Serotonin Catabolism and the Formation and Fate of 5-Hydroxyindole Thiazolidine Carboxylic Acid

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Serotonin (5-HT) functions as a neurotransmitter and neuromodulator in both the central and enteric nervous systems of mammals. The dynamic degradation of 5-HT metabolites in 5-HT-containing neuronal systems is monitored by capillary electrophoresis with wavelength-resolved laser-induced native fluorescence detection in an effort to investigate known and novel 5-HT catabolic pathways. Tissue samples from wild type mice, genetically altered mice, Long Evans rats, and cultured differentiated rat pheochromocytoma PC-12 cells, are analyzed before and after incubation with excess 5-HT. From these experiments, several new compounds are detected. One metabolite, identified as 5-hydroxyindole thiazolidine carboxylic acid (5-HITCA), has been selected for further study. In 5-HT-incubated central and enteric nervous system tissue samples and differentiated PC-12 cells, 5-HITCA forms at levels equivalent to incubated central and enteric nervous system tissue samples and acid (5-HITCA), has been selected for further study. In5-HT-contaminated central and enteric nervous system tissue samples and differentiated PC-12 cells, 5-HITCA forms at levels equivalent to 5-hydroxyindole acetic acid, via a condensation reaction between l-cysteine and 5-hydroxyindole acetaldehyde. In the enteric nervous system, 5-HITCA is detected without the addition of 5-HT. The levels of l-cysteine and homocysteine in rat brain mitochondria are measured between 80 and 140 µM and 1.9 and 3.4 µM, respectively, demonstrating that 5-HITCA can be formed using available, free l-cysteine in these tissues. The lack of significant accumulation of 5-HITCA in the central and enteric nervous systems, along with data showing the degradation of 5-HITCA into 5-hydroxyindole acetaldehyde, suggests that an equilibrium coupled to the enzyme, aldehyde dehydrogenase type 2, prevents the accumulation of 5-HITCA. Even so, the formation of 5-HITCA represents a catalytic pathway of 5-HT that can affect the levels of 5-HT-derived compounds in the body.

Significant research efforts have focused on advancing our understanding of serotonin (5-HT) function and its mechanism of release, uptake, and metabolism. The majority of this research has involved the central nervous system, where imbalances in 5-HT levels have been linked to various diseases, including Parkinson, Huntington, Alzheimer, Alzheimer-like dementia, anxiety, and depression (1), and its regulation depends on a multitude of 5-HT receptors and neurochemical pathways (2). Other 5-HT functions within the brain involve learning and memory (3, 4) and regulation of various stages of development (5). However, neither 5-HT nor its effects are limited to the central nervous system; 5-HT is found in most smooth muscles in the body and is responsible for the induction of the contractile responses of the gastrointestinal, pulmonary, and genito-urinary systems (6). Specifically, researchers estimate that 95% of the approximate 10 mg of 5-HT in the human body is produced in the enteric nervous system, which includes both the peripheral nervous system of the gastrointestinal tract, as well as the 5-HT-secreting enterochromaffin cells of the gut lining (7). In the enteric nervous system, 5-HT fulfills all criteria necessary for classification as a neurotransmitter (8). Imbalances in 5-HT levels within the enteric nervous system have been observed in association with various disorders, including irritable bowel syndrome, functional dyspepsia, non-cardiac chest pain, and gastric ulcer formation (6, 9).

Our focus is to gain insight into the pathways by which 5-HT is catabolized and the compounds into which it is converted. Because of its high biological potency, tight regulation of 5-HT levels in specific nervous system regions is necessary, and 5-HT catabolism plays an important role in this regulation. Because 5-HT conversion into these other compounds affects the overall levels of 5-HT, formation of these conversion products can be a fundamental factor in 5-HT regulation.

Scheme 1 represents the main 5-HT metabolic pathways and enzymes; however, these represent only a subset of the pathways of 5-HT metabolism, as other lower abundance serotonin metabolites, such as 5-HT sulfate, are known. In immune response pathways, compounds such as formyl 5-HT and 5-hydroxykynuremine (10) are additional serotonin metabolic products. Research performed in the 1950s to track the metabolism of radiolabeled tryptophan demonstrated a number of unknown 5-HT metabolites, proposed to result from addition branches of the monoamine oxidase (MAO) pathway (11). MAO exists in two forms, MAOa and MAOb (12); the former is the primary form of the enzyme responsible for the conversion of 5-HT, although, in the absence of MAOa, MAOb takes over the 5-HT conversion process. With a more in-depth understanding of this, and other potential tissue-specific 5-HT catabolic pathways, it may be possible to develop methods for controlling serotoninergic levels in a tissue-specific manner.

In this study, we identify unique 5-HT metabolites by analyzing 5-HT-producing tissues, before and after incubation with exogenously applied 5-HT. Specifically, we monitor 5-HT metabolite formation within the cen-
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**Scheme 1. Summary of the major 5-HT metabolic pathways in mammals.** Symbols: A, 5-HT; B, 5-HIAL; C, NAS; D, 5-HIAA; E, 5-HTOL (5-hydroxytryptophol); F, melatonin (5-methoxy-N-acetylserotonin); G, MAOa and MAOb; H, 5-HTOL (5-hydroxytryptophol); I, N-acetylseryosine; J, 5-HIAL (5-hydroxyindole acetic acid (5-HIAA)).

tral nervous system, intestines, and differentiated rat pheochromocytoma (PC-12) cells using a custom capillary electrophoresis (CE) system, observing numerous putative 5-HT metabolites. One particular unknown is observed to elute with regularity, and at a surprisingly high fluorescence intensity, comparable with that of 5-hydroxindole acetic acid (5-HIAA).

Using CE with a wavelength-resolved laser-induced native fluorescence (LINF) detection system and mass spectrometry (MS), we identify this particular component as 5-hydroxyindole thiazolidine carboxylic acid (5-HITCA) and explore the mechanism of its formation. The formation of 5-HITCA was previously reported (13–15) when 5-HT was added to homogenized brain tissues and levels of 5-HITCA were shown to markedly increase upon the addition of exogenous L-cysteine (L-Cys). We demonstrate here that in the presence of 5-HT, 5-HITCA is formed in different quantities dependent on the brain region being studied, the levels can be similar to the levels of 5-HIAA in these tissues, and that it forms without exogenously added L-Cys. Furthermore, we investigate the pathways for its formation and demonstrate that it can be detected natively in homogenized enteric nervous system samples. Data measuring the fate of exogenous 5-HITCA as a function of time provides evidence that the compound is not produced de novo but rather is formed from 5-HT. The formation of 5-HITCA is also observed in homogenized brain tissues, intestines, and differentiated rat pheochromocytoma (PC-12) cells using a custom capillary electrophoresis (CE) system, observing numerous putative 5-HT metabolites. One particular unknown is observed to elute with regularity, and at a surprisingly high fluorescence intensity, comparable with that of 5-hydroxyindole acetic acid (5-HIAA).

**Experimental Procedures**

**Materials**

Unless stated otherwise, all reagents were obtained from Sigma at analytical grade or higher, with no further purification. Modified Gray’s balanced salt solution (mGBSS) was previously described (16) and consisted of the following components (in mM): CaCl2 (1.5), KCl (4.9), KH2PO4 (0.2), MgCl2 (11), MgSO4 (0.3), NaCl (138), NaHCO3 (27.7), Na2HPO4 (0.8), HEPES (25), glucose (10), pH 7.2.

**Capillary Electrophoresis**

*CE-LINF with Sheath Flow Detection*—A previously described, laboratory-assembled CE-LINF system (17–22) was employed for these studies. In short, ~3 nl of sample was injected electrokinetically (2.5 kV) into a capillary of 50-µm inner diameter/150-µm outer diameter. Analyte molecules leaving the capillary entered a sheath flow cuvette where they were excited by 257 nm radiation from a frequency-doubled argon-ion laser; fluorescence was collected at a right angle and focused onto a spectrograph/CCD to obtain complete emission spectra. Indoles were identified based on migration time and fluorescence emission profiles as compared with those of known standards. Quantitation was performed using fluorescence intensities of known standard concentrations. Borate buffer (pH 8.8) was prepared for CE by dissolving 3.0 g of boric acid (H3BO3) and 9.2 g of sodium borate (Na2B4O7·10 H2O) in 1.0 liter of ultrapure water (Milli-Q water filtration system; Millipore, Bedford, MA).

*CE-LIF with On-column Detection*—A laboratory-assembled CE system with single-channel laser-induced fluorescence (LIF) detection with 350–356 nm excitation was employed for the separation and quantitation of thiols (see Ref. 23 for further details on the CE system). Injections took place at 8 kV (12 µA) for 8 s, corresponding to a 7.6-nl injection volume, and separations were performed at 20 kV (40–42 µA) on a 50-µm inner diameter/365-µm outer diameter column. For this separation, the CE running buffer consisted of 30 mM sodium borate, prepared by dissolving 0.03 g of boric acid (H3BO3) and 0.97 g of sodium borate dehydrate (Na2B4O7·10 H2O) in 100 ml of ultrapure water (Millipore), adjusted to pH 9.85 ± 0.05 with 0.1 M NaOH (Fisher Scientific, Fairlawn, NJ).

**Incubation of Samples with 5-HT**

Unless otherwise noted, all tissue samples were diluted to a final density of 0.097 g/ml (by wet weight) in mGBSS and homogenized by hand using a 0.1-ml microtissue grinder (Wheaton, Millville, NJ). Following incubation, samples were centrifuged at 10,000 × g for 10 min and frozen immediately on dry ice. Supernatant from each sample was thawed and analyzed via CE-LINF.

**Mouse Brain Samples**—Cerebellum, olfactory bulb, cortex, striatum, raphe nuclei, and pinal gland brain samples from adult C57BL/6 mice (Harlan) were obtained. For control samples, 50 µl of homogenate was incubated with 50 µl of mGBSS; for experimental samples, 50 µl of homogenate was incubated with 50 µl of 0.4 mM 5-HT HCl. Incubation was for 1 h at room temperature, protected from light. L-Cys incubation was done with 50 µl of homogenate, 25 µl of 0.8 µM 5-HT HCl, and 25 µl of 200 µM l-Cys. For pineal and raphe nuclei, the volume was 10 µl.

**MAOa Knock-out Mouse Brain Samples**—Entire brains from both MAOa knock-out (Tg8) and wild type (C3H) mice (24) from the colony at the University of Southern California were removed, frozen immediately, and shipped on dry ice to the University of Illinois at Urbana-Champaign, for measurements; the cerebellum, cortex, striatum, and olfactory bulb were dissected and homogenized at the University of Illinois at Urbana-Champaign. Homogenates were separated into 50-µl aliquots and then incubated with 50 µl of 0.2 mM 5-HT HCl for 1 h at room temperature, protected from light. For olfactory bulb, volumes were 20 µl.

**Rat PC-12 Cells**—Rat PC-12 cells were cultured in 35 x 10-mm tissue culture dishes (Fisher Scientific) in RPMI 1640 medium (Cambrex, Baltimore, MD) containing 0.75% horse serum, 0.25% fetal bovine serum (Clontech), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Cambrex). Cells were cultured at 37 °C in a 5%
CO₂-enriched, humidified atmosphere. To attain differentiation, cells were maintained in medium containing tetradecanoylphorbol-1,3-acetate or dibutyryl cyclic AMP for 2–3 days. PC-12 cell cultures were homogenized using a t-shaped glass rod. For undifferentiated cells, and those differentiated using chemicals, 6.6 μl of homogenate was incubated with 33.3 μl of 800 μM 5-HT HCl for 4 h with rotation at room temperature, protected from light. Samples were subsequently analyzed via CE-LINF.

**Intestinal Samples**—Duodenum, jejunum, and illeum small intestinal tissues were surgically removed from decapitated C57BL/6 adult mice (Harlan) and Long Evans rats (Harlan), then rapidly transferred to chilled mGBSS. Prior to experimental sampling or treatments, tissues were cleared of contents and rinsed in fresh mGBSS. Sections were cut chilled mGBBS. Prior to experimental sampling or treatments, tissues were surgically removed from decapitated C57BL/6 adult mice (Harlan) and Long Evans rats (Harlan), then rapidly transferred to chilled mGBSS. Prior to experimental sampling or treatments, tissues were cleared of contents and rinsed in fresh mGBSS. Sections were cut

**Characterization of 5-HITCA**

**Isolation of 5-HITCA**—Long Evans rat brain striatum homogenate (0.12–0.13 g/ml final density) was incubated with 5-HT (0.35 mM) and daidzin, an inhibitor of aldehyde dehydrogenase (ALDH) (25) (0.18 mM average concentration) for 1 h. The samples were centrifuged for 10 min (8,500 × g) and the supernatant filtered through Microcon centrifugation filters (3000 Da molecular mass cut-off, Millipore). In subsequent experiments designed to search for catabolite formation, both with and without the addition of exogenous 5-HT, tissues were first homogenized and then added to an equal volume of 400 μM 5-HT HCl solution or mGBSS. These incubations occurred at room temperature, protected from light, for a period of 1, 12, and 24 h. At the end of the incubation period, the samples were centrifuged at 10,000 × g for 10 min and frozen at −20°C; all samples were thawed and analyzed by CE-LINF within 12 h of the end of the 24-h incubation.

**Investigation of Enzymatic Formation**

**Enzyme Inactivation**—Six hemispherical cortical slices were cut from isolated brains of decapitated Long Evans rats. Samples were homogenized in 150 μl of mGBSS, with single-slice hemisphere homogenate from each animal either heated in a dry-bath incubator at 95 °C for 5 min, or treated with 33% acetonitrile, to inactivate 5-HT metabolic enzymes. Control samples were kept in mGBSS at room temperature. After cooling the heated homogenates to room temperature, 150 μl of 800 μM 5-HT HCl was added to each vial and allowed to incubate for 4 h while protected from light. Samples were centrifuged for 10 min at 10,000 × g and frozen overnight for analysis the following day.

**Thiol Reactivity Studies**

All reactions were performed in mGBSS. An equimolar final concentration of indole-3-acetaldheyde (IAL) and DL-homocysteine, L-Cys, or glutathione were mixed to achieve final concentrations of ~3 μM for each reagent. For measurement of the N-acetylcysteine reaction, ~200 μM IAL was mixed with ~200 μM N-acetylcysteine. Each sample was mixed by Vortex (Vortex Genie-2, VWR Scientific, Bohemia, NY). Reaction mixtures were allowed to sit a room temperature for 15 min and then analyzed via CE-LINF and MS.

**Measuring Homocysteine and L-Cys Levels within Mitochondria**

Mitochondria were isolated from rat brain by modification of two established procedures (26, 27). After separation by centrifugation, the pellets containing the mitochondrial fractions were rinsed free of EDTA and sucrose by re-suspending them in mGBSS three times after removal of the supernatant. Concentrations of homocysteine and L-Cys were then determined using the CE-LINF with on-column detection.

**Sources of 5-HITCA**

In initial studies, 5-HITCA was synthesized enzymatically via the conversion of 5-HT to 5-HIAL. Human MAOa superosomes were obtained from GenTest (Woburn, MA). The enzyme was kept frozen at −80 °C until use, at which time it was thawed in a 37 °C water bath; 50 μl of enzyme (0.005 μg/ml) was added to pre-mixed vials of 5-HT HCl (16.7 μM to 167 mM) and L-Cys (33.3 μM to 333 mM). Incubations were performed for 2, 4, and 6 h at 37 °C, protected from light. Upon completion of the incubation, samples were centrifuged at 10,000 × g for 3–6 min and frozen immediately in liquid nitrogen. The presence of
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5-HITCA was then confirmed via CE-LINF and samples were combined for isolation of the synthesized 5-HITCA by high performance liquid chromatography. All 5-HITCA was combined and concentrated via SpeedVac to ~200 µl, separated into 50-µl aliquots, then frozen. For later studies, synthetic 5-HITCA was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (28).

**5-HITCA Incubation Studies**

Purified 5-HITCA, synthesized using MAOa, was used for these experiments. One 50-µl vial was diluted with 150 µl of mGBSS. Tissue samples from mouse cortex, cerebellum, and olfactory bulb were homogenized to a final density of 0.097 g/ml in mGBSS. Homogenate was then separated into 40-µl aliquots and either heated at 98 °C for 10 min or left at room temperature. 40 µl of 5-HITCA solution was then added to each sample and incubated at room temperature, protected from light, for 1, 12, or 24 h. Control samples contained only 5-HITCA, or homogenate and mGBSS. Immediately upon termination of incubation, samples were centrifuged at 10,000 × g for 6 min at 4 °C, and kept frozen at −20 °C until thawed for analysis via CE-LINF.

**RESULTS**

**Localization of 5-HT Catabolite Formation**

The Central Nervous System—The cerebellum, cortex, olfactory bulb, and in some cases, the raphe nuclei, pineal, and striatum, from rat and mouse brains were homogenized and incubated with 5-HT, resulting in the appearance of known 5-HT metabolites, including N-acetylseryotonin (NAS) and 5-HIAA. A subset of these data, shown in Fig. 1, was taken from rat brain striatum. One unknown peak, identified as 5-HITCA, was observed in quantities comparable with 5-HIAA. The compound was found to form most abundantly in the cerebellum, with the amount of 5-HITCA production across the central nervous system regions studied shown in Fig. 2.

Differentiated PC-12 Cells—As shown in Fig. 3, following incubation of differentiated PC-12 cells with 5-HT, the catabolites NAS, 5-HIAA, and 5-HITCA were observed. Control samples of non-differentiated PC-12 cells incubated with 5-HT did not form these compounds. To eliminate oxidation of 5-HT and thereby emphasize enzymatically formed compounds, antioxidants were added to some of the incubation mixtures. Formation of 5-HITCA occurred in the presence of the antioxidants, ascorbic acid and trolox (a water-soluble vitamin E analog).

The Enteric Nervous System—Incubation experiments with 5-HT were performed using various regions of the small intestine from Long Evans rat and C57BL/6 mice. Upon incubation of intact tissues with excess 5-HT, we observed several unknown CE-LIF peaks that appeared or increased upon 5-HT incubation (see Fig. 4). In addition to these potential metabolites, the appearance of 5-HITCA and 5-HIAA (the end products of the MAO pathway) were observed in homogenized tissues treated with excess 5-HT. Jejunum samples, homogenized with mGBSS (and no exogenous 5-HT), showed formation of 5-HITCA (data not shown).

**Confirmation of 5-HITCA Structure**

The high abundance and reproducible presence of this particular unknown warranted its structural characterization. The peak, a negatively charged component at pH 8.8, was isolated by liquid chromatography. When the unknown fraction was subjected to ESI-IT-MS, a singly charged peak at m/z 279 (nominal molecular mass of 278 Da) was observed. High resolution scans showed an isotopic distribution indicative of a molecule containing a single sulfur atom. Further structural information was obtained from collisionally induced dissociation of the peak, resulting in two prominent fragment ions, m/z 134 and 148, as shown in Fig. 5A, suggesting that the compound was 5-HITCA. To confirm the characterization, the peak was analyzed by nanoESI-QTOF-MS to obtain an accurate mass for elemental composition determination. In this case, a common O-ring contaminant, a fragment of the plasticizer, di-(2-ethylhexyl)phthalate (29), served as an ideal internal standard for mass calibration. The di-(2-ethylhexyl)phthalate contaminant was resolved from 5-HITCA (Fig. 5B), whereby the observed mass of the contaminant was 279.1665 Da, an error of +0.0069 from the predicted value of 279.1596 Da. Based on this mass shift, 5-HITCA has an accurate mass of 279.0874 ± 0.0069 Da = 279.0805 Da. This confirmed the composition to be C13H15N2O3S1, representing a 0.6 ppm error from the observed value. Additionally, MS was utilized to ensure that the compound synthesized chemically and enzymatically for use in the 5-HITCA incubations studies was identical to the compound identified in biological samples as 5-HITCA.

**Mechanism of 5-HITCA Formation**

Investigating Enzymatic Involvement in 5-HITCA Formation—The involvement of enzymes in 5-HITCA production was characterized by inhibiting specific enzyme activities. Aliquots of three rat brain homogenates were heat-treated and then incubated with 5-HT. In contrast to controls, little or no 5-HITCA was observed in the three heated
samples (data not shown). The same results were observed when tissue samples were pretreated with acetonitrile to destroy enzymatic activity, and then incubated with 5-HT.

**Enzyme(s) Inhibitor Effects on 5-HITCA Formation**—Multiple enzyme inhibitors were sequentially tested to pinpoint the enzyme(s) involved in 5-HITCA formation. Upon incubation of rat cortex and cerebellum homogenate with 5-HIAA and NAS, no 5-HITCA was formed. Furthermore, in cortex samples, commercially available enzyme inhibitors (clorgyline for MAOa and deprenyl for MAOb) were used to block enzymes of known catabolic pathways (Scheme 1), although clorgyline also inhibits MAOb at higher levels. Co-incubation of 5-HT with clorgyline reduced or eliminated levels of 5-HITCA. This effect was not observed with deprenyl.

**MAOa Knock-out Studies**—The enzyme-inhibitor results suggested that MAOa was a significant pathway for 5-HITCA formation. To confirm the involvement of MAOa in 5-HITCA generation, brain tissues from MAOa knock-out mice were studied. CE-LINF analyses of incubated samples from MAOa knock-out brain regions showed the appearance of 5-HIAA and 5-HITCA upon 5-HT incubation. This indicated that the MAOa enzyme was not mandatory to 5-HITCA formation. A small amount of NAS was observed, as well as 5-HT, which was left unconverted. To account for the decrease in the 5-HT signal that was not due to an increase in 5-HIAA, 5-HITCA, or NAS, we searched for other 5-HT catabolites known to exist in animals, such as 5-HT sulfate and γ-Glu-5-HT (20); however, none were detected.

**Role of L-Cysteine in 5-HITCA Formation**—The presence of additional L-Cys increased the production of 5-HITCA by 8-fold. Rat brain mitochondria were isolated via centrifugation and then incubated with 5-HT to synthesize 5-HIAL. This mixture was separated by liquid chromatography and the fraction corresponding to 5-HIAL, as determined by ESI-MS and CE-LINF, was then immediately incubated with L-Cys. The properties of the resulting compound were identical to that of 5-HITCA in terms of CE migration time, fluorescence emission spectrum, and MS fragmentation patterns.

The levels of L-Cys and homocysteine were measured in the mitochondria of the brain via CE-LIF with on-column detection. The estimated measured mass of mitochondria for this preparation was 31 mg, resulting in a range of concentrations (n = 3) of 76–140 μM for L-Cys and 1.9–3.4 μM for homocysteine.

**Other 5-HIAL Reactions**

IAL, a commercially available compound with a structure similar to 5-HIAL, was used for this set of experiments. Reactions were performed in a medium of mGBSS using IAL and excess glutathione, N-acetyl-Cys, homocysteine, and L-Cys, all compounds that contain a free thiol group. These samples were then analyzed via CE-LINF. The sample containing IAL and L-Cys formed a product with a migration time and fluorescence spectrum similar to 5-HITCA. The product was later confirmed by MS to be the IAL analog of 5-HITCA, indole-thiazolidine-carboxylic acid (ITCA). A reaction was also observed to take place between homocysteine and IAL. However, no reaction was seen with IAL and glutathione or N-acetylcysteine.

**The Fate of 5-HITCA in the Brain**

Several regions of the mouse brain, including the cortex, cerebellum, and olfactory bulb, were incubated with 5-HITCA for 1-, 12-, or 24-h
periods and then analyzed using CE-LINF. To assess the stability of 5-HITCA, samples of 5-HITCA were held at room temperature and measured at the same three intervals. In the 5-HITCA-incubated tissue samples, the concentration of 5-HITCA decreased over time while the concentration of 5-HIAA increased (Fig. 6A). Whereas 5-HIAL was observed to form in both the 5-HITCA control and -incubated samples, 5-HIAL was observed at higher concentrations in the control samples than in those containing tissue (Fig. 6B).

DISCUSSION
To investigate 5-HT catabolism, our approach has been to incubate various tissues with 5-HT. A significant percentage (30–50%, data not shown) of the 5-HT added in the incubation solutions is converted to something other than 5-HT, but is not detected as known 5-HT metabolites. Possible fates for this undetected 5-HT include conversion into unknown metabolites, formation of serotonin-protein complexes (30), or involvement in reactions relating to the opening of the ring structure, resulting in molecules that are undetectable via our native fluorescence spectroscopic detection system.

Incubation of samples with excess 5-HT may facilitate the formation of metabolites in several ways. First, in the presence of large quantities of 5-HT, the amount of a compound into which 5-HT is being converted is expected to increase. Second, the known 5-HT catabolic pathways may become overloaded, allowing lesser-used pathways to convert more 5-HT, thereby increasing levels of 5-HT metabolites that might not be readily observed under other conditions. This can be important when a small brain region, dominated by surrounding regions lacking these metabolic pathways, has distinct enzymes that have unique functional consequences; the pineal gland being a pertinent example related to 5-HT metabolism. Last, by using knock-out mice lacking MAOa, additional pathways may be revealed.

Known metabolites were determined by comparing their CE migration times and fluorescence emission spectra with those of known standards, including 5-HT, NAS, tryptophan, tyrosine, and 5-HIAA. Incubation of central nervous system samples with 5-HT showed a peak eluting just before 5-HIAA, with an intensity nearly equal to that of 5-HIAA. Because of its relatively high abundance, it was of interest to identify and characterize this peak, which we identified as 5-HITCA. The large quantity of 5-HITCA formed following 5-HT incubation led us to characterize its structure. Based on increased formation of 5-HITCA when L-Cys was added to the 5-HT tissue incubation, it appeared that the structure was likely an indole ring with an attached L-Cys moiety. Using MS, we determined the structure, 5-hydroxyindole-thiazolidine-carboxylic acid, whereby a cyclized L-Cys accounted for the minimal fragmentation observed upon collisionally induced dissociation (Structure 1).

Upon investigation of central nervous system samples from MAOa knock-out mice, the presence of 5-HITCA, as well as 5-HIAA, was observed at levels similar to those seen in wild type animals. It is inter-
incubation of intact tissues, the intestinal samples appeared highly complex, at least four unassigned indole-containing compounds were formed following excess 5-HT incubation. When homogenized tissue samples were allowed to stand at room temperature for 24 h without the addition of exogenous 5-HT or l-Cys, 5-HITCA was observed. Interestingly, 5-HIAA was observed natively in intestine samples, suggesting that MAO is present and functioning in these regions.

How is HITCA formed? Collectively, the results obtained from incubations of heat- and acetonitrile-treated homogenate, as well as those from incubations of differentiated rat PC-12 cells in the presence of antioxidants, suggested that at least one enzymatic step is involved in the formation of 5-HITCA. Further study using enzyme inhibitors for MAO and ALDH2 revealed that 5-HITCA formation required a functional MAO pathway. Incubations with 5-HIAA did not result in 5-HITCA formation, giving evidence that 5-HITCA was formed upstream of 5-HIAA. Enzyme inhibition experiments provided evidence that ALDH2 was downstream in the metabolic pathway. All of these factors combined indicated that the molecule, 5-HITCA, was formed from the immediate MAO product, 5-HIAL. Also, the addition of l-Cys to the incubation mixture increased the amount of 5-HITCA produced by a factor of eight, making 5-HITCA two to five times more abundant than 5-HIAA in these samples, and indicating that l-Cys was involved in the production of 5-HITCA. Because 5-HITCA results from the condensation reaction between 5-HIAL and l-Cys, the formation of this compound from 5-HT requires two steps: the enzymatic production of 5-HIAL, followed by the condensation reaction of 5-HIAL and l-Cys.

However, the question remained as to why 5-HITCA was observed only upon 5-HT incubation in the central nervous system and natively in the enteric nervous system only 12 h after homogenization; further investigation was required to determine the possible fate of 5-HITCA. Fig. 6a shows that in 5-HITCA-incubated tissue samples, the decrease of 5-HITCA was accompanied by an increase in 5-HIAA. Because 5-HIAA did not appear in the control samples containing 5-HITCA, these results suggest that 5-HITCA is being converted to 5-HIAA.

How is 5-HITCA converted to 5-HIAA and is this conversion enzymatic? To answer these questions, control samples containing 5-HITCA with no tissue (and therefore no active enzymes) were left at room temperature for the duration of the experiment. Measurements of these control samples, and those of the 5-HITCA-incubated tissue samples, were taken at the same time intervals (1, 12, and 24 h). The presence of 5-HIAL was observed in both the control and tissue samples. When the levels of 5-HIAL in the control samples were compared with the levels of 5-HIAL in the tissue incubation samples, more of the compound was shown to accumulate in the control samples (Fig. 6b). This suggests that in the presence of brain homogenate, 5-HIAL is further converted via ALDH2 to 5-HIAA. Most importantly, this indicates that the conversion of 5-HITCA to 5-HIAL is non-enzymatic. Thus, under physiological conditions, a 5-HIAL sink exists that continuously pulls the 5-HITCA:5-HIAL equilibrium in the direction of 5-HIAL and prevents the accumulation of 5-HITCA. For this reason, levels of 5-HITCA are below our limits of detection (100 nM) and the compound is not observed without 5-HT incubation in the central nervous system. As the levels of 5-HT in the intestines are an order of magnitude higher, 5-HITCA levels in intestinal samples are high enough to detect with no exogenously added 5-HT or l-Cys. These results reveal the likelihood that the conversion of 5-HITCA occurs via an equilibrium that exists between 5-HITCA and 5-HIAL rather than a direct enzymatic conversion of 5-HITCA to 5-HIAA, and explain why native 5-HITCA is difficult to detect.
Susilo and colleagues (14, 15) reported the formation of ITCA from incubations of brain homogenates with tryptamine, later documenting the formation of 5-HITCA from 5-HT in brain homogenates, and Singh and Dryhurst (13) performed a follow-up study on the mechanism of formation. As discussed herein, the current study expands on these prior works, incorporating knowledge based upon the formation pathway in specific central and enteric nervous system structures, the involvement of various enzymes, as well as the potential participation of other thiols.

Based on this mechanism of 5-HITCA formation, we ask here whether condensation reactions between 5-HIAL and other thiols present in these tissues account for the other metabolites observed upon 5-HT incubation. We found that both homocysteine and L-Cys react with IAL, thereby implying that a reaction occurs between homocysteine and 5-HIAL. However, we were unable to match the CE migration time and fluorescence spectrum of such a homocysteine-HIAL compound with any of the unknown metabolites that appeared upon 5-HT incubation. Additionally, we explored the possibility of a reaction between 5-HIAL (using the analog IAL) and two other thiols, glutathione and N-acetylcysteine; however, we did not observe product formation from either of these reactions. Structural features common to L-Cys and homocysteine, but not to glutathione and N-acetylcysteine, include a free amine group positioned two or three carbons away from the free thiol. These results suggest that this combination of a free amine and a free thiol group is necessary for the reaction with 5-HIAL to occur. In contrast to our findings, Singh and Dryhurst (13) showed that 5-HITCA analogs formed with glutathione, and that little or no condensation product was observed unless the homocysteine was incubated with both 5-HT and L-Cys or glutathione. Although the presence of L-Cys did increase the amount of 5-HITCA formed, upon 5-HT incubation without additional L-Cys-containing compounds, the metabolite formed at levels equivalent to 5-HIAL in central nervous system tissues, as well as in differentiated rat PC-12 cells. In homogenized samples from the jejunum, 5-HITCA appeared without the addition of either exogenous 5-HT or L-Cys-containing compounds.

Are the levels of homocysteine and L-Cys in the rat brain high enough to allow formation of significant amounts of condensation products? The concentrations of homocysteine and L-Cys within the brain mitochondria were analyzed. L-Cys concentrations were 76–140 μM, suggesting that 5-HIAL once produced via MAO and released into the cytoplasm, reacts with L-Cys to form 5-HITCA, before coming in contact with ALDH2. Concentrations of homocysteine were much lower, suggesting little competition exists between homocysteine and L-Cys for reaction with 5-HIAL, while also providing an explanation as to why the homocysteine-HIAL condensation product was not observed upon 5-HT incubation. Finally, in contrast with earlier findings suggesting a C-S lyase is responsible for the metabolism of 5-HITCA (15), we found that an equilibrium exists between 5-HITCA and 5-HIAL; and that this equilibrium is continuously being pulled in the direction of 5-HIAL, thereby explaining the lack of 5-HITCA accumulation in the brain.

Having knowledge of the existence of 5-HITCA and its mechanism of formation, as well as its potentially tissue-specific nature and mechanism of conversion, will direct future work toward exploration of the possible functions of 5-HITCA. Because its observed regional-specific formation depends on L-Cys, MAOa, and ALDH2 or ALDH levels, the possibility of regional accumulations of 5-HITCA cannot be discounted.

Of course, the other unknown indoles discovered in the enteric nervous system after 5-HT incubation also provide interesting targets for further study. Based on the success of the combined approach of selective detection offered by CE-LIF, and the chemical-rich information of liquid chromatography-MS for characterizing metabolites in the mammalian brain, other 5-HT metabolites in both the central and enteric nervous systems will be examined.

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