Sex Differences in the expression of serotonin synthesizing enzymes in mouse trigeminal ganglia

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### Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Aromatic amino acid decarboxylase</td>
<td>AADC</td>
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<td>Dorsal root ganglion</td>
<td>DRG</td>
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<tr>
<td>Guanosine triphosphate cyclohydrolase</td>
<td>GTP-CH</td>
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<td>Gene of interest</td>
<td>GOI</td>
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<tr>
<td>Phosphoglycerate kinase</td>
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<td>Serotonin</td>
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<td>Serotonin transporter</td>
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<td>Trigeminal ganglion</td>
<td>TG</td>
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<tr>
<td>Tryptophan hydroxylase 1</td>
<td>TPH1</td>
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<tr>
<td>Tryptophan hydroxylase 2</td>
<td>TPH2</td>
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<tr>
<td>Vesicular monoamine transporter 2</td>
<td>VMAT2</td>
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<td>House keeping gene</td>
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Abstract

Migraine headaches are more prevalent in women and often occur during the early phases of the menstrual cycle, implying a link between migraine and ovarian steroids. Serotonin (5-HT) and its receptors have been proposed to play a key role in the pathophysiology of migraine. The trigeminal ganglion (TG) has been proposed as a site for 5-HT synthesis based on the expression of the rate limiting enzyme in peripheral 5-HT synthesis, tryptophan hydroxylase 1 (TPH1) in female rodent trigeminal ganglia. Tryptophan hydroxylase levels vary over the estrus cycle, however, the expression and potential regulation of other enzymes involved in 5-HT synthesis has not been reported in this tissue. C57/BL6 mice of both sexes expressed TPH1 and aromatic amino acid decarboxylase (AADC), the two key enzymes involved in 5-HT synthesis. Levels of both enzymes were significantly higher in juvenile males compared to females. In naturally cycling females TPH1 and AADC expression was highest during proestrus when compared to the other phases of the cycle, and this regulation was mirrored at the mRNA level. In-situ hybridization experiments detected TPH1 and AADC mRNA in presumptive neurons in the trigeminal ganglion. Both key enzymes involved in the synthesis of 5-HT are expressed in mouse trigeminal ganglion, and are localized to neurons. The levels of these enzymes are dependent on gender and estrus cycle stage, suggesting that ovarian steroids might play a role in the regulation of sensory neuron 5-HT synthesis.

Key words
Migraine, tryptophan hydroxylase, aromatic amino acid decarboxylase, estrus cycle, trigeminal neuron, pain
5-HT has been postulated to have a direct role in the pathogenesis of migraine and changes in 5-HT levels in the plasma and platelets have been reported during and between migraine attacks, however, a causal link between 5-HT release and migraine attacks has not been firmly established (Lance 1981; Goadsby et al., 2002). The efficacy of selective 5-HT receptor agonists (5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$) that act on the trigeminovascular system to relieve migraine headaches also suggests that 5-HT could modulate the pathogenesis of migraine. However, the source of 5-HT that acts on these receptors in the trigeminovascular system remains to be unequivocally established, with platelets, sympathetic and sensory neurons suggested as possibilities (reviewed in Lincoln, 1995). Migraine attacks are 2-3 times more prevalent in females compared to males with attacks being more frequent during or just prior to the onset of menstruation, a time when estrogen and progesterone levels are falling rapidly (MacGregor, 2010). This suggests that changes in ovarian steroid levels are linked to some migraine attacks, and they are likely acting through multiple mechanisms (Gupta et al., 2011).

Serotonin is synthesized from the essential amino acid L-tryptophan. The rate limiting enzyme, tryptophan hydroxylase converts L-tryptophan to 5-hydroxytryptophan which is the converted to 5-HT catalyzed by AADC. Serotonin is then packaged into vesicles using the vesicular monoamine transporter 2 (VMAT2). Extracellular 5-HT is taken up into the cells using the membrane bound serotonin transporter (SERT) and excess 5-HT is metabolized into 5-hydroxyindoleacetic acid (5-HIAA) using monoamine oxidase and aldehyde dehydrogenase. 5-HIAA is excreted into the urine.
The expression of TPH1 mRNA and protein in the TG was first reported in female rat and mouse which led to the hypothesis that the trigeminal ganglia could be a possible source for 5-HT synthesis (Berman et al., 2006). The expression of the other enzymes involved in the 5-HT synthesis pathway has not been investigated in the trigeminal ganglia. The aims of this study are to examine the presence of other components of the 5-HT system in mice, compare their expression between sexes and confirm estrus-related changes in protein expression in naturally cycling mice.

**Experimental procedures**

**Animals**

Juvenile (approximately 6 weeks old) and adult (approximately 13 weeks old) male and female C57/BL6 mice were used in these experiments. All experiments were approved by the Joint University of Technology/Royal North Shore Animal Ethics Committee, University of Sydney Animal Ethics Committee or the Macquarie University Animal Ethics Committee. Mice were housed in the holding rooms at the Pain Management Research Institute, Royal North Shore Hospital, the Brain and Mind Research Institute at the University of Sydney or Macquarie University and were subjected to a 12:12 hours light:dark cycle with unlimited access to food and water.

**Estrus cycle**

Estrus cycle studies were conducted using 13 week old adult female C57/BL6 mice. To determine the cycle stage vaginal smears were obtained on the day of the experiment by injecting 20µl of sterile saline into the animal’s vagina and by drawing out the liquid and placing it on a slide. Smears were obtained between 8 and 10 AM,
immediately prior to the animal being sacrificed. Smears were visualized on a Nikon TMD inverted microscope with phase contrast optics. Based on the majority of cells present in each smear mice were grouped into diestrus (presence of leukocytes), proestrus (round nucleated cells) and estrus (cornified cells).

**mRNA Quantification methods**

Mice were rendered unconscious with isoflurane, decapitated and the trigeminal ganglia were dissected. The tissue was homogenized in TRIZOL reagent (Sigma, Australia) and total RNA extracted using standard methods. RNA integrity was tested with a Nanodrop (Thermofisher Scientific, Australia) with a 280/260 ratio of 1.9-2 considered satisfactory. For real time PCR experiments, reverse transcription was performed on 1.0 µg of mRNA. For a list of primers used for the gene of interest (GOI) and the house keeping gene (HKG) refer to Table 1. The appropriate HKG was chosen by testing a range of house keeping genes including β-actin, phosphoglycerate kinase (PGK), RNA polymerase and hypoxantine-guanine phosphoribosyltransferase. Ideally the HKG and the GOI should amplify at similar cycle numbers and PGK amplified at cycle number 20. This was close to our GOI and therefore PGK was chosen for these experiments. The level of PGK was not different between male and female mice (P>0.3) or between females in different cycle stages (p>0.1). Quantitative PCR was performed using a Rotogene 3000 or 6000 (Qiagen, Australia). SYBR green dye (Invitrogen, Australia) was used to visualize double stranded DNA products. Amplification was done over 40 cycles with each cycle consisting of the following steps: an initial hold step of 95°C for 10 minutes then 40 cycles of denaturation at 95°, annealing at 55°C and an extension step at 72°C each for 20 seconds. The melt curves were generated by a change in temperature from
72-95°C with 1°C increments. Transcript levels were quantified using the 2 standard curve method.

**Western blot**

For western blots trigeminal ganglia were homogenized in lysis buffer (50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 0.1% protease inhibitor, 0.1% Tween-20) and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected and BCA assay was used to determine the amount of protein in each sample. Samples were mixed with a loading dye and run on 10% Bis-Tris gels (Invitrogen) at 200V for 50 minutes. The 7-minute transfer device (iBLOT, Invitrogen) was used to transfer proteins to PVDF membranes. Membranes were incubated in the primary antibodies sheep anti - TPH1 (1:1000, Chemicon AB1541, USA), rabbit anti-DOPA decarboxylase (1:2000, Abcam ab3905, USA) and rabbit anti-β-actin (1:2000, Sigma A5060, Australia) overnight at 4°C. Primary antibody was washed off with TBST (0.1% Tween-20, pH 7.4) followed by an hour incubation with the secondary antibody anti-rabbit HRP (GE Healthcare NA9340, 1:10000) or anti-sheep HRP (Chemicon AB324P, 1:4000) at room temperature. Membranes were incubated in Enhanced Chemiluminescent Reagent (Amersham, USA) and signals were detected by X-ray film. Developed films were scanned and quantified using ImageJ (NIH) software by an investigator blind to the nature of the sample. The density of the signal for the protein of interest was normalised to its β-actin loading control. Other antibodies used were sheep anti-SERT (SC1458, Santa-Cruz Biochemicals, USA), rabbit anti-SERT (Chemicon AB1594), rabbit anti-TPH2 (AB15572, Chemicon, USA) and rabbit anti-VMAT2 (AB1767, Chemicon).

**In situ hybridization**
Following transcardial perfusion of the mice (4% paraformaldehyde, Sigma) trigeminal ganglia were removed and stored at -80°C. TG were cut into 10 \( \mu \)m sections using a cryostat (Leica, Australia), collected on slides and stored at -80°C. After warming to room temperature, slides were washed in PBS containing 0.1% Tween-20 and sections were hybridized with 200ng/ml of the riboprobes in the prehybridization mix (50% formamide (Sigma), 5x saline/sodium citrate buffer (SSC buffer, Invitrogen), 1x Denhardt’s solution (Sigma), 500\( \mu \)g/ml herring sperm DNA (Promega, Australia) and 5% dextran sulphate (Sigma), 0.1% Tween-20) at 58°C over night. The prehybridization solution was washed off with 4x10 minute washes at 58°C in 4x SSC buffer followed by a 5 minute wash in 0.1M Maleic acid (Sigma) containing 0.1% Tween-20. Slides were incubated with the anti-dioxygenin antibody (1:1000, Roche, Australia) at 4°C O/N. The antibody was washed off with 0.1M Maleic acid containing 1% levamisole (sigma). Staining was developed with the NBT/BCIP (Roche) and detection was stopped by washing slides in the STOP solution (0.1M tris-HCl containing 1mM EDTA). For control experiments, slides were incubated with the sense probe overnight at 58°C and underwent the same wash steps and antibody incubation steps. Slides were coverslipped with fluoromount-G (SouthernBiotech, USA) and kept at 4°C.

**Microscopy**

Slides were visualised under a Zeiss Z2 microscope (Australia) and cells that were positively labelled were counted. The ruler tool was used to calculate the diameter of the positively labelled cells and from that the cell surface area was calculated and cells were grouped into small (< 300 \( \mu \)m\(^2\)), medium (300 \( \mu \)m to 600 \( \mu \)m\(^2\)) and large size (> 600 \( \mu \)m\(^2\)). There is limited information about the expression of specific
markers of nociceptors in mouse trigeminal ganglion, however populations of small to medium sized cells in the ganglia have been identified as nociceptors by virtue of their projections and neurotrophic receptor expression (Mosconi et al., 2001). Functional studies in acutely isolated mouse TG neurons show capsaicin-sensitive neurons with broad action potentials (two markers of potential nociceptors) predominate in the small to medium sized cell group (Borgland et al., 2001; Fioretti et al., 2011). Sections analyzed were obtained at random from central regions of the ophthalmic/maxillary division of the ganglia. We did not attempt to double label sections with a pan-neuronal marker, and it is not possible to accurately identify or count non-labelled neuronal profiles in these conditions.

Statistics

To compare levels of enzyme and protein expression between juvenile male and female mice the Kruskal Wallis test was used and P<0.05 was considered significant. One-way ANOVA was used when comparing the enzyme and protein changes across the estrus cycle with P<0.05 considered significant. Data are expressed as mean ± standard error mean.
Results

Changes in enzyme expression in juvenile animals

cDNA from male mouse trigeminal ganglia was amplified using standard PCR and this showed that mRNA for key enzymes involved in the synthesis and transport of 5-HT were expressed in the trigeminal ganglion, with brainstem cDNA used as a positive control (Figure 1a). In order to assess potential differences between male and female enzyme expression mRNA levels for TPH1, TPH2, AADC, SERT and VMAT2 were compared using real time PCR on cDNA from the trigeminal ganglion of juvenile mice of both sexes (n=8 for each sex, Figure 1b). Levels of TPH1 mRNA were 2-fold higher in male when compared to the females (**P<0.001) and the expression of 5-HT transporter mRNA was also significantly higher in males compared to females of the same age (*P<0.05).

Changes in protein expression in juvenile animals

TPH1 and AADC were readily visualized by western blot in trigeminal ganglion samples but we were unable to detect VMAT2 and SERT (Figure 2). A band was occasionally detected for TPH2 in trigeminal ganglion but it was too faint to accurately quantify, however we verified expression of TPH2 using the same antibody in samples from midbrain. The TPH1 antibody showed a band of similar molecular weight in midbrain and trigeminal. TPH1-like protein levels in the trigeminal ganglion of juvenile male mice was significantly higher than that in age matched females (Figure 2, ***P<0.001, n=8). Similar results were observed for AADC with
male mice displaying significantly higher protein levels compared to female mice (Figure 2, **P<0.01).

**Changes in enzyme levels across the estrus cycle**

Mature female mice had their estrus staged based on vaginal smears. Mice were grouped into 3 stages: diestrus, proestrus and estrus. We compared the mRNA levels during the different phases of the cycle using a One-way ANOVA followed by Tukey’s post-hoc test. There was significantly more mRNA for TPH1 (P<0.05), TPH2, AADC and SERT (P<0.001) during proestrus compared with estrus. AADC and TPH2 levels were also greater in proestrus compared with diestrus, and diestrus compared with estrus. VMAT2 mRNA levels did not change across the cycle (Figure 3).

We examined levels of TPH and AADC using western blot. The amount of protein for both enzymes was significantly higher in proestrus than either estrus or diestrus (Figure 4, One-way ANOVA followed by Tukey’s ***P<0.001, n=8 for each protein).

**Detection of TPH1 and AADC using in situ hybridization in the trigeminal ganglion**

We investigated expression of TPH1 and AADC in the trigeminal ganglion using in situ hybridization. The cell profiles labelled following the in situ hybridization were large with an obvious nucleus, and are consistent with those of sensory neurons. We counted 252 and 257 TPH1 and AADC positively labelled cells in the trigeminal ganglia of 6 male mice (2 non-adjacent sections analyzed per animal, Figures 5 and 6). The diameter of the positively labelled cells was measured along 2 perpendicular axes using the ruler tool in the Axiovision software and the notional cell diameter...
calculated. TPH1 labelled cells had a mean diameter of 19.3 ± 0.9 μm. AADC-positive cells had a mean diameter of 21.8 ± 1.1 μm. Cells were grouped based on their notional cross-sectional area into small (≤300μm²), medium (≤600μm²) and large (>600μm²) sized neurons. With the exception of one cell in each group, all the labelled cells were small or medium sized. Control experiments were run in parallel with the ISH experiments where the sense probe (SP6) was added to the prehybridization mix or sections were only incubated in the prehybridization solution where no binding should occur (figures 5 and 6). In these conditions no positively labelled profiles were observed.

**Discussion**

The principle findings of this study are that mice of both sexes express TPH1 and AADC and importantly, the site of expression of these enzymes is likely to be the trigeminal neurons themselves. We have confirmed previous work showing that TPH1 protein levels in TG are altered across the estrus cycle (Berman et al., 2006), and have extended these findings by demonstrating that levels of AADC protein are regulated in similar manner. While previous work had shown serotonin-like immunoreactivity in trigeminal ganglion neurons (Berman et al., 2006), the localization of TPH1 and AADC mRNA to an apparently similar population of small to medium-sized trigeminal neurons suggests that the enzymes may be co-localized, and may potentially participate in the synthesis of 5-HT. Proof of this awaits studies of protein co-localization and the demonstration of *de novo* 5-HT synthesis by trigeminal ganglion neurons.
Serotonin is a significant modulator of nociception in the trigeminal system. Injection of 5-HT into facial muscles is painful (Ernberg et al., 2000) and local application of 5-HT receptor antagonists attenuates both experimentally-induced pain and some symptoms associated with chronic pain (Chrisitidis et al., 2007; 2008). Migraine pain is relieved by agonists selective for a subset of 5-HT\(_1\) receptors (Olesen & Ashina 2011) and at least some of this therapeutic activity is likely to be mediated by actions of the drugs at sites outside the brain, including cranial blood vessels and trigeminal sensory neurons (Goadsby et al., 2002, Levy et al., 2004).

However, the cellular processes and anatomical sites of action contributing to the biological activity of 5-HT that might be released from trigeminal sensory neurons is unknown and we can only speculate as what the consequences of such release might be. If 5-HT was being released from dural afferents and acting on 5-HT\(_{1B}\) or 5-HT\(_{1B1D}\) receptors on blood vessels or sensory nerves then it could ameliorate some of the sensory inputs contributing to migraine in a manner analogous to triptans. However, 5-HT has complex effects on sensory neuron function, acting through a suite of receptors to modulate the activity of K-channels (Todorovic and Anderson, 1992), calcium channels (Del Mar et al., 1994), tetrodotoxin-resistant sodium channels (Cardenas et al., 1997), the hyperpolarization-activated cation current Ih (Cardenas et al, 1999), TRPV1 (Ohta et al, 2006) and intracellular calcium (Loyd et al, 2011), any of which could act to modulate the excitability of sensory neurons. Within the trigeminal system, the application of 5-HT alone or as part of an “inflammatory soup” sensitizes meningeal nociceptors (Zhang et al., 2007), while 5-HT also inhibits primary afferent neurotransmission in the trigeminal dorsal horn (Travagli and Williams, 1998). As noted, 5-HT also causes vasoconstriction of
cranial blood vessels that may oppose the CGRP-mediated vasodilation associated with migraine. If sensory neurons synthesized 5-HT, they could contribute to any of these processes, particularly if they were afferents activated by blood vessel dilation or by muscle activity. The potential net effect of sensory neuron derived 5-HT presumably depends not only on where and under what circumstances release occurs, but also on which 5-HT receptors are present and what other pro- or anti-nociceptive mediators such as histamine, bradykinin or CGRP are in the microenvironment.

The role of peripheral 5-HT in mediating gender differences in nociception is largely unexplored. The apparent regulation of enzymes involved in 5-HT synthesis across the rodent estrus cycle (Berman et al., 2006, this study) and our finding that juvenile male mice have more TPH1 suggests a relationship between sex hormones and 5-HT generation, although this remains to be confirmed with direct measurement of 5-HT turnover in TG. It should be noted that Berman and colleagues (2006) did not find a direct effect of estrogen on TPH1 levels in TG neurons in culture, suggesting that the relationship between sex hormones and TPH1 levels in vivo may be complex, perhaps involving rapid and/or transitory actions of estrogen, or particular concentrations of hormone. Sex steroid-dependent regulation of TPH mRNA levels in the central nervous system of rodents (Gundlah et al., 2005; Hiroi et al., 2006; Donner & Handa, 2009) and primates has been reported (Pecins-Thompson et al., 1996; Bethea et al., 2000; Sanchez et al., 2005), with both estrogen and progesterone treatment increasing TPH expression in macaques. Conflicting results have been obtained regarding the effects of estrogen on brain TPH2 expression in mice, with some studies reporting increases (Hiroi et al., 2006; Donner & Handa, 2009) and others no change (Clark et al., 2005). The clinical observations that migraine headaches are more common
during the time when estrogen levels are falling coupled with the observations of reduced TPH1 and AADC levels at these times implies that a deficit in trigeminal 5-HT might be associated with menstrual migraine. Reports that 5HT receptor levels may be regulated during the estrus cycle raise the possibility of both reduced amounts of sensory neuron 5-HT and reduced amounts of receptor being expressed in the trigeminal system (Rubinow et al., 1998). A co-incident loss of inhibitory (5-HT1B/D) receptor and ligand could exaggerate the loss of inhibitory serotonergic control of trigeminal nociceptive processing produced by less 5-HT. While Berman and colleagues (2006) did not find estrus-dependent regulation of 5-HT1 receptor mRNA in rat using semi-quantitative PCR, our preliminary investigations show that in mouse TG 5-HT1B and 5-HT1D receptor mRNA is significantly lower during estrus compared with other phases of the estrus cycle (Asghari and Connor, unpublished observations). However, it is likely that estrogen and progesterone also regulate the expression and function of other proteins involved in trigeminal neuron nociception via genomic and non-genomic mechanisms, all potentially contributing to sex and estrus-related pain mechanisms (Scheff and Gold, 2011; Gupta et al., 2011).

In addition to TPH1 and AADC, mRNA for TPH2, SERT and VMAT2 were readily detected in homogenized TG. We were, however, unable to detect the presence of these proteins by Western blot of the TG, although robust signals for TPH2 and VMAT2 were found following Western blotting of midbrain homogenates. We were unable to detect SERT by Western blot, despite use of several antibodies and extraction buffers. A faint band of appropriate molecular weight was sometimes seen in TG homogenates exposed to TPH2 antibodies, however under our experimental conditions this was not a reliable finding. It is possible that TPH2, SERT and VMAT
are expressed in TG, either at levels below the detection of our techniques or in a
form not recognized by the antibodies we used. There is some evidence that TPH2 is
not exclusively expressed in the brain; expression of TPH2 mRNA in dorsal root
ganglia (DRG) neurons has been reported (Tegeder et al., 2006) and TPH2 is thought
to be responsible for 5-HT synthesis by intrinsic gut neurons (Neal et al., 2009).

Our data, which suggest that TPH1 is the predominant isoform expressed in TG, is in
contrast to studies in DRG. Tegeder et al. (2006) reported TPH2 mRNA in mouse
DRG and subsequently used in situ hybridization to show that a few lumbar DRG
neurons expressed TPH2 mRNA after injury to the sciatic nerve. (Wijnvoord et al.,
2010). Interestingly, nerve injury increased the 5-HT content of the DRG, which
suggests that there is functional TPH in the DRG (Wijnvoord et al., 2010). This study
included a statement that TPH1 was not expressed in DRG, but no data was presented.

Our data does not establish that trigeminal neurons synthesize 5-HT. The in situ
hybridization experiments indicate that TPH1 and AADC mRNA are expressed in an
almost identical population of small to medium sized neurons, however, it is not
possible from these studies to prove co-expression, or to estimate the proportion of the
total neuronal population that express the enzymes. While we did not count the
number of unlabelled neuronal profiles in TG ganglion sections, and thus cannot
provide information as to what proportion TG neurons may express TPH1 and/or
AADC, we performed in situ hybridization experiments in the brainstem with our
probes and found that the large proprioceptive trigeminal afferents of the MeV
nucleus did not express either transcript, showing that not all trigeminal neurons can
potentially synthesize 5-HT (Asghari and Connor, unpublished observations). It is
clear, however, that the mRNA and protein signals detected by PCR and Western blot in our study likely arise from a neuronal source, and not from mast cells or small satellite cells in the TG. Other than the studies cited above (Berman et al., 2006; Tegeder et al., 2006; Wijnvoord et al., 2010) there is little other data that speak to question of whether sensory neurons, including TG neurons, can make 5-HT. Nerve fibers in the cranial dura staining positive for either 5-HT (see Lincoln 1995, and references therein) or TPH-like immunoreactivity (Chedotal & Hamel 1990; Mathiau et al., 1994) have been reported by many labs. However, their origin remains unresolved and they have been suggested to represent axons from sensory, sympathetic or even raphe neurons (Lincoln 1995). AADC is also require for 5-HT synthesis, a single study reported a few AADC-immunoreactive cells in adult mouse DRG (Brumovsky et al., 2006), while another in guinea pig found no AADC immunoreactive neurons in DRG or TG (Kummer et al., 1990). By contrast, tyrosine hydroxylase has been reported to be expressed in a cells of the dorsal root, nodose and petrosal ganglia (Katz et al., 1983; Price and Mudge, 1983; Kummer et al., 1990; Brumovsky et al., 2006) although its function in DRG remains obscure.

The biotransformation of tryptophan by TPH requires the co-factor tetrahydrobiopterin (BH$_4$), which is synthesized locally. BH$_4$ is also required for nitric oxide synthase activity, and the rate limiting enzyme in the BH$_4$ synthesis pathway, guanosine triphosphate cyclohydrolase (GTP-CH), is widely but not universally expressed in neurons, including neurons in the DRG (Tregeder et al., 2006). We detected mRNA for GTP-CH in TG, and preliminary experiments indicated that mRNA levels did not differ between male and female mice.
Our study appears to be the first to report estrus cycle related changes in AADC levels in neuronal tissue. AADC activity and mRNA have been previously reported to differ markedly between sexes in kidney and intestine but not brain (Lopez-Contreras et al., 2008). Interestingly, there is evidence that AADC activity in the rat uterus differs during different phases of the estrus cycle, with significantly lower levels of activity during estrus, a finding consistent with this study, where the lowest level of AADC protein was found during estrus (Snyder et al., 1966). It is noteworthy that levels of AADC mRNA did not differ between juvenile mice of each sex, but that protein levels were higher in age matched males than females. This suggests that AADC may be regulated via mechanisms in addition to those that affect DNA transcription or mRNA stability.

**Conclusions**

This study offers strong support to the relatively new idea that trigeminal ganglion neurons may synthesize the neurotransmitter 5-HT. We have shown that TPH1 and AADC are expressed in mouse trigeminal ganglion, confirming and extending the work of Berman et al., 2006. Further, we have shown that transcripts for TPH1 and AADC are apparently localized to neurons, and appear to have a widespread expression. The levels of TPH1 and AADC enzymes are dependent on sex and estrus cycle stage, suggesting that ovarian steroids may regulate the potential of trigeminal sensory neurons to synthesize 5-HT.

**Competing interests**

The authors declare they have no competing interests.
Authors' contributions

RA was involved in the design and execution of all the experiments, in data analysis and drafting the manuscript, ML was involved in the design and execution of the \textit{in situ} hybridization experiments, PP was involved in the study design and interpretation, MC conceived the study, participated in its design, analysis and interpretation and drafted the manuscript. All authors have read and approved the final manuscript.

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Figure Legends

**Figure 1:** Sex differences in expression of mRNA for enzymes involved in 5-HT synthesis and transport in juvenile mouse trigeminal ganglion. A) A representative gel showing amplified cDNA products for tryptophan hydroxylase 1 (TPH1), tryptophan hydroxylase 2 (TPH2), aromatic amino acid decarboxylase (AADC) and the serotonin transporter (SERT) in mouse trigeminal ganglion and midbrain. The first lane in for each species is from a male mouse, the second from a female, and the third (and fourth) from a midbrain sample. Products were visualized with ethidium bromide. NTC is no template control. B) Bar chart illustrating the relative levels of mRNA for each gene of interest (GOI) in trigeminal ganglia from juvenile (6 week old) male and female mice. mRNA levels were quantified as described in the Methods, and are expressed relative to the amount of the housekeeping gene phosphoglycerate kinase (PGK). mRNA levels for the vesicular monoamine transporter 2 (VMAT2) were also compared. There was significantly more TPH1 and SERT mRNA in trigeminal ganglia from juvenile males when compared with juvenile females (* = P < 0.05, *** = P < 0.001, n= 8 animals for each condition).
Figure 2. Sex differences in expression of enzymes involved in 5-HT synthesis in juvenile mouse trigeminal ganglion. A) Representative western blots using antibodies directed against tryptophan hydroxylase 1 (TPH1), β-actin and aromatic amino acid decarboxylase (AADC) protein in 6-week old male C57 BL/6 mice. Western blotting was performed as outlined in the Methods. Each lane represents a different mouse, the TPH1, β-actin and AADC immunoreactivity was assessed in different aliquots of the same sample. The middle lane on each blot is a molecular weight marker, as indicated. Protein samples from male and female animals were run together, and each sample was run at least twice for each protein. B) Bar chart illustrating the relative levels of immunoreactivity for TPH1 and AADC in trigeminal ganglia from juvenile (6 week old) male and female mice. Protein levels were quantified as described in the Methods, and are expressed relative to the amount of β-actin in each sample. There was significantly more TPH1- and AADC-like immunoreactivity in trigeminal ganglia from juvenile males when compared with juvenile females (** = P < 0.01, *** = P < 0.001, n = 8 animals for each condition).
Figure 3- Estrus-cycle associated changes in expression of mRNA for enzymes involved in 5-HT synthesis and transport in the trigeminal ganglion of mature mice. Bar charts illustrating the levels of mRNA for A) tryptophan hydroxylase 1 (TPH1); B) tryptophan hydroxylase 2 (TPH2); C) aromatic amino acid decarboxylase (AADC); D) the vesicular monoamine transporter 2 (VMAT2) and E) the serotonin transporter (SERT) determined at different stages of the estrus cycle in trigeminal ganglion. mRNA levels were normalized to the housekeeping gene phosphoglycerate kinase (PGK) and are reported as mean ± s.e.m. of tissue from 8 animals for each stage. Tissue was isolated and mRNA levels determined by quantitative PCR, as outlined in the Methods. With the exception of VMAT2, the proestrus stage had higher mRNA levels for the genes of interest than estrus, and with the exception of TPH1 and VMAT2, proestrus had higher levels of mRNA expression that diestrus. * = P < 0.05; ** = P < 0.01; *** = P < 0.001.
Figure 4. - Estrus-cycle associated changes in levels of enzymes involved in 5-HT synthesis in the trigeminal ganglion of mature mice. Bar charts illustrating the relative levels of immunoreactivity for antibodies directed against A) tryptophan hydroxylase 1 (TPH1) and B) aromatic amino acid decarboxylase (AADC) in trigeminal ganglia from mature female mice euthanased at different stages of their estrus cycle. Protein levels were quantified as described in the Methods, and are expressed relative to the amount of β-actin in each sample. Each sample was tested at least twice for each protein. There was significantly more TPH1- and AADC-like immunoreactivity in trigeminal ganglia from mice in proestrus than either disestrus or estrus. (** = P < 0.001, n = 8 animals for each condition).
Figure 5: *In situ* hybridization for tryptophan hydroxylase 1 in trigeminal ganglion. In situ hybridization was carried out as described in the Methods. The panels illustrate a low power image of a trigeminal ganglion slice incubated with a probe to tryptophan hydroxylase 1 (TPH1), a high power image of a subsection of the same slice, and a low power image of a slice of ganglion incubated with the corresponding sense probe. Incubation with the TPH1 probe results in widespread staining of neuronal profiles, while the sense probe produces no labelling. This picture is representative of ganglia from 6 male mice.
Figure 6: *In situ* hybridization for aromatic amino acid decarboxylase in trigeminal ganglion. In situ hybridization was carried out as described in the Methods. The panels illustrate a low power image of a trigeminal ganglion slice incubated with a probe to aromatic amino acid decarboxylase (AADC), a high power image of a subsection of the same slice, and a low power image of a slice of ganglion incubated with the corresponding sense probe. Incubation with the AADC probe results in widespread staining of neuronal profiles, while the sense probe produces no labelling. This picture is representative of ganglia from 6 male mice.
A  mRNA for 5-HT related proteins

B  mRNA levels in young mice (qPCR)

Relative amount of mRNA ratio GOI:PGK

**Figure 1**
Figure 3

A

TPH

60 kD
50 kD
40 kD

β-actin

50 kD
40 kD

AADC

60 kD
50 kD
40 kD

B

Ratio of protein:β-actin

Male
Female

TPH
AADC

**

**

Click here to download Figure: Figure 2
Figure 5

Trigeminal Ganglion

TPH1
10 x

TPH1
40x

TPH1 sense
4 x
Figure 6

Trigeminal Ganglion

AADC 10 x

AADC 40x

AADC sense

50 μm

10 μm

100 μm
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