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Extracts of Retina and Brain That Excite Afferent Fibers Innervating Hair Cells Contain a Compound Related to Hydroxyphenylglycine-*N*-carbamoyl

W.F. Sewell^{1,2,3,*}, E.A. Mroz^{1,3}, and J.E. Evans⁴

¹Eaton-Peabody Laboratory, Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts

²Program in Neuroscience, Harvard Medical School, Boston, Massachusetts

³Department of Otolaryngology and Laryngology, Harvard Medical School, Boston, Massachusetts

⁴Proteomics and Mass Spectrometry Facility, University of Massachusetts Medical School, Worcester, Massachusetts

Abstract

To identify new neurotransmitter and modulator candidates that might be important in transmission from sensory hair cells to afferent nerves, we examined extracts of neural tissue for compounds that excite afferent fibers innervating hair cells. Here, we describe the extraction and purification from retina and brain of a potent, unstable, excitatory compound with pharmacological activity similar to glutamate on afferent fibers innervating hair cells. This compound, however, was clearly distinguished from glutamate, other common amino acids, and known endogenous glutamate-receptor agonists. After derivatization and analysis by gas chromatography–mass spectrometry, the major compound found in highly purified neuroactive chromatographic fractions had the same gas chromatographic elution time and mass spectrum as the compound formed by derivatization of *L-p*-hydroxyphenylglycine-*N*-carbamoyl. Hydroxyphenylglycine-*N*-carbamoyl, however, did not copurify with the neuroactive compound and was not neuroactive. We thus hypothesize that the detected compound was produced from a precursor, structurally related to *L-p*-hydroxyphenylglycine-*N*-carbamoyl, that was a major component of the neuroactive chromatographic fractions. Because several compounds related to hydroxyphenylglycine are known to act on glutamate receptors, such a compound is an interesting candidate to be an endogenous glutamate-receptor ligand in the mammalian nervous system.

Keywords

hearing; cochlea; neurotransmitter; endogenous; hydroxyphenylglycine-*N*-carbamoyl; glutamate

INTRODUCTION

In an attempt to identify new neurotransmitters or endogenous modulators that might be important in the function of hair-cell organs, we previously examined extracts of hair-cell tissue for compounds capable of exciting afferent fibers and innervating hair cells of the lateral-line organ of *Xenopus laevis*. Hair cells at rest spontaneously release neurotransmitter because of

*Correspondence to: William F. Sewell, Ph.D., Eaton-Peabody Laboratory, Massachusetts Eye and Ear Infirmary, 243 Charles St., Boston, MA 02114. E-mail: wfs@epi.meei.harvard.edu

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the resting activation of voltage-dependent calcium channels. The released neurotransmitter activates glutamate receptors on afferent fibers to produce a spontaneous rate of discharge in the afferent fibers (Sewell, 1996). In *Xenopus*, one can monitor afferent discharge for hours from the same set of synapses while applying compounds to the synapse. When we applied extracts of inner ear tissue to the synapse, we detected a potent excitatory compound capable of increasing afferent discharge rate when present at the synapse (Sewell and Mroz, 1987). However, even when the inner ears of 6,000 fish were processed, we were left with too little material for identification of the neuroactive compound after purification because of sample consumption in bioassay and sample instability (Sewell and Mroz, 1990). Reasoning that other neural tissues may contain this neuroactive compound, we examined extracts of brain and retina for similar neuroactive substances. Here, we show that those extracts contain a compound similar to that found in hair-cell tissue. With the larger amounts of starting material available from retina and brain, we have determined that the major compound detected with gas chromatography–mass spectrometry (GC-MS) is related to hydroxyphenylglycine-*N*-carbamoyl. Though hydroxyphenylglycine-*N*-carbamoyl is not bioactive, hydroxyphenylglycine and several related compounds are known to interact with metabotropic glutamate receptors. These results raise the possibility that an endogenous ligand related to hydroxyphenylglycine may be active at the hair-cell synapse.

MATERIALS AND METHODS

Tissue extraction

Bovine retinas were obtained from Pel-Freez Biologicals (Rogers, AK), J.A. Lawson (Lincoln, NE), or Arena Bros. (Hopkinton, MA). In the first two cases, the supplier performed the dissection; in the third, we did. Retinas (obtained from J.A. Lawson) were from eyes that had been dark-adapted on ice for several hours before dissection and freezing. Otherwise, retinas were generally dissected within an hour of death and kept on dry ice from the time of dissection until arrival in our laboratory. Frozen bovine brains were obtained from Pel-Freez.

Each batch of 20 g of retina (about 40 retinas) was extracted in 100 ml boiling water for up to 10 min and centrifuged in a Hermle 220.72 V04 swinging bucket head at 5,000 rpm (3,600g_{max}) at 4°C for 30 min. The supernatant was lyophilized, and the pellet from centrifugation was discarded. The powder from lyophilization was reextracted with 100 ml ethanol (85%) and centrifuged for 20 min. This supernatant was rotary-evaporated under reduced pressure (at 35°C) to a volume of ~3 ml. Water was added to adjust the volume to 6 ml, the pH adjusted to 7 with 2.5 M NaOH, and this solution was partitioned against 6 ml of ethyl acetate at room temperature. The lower, aqueous phase from this partitioning was reduced in volume (by rotary evaporation) to remove residual ethyl acetate and then passed through ion-exchange resins (see later). Bovine brain was treated similarly, but on a larger scale.

Variations included initial extraction with ethanol, extraction of retinas at a larger scale (up to 270 g), use of brain acetone powder (Sigma) as starting material, extraction of brains with acetone, and rotary evaporation rather than lyophilization of the initial extract. Also, some brains were extracted using the Folch method (Folch et al., 1957). When feasible, samples were handled under argon in a dimly lit room.

Ion-exchange treatments of extract

The volume of the aqueous phase from ethyl-acetate partitioning was increased to 7 ml with water, its pH adjusted to 7, and passed through a 14-ml column of cation-exchange resin (AG50WX12 100-200 mesh, Na⁺ form, BioRad Laboratories), followed by 7 ml of water. The collected 14 ml solution was adjusted to pH 7.0 and passed through a 14-ml column of anion-exchange resin (AG1 X8 100-200 mesh, acetate form). This was followed by 7 ml water, and

the pooled 21 ml of the collected solution was frozen and lyophilized for subsequent chromatographic fractionation. Treatment with the cation-exchange resin removed potassium, which could alter the bioassay, from the fractions.

Variations included omission of anion-exchange treatment and dilution to 70 ml before application to the anion-exchange resin for more complete removal of glutamate. Volumes of ion-exchange resins and applied samples and rinses were scaled proportionately to the extracted tissue weight when large scale extractions were performed.

Chromatography

Gel permeation—The lyophilized sample after ion-exchange treatment of an extract of 20 g retina was taken up in 3 ml water; Hepes was added to a final concentration of 10 mM and pH adjusted to 7.5 with 2.5 M NaOH. This solution was applied to a 50-ml (2.5×10 cm) Sephadex G10 column eluted with 20 mM NaCl, 10 mM Hepes, and 5 mM NaOH at pH 7.5, at a linear-flow velocity of 2 cm/h; 1.1-ml fractions were collected. For large scale extractions, larger columns and eluent volumes were used proportionately, maintaining linear velocity of the eluent at 2 cm/h.

High-performance liquid chromatography cation-exchange chromatography—One milliliter of concentrated, pooled eluate from Sephadex G10 containing not more than 750 μ mol of sodium was adjusted to pH 4 (titrated with formic acid) and injected on a 0.46×25 cm HEMA-IEC-BIO 1000 SB column (10- μ m particle size) with a corresponding guard column (Alltech). The mobile phase was 5 mM sodium formate, adjusted to a pH of 4 with formic acid, at a flow rate of 1.67 ml/min; 5-ml fractions were collected.

HPLC reverse-phase chromatography—Up to 1 ml of sample, titrated to a pH of 4 with acetic or formic acid, was injected onto a 0.46×25 cm Alltech Adsorbosphere HS column (5- μ m particle size) with a corresponding guard column. Gradient elution at 1 ml/min was performed from 5 to 95% methanol in 5 mM sodium acetate (or formate), pH 4. After 10 min, at 5% methanol, a 15-min linear gradient to 95% methanol was begun, and 1-ml fractions were typically collected. One half of each chromatographic fraction to be examined was lyophilized and prepared for bioassay; the other half was taken for chemical analysis or for mass spectrometry.

Bioassay

We used our extensively characterized preparation of the lateral-line organ of immediately postmetamorphic *Xenopus laevis* to test for glutamate-like neuroactive compound in chromatographic fractions (Mroz and Sewell, 1989; Sewell and Mroz, 1987). This in-vitro preparation consisted of a piece of skin containing neuromasts of the lateral-line organ, placed with its inner surface up on a sheet of filter paper. The associated lateral-line nerve was drawn up into a suction electrode. All but two or three neuromasts were destroyed, allowing us to detect individual action potentials in the afferent fibers of the nerve, while providing a high-enough overall firing rate to reduce variability in the responses. “Spontaneous” discharge in these fibers occurs in response to transmitter release from the hair cell in the absence of mechanical stimulation and is thought to be mediated by glutamate receptors (Sewell, 1996). The inner surface of the skin, in diffusional continuity with the afferent synapse, was continually perfused with a buffered salt solution (120 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl_2 , 20 mM Hepes, and 10 mM NaOH; pH 7.5) at a flow rate of 60 μ l/min.

Samples to be tested for neuroactivity were individually adjusted to the same salt concentrations and pH as those of the superfusion buffer. We measured osmolality of the adjusted samples. Some samples after gel-permeation chromatography had elevations of 10–

30 mosm, mostly due to taurine. Purification steps after gel-permeation chromatography separated taurine and other neutral amino acids from the bioactivity. Sample volumes of 100 μ l were applied via a sample injection loop into the continuing flow of superfusion fluid; thus, they were present for somewhat more than one minute at the tissue. "Excitatory" neuroactivity of a sample in this bioassay refers to an alteration of the afferent-nerve firing rate similar to that typically seen with known glutamate-receptor agonists: an excitation at low concentrations and an excitation/inhibition at higher concentrations. Twenty or more samples, enough to test most or all fractions of interest from a single chromatography run, were routinely tested on a single preparation. Each sample was typically tested on three separate bioassay preparations.

Modifications—At early stages of the purification, the perfusion medium and all samples to be bioassayed contained bicuculline methiodide at 0.3 mM to remove any interference from GABA, which is present in high concentration in crude retina extracts and is capable of producing excitation of afferent discharge (Mroz and Sewell, 1989). This concentration of bicuculline had little effect on spontaneous afferent discharge, but blocked effects of GABA up to 3 mM.

If acetate or formate concentrations above 10 mM were expected in samples to be applied to the bioassay, the excess acetate or formate was removed by adjusting the pH to between 3 and 4 with HCl, followed by lyophilization. Similarly, if ammonium ion concentrations above 0.2 mM were expected, the excess was removed by lyophilization after alkalization with NaOH.

Chemical assays

The concentrations and chromatographic elution patterns of known substances (protein, glutamate, GABA, glutamine, glucose, sodium, and Hepes) from gel-permeation chromatography were monitored with published photometric, colorimetric, or fluorometric procedures (Sewell and Mroz, 1987,1990). Additional tests for these substances were performed when required to rule out their contributing to neuroactivity of fractions found in later stages of purification.

Gas chromatography–mass spectrometry

Derivatization for GC-MS analyses—Two different derivatization schemes were used to obtain volatile products for analysis by GC-MS. One method was *tert*-butyldimethylsilylation (TBDMS), using *N*-methyl-*N*-(*tert*-butyl-dimethylsilyl)-trifluoro-acetamide (MTBSTFA; Pierce Chemical), of sites bearing active hydrogens on OH, SH, and NH groups. Samples were dried into 100- μ l Reacti-vials (Pierce Chemical), and 25 μ l each of dimethylformamide and MTBSTFA was added. The mixture was heated at 105°C for 30 min and cooled to room temperature before direct-injection GC-MS analysis.

The second derivatization method produced thermally stable and volatile electron-capturing derivatives from multifunctional compounds. In this derivatization scheme, aldehyde and ketone groups were first methoximated, hydroxyl groups and amino groups (other than those on carbons alpha to carboxyl groups) were acetylated, carboxyl groups were pentafluorobenzylated (providing highly sensitive detection by electron-capture negative chemical ionization GC-MS), and remaining amino groups acetylated (de Jong et al., 1986). The first step, methoximation of oxo groups, was performed by dissolving the dried sample in 50 μ l of 2% methoxylamine hydrochloride in pyridine and allowing it to react overnight at room temperature. After evaporation of the pyridine under a stream of nitrogen at 45°C, amines and phenolic hydroxyls that are susceptible to pentafluorobenzylation were blocked by acetylation in 250 μ l of methanol containing 50 μ l of acetic anhydride and 5 μ l of triethylamine for 10 min at room temperature. After evaporation of this mixture, the residue was dissolved in 250 μ l of acetonitrile containing 10 μ l of pentafluorobenzyl (PFB) bromide (Pierce

Chemical) and 5 μ l of triethylamine and reacted for 10 min at 60° to form PFB esters of carboxyl groups. Finally, after evaporation of that mixture, compounds were peracetylated by reaction for 60 min at 60° in 250 μ l of 1:1 (v:v) acetic anhydride–pyridine. The solvent was removed by nitrogen evaporation and the residue partitioned between 200 μ l of ethyl acetate and 300 μ l of water. The aqueous water (lower) phase was discarded, and the ethyl acetate phase was dried under a stream of nitrogen at 40°C and redissolved in ethyl acetate for analysis by GC-MS. This method, producing *O*-methoxime-*N,O*-acetyl-PFB ester derivatives, is referred to here as the “PFB derivatization.” In some instances, noted in the Results section, acetylation was performed with perdeutero acetic anhydride (Cambridge Isotope Laboratories, North Andover, MA).

Gas chromatography and mass spectral analysis—A Finnigan 4500 GC-MS system equipped with a Teknivent Vector/Two data system was used for these analyses. Gas chromatography of derivatized samples was performed using a 30 m $\times$ 2.5 mm DB5-MS fused silica-capillary column, with a 1- μ m phase thickness (J&W Scientific), connected directly to the MS ion source. Direct injections of 1 μ l of derivatized sample solutions were performed with the injector at 300°C and hydrogen as the carrier gas at 10 psi. The column was programmed from 100 to 160°C at 30°/min and then to 330° at 5°/min with the transfer lines maintained at 300°C. Electron-impact (EI) positive-ion GC-MS of TBDMS derivatives was performed with the ion source at 190°C. Negative chemical ionization (NCI) for MS of pentafluorobenzylated samples was performed with the ion source at 170°C and methane as the moderator gas at 0.2 torr. GC-MS data were collected starting 5 min after injection and continuing until the end of the GC run, from *m/z* 100 through 600, with a cycle time of 0.7 s.

All chemicals, solvents, and reagents used were of A.C.S. analytical reagent grade or better, except ethanol which was USP. Nippon Kayaku (Tokyo, Japan) generously provided *L-p*-hydroxyphenylglycine-*N*-carbamoyl. The identity of the material was confirmed by direct-probe introduction EI and ammonia chemical-ionization mass spectral analysis.

RESULTS

Glutamate-like neuroactive compound was extracted from retina

Extracts of retina contained a neuroactive compound similar to that found in extracts of inner ear. The neuroactive compound was detected with a bioassay, based on discharge rate in afferent fibers innervating hair cells in the lateral-line organ of *Xenopus laevis*. Responses of the bioassay to the extracted neuroactive compound were very similar to the responses to exogenously applied glutamate (Fig. 1). However, as described later, these purified extracts contained neither glutamate nor any other known glutamate-receptor agonist.

Neuroactive compound purified similarly in retina and hair-cell extracts

We initially used the same extraction (boiling water), pretreatment (cation-exchange resins at neutral pH), and first chromatographic separation (Sephadex G-10 gel-permeation chromatography) as we had used on extracts of inner ear. In the presence of 300 μ M bicuculline, to block effects of GABA, the extracts of retina displayed an excitatory neuroactivity with similar chromatographic properties as found for inner ear extracts. The neuroactive compound eluted near a large peak of ninhydrin-positive material (Fig. 2) (which included taurine and glutamine), but was separated from many other potentially active compounds, including glutamate, aspartate (which elutes with glutamate), and ATP (which elutes between protein and glutamate). Bioassay responses to fractions from the G10 column are shown in Figure 3. Most neuroactivity eluted in fractions 25, 26, and 27, and was observed, at lower magnitude, when the samples were diluted 2-fold (second panel).

The time course of the bioassay response can be better appreciated at the expanded time scale in the lower panel of Figure 3. The high concentration of taurine in these samples was sufficient to produce osmotic suppression of discharge, which was the probable cause of much of the suppression. After taurine was removed in subsequent chromatographic steps, the bioassay responses more closely resembled those seen in hair-cell extracts.

Additional prechromatographic treatments, described in the Methods, removed several compounds that interfered with subsequent chromatographic steps. Reextraction of the boiling-water extract with ethanol removed protein. Partitioning at neutral pH between water and ethyl acetate minimized lipids while leaving the neuroactive compound in the aqueous phase. Anion-exchange pretreatment prior to gel-permeation chromatography greatly reduced levels of glutamate, aspartate, and other anions. For all these treatments, we examined bioassay responses to fractions from gel-permeation chromatography and verified that recovery of neuroactive compound was unimpaired. We also compared boiling-water extraction (followed by reextraction in ethanol) to cold-ethanol extraction, and found no substantial differences in neuroactivity after gel-permeation chromatography. This result suggests that the neuroactive compound is stable to brief periods of heating at 100°C. Several other extraction procedures (petroleum ether/acetone, acetone, and Folch extraction) seemed to produce no greater yields of the neuroactive compound than did boiling water or ethanol extraction.

Substantial purification of the neuroactive compound by cation-exchange HPLC

The main contaminants remaining after the pretreatment and gel-permeation chromatography steps (primarily water-soluble, neutral amino acids including glutamine, alanine, glycine, and taurine) were separated from the neuroactive compound by cation-exchange high performance liquid chromatography (HPLC) at pH 4. The neuroactive compound was retained for nearly one hour (100 ml of eluent) on this column, while most amino acids were washed through or were only slightly retained. The neuroactive compound was eluted broadly on the trailing edge of a large UV (214 nm) absorbance peak, which we identified as phenylalanine (Fig. 4). A small amount of the neuroactive compound was also observed in fraction 4.

The experiment illustrated in Figure 4 was repeated with similar results 15 times: eight times at pH 2.5 and seven times at pH 4.0. This difference in pH made little or no difference in the elution time or the recovery of the neuroactive compound. Elution times for both the neuroactive compound and phenylalanine could vary among chromatographic runs over a range 20% above or below the typical elution time of 54–60 min. Nevertheless, in all cases, these elution times varied in parallel. The neuroactive compound always eluted on the trailing edge of the phenylalanine peak. Furthermore, neuroactive fractions, when pooled and rerun on this column, still showed neuroactivity eluting on the trailing edge of the phenylalanine peak.

This behavior on cation-exchange HPLC at pH 4 was unusual. Acidic amino acids and most neutral α -amino acids (with carboxyl groups having pK's less than three) were eluted from this column within 20 min or less; GABA and basic amino acids were eluted after 90 min or more. Only zwitterions with aromatic groups (phenylalanine) or having distinctive pK's of carboxyl groups (e.g., β -amino isobutyrate, pK 3.4) were found to elute between the neutral α -amino acids and the basic amino acids.

The interactions of the neuroactive compound and phenylalanine with this column were not completely ionic but seem to have substantial hydrophobic character. In three cases, 5% methanol in the eluate reduced the retention times of both the neuroactive compound and phenylalanine to 20 min.

Neuroactivity coeluted with a UV-absorbance peak on reverse-phase HPLC

On gradient-elution reverse-phase (C18) HPLC at pH 4.0, the neuroactive compound was retained until the gradient reached 70–80% methanol. Responses of the bioassay to fractions from a reverse-phase HPLC separation of retina extract are plotted in Figure 5. The characteristic bioassay response was seen primarily with fraction 23, which also contained a peak of UV absorbance (214 nm). The retention time of the neuroactive compound on this column varied between chromatographic runs from 22 to 24 min.

To examine whether the earlier pretreatment and purification steps had led to large losses of neuroactivity, we subjected the crude retina extract (dried ethanol extracts simply partitioned between water and ethyl acetate) to this reverse-phase HPLC step. The amount of neuroactive compound eluting from the HPLC column was of the same magnitude as that found after the more extensive purification from similar masses of retina. Thus, there appeared to be no major losses of neuroactivity in the early stages of the purification scheme. Elimination of the cation-exchange HPLC step did not seem to significantly reduce sample purity (based on UV absorbance on reverse-phase HPLC), so later tissue preparations were taken from gel-permeation chromatography directly to the reverse-phase HPLC separation.

Retention of the neuroactive compound on the reverse-phase column depended on pH. At pH 7.0, the neuroactive compound was not retained but was detected in the wash through. If fractions that washed through at pH 7.0 were adjusted to pH 4.0 and reapplied, the neuroactive compound was then retained on, and eluted from, the column.

Two types of control samples verified the biological specificity of the result. Neither concentrated mobile phase nor biologically inactive fractions from previous chromatographic steps showed neuroactivity after elution from the reverse-phase HPLC column.

Recovery of neuroactive compound from retinas varied, but compared to that from hair cells

Figure 6 illustrates results from a series of 13 nearly identical purification preparations after reverse-phase HPLC, in which extracts were applied to the bioassay at the same concentration. In more than half of the preparations, no neuroactivity was recovered. In most of these, there was little or no absorbance recovered at elution times associated with neuroactivity. Neuroactivity was never observed in the absence of a UV-absorbance peak, eluting between 22 and 24 min. However, the neuroactive compound recovered did not correlate well with the amount of UV-absorbance at 214 nm.

Because the neuroactive compound was found to be unstable in the highly purified samples, other components of the extract may have stabilized this compound. This suggested that the sample may have been light- or oxygen-sensitive. To preserve the neuroactive compound, we tried handling samples under argon and in dim light, collecting eluate into tubes containing ascorbic acid, and not monitoring UV absorbance (to minimize exposure of eluate to intense light). None of these precautions preserved the neuroactivity substantially. Loss of neuroactivity was more apparent when the sample was exposed to strong acid conditions.

While recovery of the neuroactive compound from the retina was variable, in some preparations, the retina was as rich a source as the inner ear. An example is shown in Figure 7, where we have plotted neuroactivity as a function of concentration of the extracted tissue for an extract of bovine retina and for goldfish inner ears (Sewell and Mroz, 1987). While the data of Figure 7 indicate that it is possible to obtain high recoveries of the neuroactive compound from bovine retina, most preparations, presumably due to both the biological and the chemical instability of the active component, yielded recoveries 15–20% of the maximum shown in Figure 7.

Similar neuroactivity was found in brain

We were also able to purify this neuroactive compound from bovine brain. Brain extracts contained a large number of neuroactive compounds, most of which suppressed discharge rate. These suppressive substances were not separated from the excitatory compound by gel-permeation chromatography, so we usually did not assay brain extracts until after one of the HPLC steps. The suppressive substances were much more strongly retained on reverse-phase HPLC than was the excitatory neuroactive compound. Upon cation-exchange and reverse-phase HPLC, neuroactivity could sometimes be detected in brain extracts, with the same chromatographic elution times as for retina extracts.

There were two major problems in using brain as a source: a large amount of lipid, interfering in most purification steps, and highly inconsistent recovery of excitatory neuroactivity. Although on occasions large amounts of neuroactive compound could be extracted from brain, in the majority of extractions very little was recovered. A loss of neuroactivity from tissue soon after death might also account for the inconsistency of brain as a source.

The major compound in neuroactive fractions and L-p-hydroxyphenylglycine-N-carbamoyl, derivatized with PFB, had identical GC elution times and mass spectra

An ethanol extract of 143 g of retina was purified through gel-permeation chromatography, cation-exchange HPLC, and reverse-phase HPLC to yield a neuroactive fraction with a coincident peak of UV absorbance at 214 nm eluting at 23.5 min (Fig. 8, left panels). From the reverse-phase HPLC separation, fraction 24 (neuroactive) and fractions 23 and 25 (not neuroactive) were each prepared with PFB derivatization and analyzed by GC-MS. The major component exclusively found in the neuroactive fraction (fraction 24) was detected by GC-MS eluting in GC at 29 min (Fig. 8, right panel). The mass spectrum of this derivatized compound (Fig. 9, upper-right panel) showed an intense peak at m/z 275, consistent with the loss of PFB (181 u) from a MW 456 compound. The loss of PFB strongly suggests the presence of a carboxyl group. Also present are ions at m/z 233 (a loss of 42 from m/z 275), indicating the presence of an acetyl in addition to the PFB, and m/z 217 (a loss of 58 from m/z 275), suggesting the presence of an acetylated hydroxyl group. This compound was not observed when inactive fractions from cation-exchange HPLC (eluting before and after the neuroactive fraction) were chromatographed by reverse-phase HPLC.

Coelution of the neuroactive compound with this MW 456 compound from reverse-phase HPLC was observed in six different analyses from several tissue preparations. These included a Folch extract of 140 g of retina, a Folch extract of 145 g of brain powder, an ethanol extract of 600 g of retina, and the ethanol extract whose results are shown in Figure 8.

On the basis of these results and those from TBDMS derivatization (described later), we hypothesized a candidate compound to be hydroxyphenylglycine-N-carbamoyl (structure shown in Fig. 12). PFB derivatization of L-p-hydroxyphenylglycine-N-carbamoyl produced a product with elution time, molecular weight, and mass spectrum identical to that of the neuroactive fractions from HPLC-purified retina extracts (Fig. 9). In both cases, the predominant derivatization product had a molecular weight of 456, indicating that a dehydration reaction occurred during derivatization of L-p-hydroxyphenylglycine-N-carbamoyl.

Neuroactive fractions and L-p-hydroxyphenylglycine-N-carbamoyl, derivatized with TBDMS, had identical GC elution times and mass spectra

HPLC fractions from retina extracts were derivatized with TBDMS and analyzed by GC-MS. A prominent peak in the total ion current (TIC), unique to the neuroactive fraction, eluted at 20.7 min (Fig. 10, left panel). A molecular weight of 552 was determined for the derivatized

compound in this peak, on the basis of an m/z 495 ion (M-57) under electron-impact GC-MS and an m/z 553 (MH^+) ion under positive-ion ammonia chemical ionization GC-MS (data not shown). This fragmentation pattern is typical of TBDMS derivatives (Donike and Zimmerman, 1980).

TBDMS derivatization of *L-p*-hydroxyphenylglycine-*N*-carbamoyl produced the same product as the neuroactive fraction (Fig. 10, right panels), based on GC elution and mass spectra. Because neuroactive fractions contained relatively high amounts (2.5 μ mol) of sodium formate, derivatization of *L-p*-hydroxyphenylglycine-*N*-carbamoyl was performed under similar conditions. Otherwise, the derivatized product suggested that dehydration occurred during derivatization.

Thus, both *L-p*-hydroxyphenylglycine-*N*-carbamoyl and the major substance detected by GC-MS in highly purified neuroactive fractions from extracts of retina yield the same products upon both TBDMS and PFB derivatization.

Hydroxyphenylglycine-*N*-carbamoyl itself is not in the neuroactive fractions, and is not neuroactive

There is little doubt that the major compound detected in neuroactive fractions after derivatization is identical to that found in derivatized *L-p*-hydroxyphenylglycine-*N*-carbamoyl. The identical elution times and mass spectra provide strong evidence for this assertion. However, the chemical properties of hydroxyphenylglycine-*N*-carbamoyl are such that it does not copurify with the neuroactive compound. Unlike the excitatory neuroactive compound, it is not retained by the cation-exchange HPLC column at pH 4, and it elutes well before the neuroactive substance by reverse-phase HPLC (Fig. 11). Hydroxyphenylglycine-*N*-carbamoyl had no effect on afferent-fiber discharge rate even when applied at concentrations as high as 3 mM.

DISCUSSION

A small excitatory amino acid related to hydroxyphenylglycine-*N*-carbamoyl may be neuroactive at the hair cell synapse

The major reaction product detected in these highly purified neuroactive extracts by GC-MS, whether derivatized with TBDMS or PFB, is identical to the corresponding derivatization product obtained from *L-p*-hydroxyphenylglycine-*N*-carbamoyl. Since hydroxyphenylglycine-*N*-carbamoyl itself is neither present in the purified neuroactive samples nor neuroactive, we hypothesize that neuroactive extracts contain a compound similar enough to hydroxyphenylglycine-*N*-carbamoyl to produce the same derivatized product. On the basis of reverse-phase HPLC, the neuroactive compound in the tissue extracts would appear to be more hydrophobic than hydroxyphenylglycine-*N*-carbamoyl, at least at pH 4. One hypothesis is that the neuroactive compound may be structurally similar to hydroxyphenylglycine-*N*-carbamoyl but is altered during derivatization for GC-MS.

Given our hypothesis that the neuroactive compound is related, but not identical, to hydroxyphenylglycine-*N*-carbamoyl, what can we deduce about the chemical nature of the neuroactive compound from its chromatographic behavior? Its elution from Sephadex G-10 gel-permeation chromatography (near glutamine and glucose) suggests a molecular weight of no more than a few hundred Daltons. Its failure to partition into ethyl acetate from water indicates significant polarity at neutral pH.

One of the UV-absorbing compounds in neuroactive fractions, which has a UV spectrum similar to that of hydroxyphenylglycine-*N*-carbamoyl, is a candidate neuroactive compound. If we assume the compound has the same extinction coefficient as hydroxyphenylglycine-*N*-

carbamoyl, then after all extraction and purification steps, we estimate a recovery of less than 15 nmol/g retina. We show (Fig. 7) that the concentration of neuroactive compound is similar in retina and hair-cell tissue from goldfish inner ears. We have previously calculated (Sewell and Mroz, 1987) that we have 15 million hair cells per gram of goldfish inner ear in our extracts. Thus, we may assume 15 nmol neuroactive compound/15 million hair cells, or 10^{-9} moles in 10^6 hair cells (i.e., 10^{-15} moles/hair cell. Volume of a hair cell is 1–3 pL (10–12 l), indicating a concentration of neuroactive substance in hair cells of 0.3 to 1 mM. The UV data also suggest that the compound is relatively potent, in that the concentration in bioassayed, neuroactive samples is typically under 50 μ M.

A compound related to hydroxyphenylglycine is a reasonable candidate to be an agonist for the glutamate receptor. However, we cannot determine with our bioassay whether the compound is actually acting on the glutamate receptor. Antagonists for the glutamate receptor block afferent discharge rate, eliminating the ability to observe a change in discharge rate. Nevertheless, the effects on the discharge rate of the extracted neuroactive substance were very similar to effects of applied glutamate on discharge rate.

Several known agonists and antagonists for a variety of glutamate receptors contain similar structures (Fig. 12). Hydroxyphenylglycine and a number of closely related compounds are agonists for the metabotropic glutamate receptor (Thomsen et al., 1994). Several compounds with hydroxyphenyl groups and hydrophobic side chains, including jorotoxin and philanthotoxin, interact with nonNMDA and NMDA glutamate receptors (Eldefrawi et al., 1988; Kawai et al., 1984; Ragsdale et al., 1989).

The neuroactive compound extracted and purified from retina and brain is likely to be the same as that extracted from hair cells

Retina, because it is available in large quantities, has proven to be a better source than inner ear for the neuroactive compound. Hair cells and several cell types in the retina store and release neurotransmitter at ribbon synapses (Smith and Sjostrand, 1961), leading us to postulate that they might use the same neurotransmitter or modulators. With retina as a source, we were able to purify the neuroactive compound substantially while having material left over for chemical analysis. Frozen bovine brain was not as good a source because of inconsistent quantities of extractable material and large quantities of lipid.

The neuroactive compound extracted from retina appears to be the same as that extracted from the inner ear. Pharmacologically, both showed glutamate-like effects on discharge rate. Both displayed identical chemical behavior in initial extraction, ion-exchange treatment, and gel-permeation chromatography. Elution properties from cation-exchange chromatography are also consistent with the two being identical. Our recovery of only 10% after cation-exchange HPLC of inner ear tissue is consistent with the small quantity of neuroactive compound eluting early from cation-exchange of retina extracts. In our previous work, we did not carry out elution long enough to have recovered the major portion of the neuroactive compound. Neuroactive compound extracted from brain was, in every way tested, chemically identical to that from retina. The presence in retina and brain of a neuroactive compound that can excite afferent fibers innervating hair cells suggests that this compound may be widely distributed in the nervous system.

Neuroactive compound not one of the many identified endogenous ligands for the glutamate receptor

A number of endogenous ligands for the glutamate receptor could potentially affect discharge in afferent fibers innervating hair cells. However, we have ruled out most known endogenous ligands as candidates, based on their chromatographic behavior. These include glutamate,

aspartate, quinolinic acid (a tryptophan metabolite that interacts with glutamate receptors in the brain (Stone, 1993) and affects activity in the auditory nerve (Yellon et al., 1994)), *N*-acetylaspartylglutamate (Zaczek et al., 1983), amino adipate (a lysine metabolite known to interact with transmitter action and release in the inner ear (Bledsoe and Bobbin, 1982; Prigioni et al., 1984; Soto and Vega, 1988)), several compounds related to cysteine and methionine (cysteic acid, cysteine sulfinic acid, homocysteic acid, some of which are known to excite afferent fibers innervating hair cells of the lateral-line organ (Bledsoe et al., 1983; Mroz and Sewell, 1989)), ergothioneine (present in high concentrations in the optic nerve and cerebellum (Crossland et al., 1966)), and guanosine monophosphate (identified in extracts of porcine brain as an endogenous inhibitor of kainate binding (Migani et al., 1997)).

The neuroactive compound is an alternate candidate to glutamate as a neurotransmitter

One of our original motivations for this work was that glutamate itself did not appear to be a good candidate to be the hair-cell neurotransmitter, though there is compelling evidence that glutamate receptors mediate transmission between the hair cell and the afferent fiber in the auditory (Eybalin, 1993; Matsubara et al., 1996; Ravindranathan et al., 2000; Ruel et al., 1999; Sewell, 1996), vestibular (Soto et al., 1994; Soto and Vega, 1988; Valli et al., 1985; Vega and Soto, 2003; Zucca et al., 1992), and lateral-line (Bailey and Sewell, 2000; Bledsoe et al., 1983) organs. The neuroactive compound we have isolated is an alternative to glutamate as a candidate to be the hair-cell neurotransmitter. It can excite afferent fibers innervating hair cells. However, we do not know the mechanism for the increase in discharge rate. The temporal pattern of the increase is very similar to that of glutamate receptor agonists, giving credence to the idea that it is acting at the glutamate receptor. However, it is difficult to critically test the hypothesis with the current bioassay, which relies on spontaneous discharge in afferent fibers, which is produced by transmitter release by the hair cells. Blocking glutamate receptors blocks spontaneous discharge, effectively eliminating a fundamental feature of the bioassay. While an action of the neuroactive compound on glutamate receptors of the afferent nerve fiber is a plausible hypothesis, other possibilities abound, including metabolic effects, an action on the hair cell to alter spontaneous transmitter release, and an action on other receptors on the afferent fiber.

The compound similar to hydroxyphenylglycine-*N*-carbamoyl that we have detected is a compelling candidate for the source of this glutamate-like neuroactive compound. It is the major compound in these extracts that copurifies with the bioactivity through multiple chromatographic steps. Although UV-spectrometry and GC-MS show that there are still several other compounds in the neuroactive fraction, these are present in very small quantities. By continuing to correlate neuroactivity with compounds in the purified extracts, it will be possible to determine whether this compound is indeed neuroactive, a first step in assessing its role in neural function. Whether or not it proves to be the hair-cell transmitter, its characterization as a potent neuroactive compound isolated from nervous tissue ensures it neurobiological importance.

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Abbreviations

EI	electron impact
GC-MS	gas chromatography–mass spectrometry
NCI	negative chemical ionization
PFB	pentafluorobenzyl
TBDMS	<i>tert</i> -butyldimethylsilylation
TIC	total ion current

Fig 1.

The neuroactive compound extracted from retina produced effects similar to glutamate on afferent fiber discharge in the lateral-line organ. These nerve fibers discharge spontaneously because of release of transmitter from the hair cell in the absence of mechanical stimulation. Both the neuroactive compound and glutamate produce an initial increase in discharge, followed by a long suppression. Samples in a volume of 100 μ l were administered at time 0 and applied at a rate of 1 μ l/s. There was a delay of 10–20 s for the administered sample to reach the preparation from the sample loop. Discharge rates were normalized to have the same preinjection baseline rate.

Fig 2.

The neuroactive compound eluted in gel-permeation chromatography near glutamine and a large peak of ninhydrin-positive material. Illustrated are the elution profiles of several common components of the extract after gel-permeation chromatography. The data presented were from a boiling-water extract of 17 g of retinas, reextracted with 80% ethanol, dried and partitioned between water and ethyl acetate, and treated at neutral pH with cation- and anion-exchange resins. Values are normalized to the peak concentration of each compound analyzed as follows: protein, filled triangles = 0.37 mg/ml; glutamate, open triangles = 0.03 mM; glutamine, open squares = 2.55 mM; ninhydrin, open circles = 38.2 mM; neuroactivity, filled circles = 57% increase in discharge rate; sodium, crosses = 288 mM.

Fig 3.

Neuroactivity was detected in fractions eluting from Sephadex G10 gel-permeation chromatography. Neuroactivity was seen primarily in fractions 25–27. Fractions whose responses are shown in the upper panel were diluted to 50% for assay in the middle panel. The lower panel shows responses to fraction 25 on an expanded time scale for both diluted (gray trace) and full-strength (black trace) application.

Fig 4.

Neuroactive compound eluted after 1 h (fractions 20 and 21) from cation-exchange HPLC on the trailing edge of an absorbance peak associated with phenylalanine. Neuroactive fractions from gel-permeation chromatography were applied to a cation-exchange HPLC column, and the collected eluate was tested for neuroactivity. The upper panel illustrates elution of UV-absorbing (214 nm) material from the cation-exchange HPLC column. The lower panel illustrates the response of the bioassay to samples prepared from fractions taken from the HPLC column. The bioassay response to pooled fractions 20 and 21 is illustrated with an expanded time scale in Figure 1.

Fig 5.

Neuroactive compound coeluted with an isolated UV-absorbing peak during gradient-elution reverse-phase C18 HPLC. Bioassay responses for fractions 21 through 30 are illustrated in the upper panel. The bioassay response to fraction 23 (eluting between 22 and 23 min, at 80% methanol) is shown on an expanded time scale in the bottom panel. The bottom-right panel shows UV absorbance in the column eluate. (In the top panel, the first injection is an artificial perilymph injection with low (nominally 0 mM) calcium ion and is used as a control to ensure that the stitch we are monitoring is being perfused.)

Fig 6.

Neuroactivity was not seen in the absence of UV absorbance (214 nm). Absorbance (214 nm) is plotted vs. neuroactivity after reverse-phase HPLC for 13 preparations, for each of 20 g of bovine retina. In two preparations, neuroactivity and absorbance were recovered from two sequential fractions of the eluate; these four data points are plotted in gray.

Fig 7.

Retina and hair-cell extracts could contain similar concentrations of the neuroactive compound. Relation between neuroactivity and the mass of tissue extracted are compared between extracts of goldfish inner ear (open circles) and of bovine retina (filled circles). Concentration of extracted tissue was determined by taking into account the starting material and the dilutions and concentrations of the extract during the purification process. Neuroactivity was determined by measuring the increase in discharge rate and averaging responses to all bioassays (usually three) of the same sample. Data from goldfish are replotted from Sewell and Mroz (1987).

Fig 8.

GCMS of PFB-derivatized fractions from reverse-phase HPLC detected a large single component specific to the neuroactive fraction. Top left: Chromatogram of optical absorbance at 214 nm during reverse-phase HPLC shows a peak at 23.5 min. Fractions were collected at 1-min intervals and applied to the bioassay (bottom left). Dashed lines indicate injection times of each sample. Excitatory neuroactivity is solely seen in fraction 24. Right panel: TIC chromatogram from GC-MS analysis of PFB-derivatized fractions shows a large peak exclusive to the neuroactive fraction eluting at 29 min. MS data was obtained under negative-ion, electron-capture, chemical ionization conditions.

Fig 9. PFB-derivatized *L-p*-hydroxyphenylglycine-*N*-carbamoyl and neuroactive fractions have identical mass spectra and elution times on GC-MS. TIC chromatograms are plotted in the left panel for *L-p*-hydroxyphenylglycine-*N*-carbamoyl (lower trace) and a neuroactive fraction from reverse-phase HPLC (upper trace). Corresponding mass spectra are shown at the right panel.

Fig 10.

A compound exclusive to the neuroactive fraction has the same GC elution time and mass spectrum as hydroxyphenylglycine-*N*-carbamoyl after TBDMS derivatization. Left panel: Neuroactive (23) and adjacent nonneuroactive (22 and 24) fractions from reverse-phase HPLC of the retina extract were derivatized with TBDMS and analyzed on GC-MS (EI ionization). The upper-right panel shows the mass spectrum of the GC peak (eluting at 20.65 min) specific to the neuroactive fraction. This mass spectrum for derivatized *L-p*-hydroxyphenylglycine-*N*-carbamoyl is shown in the lower right panel.

Fig 11.

Hydroxyphenylglycine-*N*-carbamoyl (dotted line) is not retained by reverse-phase HPLC. The neuroactive compound (solid line) is retained for 23 min.

Fig 12.

The hypothesized structure of the neuroactive compound is similar to structures of several compounds known to interact with glutamate receptors.