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Metabolomics Analyses of 14 Classical Neurotransmitters by GC-TOF with LC-MS Illustrates Secretion of 9 Cell-Cell Signaling Molecules from Sympathoadrenal Chromaffin Cells in the Presence of Lithium

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Abstract

The classical small molecule neurotransmitters are essential for cell-cell signaling in the nervous system for regulation of behaviors and physiological functions. Metabolomics approaches are ideal for quantitative analyses of neurotransmitter profiles, but have not yet been achieved for the repertoire of 14 classical neurotransmitters. Therefore, this study developed targeted metabolomics analyses by full scan gas chromatography / time of flight mass spectrometry (GC-TOF) and hydrophilic interaction chromatography-QTRAP mass spectrometry (HILIC-MS/MS) operated in positive ionization mode for identification and quantitation of 14 neurotransmitters consisting of acetylcholine, adenosine, anandamide, aspartate, dopamine, epinephrine, GABA, glutamate, glycine, histamine, melatonin, norepinephrine, serine, and serotonin. GC-TOF represents a new metabolomics method for neurotransmitter analyses. Sensitive measurements of 11 neurotransmitters were achieved by GC-TOF, and three neurotransmitters were analyzed by LC-MS/MS (acetylcholine, anandamide, and melatonin). The limits of detection (LOD) and limits of quantitation (LOQ) were assessed for linearity for GC-TOF and LC-MS/MS protocols. In neurotransmitter-containing dense core secretory vesicles of adrenal medulla, known as chromaffin granules (CG), metabolomics measured the concentrations of 9 neurotransmitters consisting of the catecholamines dopamine, norepinephrine, and epinephrine, combined with glutamate, serotonin, adenosine, aspartate, glycine, and serine. The CG neurotransmitters were constitutively secreted from sympatho-adrenal chromaffin cells in culture. Nicotine- and KCl-stimulated release of the catecholamines and adenosine. Lithium, a drug used for the treatment of

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Author Contributions. VH and OF designed the study and organized laboratory staff to conduct the experiments. TT and SP conducted the chromaffin granule and chromaffin cell experiments. TK, MP,CT, and SS designed and performed the metabolomics experiments. Figures and Tables were prepared by TK, MP, SP, and VH. SP and CL conducted a literature search of neurotransmitters and lithium. VH and TK wrote the manuscript, which was edited and reviewed by all authors.

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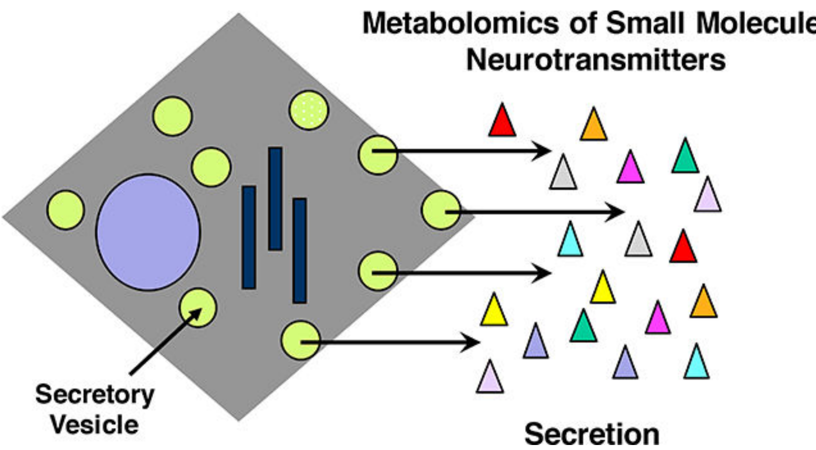
bipolar disorder, decreased the constitutive secretion of dopamine and norepinephrine, and decreased nicotine-stimulated secretion of epinephrine. Lithium had no effect on other secreted neurotransmitters. Overall, the newly developed GC-TOF with LC-MS/MS metabolomics methods for analyses of 14 neurotransmitters will benefit investigations of neurotransmitter regulation in biological systems and in human disease conditions related to drug treatments.

Graphical Abstract



Keywords

classical neurotransmitters; metabolomics; mass spectrometry; secretion; adrenal medulla; lithium

Introduction

Neurotransmitters are fundamental for chemical cell-cell signaling in the nervous system for control of behavioral and physiological functions. Neurotransmitters are composed of the classical small molecule neurotransmitters (1, 2) and the endogenous neuropeptides (1, 3). Multiple neurotransmitters function together in the control of nervous system functions. It is, therefore, necessary to define the quantitative profiles of the spectrum of neurotransmitters to understand how they participate in neurotransmission and cell-cell signaling.

Mass spectrometry (MS) approaches are ideal for identification and quantitation of neurotransmitters. Indeed, peptidomics mass spectrometry have been achieved to define profiles of neuropeptides with respect to their structural identification and quantitation (4–6). Extensive development of targeted and global untargeted analyses of neuropeptide profiles has been achieved in the field (7–9).

Investigation of classical neurotransmitter regulation requires evaluation of the major repertoire of these small molecules which can be achieved by metabolomics mass spectrometry methods. However, to the best of our knowledge based on extensive literature evaluation, there are currently no publications that have reported identification and quantitation of the major spectrum of 14 classical neurotransmitters, as analyzed by this study. While many studies have conducted MS analyses of ‘neurotransmitters’, most have

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only analyzed 3–6 neurotransmitters (10–30) and a few have analyzed 7–8 neurotransmitters (31–34). Many of these studies of 'neurotransmitters' include metabolites, rather than only the bona fide neurotransmitters. For these reasons, there is a need to develop metabolomics methodology for quantitative profiling of the major repertoire of 14 classical neurotransmitter molecules, the goal of this study.

Therefore, this study developed targeted MS identification and quantitation of 14 classical neurotransmitters by GC-TOF (gas chromatography time-of-flight), combined with LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) analyses. GC-TOF is appropriate for analyses of low molecular weight molecules (35, 36), but has not been used in prior neurotransmitter analyses which have largely utilized LC-MS and LC-MS/MS (10–34). Results of this study found that GC-TOF, combined with LC-MS/MS, provides an improved metabolomics strategy for analyses of 14 classical neurotransmitters consisting of acetylcholine, adenosine, anandamide, aspartate, dopamine, epinephrine, GABA, glutamate, glycine, histamine, melatonin, norepinephrine, serine, and serotonin. GC-TOF provided sensitive LOD (limit of detection) and LOQ (limit of quantitation) for 11 neurotransmitters, while 3 other neurotransmitters (acetylcholine, anandamide, and melatonin) were detected only by LC-MS/MS for identification and quantitation.

These metabolomics protocols were used to evaluate neurotransmitters in secretory vesicles of sympathoadrenal chromaffin cells which are involved in stress responses (37–41). The dense core secretory vesicles from adrenal medulla, known as chromaffin granules (CG), have been utilized as a model of neurotransmitter neurochemistry and secretion (42). Nine neurotransmitters in CG were identified and quantitated by metabolomics analyses. Results demonstrated a broad range of neurotransmitter levels that spanned more than 6,000-fold for the lowest to highest levels of these small molecule. These 9 neurotransmitters were secreted from adrenal medullary chromaffin cells (in primary culture) under constitutive and stimulated (nicotine and high KCl) conditions.

The adrenal medulla is involved in stress-related disorders which impact mental health conditions including bipolar disorder and other related illnesses (43–46). Lithium, a widely used drug for the treatment of bipolar disorder (47–50), modulates sodium and calcium channels of chromaffin cells (51–53). Regulation of sodium and calcium channels, essential for neurotransmitter secretion, suggested that lithium may modulate neurotransmitter secretion from chromaffin cells. Indeed, metabolomics analyses of this study showed that lithium treatment of chromaffin cells regulated constitutive and stimulated secretion of catecholamine neurotransmitters.

Overall, this study demonstrates effective GC-TOF and LC-MS/MS protocols for identification and quantitation of 14 classical neurotransmitters over a broad range of concentrations, and can measure changes in neurotransmitter profiles under different conditions. These newly developed GC-TOF with LC-MS/MS metabolomics methods have application to investigations of neurotransmitter regulation in biological systems and in human disease conditions related to drug treatments.

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Results

Metabolomics analyses of classical neurotransmitters by GC-TOF and LC-MS/MS

The 14 classical neurotransmitters analyzed in this study consisted of acetylcholine, adenosine, anandamide, aspartate, dopamine, epinephrine, GABA (γ-amino butyric acid), glutamate, glycine, histamine, melatonin, norepinephrine, serine, and serotonin. The molecular weights (molecular mass) and structures of these neurotransmitters are shown in Table 1. The low molecular weights of these molecules are in the range of 110 daltons to 350 daltons, and most are the range of 100–200 daltons. The neurotransmitters listed in Table 1 were evaluated for identification and quantitation by targeted GC-TOF (TIC) and LC-MS/MS (MRM).

GC-TOF provided sensitive measurements of 11 neurotransmitters consisting of adenosine, aspartate, dopamine, epinephrine, GABA, glycine, glutamate, histamine, norepinephrine, serine, and serotonin with limits of detection (LOD) of 0.025 μg/ml to 0.25 μg/ml (Table 2). The limits of quantitation (LOQ) for these neurotransmitters were in the range of 0.01 μg/ml to 0.5 μg/ml (Table 2). The linearity of measurements for these 11 neurotransmitters were assessed by 6-point calibration curves in the range of 0.05 μg/ml to 2.5 μg/ml. Calibration curves (Supplemental Figure 1a, b) displayed linear relationships of neurotransmitter concentrations and quantitative values, with R^2 values of greater than 0.99 (0.993 to 0.999) for adenosine, aspartate, dopamine, epinephrine, GABA, histamine, norepinephrine, serine, and serotonin, and R^2 value of 0.984 for glutamate. GC-TOF analyses was also conducted for glycine in the range of 0.025 μg/ml to 1.0 μg/ml, which showed a linear calibration curve with R^2 value greater than 0.99 (Table 2). These R^2 values indicate the linearity of the concentrations of 11 neurotransmitters measured by GC-TOF.

LC-MS/MS analyzed 3 neurotransmitters consisting of acetylcholine, anandamide and melatonin, since they were not detected by GC-TOF. LC-MS/MS data indicated the limits of detection (LOD) in the range of 0.01 μg/ml to 0.025 μl/ml, and limits of quantitation (LOQ) in the range of 0.025 μg/ml to 0.5 μg/ml (Table 2). The retention times and slope measurements for calibration plots were determined (shown in Table 2). Calibration curves (Supplemental Figure 2) showed linear relationships of concentrations of acetylcholine, anandamide, and melatonin with measurements, displaying R^2 values of 0.999, 0.999, and 0.997, respectively. These R^2 values illustrate the linearity of concentrations of the 3 neurotransmitters measured by LC-MS/MS.

It is noted that GC-TOF and LC-MS/MS were assessed for all 14 neurotransmitters to determine the best protocol for each neurotransmitter. Three neurotransmitters consisting of glycine, glutamate, and serine were readily detected only by GC-TOF, but not by LC-MS/MS under the conditions used in this study. Eight neurotransmitters consisting of adenosine, aspartate, dopamine, norepinephrine, epinephrine, GABA, histamine, and serotonin were identified by GC-TOF and LC-MS/MS. GC-TOF measured lower levels (LOD) of dopamine, norepinephrine, epinephrine, and serotonin than LC-MS/MS. GC-TOF and LC-MS/MS provided similar orders of magnitude of LOD values for adenosine, aspartate, and GABA. Three neurotransmitters consisting of acetylcholine, anandamide, and melatonin were detected only by LC-MS/MS, not by GC-TOF. These data demonstrated the

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effective metabolomics analyses of 11 neurotransmitters by GC-TOF, and 3 neurotransmitters by LC-MS/MS (Table 2).

Neurotransmitters in sympathoadrenal dense core secretory vesicles, chromaffin granules (CG), of the sympathetic nervous system

Adrenal medullary chromaffin granules (CG) are the dense core secretory vesicles (DCSV) that store and release neurotransmitters in response to stress (37–39). Isolated CG were utilized for identification and measurements of small molecule neurotransmitters by the GC-TOF and LC-MS/MS methods developed by this study. Results showed that the CG contain 9 neurotransmitters consisting of adenosine, aspartate, dopamine, epinephrine, glutamate, glycine, norepinephrine, serine, and serotonin (Table 3). The neurotransmitter concentrations ranged from the high levels of epinephrine, norepinephrine, and adenosine of 1.4–12.9 μg/mg (μg neurotransmitter per mg CG protein), to low levels of dopamine at 0.024 μg/mg, and very low levels of aspartate, glutamate, glycine, serine, and serotonin of 0.002 to 0.006 μg/mg. The range of neurotransmitter concentrations measured spanned a 6,450-fold range. The high sensitivities of the metabolomics protocols allowed measurements of very low to high levels of these neurotransmitter molecules.

Epinephrine was present at the highest concentration in the chromaffin granules, of 12.9 μg/mg, compared to the others. The neurotransmitters dopamine and norepinephrine are precursors of epinephrine, synthesized from tyrosine. The levels of dopamine and norepinephrine were 0.024 μg/mg and 2.89 μg/mg, respectively, which indicate that the biosynthetic pathway of tyrosine to dopamine, to norepinephrine, and to epinephrine results in accumulation of high levels of epinephrine.

Adenosine was present in the CG at 1.43 μg/mg, a concentration of similar order of magnitude as norepinephrine. The finding of adenosine is significant since it has not been previously reported to be present in CG.

The concentrations of aspartate, glutamate, glycine, serine, and serotonin in the CG were present in the range of 0.002 to 0.006 μg/mg. The levels of these neurotransmitters were substantially lower than the catecholamines epinephrine, norepinephrine, and dopamine, and lower than adenosine. The presence of aspartate, glycine, and serine in CG has not been previously reported. The neurotransmitters anandamide, acetylcholine, GABA, histamine, and melatonin were not detected in the CG with the current methods.

Constitutive, basal secretion of neurotransmitters from chromaffin cells in the absence and presence of lithium

CG neurotransmitters secreted from adrenal medullary chromaffin cells in primary culture were evaluated. Constitutive (basal) and stimulated secretion occurs for chromaffin granule components. Secretion was studied in the absence and presence of lithium chloride (1 mM) for 72 hours. Lithium at 1 mM is the therapeutic concentration used for treatment of bipoloar disorder (54); 72 hours was used because lithium treatment occurs for days, and this time-frame provides measurable amounts of secreted neurotransmitters from chromaffin cells.

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Constitutive, basal secretion of neurotransmitters during a 60 minute period was assessed by metabolomics measurements of the secretion media. Basal secretion of the catecholamines dopamine, norepinephrine, and epinephrine into the media was measured as concentrations of 0034 μg/ml, 0.58 μg/ml, and 1.02 μg/ml, respectively (Figure 1a). Secretion of the catecholamines was accompanied by basal secretion of adenosine, aspartate, glutamate, glycine, serine and serotonin; the secretion of these 6 neurotransmitters was not affected by lithium (Figure 1b). Concentrations of these constitutively secreted neurotransmitters ranged from the highest value of 1.008 μg/ml for epinephrine, to moderate levels of about 0.121 to 0.574 μg/ml for norepinephrine, serotonin, aspartate, and serine, and to lower levels of approximately 0.004 to 0.016 μg/ml for dopamine, adenosine, and glutamate (Figure 2, and Supplemental Table 1). These data showed that the 9 neurotransmitters present in chromaffin granules undergo basal secretion from chromaffin cells.

Lithium treatment (1 mM $LiCl_2$ for 72 hours) significantly decreased levels of constitutively secreted dopamine and norepinephrine, but had no effect on levels of constitutively secreted epinephrine (Figure 1a). Lithium decreased basal secretion of dopamine by 34%, and decreased basal secretion of norepinephrine by 42%. Secretion of adenosine, aspartate, glutamate, glycine, serine and serotonin was not affected by lithium (Figure 1b).

Stimulated secretion of catecholamine neurotransmitters by nicotine and KCl depolarization: effects of lithium treatment

Metabolomics analyses was conducted for stimulated secretion of neurotransmitters induced by nicotine (55–58) and high KCl depolarization (57, 59). These secretion studies also examined the effects of lithium, related to regulation of catecholamines of bipolar disorder (60–64).

Nicotine and KCl stimulated secretion of dopamine, norepinephrine, epinephrine (Figure 3), and adenosine (Figure 4). But nicotine and KCl had no stimulatory effect on the secretion of the other 5 neurotransmitters consisting of aspartate, serotonin, glutamate, glycine, and serine (Figure 4). The different profiles of neurotransmitters secreted in stimulated conditions suggests involvement of subpopulations of secretory vesicles (57, 65, 66) that may be differentially regulated by secretagogues.

Lithium treatment of these cells (72 hours) resulted in reduction of nicotine-stimulated epinephrine secretion; but lithium had no effect on KCl-stimulated epinephrine secretion (Figure 3c). Lithium had no effect on nicotine- or KCl-stimulated secretion of norepinephrine or dopamine (Figure 3b a, respectively). And lithium had no effect on nicotine- or KCl-stimulated secretion of adenosine, aspartate, glycine, glutamate, serine, or serotonin (Figure 4). These data demonstrate that under stimulated secretion conditions, lithium selectively reduced nicotine-stimulated secretion of epinephrine from chromaffin cells.

Discussion

The classical small molecule neurotransmitters are essential for cell-cell signaling in the nervous system for the regulation of behaviors and physiological functions. To investigate

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the the major repertoire of neurotransmitter profiles, effective and sensitive methods for identification and quantitation of the spectrum of the 14 classical neurotransmitters are necessary. For this reason, this study developed and optimized GC-TOF and LC-MS/MS mass spectrometry approaches for sensitive detection and quantitation of these 14 neurotransmitters consisting of acetylcholine, adenosine, anandamide, aspartate, dopamine, epinephrine, GABA, glutamate, glycine, histamine, melatonin, norepinephrine, serine, and serotonin. GC-TOF, with LC-MS/MS, represents a new metabolomics approach for analyses of neurotransmitters.

Novel findings from this study showed that targeted GC-TOF (TIC), with HILIC LC-MS/MS (MRM), protocols provided (1) identification and quantitation of the major repertoire of 14 classical neurotransmitters, and (2) GC-TOF is a new metabolomics method for identification and quantitation of 11 of the 14 neurotransmitters. LC-MS/MS was utilized for analyses of 3 neurotransmitters consisting of acetylcholine, anandamide, and melatonin, since these were not detected by GC-TOF. The combined GC-TOF and LC-MS/MS methods display sensitive ranges for neurotransmitter measurements, based on LOQ and LOD properties. The limits of detection (LOD) were in the range of 0.01 μg/ml to 0.25 μg/ml. The limits of quantitation (LOQ) values were in the range of 0.01 μg/ml to 0.5 μg/ml. Calibration curves for the neurotransmitters showed R^2 values of 0.98 to greater than 0.99, which confirmed the linearity of neurotransmitters concentrations measured by these metabolomics methods.

The newly developed metabolomics protocols allowed identification with quantitation of 9 neurotransmitters in dense core secretory vesicles (DCSV) from adrenal medulla of the sympathetic nervous system. The sympatho-adrenal DCSV, known as chromaffin granules (CG), store and secrete a multitude of neurotransmitters in response to stress (37, 38). Metabolomics analyses showed that the levels of the 9 neurotransmitters in CG spanned a broad range of 6,450-fold. This is a tremendous range of neurotransmitters contained within the CG.

Results demonstrated the different levels of the catecholamines dopamine (0.024 μg/ml), norepinephrine (2.89 μg/ml), and epinephrine (12.9 μg/ml in CG. Since dopamine is converted to norepinephrine, and norepinephrine is converted to epinephrine, this biosynthetic pathway indicates the accumulation of epinephrine compared to the lower precursor levels of dopamine and norepinephrine in CG.

The neurotransmitter adenosine is present at 1.43 μg/mg in CG. Adenosine represents a newly identified neurotransmitter in CG, since only the related ATP (67) and dinucleotide adenosine (68, 69) molecules have been reported in CG.

The concentrations of aspartate, glutamate, glycine, serine, and serotonin in CG were present in the range of 0.002 μg/mg to 0.006 μg/mg, which are much lower than the catecholamines. Measurement of aspartate, glycine, and serine demonstrated newly identified neurotransmitters in CG.

The presence of the 9 neurotransmitters in CG predicted their secretion from chromaffin cells in primary culture. Indeed, the CG neurotransmitters were secreted constitutively from

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chromaffin cells. Epinephrine was secreted at the highest level (1008 ng/ml), followed by substantial levels of secreted norepinephrine (574 ng/ml) and serotonin (309 ng/ml). Moderate levels of aspartate (121 ng/ml), serine (127 ng/ml) and glycine (65 ng/ml) were secreted constitutively. Low levels of adenosine (16 ng/ml), dopamine (3.8 ng/ml), and glutamate (12 ng/ml) were also secreted constitutively.

Stimulated secretion of neurotransmitters from chromaffin cells can be induced by nicotine and high KCl depolarization (59). Both nicotine and KCl stimulated the secretion of dopamine, norepinephrine, epinephrine, and adenosine above basal constitutive secretion levels (Supplementary Table 1). But these stimulators had no effect on the basal secretion of the other 5 neurotransmitters consisting of aspartate, serotonin, glutamate, glycine, serine. The different profiles of neurotransmitters secreted during the two nicotine- and KCl-stimulated conditions suggests the presence of subpopulations of secretory vesicles (57, 65, 66) that may be differentially regulated by secretagogues.

The adrenal gland of the sympathetic nervous system is a key responder to stress through the secretion of the catecholamine and related neurotransmitters (37, 38). Stress-related disorders involve mental health conditions including bipolar disorder and related (43–46). Lithium, a drug widely used for the treatment of bipolar disorder, has been found to regulate chromaffin cell sodium and calcium channels (51–53). Since these ion channels are necessary for neurotransmitter secretion, lithium may regulate neurotransmitter secretion from chromaffin cells. Data from this study showed that lithium reduced basal, constitutive secretion of dopamine and norepinephrine. Lithium treatment also reduced nicotine-stimulated secretion of epinephrine from chromaffin cells. But lithium had no effect on KCl-stimulated epinephrine secreiton. Lithium also had no effect on nicotine- or KCl-stimulated secretion of the other 8 neurotransmitters consisting of norepinephrine, dopamine, adenosine, aspartate, glycine, glutamate, serine, and serotonin. These findings indicate the selective regulation by lithium of the catecholamines dopamine, norepinephrine, and epinephrine, and lack of effects on the other 6 neurotransmitters secreted from chromaffin cells. This finding complements prior studies showing that lithium modulates the dopamine and norepinephrine catecholamines (60–64), as well as excitatory and inhibitory neurotransmitter systems (70–74).

Overall, targeted metabolomics analyses of the repertoire of 14 classical neurotransmitters by GC-TOF, with LC-MS/MS, protocols provides the identification and quantitation of these classical neurotransmitters over a broad range of concentrations. As shown in this study, metabolomics measured the levels of 9 neurotransmitters in dense core secretory vesicles (CG) which are secreted from adrenal medullary chromaffin cells under constitutive and stimulated conditions. Further, lithium selectively regulates secretion of the catecholamine neurotransmitters from these cells. These metabolomics protocols have broad application to investigations of neurotransmitter regulation in biological systems and in human disease conditions related to drug treatments.

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Methods and Materials

Standards for neurotransmitter molecules

Standards for the neurotransmitters were obtained from commercial vendors for use as calibration standards in metabolomics protocols. The neurotransmitter reference standards consisted of acetylcholine, adenosine, anandamide, aspartate, dopamine, epinephrine, GABA, glutamate, glycine, histamine, melatonin, norepinephrine, serine, and serotonin and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chromaffin granules (CG) isolated from sympathoadrenal medulla tissue

Chromaffin granules (known as dense core secretory vesicles) were purified from fresh bovine adrenal medulla (from Sierra Medical Sciences, Whittier, CA) by sucrose density gradient centrifugation as we have described previously (75). The high purity of the isolated CG has been demonstrated by enzyme markers of subcellular organelles (75–77). The homogeneity and integrity of the purified CG have been confirmed by electron microscopy (78). Protein content of the purified CG was measured by the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA).

Extraction of chromaffin granules for metabolomics analyses

The isolated CG were subjected to freeze-thaw, two times, followed by addition of pre-chilled extraction solvent (0.5 mL), consisting of acetonitrile:isopropanol:H_20 at 3:3:2 (vol/vol/vol) degassed with nitrogen, and cooled to −20° C (in ThermoElectron Neslab RTE 740 cooling bath). Cell grinding of CG was performed twice at –20° C in a Geno/Grinder homogenizer and cell lyser (SPEX SamlePrep, Metuchen, NJ). Addition of an additional 0.5 mL extraction solvent and tissue grinding was conducted. After shaking at 4° C for 5 minutes, samples were centrifuged for 2 minutes at 14,000 rcf using the Eppendorf 5415 D centrifuge. One 0.5 mL aliquot was evaporated to dryness in a Labconco Centrivap cold trap concentrator; the other 0.5 mL aliquot was stored at −20° C.

Secretion of neurotransmitters from chromaffin cells (in primary culture) in the presence of lithium

Primary cultures of chromaffin cells were prepared from fresh bovine adrenal medulla, as we have described previously (59). Cells were plated in 6-well plates at 1.5×10^6 cells/well and kept in culture at 37° C in 6% CO_2 and 94% air. After 6 days in culture, cells were treated with control media or media containing lithium chloride (1 mM) media for 72 hours Cells were then subjected to basal, constitutive secretion for 60 minutes in standard release media buffer (SRMB, consisting of 25 mM HEPES pH 7.3, 118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 2.2 mM $CaCh_2$, 1.2 mM $MgSO_4$, and 0.5 μg/ml BSA), followed by collection of media. Cells were then incubated in nicotine (10 μM) or KCl (50 mM) in SRMB for 60 minutes, and the secretion media was collected. KCl and nicotine were utilized to stimulate regulated secretion of neurotransmitters. The secretion media samples were centrifuged at 600 x g for 5 minutes at 4° C to remove residual cells, and the supernatant was collected as the secretion media for neurotransmitter measurements by

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metabolomics methods. Experiments were conducted in replicate samples (at least quadruplicate or greater number of replicates).

Neurotransmitter metabolomics analyses conducted by GC-TOF and LC-MS/MS

CG extracts were prepared for targeted metabolomics experiments. For this purpose, 6-point standard calibration curves in in the range of 2.5 to 5000 ng reference material were measured and limit of detection (LOD) and limit of quantification (LOQ) for each neurotransmitter molecule under investigation were calculated. Three metabolites (acetylcholine, anandamide, and melatonin) were reported in LC-MS/MS multiple reaction monitoring (MRM) mode, and all other metabolites were measured in GC-MS total ion current (TIC) mode.

GC-MS (gas chromatography - mass spectrometry) analyses in total ion current (TIC) mode was conducted similar to previously published methods (35, 79, 80). In short, samples were derivatized by O-Methylhydroxylamine hydrochloride (MeOX) and subsequently silylated by N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). A mixture of fatty acid methyl esters (FAME) retention index markers was added. Measurement was performed on a 6890 Agilent GC coupled to Leco Pegasus IV TOF MS mass spectrometer. A Restek RTX-5Sil MS 30 m length; 0.25 mm i.d.; 0.25 um film 95% dimethyl/5% diphenyl polysiloxane with 10m guard column was used for separation. The injector had an initial temperature of 50°C with 0.5 min equilibration time. The injector temperature ramp was 12°C/sec to 275°C; the initial hold time was 3 min. The injector was operated in splitless mode with 25-sec purge time and 40 ml/min purge flow and then 1 ml/min helium carrier gas flow. The GC was operated at 1 ml/min constant flow of Helium. Oven ramp was set to 50°C (1 min hold) to 330°C at 20°C/min, 5 min hold before cool-down for a total of 22 min run time. The Transfer line temperature was set to 280°C. The mass spectrometer was operated at 70 eV with an ion source temperature of 250°C in positive ionization mode. Mass spectral acquisitions were performed from 85 to 500 Da with an acquisition rate of 17 spectra/s. A total of 0.5 μl sample material was injected on a baffled glass liner. Data analysis was performed with the Leco ChromaTOF and BinBase software. Calibration curves and LOD and LOQ for neurotransmitters were calculated in Microsoft Excel.

LC-MS/MS (liquid chromatography - tandem mass spectrometry) was conducted by a hydrophilic interaction chromatography method (HILIC) using a Waters XBridge Amide 4.6 × 100 mm, 3.5 pm column. An Agilent 1200 Binary Pump SL was coupled to a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Mobile phase A buffer was 10 mM ammonium formate in H_2O, 0.125% formic acid, and mobile phase B buffer of 10 mM ammonium formate in 95%/5% acetonitrile:H_2O, 0.125% formic acid. The LC gradient, at a flow rate of 0.3 ml/min, was 100% B at 0–3 minutes (min.), 70% B at 10 min., 40% B at 14 min., 30%B at 15 min., and 100% B at 19 min. The column was then equilibrated with a total runtime of 25 min. The column temperature was set to 45° C. The mass spectrometer was operated in positive ionization multiple reaction monitoring (MRM) mode. Only three neurotransmitters were analyzed by LC-MS/MS (MRM): acetylcholine (146>87), anandamide (348>62) and melatonin (233>174) (see Table 2), since these were not detected by GC-TOF. These neurotransmitter molecules were

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assessed for retention time, limit of detection limit (LOQ), limit of quantitation (LOQ), and slope of calibration plots. Data analysis was performed with the Analyst 1.5.1 software.

Statistical analyses

Statistical significance of neurotransmitters secreted from chromaffin cells under different conditions utilized student's t-test and ANOVA analyses with $p < 0.05$ for statistical significance (using GraphPad Prism program). Secretion data of the nine neurotransmitters from chromaffin cells under different conditions (basal, nicotine, and KCl in the absence and presence of lithium) are shown in Supplemental Table 1, which provides mean, standard error of the mean (sem), standard deviation (sd), and p values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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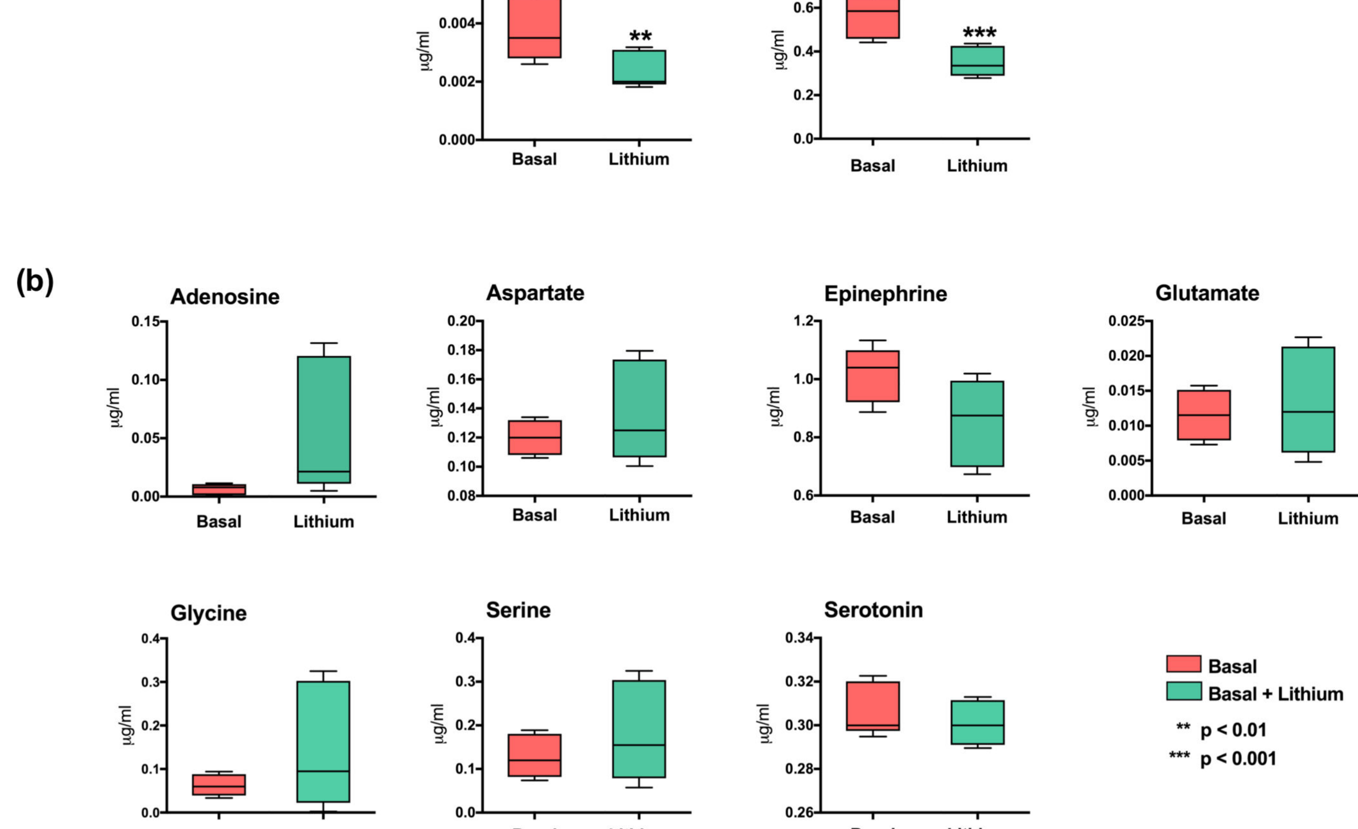

Figure 1. Constitutive Secretion of Nine Neurotransmitters from Chromaffin Cells in the Absence and Presence of Lithium.

(a) Dopamine and norepinephrine constitutive secretion regulated by lithium. Basal, constitutive secretion of dopamine and norepinephrine from chromaffin cells was conducted for a period of 60 minutes in the absence or presence of lithium (1 mM). The secretion media was subjected to the metabolomics methods developed in this study to measure the concentrations of these two neurotransmitters in the media. Results are shown as box and whisker plots showing the mean ± sem (standard error of the mean), and mean ± 1.96 sem (n=8 per group, without and with lithium). Lithium treatment significantly reduced the basal secretion of dopamine and norepinephrine compared to no treatment. **p< 0.01, ***p<0.001, for comparison of lithium and no lithium conditions, by students' t-test.

(b) Basal secretion of seven neurotransmitters in the absence and presence of lithium. Basal, constitutive secretion of the neurotransmitters adenosine, aspartate, epinephrine, glutamate, glycine, serine and serotonin from chromaffin cells (in primary culture) was conducted for a period of 60 minutes, in the absence or presence of lithium (1 mM). Neurotransmitter concentrations were measured by the metabolomics methods developed in this study. Results are shown as box and whisker plots of the mean ± sem (standard error of the mean), and mean ± 1.96 sem (n=8 per group).

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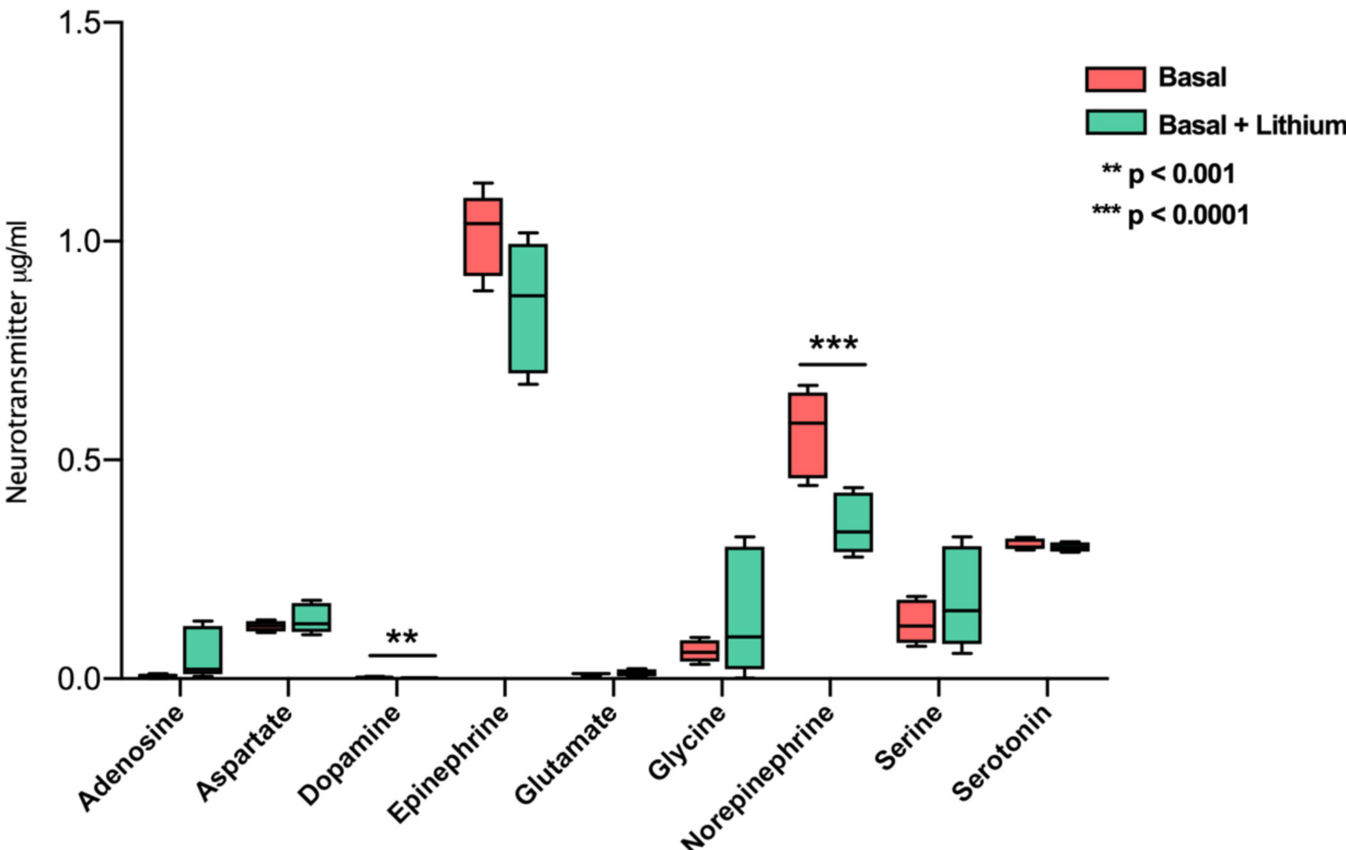

Figure 2. Broad Range of Secreted Quantities of Nine Neurotransmitters Released from Chromaffin Cells.

Chromaffin cells in culture secrete neurotransmitters under basal, constitutive conditions (60 min.). Comparisons of the range of neurotransmitter levels secreted were assessed for the nine secreted neurotransmitters consisting of adenosine, aspartate, dopamine, epinephrine, glutamate, glycine, norepinephrine, serine, and serotonin. The graphs display neurotransmitter concentrations as box and whisker plots for mean $\pm$ sem and $\pm$1.96 sem, shown (n=8). **p< 0.001, ***p<0.0001, for comparison of lithium and no lithium conditions, by students' t-test.

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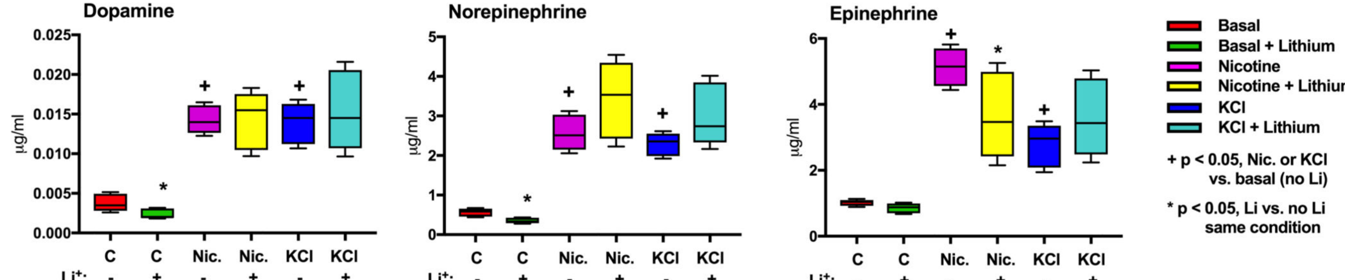

Figure 3. Nicotine- and KCl-Stimulated Secretion of Catecholamine Neurotransmitters in the Absence and Presence of Lithium.

Chromaffin cells were subjected to stimulation of regulated secretion by incubation with nicotine (10 µM) or KCl (50 mM) for 60 minutes, after treatment without or with lithium (1 mM, for 72 hours), and the secretion media was collected for measurements of the catecholamines dopamine, norepinephrine, and epinephrine by the metabolomics methods developed in this study. The results for neurotransmitter secreitons are shown in for dopamine, norepinephrine, and epinephrine panels. Box and whisker plots display mean $\pm$ sem, and $\pm$1.96 sem. Nicotine and KCl significantly increased the amounts of secreted dopamine, norepinephrine, and epinephrine (+$p<0.05$). Lithium treatment significantly reduced nicotine-stimulated epinephrine secretion (*$p< 0.05$, student's t-test).

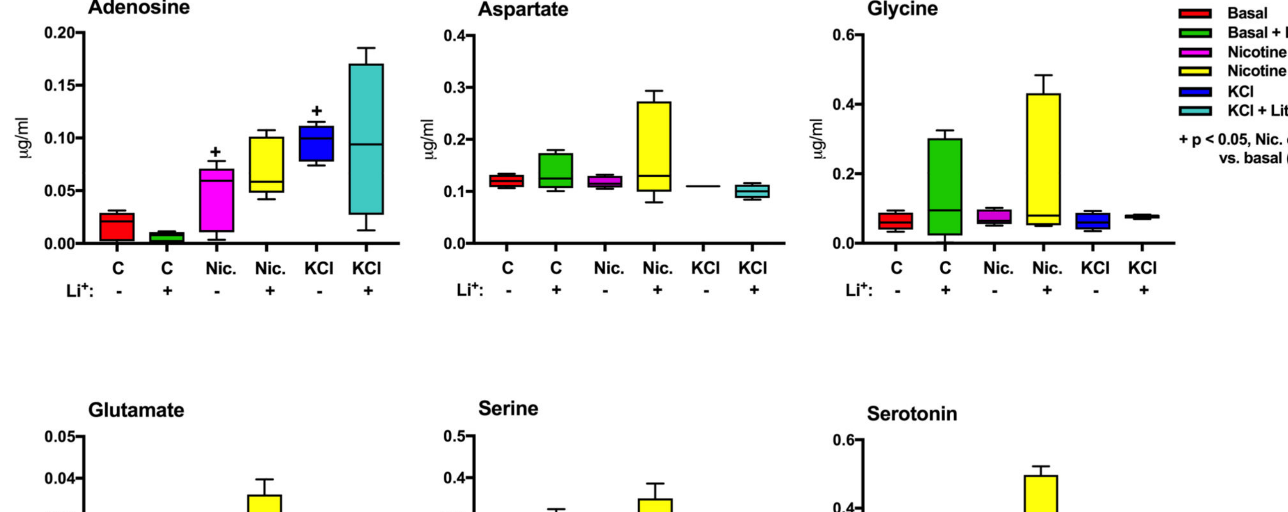

Figure 4. Secretion of Adenosine, Aspartate, Glutamate, Glycine, Serine, and Serotonin from Chromaffin Cells in the Presence of Nicotine and KCl, with Lithium Treatment.

Chromaffin cells were subjected to stimulation of regulated secretion by incubation in nicotine (10 μM) or high KCl (50 mM), in the absence or presence of lithium (1 mM) for 60 minutes. The metabolomics methods developed in this study measured neurotransmitter concentrations in the media for adenosine, aspartate, glycine, glutamate, serine, and serotonin (each shown as a separate graph). Results are shown as box and whisker plots for the mean $\pm$ sem, and $\pm$1.96 sem. Nicotine and KCl significantly stimulated the secretion of adenosine (+$p<0.05$, student's t-test).

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Table 1.

Small Molecule Classical Neurotransmitters.

Properties of fourteen neurotransmitters are provided with respect to molecular formula, molecular weight, and structure. These fourteen small molecules comprise the majority of the classical neurotransmitters which consists of a total of about seventeen molecules. The fourteen neurotransmitters listed in this table are those which could be detected and quantitated by GC-TOF and LC-MS/MS in this study.

Neurotransmitter	Molecular Formula	MW	Structure
Acetylcholine	$C_7H_{16}NO_2$	146.207	
Adenosine	$C_{10}H_{13}N_5O_4$	267.241	
Anandamide	$C_{22}H_{37}NO_2$	347.53	
Aspartate	$C_4H_7NO_4$	133.103	
Dopamine	$C_8H_{11}NO_2$	153.178	
Epinephrine	$C_9H_{13}NO_3$	183.204	
GABA	$C_4H_9NO_2$	103.12	
Glutamate	$C_5H_8NO_4$	147.129	
Glycine	$C_2H_5NO_2$	75.067	

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Neurotransmitter	Molecular Formula	MW	Structure
Histamine	$C_5H_9N_3$	111.15	
Melatonin	$C_{13}H_{16}N_2O_2$	232.278	
Norepinephrine	$C_8H_{11}NO_3$	169.178	
Serine	$C_3H_7NO_3$	105.093	
Serotonin	$C_{10}H_{12}N_2O$	176.215	

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Table 2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ) of Neurotransmitters.

Metabolomics analyses of 14 neurotransmitters was optimized by GC-TOF or LC-MS/MS methods, with retention times indicated, for detection and quantitation. The limit of detection (LOQ) and limit of quantitation (LOQ) of neurotransmitters were determined. Calibration plots of neurotransmitter standards were characterized by slope and R^2 values. Eleven neurotransmitters displayed optimal identification and quantitation by GC-TOF. Three neurotransmitters displayed optimal identification and quantitation by LC-MS/MS.

Neurotransmitter	Method	RT (sec)	LOD (μg/ml)	LOQ (μg/ml)	slope	R^2
Adenosine	GC-TOF	842.556	0.25	0.5	464.6	0.9931
Aspartate	GC-TOF	520.684	0.10	0.5	4238	0.9964
Dopamine	GC-TOF	703.788	0.025	0.1	39075	0.9962
Epinephrine	GC-TOF	668.625	0.25	0.1	31276	0.9964
GABA	GC-TOF	526.858	0.025	0.1	33974	0.9999
Glycine	GC-TOF	438.07	0.05	0.01	47851	0.9984
Glutamate	GC-TOF	556.729	0.25	0.5	1415.5	0.9847
Histamine	GC-TOF	644.988	0.10	0.5	3061	0.9956
Norepinephrine	GC-TOF	724.603	0.05	0.25	8579.5	0.9947
Serine	GC-TOF	458.415	0.05	0.1	5290.8	0.9953
Serotonin	GC-TOF	812.391	0.10	0.25	8320.8	0.9992
Acetylcholine	LC-MS	13.97	0.025	0.1	159	0.9995
Anandamide	LC-MS	3.91	0.025	0.50	52.6	0.9999
Melatonin	LC-MS	4.40	0.01	0.025	1460	0.9974

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Table 3.

Neurotransmitter Concentrations in Neurosecretory Chromaffin Granules.

Concentrations of small molecule neurotransmitters, in isolated chromaffin granules, were measured by GC-TOF or LC-MS/MS. Neurotransmitter concentrations are expressed as μg neurotransmitter per mg protein of chromaffin granules. Neurotransmitter values reported for these biological samples were within the linear dynamic range for measurement of each neurotransmitter.

Neurotransmitter	Concentration (μg/mg)
Adenosine	1.429
Aspartate	0.006
Dopamine	0.024
Epinephrine	12.9
Glutamate	0.005
Glycine	0.002
Norepinephrine	2.89
Serine	0.002
Serotonin	0.006