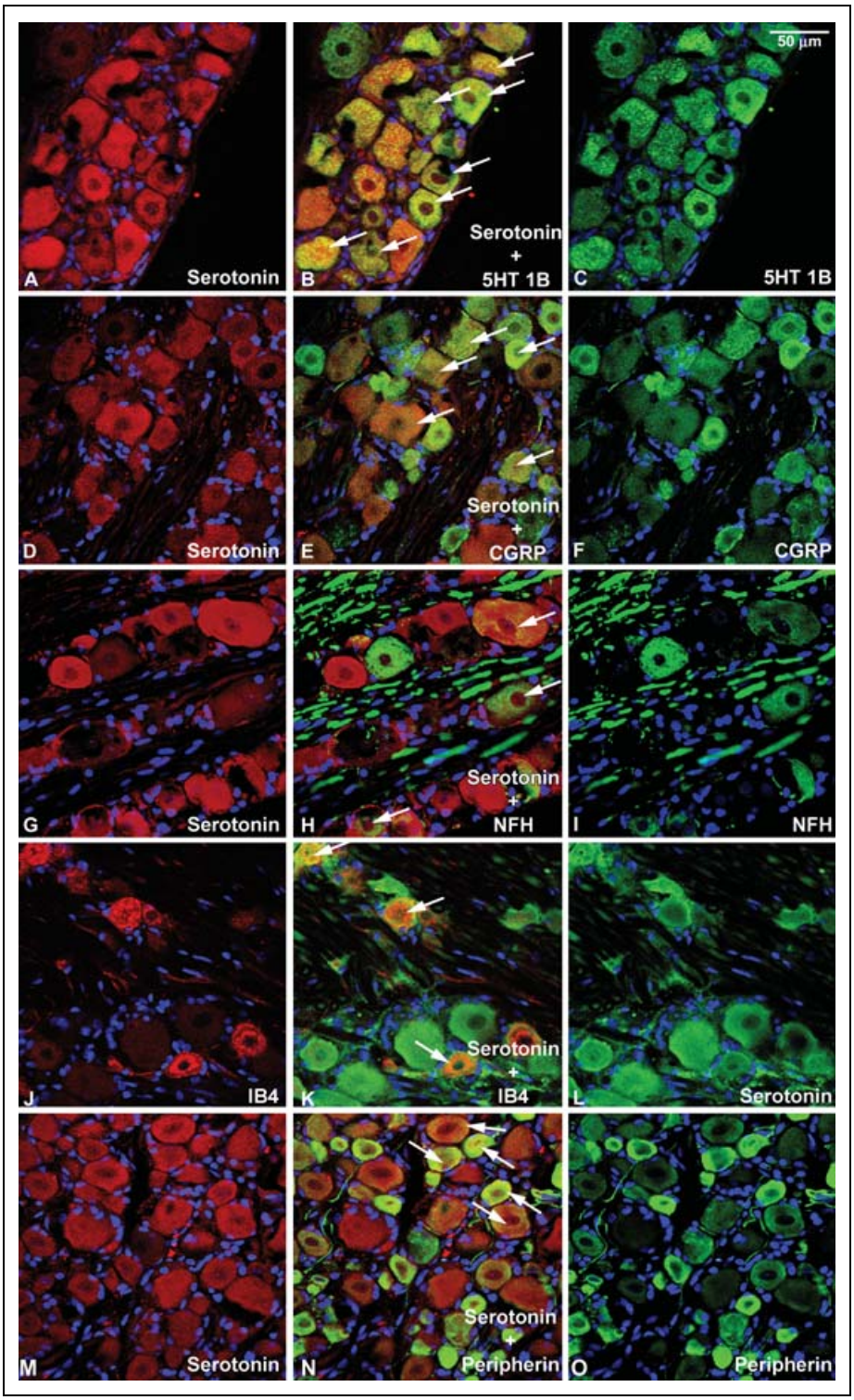

Fig 5.—Colocalization of serotonin and NF-H, peripherin, CGRP, and IB4 in female Sprague-Dawley rat trigeminal ganglia. Serotonin was present in all of the major trigeminal neuronal subtypes identified. Panels on the left are single-labeled for serotonin (A, D, G, and M) or IB4 (J). The middle row of panels contains merged images of the left and right columns. Double-labeled neurons appear yellow or orange. The right row of panels are single-labeled for 5HT-1B (A), CGRP (F), NF-H (I), serotonin (L), or peripherin (O). In panels B, E, H, K, and N, arrows indicate examples of double-labeled neurons. Scale bar (50 μm), shown in the top right corner of C, applies to all images.

the trigeminal ganglion,[48-50] and that these axons do not disappear after superior cervical ganglionectomy. Thus, it is possible that sympathectomy may reduce the serotonin content of the trigeminal axon terminals without affecting them structurally.

Both serotonin and TPH-containing axons have been described in rat dura, but the results of several studies are inconsistent. Perivascular serotonin-immunoreactive axons were demonstrated in the dura of male Sprague-Dawley rats.[51] In a different study, serotonin-positive fibers that did not contain TPH were observed in male Sprague-Dawley rat dura. At the time, those fibers were thought to be sympathetic axons that took up serotonin released from other cells.[52] In third study, TPH-positive but serotonin-negative axons were demonstrated in the dura of male Sprague-Dawley rats.[53] These inconsistencies may result from exclusive use of male Sprague-Dawley rats in previously published studies. Our data suggest that the TPH, serotonin-containing axons reported by others are authentic serotonergic axons originating from trigeminal ganglia.

TPH is transported from cell body to nerve terminals to allow local synthesis of serotonin in axon terminals.[54] Our data suggest that the inconsistent results in males may reflect a lower concentration of TPH and serotonin in dural axon terminals than is present in females. Other studies have suggested that trigeminal neurons contain serotonin. Serotonin-immunoreactive basket-like axonal structures surrounding cell bodies (pericellular axonal baskets) have been demonstrated in trigeminal ganglia, but the cells of origin of these axons were not apparent.[25] In dorsal root ganglia, axonal baskets increase after nerve injury, and many of them originate from nearby sensory neurons,[55-57] suggesting that they may also originate locally within the trigeminal ganglion. It is not known whether these serotonergic axonal baskets respond to nerve injury or peripheral inflammation in female rodents.

Previous studies have also demonstrated serotonin-containing axons in the spinal trigeminal nucleus. Those axons form a dense plexus in layers I and II, precisely overlapping the distribution of primary afferent CGRP-containing axons.[58] The serotonin-containing axons in layers I and II of the spinal trigeminal nucleus were also assumed to originate from the dorsal raphe nucleus.[59] The raphe-trigeminal axons, however, are much less dense than the serotonin-containing terminals, and their pattern of termination is different, as they are absent from layer II,[59] while serotonin-containing terminals are densely distributed in that layer.[58] Taken together, these observations suggest that many of the serotonergic axon terminals in lamina II of the spinal trigeminal nucleus, a major termination layer of nociceptors,[60,61] may be primary afferent terminals.

In humans, it has been reported that only 5HT-1B is present on dural arteries and only 5HT-1D is present on trigeminal sensory neurons,[62] but other reports have demonstrated both receptor subtypes in human trigeminal neurons.[63] In rodents, both 5HT-1B[64,65] and 5HT-1D are present in trigeminal neurons,[66] and 5HT-1D is present on primary afferents in the spinal trigeminal tract.[67] In the present study, we found that expression of both receptors was constant across the estrous cycle. Our data suggest that cyclical changes in numbers of these receptors are unlikely to contribute to menstrual migraine. Conversely, expression of these receptors might be regulated by meningeal inflammation, which in turn is hormonally modulated. The precedent for this speculation is that in dorsal root ganglia, 5HT-1B mRNA is upregulated in a model of inflammatory pain.[68] It is not known whether meningeal inflammation has a similar effect on 5HT-1B or 5HT-1D mRNA, or whether ovarian steroids modulate that response.

Our data are also relevant to studies implicating nitric oxide in migraine pathogenesis. Serotonin depletion increases pial microvascular dilation in response to intravenous nitroglycerin[69,70] without changing NO evoked c-fos immunoreactivity in trigeminal nucleus caudalis, which is viewed as a nociceptive response.[70] Our data suggest a mechanism for those results. About half of rat trigeminal ganglion neurons, including both small- and medium-sized neurons, contain nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), a marker of nitric oxide synthase.[71] It is likely that NADPH-d and serotonin are colocalized and that serotonin and nitric oxide are coreleased from the same neurons. Depletion of serotonin,

a vasoconstrictor, would leave the vasodilator nitric oxide unopposed.

Phenotype of Serotonin-Containing Trigeminal Neurons.—We identified the phenotype of serotonin-containing neurons using a panel of markers that correlate with function. Serotonin was present in all of the subtypes of trigeminal neurons including those that also contained NF-H, a marker of neurons with myelinated axons, CGRP-containing neurons, IB4-binding neurons, and peripherin-containing neurons. From the other perspective, these populations all included many neurons containing serotonin. These data indicate that serotonin-containing neurons comprise subpopulations of each of the major phenotypic groups of trigeminal neurons, including nociceptors and non-nociceptive sensory neurons.

Role of Serotonin Autoreceptors and Heteroreceptors.—Extensive colocalization between serotonin and 5HT-1B in trigeminal neurons suggests that the 5HT-1B receptors may function as autoreceptors. In other neurons, 5HT-1B autoreceptors control release of serotonin from terminals and cell bodies by inhibiting presynaptic serotonin release.[72] Triptans act by activating 5-HT autoreceptors present on sensory fibers innervating blood vessels in the dura,[73] but the source of these sensory fibers has not been identified. Treatment with triptans should activate these autoreceptors, thus inhibiting serotonin release from trigeminal axon terminals in the periphery and in the brainstem. This mechanism would suggest a pronociceptive function of serotonin in trigeminal neurons.

The presence of 5HT-1B in trigeminal neurons lacking serotonin suggests that 5HT-1B receptors also act as heteroreceptors. Triptans have been proposed to function as ligands for serotonin heteroreceptors at both the peripheral and central processes of trigeminal neurons. Triptans inhibit release of CGRP from trigeminal axon terminals in meninges,[74] and may also inhibit CGRP release from central trigeminal axon terminals in subnucleus caudalis, as CGRP immunoreactive trigeminal fibers in the spinal trigeminal tract coexpress 5HT-1D receptors.[67] Triptans, serotonin receptor agonists, can decrease CGRP release from sensory neurons.[75] Our data suggest that serotonin derived from trigeminal neurons might be capable of decreasing CGRP release in the same manner.

Hormonal Regulation of TPH and Relevance to Menstrual Migraine.—Our data demonstrating hormonal regulation of TPH are in agreement with several studies demonstrating that ovarian steroids increase TPH mRNA and protein in the central nervous system.[76-79] Both estrogen and progesterone are involved in TPH regulation,[77,80] and estrogen effects on TPH-1 are mediated via ER β.[81] This mechanism may also occur in trigeminal neurons, which express both ERα and ERβ.[11] Determining the relative contributions of estrogen and progesterone on TPH regulation and the specific hormone receptors involved will require further studies.

Our data demonstrate that TPH mRNA and protein are regulated during the natural estrous cycle in female mice. If similar regulation by ovarian steroids occurs in women, serotonin may be present at higher levels in women than in men, but the levels may also change across the menstrual cycle, contributing to menstrual migraine. These data also draw attention to the TPH-1 gene as a potential contributor to migraine pathogenesis. TPH-1 polymorphisms are associated with unipolar depression, bipolar disorder, and suicidal behavior.[82-86] Interestingly, depression and migraine demonstrate significant comorbidity.[87] In light of the present findings, it would be interesting to determine whether polymorphisms in TPH occur in migraineurs as well as in patients with depression.

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